GENES AND NEURONS INVOLVED IN *DROSOPHILA* LARVAL FORAGING BEHAVIOUR

THE ROLE OF *dRanBPM* IN THE *DROSOPHILA* LARVAL CNS AND ITS EFFECT ON FORAGING BEHAVIOUR

By

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Abstract

The foraging stage of larval development is characterized by an attraction to food resources and an increased consumption of nutrients. Food attraction is partially mediated by an attunement to a light source, visible as distinct locomotor patterns that larvae exhibit in light and dark environments. Loss of the *Drosophila Ran Binding Protein in the Microtubule Organizing Center* (*dRanBPM*) leads to a disruption of locomotion in response to light, a reduction in larval feeding and larval size, and a decrease in viability. The long isoform of dRanBPM has been shown to localize to the Mushroom Body (MB) of the larval CNS, an important structure involved in learning and memory behaviours. Various neuronal markers failed to show any severe morphological abnormalities due to loss of *dRanBPM* gene function in the larval CNS. In addition to a decrease in food consumption, *dRanBPM* mutants also showed a decreased attraction to a food source. These results are supplemented by evidence that *dRanBPM* affects sensory modalities associated with olfaction and phototaxis. The role of the MB in larval behaviours associated with loss of *dRanBPM* gene function has yet to be fully elucidated. *dRanBPM* mutant behavioural phenotypes, such as larval response to light, have been recapitulated through MB silencing studies. My results suggest further that decreasing but not increasing excitability in the γ neurons of the MB affects response to light and feeding behaviours.

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List of Abbreviations

dRanBPM	*Drosophila Ran Binding Protein in the Microtubule Organizing Center*
dRanBPM	Drosophila Ran Binding Protein in the Microtubule Organizing Center
for	*foraging*
dnpf	*Drosophila Neuropeptide F*
dNPF	*Drosophila* Neuropeptide F
SEG	Subesophageal Ganglion
CNS	Central Nervous System
MB	Mushroom Body
KCs	Kenyon Cells
Phospho H3	Phosphorylated Histone 3
BrdU	Bromodeoxyuridine
dfmr1	*Drosophila Fragile X Mental Retardation 1*
dFMRP	Drosophila Fragile X Mental Retardation Protein
mbd	*mushroom bodies deranged*
mbr	*mushroom bodies reduced*
dnc^1	$dunce^1$
PDE	cAMP phosphodiesterase
GFP	Green Fluorescent Protein
Fas II	*fasciclin II*
PHM	Peptidylglycine alpha-hydroxylating Monooxygenase
PAL	Peptidyl- alpha-hydroxyglycinealpha-amidating Lyase
DIMM	DIMMED
yw	$yellow^- white^-$
snpf	*Short neuropeptide F*
sNPF	Short Neuropeptide F
EKO	Electrical Knock Out
VR1	*vanilloid receptor subtype 1*
TNT-VIF	Active Tetanus Toxin
TNT-G	Inactive Tetanus Toxin
NMJ	Neuromuscular Junction
OR	*Oregon-R*
mbm^1	$mushroom\ body\ miniature^1$
HU	Hydroxy Urea
Nb	Neuroblast
NaChBAC	*bacterial sodium channel*
TrkB	Tropomyosin-Related Kinase Receptor B
BDNF	brain-derived neurotrophic factor
DNT1	*Drosophila* Neurotrophin 1
sbl	*smellblind*
PNs	Projection Neurons

Chapter 1: Introduction

1.1 Drosophila Genetics and Behaviour

The model organism, *Drosophila Melanogaster*, has been used extensively in the study of the genetic basis of complex behaviours. Genetic analysis of behaviour is largely rooted in the neurosciences, in that it attempts to uncover the genes responsible for the development of neural circuits that regulate behavioural output (reviewed by Manoli et al., 2006). The morphology of *Drosophila* and it's development have been extremely well characterized. The availability of established genetic techniques in combination with a fully sequenced genome, in addition to it's conserved genetic makeup and neuronal processes, make *Drosophila* a useful model for the study of behaviour (reviewed by Featherstone and Broadie, 2000).

Genes that influence behaviour are often pleiotropic in that they affect more than one trait (reviewed by Kendler and Greenspan, 2006). The *foraging* (*for*) gene was originally identified and characterized for its role in food-search behaviours in *Drosophila* (Sokolowski et al., 1997). Larvae carrying the for^{R} allele, called rover larvae, travelled over larger distances in search of food than did sitter larvae carrying the for^{S} allele (Sokolowski et al., 1997; Kaun et al., 2007). Additional research on the *for* gene has revealed that it is also involved in larval behaviours associated with olfactory and visual reward learning (Kaun et al., 2007) and in adults it is involved in stimuli habituation (Engel et al., 2000) and visual operant conditioning (Wang et al., 2008).

The ability of a single gene product to influence such a wide array of behaviours also highlights the complexity of behaviour itself. To facilitate the act of foraging,

Drosophila larvae must become attuned to a light source (Grossfield, 1978), move towards a food medium (Sokolowski et al., 1997), and consume an adequate amount of nutrients (Sewell et al., 1975). Governing these actions are higher brain centers, such as a center for learning and memory called the mushroom bodies (MBs) (reviewed by Fahrbach, 2006), which influence how visual, olfactory and chemosensory cues are integrated to motivate foraging behaviours. Using behavioural assays to measure the effects of genes and pathways on each of these different aspects of foraging behaviour, one can begin to understand how these processes converge to generate the more complex series of events known as foraging.

The overall purpose of this thesis is to describe how different aspects of foraging behaviour can be affected by a pleiotropic gene, *Drosophila Ran Binding Protein in the Microtubule Organizing Center* (*dRanBPM*) and by neural circuits and structures of the larval CNS.

1.2 Drosophila Larval Foraging Behaviour

The *Drosophila* larval foraging behaviour begins shortly after hatching, when *Drosophila* larvae are in their 1st instar and lasts until mid 3rd instar. During this period, larvae continuously feed (Sewell et al., 1975) and this action heavily impacts the success of their development. Larval feeding has been shown to not only affect the larval rate of development but also effects the timing of pupal formation (Stern and Emlen, 1999), adult emergence (Bakker, 1969), and overall viability (Ohnishi, 1979). Larval feeding

until early 3rd instar is also critical to the attainment of a critical weight, a measurement of body size that is sufficient to cue larval progression into the wandering stage (King-Jones, 2005). In the wandering stage, larva display food avoidance behaviours and expel their guts before they begin pupation (Wu et al., 2003). Thus, food consumption during the foraging stage ensures that the larva has attained an adequate amount of nutrition that will allow it to undergo metamorphosis (Mirth and Riddiford, 2007).

The availability of a food source and the amount of time larvae spend actively seeking out food resources, govern how much time the larvae will spend feeding. Food search behaviours are facilitated through the presence of environmental cues such as odorants, chemicals, and guided through the presence or absence of light. Larvae exhibit attraction and repulsion to different odors, and these effects are conserved across different *Drosophila* strains and are maintained into the wandering stage of larval development (Monte et al., 1989). Recently it has been shown that sensing olfactory cues confers a larval survival advantage. Asahina et al., (2008) used a survival assay in which larvae were exposed to high larval densities and forced to compete for limited food resources. Throughout the assay, which began shortly after larval hatching and extended through to pupation, larvae were forced to seek out multiple food sources in darkness over the course of their development in order to survive. Larval mutants with complete olfactory impairment, *Or83b*, and those with partial olfaction impairment, *Or42a*, showed severely reduced survival and an inability to move from the initial food source to the secondary source. These results suggest when food resources are limited and larval

competition is high, a functioning olfactory system and the ability to detect olfactory cues are necessary for survival.

Larval attraction to a food source can also be modulated by the presence of certain chemicals such as sugar. Studies suggest that activation and synaptic transmission of *Drosophila* neuropeptide F (dNPF), a neuromodulator that is thought to play a role in foraging behaviours (DiBona, 2002; Tecott and Heberlein, 1998), can be directly affected by the presence of sugars in the food source (Shen and Cai, 2001). 3rd instar larvae fed yeast paste which contained increasing concentrations of glucose, had increased *dnpf* expression and increased synaptic transmission into the subesophageal ganglion (SEG) region of the larval central nervous system (CNS). Upregulation of *dnpf* in the presence of glucose is also consistent with the fact that its expression is highest during larval feeding stages. Once larvae become older and start to exhibit behaviours characteristic of the wandering stage of development, *dnpf* RNA levels have been shown to decrease (Wu et al., 2003). 3rd instar larvae with ablated dNPF neurons show little motivation to feed when presented with a medium of glucose. In contrast, over expression of *dnpf* prolongs the feeding stage in 3rd instar larvae by 12-24 hours (Wu et al., 2003). Therefore, *dnpf* expression can not only be modified through glucose concentration in the food, but developmental stage can also govern the impact of *dnpf* on the feeding response.

The larval foraging stage is also characterized by photophobic behaviour, in which the larvae are attracted to dark environments and repelled by light (Gody-Herrera et al., 1994). Larvae appear to burrow deeper into a food source that is surrounded by

darkness, than in a food source exposed to light (Godoy-Herrera, 1986). In addition the preference for darkness during foraging stages may cue the larvae towards a food source, since burrowing into food creates a dark environment (Godoy-Herrera et al., 1994). Upon the cessation of feeding, wandering larvae exhibit photoneutrality (Godoy-Herrera et al., 1992, 1994) in which they exit the dark food source and move away from it. There is evidence that this period of photoneutrality may cease once larvae are ready to pupate (Schnebel and Grossfield, 1986). When given a choice between dark and lit environments, wandering larvae prefer to undergo metamorphosis in the dark (Manning and Markow 1981). Larvae also tend to pupate higher above the food medium when vials are placed in the dark as opposed to lit environments (Schnebel and Grossfield, 1986). Although it is not yet known what triggers this preference for pupation environment, it is speculated that circadian rhythmicity coupled with hormonal cues like ecdysone (White et al., 1997, 1999) and neuropeptides like dNPF may make wandering larvae sensitive to their photic environment during this critical time (Paranjpe et al., 2004).

1.3 *Drosophila* Larval Response to Light

A taxis is the movement of the main axis of an individual in the direction of a particular stimulus in the environment. The movement to orientate oneself towards or away from a light source is referred to as phototaxis (Wehner, 1981). Positive phototaxis occurs when larvae move towards the light, and negative phototaxis is the movement away from a light source. As mentioned in the previous chapter, foraging larvae exhibit

photophobic behaviour, in which they exhibit preference for dark environments (Lilly and Carlson, 1990). They also display negative phototaxis in which larvae position themselves to face dark environments (Grossfield, 1978; Godoy-Herrera et al., 1992). In contrast, wandering larvae exhibit photoneutrality and measurements of phototaxis resulted in a random response by larvae at this stage (Godoy-Herrera et al., 1992, 1994; Sawin-Mccormack et al., 1995). The discovery that larva at different stages exhibit these different forms of taxis, have come from experiments which present larva with both light and dark environments simultaneously and allow them to chose one environment over the other. An alternative to this approach was presented by Busto et al. (1999), who developed an "ON/OFF" assay in which larvae are exposed to light and dark environments sequentially. Using this assay, one can assess the locomotion, as opposed to simply the body positioning or taxis which was assessed in previous assays. The results from this study showed that in light pulses, wild type foraging larvae travel a shorter distance than in dark pulses. This shorter distance observed is the result of increased larval pausing and direction change throughout the course of the assay. A higher frequency of head swinging behaviours is also displayed, in which larva are seen moving their heads from side to side (Busto et al., 1999; Scantlebury et al., 2007). Head swinging allows the larvae to compare the relative intensities of light on either side of them, thus helping the larva change direction and migrate towards a dark place (reviewed by Sawin et al., 1994).

Larval locomotion is achieved by a wave of peristaltic contractions that run anterior to the posterior portion of the larval body. The longitudinal and oblique muscles

that comprise the larval body wall, function to retract and extend the head and pharynx and to lengthen and constrict the length of the body, respectively. The muscles are innervated by motor neurons of the abdominal and thoracic ganglion. These ganglion are joined by paired longitudinal nerve cords which connect to the SEG and other brain regions. Sensory information is received by regions of the CNS such as Bolwig's organ which are the central components of the larval visual system (Bolwig, 1946). The Bolwigs organ is a pair of symmetrical cell clusters composed of 12 photoreceptors. The photoreceptor cells extend their axons forming Bolwig's nerve which innervate the larval brain hemispheres (Tix et al., 1989). Exposure of larva to light has been shown to decrease the frequency of peristaltic contractions, leaving the stride length of the larva unaffected (Scantlebury et al., 2007).

1.4 *Drosophila Ran Binding Protein in the Microtubule Organizing Center (dRanBPM)*

Drosophila Ran Binding Protein in the Microtubule Organizing Center (*dRanBPM*) was identified in a mutant screen aimed at identifying genes that play a role in the photophobic behaviours of foraging larvae, more specifically larval response to light and light modulated locomotion (Scantlebury, 2010). *Drosophila* lines, carrying a random insertion of a transposable P{lac W} element, were analyzed using an ON/OFF response to light assay for deviations in response indices from the stereotypical light response (Scantlebury, 2007). In the ON/OFF assay, larvae are exposed to 10 second

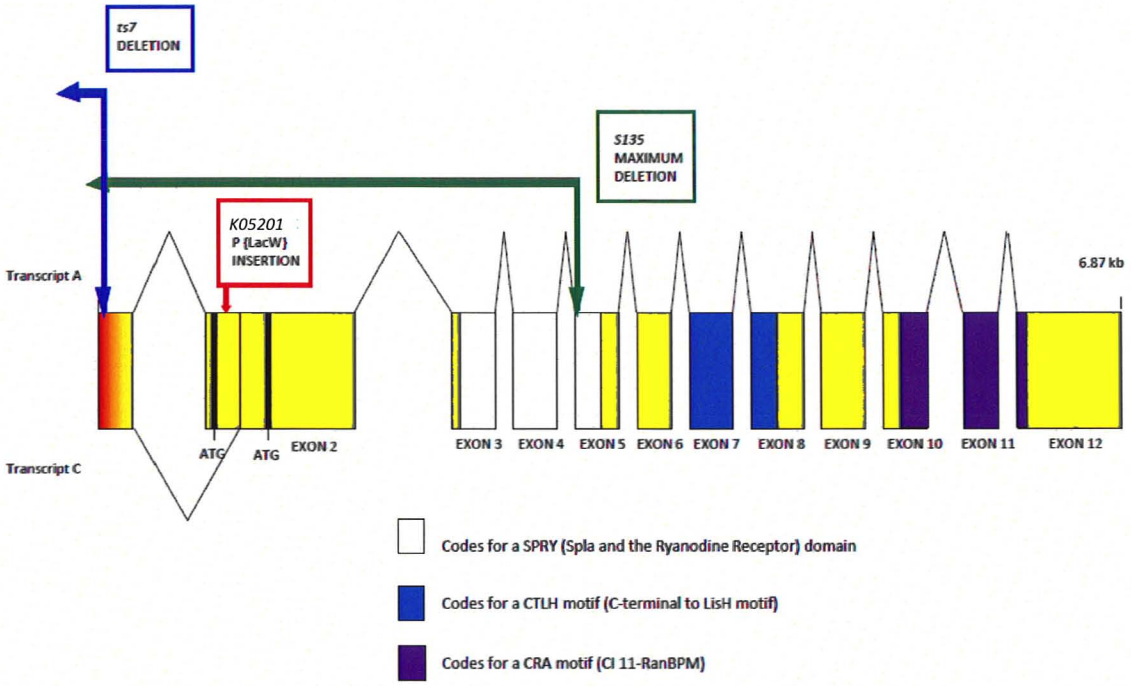
pulses of light followed by 10 second pulses of darkness over a one minute period. Response indices are then calculated by measuring the distance the larva travels in darkness minus the distance travelled in the light divided by the total distance travelled in both phases. P element disruption of the gene coding for *dRanBPM* (CG42236) resulted in a significant reduction in response indices compared to wild-type.

The *dRanBPM* gene is 6.87 kb in length and is located on the right arm of chromosome 2 and contains 12 exons. Two isoforms of dRanBPM are produced through alternative splicing. The transcript for the short isoform ($dRanBPM^{short}$) is truncated 511 nucleotides at the 5'end in comparison to the transcript for the long isoform ($dRanBPM^{long}$). The region at the 5'end contains a glutamate rich N terminal which is missing in the short isoform (Dansereau and Lasko, 2008). The predicted mass of the short isoform is 67kD and 106.4kD for the long isoform. dRanBPM shares 45% amino acid sequence similarity with its mammalian ortholog as well as a number of conserved domains. dRanBPM contains a conserved Spla and the Ryanodine Receptor Domain (SPRY), likely involved in protein-protein interactions (Ponting et al, 1997; Hilton et al., 1998). 3' to the SPRY domain is an lissencephaly homology (LisH) motif coupled with a C-terminal to the LisH (CTLH) motif which function in microtubule dynamics (Umeda et al., 2003), **Figure 1**.

As mentioned previously, the original *dRanBPM* mutant allele identified in the mutant screen ($dRanBPM^{k05201}$), caused a significant reduction in response to light.

Figure 1. The organization of the *dRanBPM* gene and its mutant alleles. The gene *dRanBPM*, is composed of 12 exons which code for transcripts A and C. Two ATG start codons are shown in exon 2. Colours code for protein domains of SPRY, CTLH/LisH and the CRA domain. The P{lacW} element which is present in the $dRanBPM^{k05201}$ mutant allele is inserted in exon 2. $dRanBPM^{s135}$ was generated from an imprecise excision of the P{lacW} element and extends upstream of exon 5. The temperature sensitive allele of $dRanBPM^{ts7}$ is an imprecise excision of an enhancer trap located 5' to the *dRanBPM* gene. This figure was adapted from Dineen (2009) and Scantlebury (2007).

Figure 1. The organization of the *dRanBPM* gene and its mutant alleles.

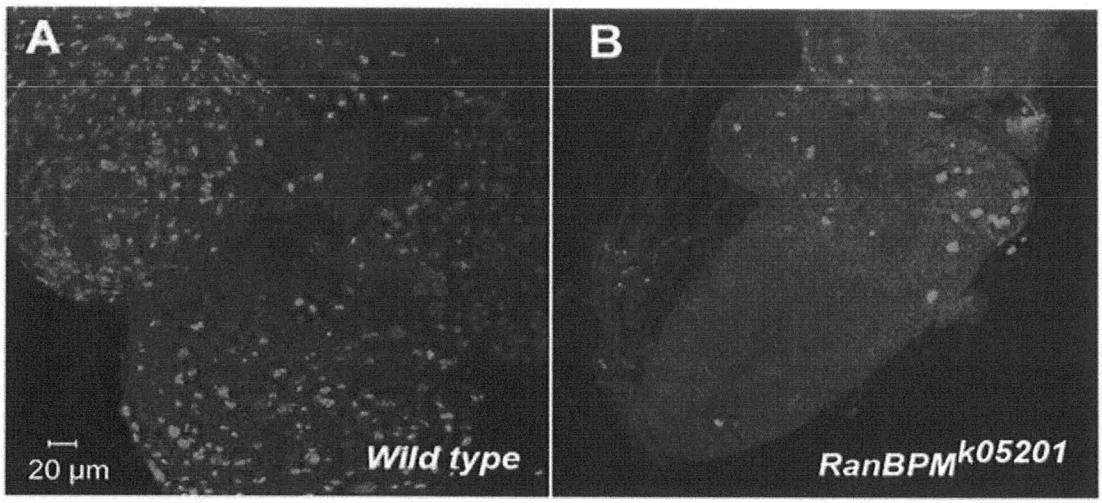


These mutant larvae also display reduced and uncoordinated movements in total darkness and in the ON/OFF assay, as evidenced through their increased levels of direction change in both phases of the assay (Scantlebury, 2010). Additional *dRanBPM* mutant alleles also cause a disruption in response to light. Homozygous and heteroallelic combinations of $dRanBPM^{k05201}$ along with additional mutant alleles of $dRanBPM^{s135}$ (created through the imprecise excision of the original P{lacW} element) and $dRanBPM^{ts7}$ (imprecise excision of an enhancer trap element) also show defects in response to light. Homozygous and heteroallelic combinations of $dRanBPM^{k05201}$ and $dRanBPM^{s135}$ mutants show a 100% reduction in viability after 3rd instar foraging, as well as a decrease in size which is likely linked to their reduction in feeding behaviour. $dRanBPM^{ts7}$ is a temperature sensitive allele allowing for 100% viability when larvae are raised at 18°C. Under normal temperature conditions these mutants die between pupal and adult stages (Scantlebury, 2007). Homozygous and heteroallelic combinations of $dRanBPM^{ts7}$ show no size phenotype and feed like wild type controls.

$dRanBPM^{k05201}$ and $dRanBPM^{s135}$ mutants fed 58-74% less than controls of the same age and $dRanBPM^{k05201}$ mutants appear to have a reduced attraction to and motivation to remain in the food source. In addition, homozygous mutants for $dRanBPM^{k05201}$ show a reduction in cell proliferation as seen by immunohistochemistry using phosphorylated Histone3 (phosphoH3) immunohistochemistry analysis (Scantlebury et al., 2010) **Figure 2**. Histone 3 is specifically phosphorylated when chromosomes condense to undergo mitosis, phosphoH3 recognizes this phosphorylated

Figure 2. *dRanBPM*^{k05201} mutants show a reduction in cellular proliferation in the larval CNS. Confocal projections of *dRanBPM*^{k05201} mutants (B) and wild type controls (A) immunolabelled with anti-phosphoH3 primary antibody and an Alexa 488 secondary. The CNS of *dRanBPM*^{k05201} mutants are smaller than wild type specimens, and have a reduction in phosphoH3 labeling indicative of reduced cellular proliferation (Scantlebury et al., 2010). Wild type images contain 10-13 sections and *dRanBPM*^{k05201} mutants contain 5-7 sections, both at 2µm intervals. All images taken by Xiao Li Zhao.

Figure 2. *dRanBPM* ^{k05201} mutants show a reduction in cellular proliferation in the larval CNS.

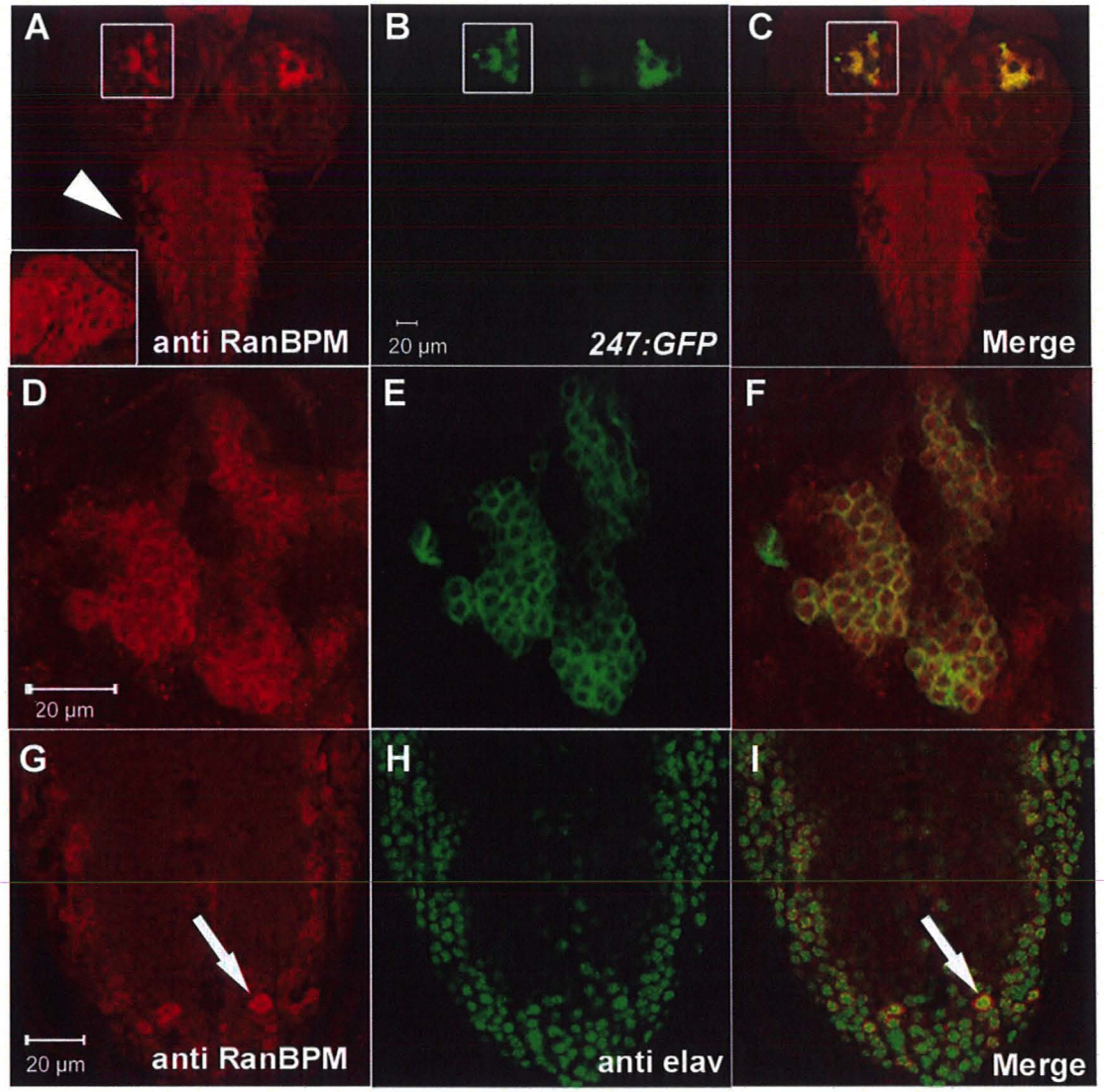


state of the histone. Studies by Britton and Edgar (1998) reported a correlation between food intake following larval hatching and the degree of cellular proliferation in the CNS. After completion of an embryonic program of proliferation which gives rise to the neurons and glia of the larval CNS, neuroblasts become quiescent and re-enter the cell cycle in the 1st instar larval phase of development. This re-entry occurs in a spatiotemporal wave-like pattern, with proliferation starting at the anterior in the brain region and travelling towards the posterior to the ventral nerve cord. Starving wild type larvae led to a dramatic reduction in proliferating neuroblasts, with bromodeoxyuridine (BrdU) incorporation levels concentrated to the anterior of the CNS, a similar pattern to what is seen in *dRanBPM* ^{k05201} mutants (Britton and Edgar, 1998).

Immunohistochemistry analysis using a dRanBPM antibody specific for the long isoform showed staining in the lobes and on the sides of the ventral nerve cord which co localized with neuronal labelling using an *elav* driver (Robinow, 1988; Berger, 2007). There was an absence of co-localization of dRanBPM^{long} with both Phospho H3 and *repo* (Xiong, 1994), indicating that dRanBPM^{long} is not located in proliferating cells or in the glia, respectively. dRanBPM^{long} showed strong immunolabelling in the kenyon cells (KCs) of the MB, labelled with the MB specific driver, MB247 (Schulz et al., 1996) **Figure 3**. The KCs are visualized as a cluster of cells located bilaterally in each brain lobe of the larval CNS (reviewed by Fahrbach, 2006). Consistent with these expression patterns, the long or short isoform of dRanBPM targeted to the nervous system was able to rescue response to light, locomotion, feeding mutant phenotypes as well as rescue the decreased viability. Further rescue experiments revealed that both isoforms expressed

Figure 3. Expression of dRanBPM^{long} in the larval CNS. Confocal images of the CNS in wildtype larvae, carrying a *UAS-CD8-GFP* transgene driven by *MB247* (**B,C**), are stained with an antibody specific to the dRanBPM^{long} and a red Cy3-conjugated secondary (**A,C**). The long isoform of dRanBPM colocalizes to the KCs of the MB as seen in the magnified images **D-F**. dRanBPM^{long} is also located in the ventral cord and colocalizes with the post mitotic neuronal marker *elav*, shown in green (**G-I**). Images in panels A-C were taken with the 20x objective, panels D-I were taken with the 63x objective. All images taken by Xiao Li Zhao.

Figure 3. Expression of dRanBPM^{long} in the larval CNS.



specifically in the MB KCs at 29°C were also able to rescue the response to light and feeding mutant phenotypes, and partially rescue locomotion. MB expression was, however, unable to rescue viability (Scantlebury et al., 2010).

Investigations in our laboratory also provide evidence for a functional interaction between dRanBPM and *Drosophila* Fragile X Mental Retardation Protein (dFMRP) towards the control of synaptic growth in the *Drosophila* neuromuscular junction (NMJ). In humans, trinucleotide repeat expansion of the 5'end of the gene encoding FMRP results in transcriptional silencing leading to Fragile X Mental Retardation Syndrome. This disorder is characterized by many behavioural abnormalities including hyperactivity, attention deficits, and impaired motor coordination and motor function (reviewed by Zhang and Broadie, 2005). FMRP has been well characterized in mammals as an RNA binding protein that functions as a suppressor for the translation of proteins involved in the regulation of synaptic structure (Brown et al., 2001).

Drosophila larvae mutant for the Drosophila Fragile X Mental Retardation gene (*dfmr1)* exhibit a reduction in locomotion (Xu, et al., 2004), whereas *dfmr1* mutant adults have reduced flying behaviours (Zhang et al., 2001) and disruptions in rhythmic behaviours such as eclosion (Dockendorff et al., 2002). Phenotypically, adult *dfmr1* mutants exhibit a midline crossing of the β lobe neurons in MBs and larval *dfmr1* mutants show overbranching and increased bouton number at the NMJ (Michel et al., 2004; Pan et al., 2004). In mammals, RanBPM has been shown to physically interact with FMRP through a CRA domain located near the C terminal of RanBPM (Menon et al.,

2004). Although no physical interaction between dFMRP and dRanBPM has been reported in *Drosophila*, evidence for a functional interaction was found through genetic epistasis analysis at the NMJ (Zahanova, 2008; Scantlebury et al., 2010).

Since double mutants of *dRanBPM*^{k05201} and *dfmr1* are lethal, we introduced one copy of d*RanBPM*^{k05201} in an *dfmr1* mutant background. We found that a reduction in function of *dRanBPM* was able to decrease the number of synaptic boutons and branches back to wild type levels (Scantlebury et al., 2010). Similar experiments were conducted to determine if the introduction of a mutant *dRanBPM*^{k05201} allele could modulate the locomotion phenotypes also exhibited by homozygous *dfmr1* mutants. Introduction of one mutant copy of *dRanBPM*^{k05201} in a homozygous *dfmr1* mutant background failed to show any differences in locomotion and response to light when compared to *dfmr1* homozygous mutants alone (Dineen, 2009). These results provide evidence that dRanBPM and dFMRP may be partners in the control of dendritic arbour formation, however do not functionally interact for the control of locomotion.

There are only two published studies that report a role for *dRanBPM* in *Drosophila*. Aside from our recently published work on its role in larval behaviours (Scantlebury et al, 2010), a study by Dansereau and Lasko (2008), reported that RanBPM^{long} is required for the arrangement and size regulation of the germline stem cell niche. In contrast, the dRanBPM ortholog in mammals has been well studied and its function well documented. The vertebrate RanBPM was first identified as binding Ran, a protein involved in the regulation of nuclear cytoplasmic transport, in a yeast two hybrid

screen (Yokoyama et al., 1995). RanBPM is a member of the group of Ran binding proteins which function in many cellular processes including nucleocytoplasmic transport and cell cycle progression in vertebrates (reviewed by Dingwall et al., 1995). RanBPM itself, however, does not appear to function in nucleocytoplasmic transport (Nishitani et al., 2001). RanBPM is thought to interact with multiple receptors as a scaffolding protein for signal transduction processes (reviewed by Murrin and Talbot, 2007). RanBPM exists as two isoforms in mammals, the larger isoform is primarily cytoplasmic (Zou et al., 2003), while the smaller isoform is concentrated to the nucleus (Nishitani et al., 2001). In mammals it is found primarily in the brain, heart and kidneys (Nishitani et al., 1999) The short isoform of RanBPM has been shown to function to initiate the reassembly of the centrosome through reorganization and nucleation of microtubules (Yokoama et al.,1998), and has also been linked to neuronal differentiation (Brunkhorst, 2005). The long isoform of RanBPM has been implicated in assisting in signal transduction pathways by functioning as an adaptor protein for various kinases. RanBPM interacts with the hepatocyte growth factor (HGF) receptor mesenchymal-epithelial transition factor (MET) to aid in Sos recruitment which is needed to activate the Ras pathway (Wang et al, 2002). In neurotrophin regulated signalling pathways it has been shown that RanBPM binds to TrkA (Yuan et al., 2006) and TrkB (Yin et al., 2010), which is suggested to promote MAPK and Akt activation. RanBPM also functions as an adaptor protein by binding to Axl and Sky which are involved in tumourigenesis (Hafizi et al, 2005). RanBPM has additionally been shown to function in cell growth and apoptosis by regulating p73α (Kramer et al., 2005), and AChE (Gong et al., 2009). It also forms associations with

adhesion molecules such as LFA-1(Denti, 2004) and CD39 (Wu et al., 2006) which function in immune response, and forms a complex with Muskelin (Valiyaveettil et al., 2008) to control cell spreading. RanBPM's multiple protein-protein interactions combined with its association in large protein complexes (Nishanti et al, 2001), support RanBPM's classification as a scaffolding protein.

1.5 The Mushroom Bodies

In *Drosophila,* the intrinsic neurons of the MBs are formed from four neuroblasts, two in each brain lobe. These neuroblasts give rise to two clusters of KCs, one in each brain hemisphere. These KCs and their associated projections are visible as two mushroom-like structures, called the MBs (Heisenberg, 2003). The neuroblasts that form the MB structure are unique in that they continue to divide throughout *Drosophila* development into pupal stages (reviewed by Fahrbach, 2006). Each MB is composed of four main parts: the Kenyon Cells (KCs), the calyces, the pedunculus and the vertical and medial lobes. The 2500 KCs of each brain hemisphere are clustered at the dorsal posterior surface in each lobe of the central brain. KCs are organized in a birth dependent order, with later born KCs in the middle, closest to the proliferating neuroblast, and earlier born KCs pushed towards the periphery (Yang et al. ,1995; Armstrong et al., 1998). This concentric organization is maintained as KCs extend their unipolar axons to the anterior of the larval CNS, thus comprising the calyx structure. The axon bundle further extends and converges to form the pedunculus (Verkhusha et al., 2001; Kurusu,

2002). The pedunculus extends ventrally toward the anterior surface of the brain, where it bifurcates into five terminal lobes. The α and α′ lobes project toward the dorsal surface, while the β, β′ and γ lobes project toward the midline of the brain. KCs are classified according to which lobe they terminate in and exhibit a unique pattern of development. The γ neurons are generated first, followed by the α′ and β′ neurons which are born in the middle of the 3rd larval instar, followed by the α and β neurons which are formed during early to late pupal stages. The γ neurons undergo further modification in the early pupal stages where they are cleaved past the initial point of bifurcation by the phagocytosis action of the glial cells. The purpose for this regression is unknown, however the γ neurons do re-extend in the adult phases but only toward the medial lobe forming a new class of unbranched KCs (Lee, Lee, and Luo, 1999).

Early evidence for the function of the mushroom body in Drosophila was based on behavioural analysis of mutants that harbored single gene variants that resulted in altered MB structure. Two mutants in particular, *mushroom bodies deranged* (*mbd*) and *mushroom bodies reduced* (*mbr*) displayed noticeable structural abnormalities including size distortions of the calyces and thinning or absence of the peduncle, as well as behavioural deficiencies in olfaction learning paradigms. (Heisenberg, 1985; deBelle and Heisenberg, 1994; reviewed by Strausfeld et al., 1998). Investigation of the behavioural outcomes associated with other MB mutants as well as measuring the behavioural effects of chemically ablating the MB neuroblasts sparked interest in the MB as a central hub for *Drosophila* adult behaviours (reviewed by Heisenberg, 1998). To date the MB has been implicated in choosing among conflicting visual cues (Tang and Guo, 2001; Xi et al.,

2008), context generalization (Liu et al, 1999), mediating aggressive behaviours (Baier et al., 2002), sleep (Joiner et al., 2006), and courtship behaviours (McBride et al., 1999). More specifically, the neuronal subtypes which comprise the medial and vertical lobes of the MB have been shown to independently function in specific adult behaviours. The γ lobe has been shown to be important for short term memory (Zars et al., 2000), while the vertical lobes function in long term memory formation (Pascual and Preat, 2001). Studies of the α and β neurons suggest that they are important in olfactory memory retrieval but not acquisition (Dubnau, et al., 2001).

Several studies have implicated the MB in aspects of adult movement. Besson and Martin (2005) provided evidence of a role of the MB in centrophobism behaviours in which adults lacking MB spent more time flying in the center of an arena than controls that prefer to fly in the periphery. Martin et al. (1998) found that adults with ablated MBs and clipped wings had reduced walking ability. More recently, Serway et al. (2009) compared seven different mutants with MB structural defects with adults with ablated MBs and found that those with structural defects were less active and walked slower than those lacking total MB function. All adults exhibited normal velocity and orientation to surroundings, suggesting that the MBs are important in modifying the amount of walking.

Studies attempting to elucidate the role of the MB in larva have mainly focused on learning and memory with respect to olfaction (Stocker et al., 2001; Selcho et al., 2009; Heisenberg et al.,1985). However, similarities in the MB structure and neuronal networks used by both *Drosophila* larvae and adults in a variety of behaviours suggest

that the MBs play similar roles during both stages of development. Early evidence of this was shown in a series of experiments by Aceves-Pina et al., (1979) who demonstrated that like adults, larvae have the ability to respond to cues, such as electric shock, and to reinforcement, as measured by an olfaction paradigm. Further, learning mutants of the genes *dunce*^{1} (*dnc*^{1}), *turnip* and *cabbage*, showed the stereotypical pattern of a reduction in olfactory learning but with conserved measures of responsiveness to cues and reinforcement. These results demonstrate that these genes affecting learning do not simply block larval sensory and motor pathways but function similar to that of adults, suggesting that these neural networks present in adults, are also present in 3rd instar larvae. The product of the gene *dnc* is the enzyme cAMP phosphodiesterase (PDE) which is a component of the cAMP pathway. Additional research involving learning mutants of *dnc*^{1}, found that PDE is localized to the MB of larvae and adult *Drosophila* (Nighorn et al., 1991) further supporting the role of the MB in learning behaviours at both developmental stages. PDE is a component of the cAMP pathway which is involved in cellular communication. Loss of PDE leads to deficiencies in operant conditioning and in learning paradigms involving olfactory cues (Dudai, 1983; Temple et.al., 1983), experience based courtship behaviours (Hall, 1986), habituation and sensitization (Duerr and Quinn, 1982). Tully (1994) investigated whether larvae trained in an odor-shock paradigm could retain what they had learned through metamorphosis into *Drosophila* adult stages. Adult *Drosophila* that learned to associate odors with shocks in their larval stages, had higher performance indices than adults that were not trained as larvae. *dnc*^{1} mutants, unable to learn as larvae, did not perform significantly different than adults that

were not trained as larvae. Studying the same paradigms as that of Aceves-Pina (1979), Heisenberg et al., (1985) showed that when connections to and from the MB are severed, these behaviours are disrupted to the same extent in both adults and larvae. These results suggest that even though the structure of the MB is rearranged through development from larvae to adult in *Drosophila*, for the most part its functional identity is maintained.

1.6 Overview and Thesis Objectives

Many genes have been identified that regulate different aspects of foraging behavior such as feeding, larval crawling, and social cooperative burrowing (de Belle et al., 1989, 1993; Ainsley et al., 2003; Wu et al., 2003). *dRanBPM* is a pleiotropic gene that influences different aspects of larval foraging behavior including feeding, response to light and locomotion. The long isoform of dRanBPM is strongly expressed in the VNC as well as the KCs of the MB, a critical structure in the CNS regulating higher order functioning such as learning and memory. Mutations in *dRanBPM* result in larvae of smaller size compared to wild type, however the impact of this size discrepancy on CNS neuronal structure has yet to be determined.

To that end my **first objective was to characterize the phenotype of the central nervous system of *Drosophila dRanBPM* mutants using different neuronal markers**. As a way of sampling the nervous system I analyzed various neuronal pathways whose expression patterns were well documented in the literature (Tanaka et al., 2008; Rodriguez-Moncalvo and Campos, 2009; Taghert et al., 2001; Park et al., 2008; Wu et

al., 2003; Johard et al., 2008) and that were relevant in behaviours that are affected in *dRanBPM* mutants. Neuronal morphology was compared between 3rd instar *dRanBPM* mutants and 3rd instar controls for the MB neurons, serotonergic neurons and cell bodies, peptidergic neurons using the *386*-GAL4 and *c929*-GAL4 drivers and for the short and long form of *Drosophila* neuropeptide F.

In feeding assays, *dRanBPM* mutants show food aversion behaviours. A lower percentage of mutant larvae remain in a food source and feed compared to controls (Scantlebury et al., 2010). Abnormal feeding behaviours have been linked to olfaction deficiencies (Aceves-Pina et al., 1979; Rodrigues, 1980; Monte et al., 1989;) and or an inability to taste the food substrate (Heimbeck et al., 1999). *dRanBPM* mutants also show a reduction in response to light using an ON/OFF assay, however their preference for dark over light environments when presented simultaneously has yet to be determined. My **second objective was to assess the *dRanBPM* mutations on larval sensory behaviors involving smell, taste, and preference for light or dark.**

Suppression of MB neurons using an active form of *tetanus toxin light chain* (*TNT-G*) caused a reduction in response to light compared to control larvae (Dineen, 2009). The MB has been previously implicated in larval foraging behaviours (Osborne et al., 2001) however it has yet to be determined how MB suppression affects larval feeding. In addition, the ability to recapitulate the response to light phenotype using MB suppression experiments provides evidence that neuronal excitability within the MB γ neurons are important for larval behaviour. To further investigate the level of neuronal

excitability required to affect response to light and feeding behaviours, it would be beneficial to hyperexcite the MB and measure the associated affects. Thus, **my final objective was to assess how MB driven suppression and excitation influence larval feeding and response to light behaviours.**

Chapter 2: Materials and Methods

2.1 Fly Stocks

Drosophila melanogaster fly lines used in these experiments were maintained on a medium of sucrose, inactivated yeast, agar, ten percent tegosept in ethanol and an acid mix consisting of phosphoric and propionic acid. Food plates were also enriched with vitamin A (Jamiesons β-carotene) at a concentration of 1.25g/ L. The wild type control strains used in these investigations consisted of *Oregon-R* (*OR*) and *yellow*$^{-}$, *white*$^{-}$ (*yw*). All fly strains were reared primarily at room temperature (23°C – 25°C) and in 25°C or 29°C incubators when noted.

2.2 *dRanBPM* strains

The primary *dRanBPM* mutant allele used in this study was *yw; dRanBPM* ^{k05201}*/CyO[y*$^{+}$*]* (BSC 10564). This allele contains a P{lacW} element with a mini w^{+} gene that disrupts the second exon of the *dRanBPM* gene. A second mutant allele, *yw; dRanBPM*^{s135}*/CyO(y*$^{+}$*)*, was generated through the imprecise excision of the P{lacW} element which also removed flanking areas of DNA around the element (performed by Veronica Rodriguez Moncalvo). The final *dRanBPM* mutant allele was created by the imprecise excision of an enhancer trap insertion located in the first exon of the *dRanBPM* gene, *yw; RanBPM* ^{ts7}*/ CyO[y*$^{+}$*]*. This temperature sensitive allele reduces larval lethality when reared at 18°C, but not at 25°C. A *dRanBPM* revertant allele, *yw; dRanBPM* ^{revertant}*/ CyO[y*$^{+}$*],* was created through the precise excision of the P{lacW} element present in the

yw; dRanBPM^{k05201}*/ CyO[y*$^{+}$*])* allele (performed by Niko Pretorius). Strains carrying only one copy of each *dRanBPM* allele over *CyO[y*$^{+}$*]* were also used as control lines.

2.3 Transgenic fly strains

The two MB specific GAL4 drivers that were used in these experiments were *MB247*-GAL4 donated by Linda Restifo and *MB201Y*-GAL4 (BSC (Bloomington Stock Center) #4440). *MB247*-GAL4 is an enhancer trap insertion located upstream of the *Dmef2* gene (Schulz et al., 1996), *MB201Y*-GAL4 is a P[GAL4] insertion (Yang et al., 1995). Both *MB247*-GAL4 and *MB201Y*-GAL4 label the γ neurons and the calyces of the MB, *MB201Y*-GAL4 labels the whole structure of the α/β neurons while *MB247*-GAL4 only labels the surface and the posterior portion of these neurons (Tanaka et al., 2008). Other GAL4 lines used in this research included *386Y*-GAL4 and *c929*-GAL4 (kindly donated by Paul Taghert) and *dnpf*-GAL4 (*Neuropeptide F*, BSC # 25681) all of which label subsets of peptidergic neurons. *386Y*-GAL4 is a p{w^{+}, GAL4} insertion in the 3' end of the *amontillado* gene that encodes a prohormone processing enzyme that acts within *Drosophila* neurons (Siekhaus and Fuller, 1999). *c929*-GAL4 is a P[GAL4] insertion located on the second chromosome within the *crytocephal* gene (Hewes et al., 2000) its expression pattern however closely mirrors that of the bHLH transcription factor, *DIMM* (Taghert et al., 2001). *dnpf*-GAL4 is a P[GAL4] insertion is located in the upstream regulatory region of the *dnpf* gene (Wen et al., 2005). Additional UAS responder strains included UAS-*TNT-G* and UAS-*TNT-VIF* (active and inactive tetanus

toxin light chain, respectively) and UAS-*EKO* (*electrical knockout*). *TNT-G* is a neurotoxin that functions to prevent neurotransmitter release into the synapse, and *TNT-VIF* contains a lack of function mutation (Sweeney et al., 1995). UAS-*NaChBAC* (*Bacterial Sodium Channel*) is located on the 2nd chromosome and causes neuronal hyperexcitability (Nitabach et al., 2006). UAS-CD8-GFP (Green Fluorescent Protein, BSC # 5130).

2.4 *MARCM* ready fly lines

Larvae of genotype *elav*-GAL4, *UAS-CD8-GFP, hsFLP,w-/FM7* (BSC stock #5146) were crossed to the double balancer line *yw; sp/TS; Ly/TL*, kindly provided by Alecia Pepper. A stock of *elav*-GAL4, *UAS-CD8-GFP, hsFLP,w-/elav*-GAL4, *UAS-CD8-GFP, hsFLP,w-; Sp/ TS; +/TL* was created by crossing females carrying *elav*-GAL4, *UAS-CD8-GFP, hsFLP,w-/+; TS/+; TL/+* with males carrying *elav*-GAL4, *UAS-CD8-GFP, hsFLP,w-/+; Sp/+; +/+*. A second stock was made by crossing males carrying *w*; FRT-Gal80* (BSC #5140) to females carrying *FM7/FM7; Star/ CyO[y+]*, provided by Flora Suen, and picking up males and females that carried *FM7/ +; FRT-Gal80/ CyO[y+]*. A female from the first stock and a male from the second stock were then crossed to create a third stock of genotype *elav*-GAL4, *UAS-CD8-GFP, hsFLP,w-/FM7; FRT-Gal80/ TS; +/TL.*

To recombine $RanBPM^{k05201}$ downstream of a *42D FRT* site, a fly line carrying *Neo(FRT)42D, UbiGFP/ CyO* (BSC # 5626) was crossed to $RanBPM^{k05201}$*/ CyO[y+]*.

Non curly flies were then crossed to *yw; S/ CyO[y$^+$]* and grown on a medium containing 0.3mg/ml of neomycin (Geneticin Selective Antibiotic liquid, GIBCO) to select for flies carrying the *Neo(FRT)42D* site. Wild type flies reared at lower concentrations of neomycin were found to be viable (data not shown). Curly winged females that survived the neomycin food were also screened for a loss of GFP using a dissection scope fitted with a GFP filter. This indicated a possible recombination event replacing the *UbiGFP* with $RanBPM^{k05201}$) and crossed to *yw; S/ CyO[y$^+$]*. To further confirm the presence of a recombination event, candidate flies were tested for viability when crossed to $RanBPM^{s135}$ (heteroallelic combinations of $RanBPM^{k05201}$ /$RanBPM^{s135}$ are 100% lethal) and then tested again for viability when grown in neomycin food (Appendix C).

2.5 Immunohistochemistry

Larval brain dissections were performed in 1X PBS (Phosphate-Buffered Saline) and fixed in 4% paraformaldehyde (pH 7.2- 7.4) for 30 minutes at room temperature. Brains were washed in 1X PBS for 15 minutes, changing the wash every 5 minutes. Brains were then transferred to a 0.5% Triton X-100 (PBT) solution and washed for 3 hours, changing the wash every 30 minutes. After washing, brains were blocked for 1 hour in 10% goat serum (NGS) in 0.5% PBT. Block was removed and primary antibodies were added in a fresh solution of 10% goat serum (NGS) in 0.5% PBT and placed at 4°C for 12 hours. After primary incubation, brains were washed in 0.5% PBT for 3 hours, changing the wash every 30 minutes. Brains were blocked for 1 hour and secondary

antibodies were added and incubated as described above. Brains were then washed in 1X PBS for 3 hours, changing the wash every 30 minutes. All Brains were mounted in 70% glycerol in 1 X PBS.

Primary antibodies used in this study included rabbit anti-5-HT (1:200) (Protos Biotech) anti-FMRF amide (1:1000) (SNPF, donated by Jan Veenstra), and mouse anti-FasII (1:2) (DSHB). Secondary antibodies used included Texas Red-conjugated goat anti-rabbit IgG (1:200), Cy3-conjugated goat anti-rabbit IgG (1:200) (Jackson); Alexa 594-conjugated goat anti-mouse IgG goat (1:200), Alexa 488-conjugated goat anti-mouse IgG (1:200) (Molecular Probes). Dissected brains were visualized using a Zeiss Axiovert 100M and brightness and contrast were adjusted using the Zeiss Laser Scanning Microscopy (LSM) 510 image software.

2.6 Behavioural Assays

Collection and Synchronization of Larvae

For behavioural assays and immunohistochemistry experiments, fly houses were stored at 25°C for no longer than a week and contained 1-3 day old female virgins and males. Prior to experimental collections, a fresh food plate (50mm x 50mm; Fisher Scientific, Houston Texas) was put on the house for one hour to collect embryos for a pre-collection. After one hour another fresh food plate was put on the house for an additional one hour collection to be used to conduct the experiments. All collections were

performed at 25°C. Collection plates were then left at 25°C or transferred to 29°C incubators as indicated. Approximately 22-23 hours after the time of collection (about 16- 17 hours for flies incubated at 29°C), newly hatched larvae were cleared from the collection plates and put back into their respective incubators for one hour. The larvae that hatched within this one hour period, were transferred to a fresh food plate. Plates to be used for the ON/OFF assay contained approximately 30 larvae per plate, whereas plates to be used for the rest of the behavioural assays contained 40-50 larvae per plate. These plates were then allowed to develop at their respective temperatures. Third instar larvae were used for each behavioural assay and were collected around 84-90 hours AEL (after egg laying) for those reared at 25°C, and those reared at 29°C were collected around 66-70 hours AEL.

Locomotion and ON/OFF Response to Light Assay

Locomotion assays and the ON/OFF Response to Light Assay were conducted similar to that described in Busto et al., 2009 and Scantlebury et al., 2007; 2010. Third instar larvae were removed from a collection plate and washed in 1 x PBS and water to remove food particles. A single larva was then placed in the center of a plastic petri dish (100mm x 15 mm, Falcon) which contained 15mL of solidified 1% agar solution. The larva were left to wander the plate for 1 min to become familiar with the surface of the plate, before being returned to the center of the plate with a paint brush. Locomotion behaviour was assayed for 30 seconds under a red safelight (20W lamp with GBX-2 filter). Following this assay, the larva was returned to the center of the plate for the

ON/OFF assay. In the ON/OFF assay, larvae are exposed to 10 second pulses of light followed by 10 second pulses of darkness for a one minute duration. The light source used in this assay was positioned above the testing arena and pulses were controlled by a serial microcontroller (MacIO,MacBrick, Netherlands) and a relay unit (AZ696) interfaced with a Macintosh G3 running a custom macro program in NIH image (1.62f) image processing software. A monochrome digital firewire camera (PL-A641,Pixelink, Ottawa, Canada) was also positioned above the testing arena and contained a macro zoom video lens (18-108,F2.5 MVZL, OPTEM International, Fairport, NY). Movies were recorded using Pixelink Capture Software which was run on a Macintosh G4/733 MHz workstation running MacOS 9.1. Tracing of larval paths in the locomotion and ON/OFF assay was achieved using the Dynamic Image Analysis System (DIAS, 3.2, Solltech, Inc., Iowa, USA). Data pertaining to speed, direction change, and locomotion distances were extracted from the larval path tracings using DIAS (Soll, 1995).

Olfactory Assay

To measure larval olfactory response to attractive and repulsive odours, a plate assay was used as described in Lilly and Carlson (1989) and which was based on the assay used by Aceves -Pina and Quinn (1979). 3rd instar foraging larvae were staged as described previously, picked from food plates with a paint brush and washed by transferring them into a dish containing 1XPBS, and then into a dish containing ddH20. Prior to the start of the assay, a plastic petri plate (100 mm x 15 mm, Falcon) was filled with 15mL of 1% agarose and allowed to cool. Two filter discs (pre cut to 1/4 inch

diameter) were then placed with forceps on opposing ends of the plate. 25-30 larvae were placed, using a paintbrush, in the center of the plate prior to the deposit of the odorants. 20µl of ddH20 (the control side of the plate) was first added to one of the filter discs, followed by the addition of 20µl of either 10x diluted propionic acid (for the attraction assay) or propionic acid at a regular concentration of 13.4mol/L (for the repellent assay). The addition of either concentration of propionic acid signalled the start of the assay. Larvae were allowed to wander the plate for 10 minutes, with recordings taken every 5 minutes. At each time point the position of each larvae were counted in relation to their location on the plate. Their position on either the stimulus side (propionic acid) or the control side (ddH20) was noted, and a response index was calculated (number of larvae on the stimulus side minus the number of larvae on the control side divided by the total number of larvae). In addition, the position of each larva from the center of the stimulus disc of filter paper was measured as being either 13mm or 22mm.13mm was approximately the entire surface area of the filter paper, and 22mm represented a region where larvae are known to congregate in the repulsion assay (Lilly and Carlson, 1989). Larvae that did not migrate more than 1cm from the middle of the plate were removed from the analysis.

Contact Chemosensory Assay

The design for the contact chemosensory assay was adapted from that described in Lilly and Carlson (1989), which was based on that used in Tompkins (1979). Petri dishes (100 mm x 15 mm: Fisher Scientific) used in this assay were pre sectioned into

four quadrants. 10ml of 1% agar was added to opposing quadrants (the control quadrants), and 10ml of 1% agar containing 1M of NaCl (the stimulus quadrants) were added to the remaining two quadrants. Quadrants were then left to solidify, approximately 1 hour. 15ml of 1% agar at a temperature of 55°C was then added to the entire plate in order to cover the plastic quadrant dividers. Plates were left to cool for 2 hours to allow for the diffusion of the NaCl through the top layer of agar. The assay was conducted immediately following. 25- 30 larvae were washed as described previously and were placed in the center of the plate where the dividers converged. Larvae were left to wander the plate for up to 15 minutes, with recordings taken every 5 minutes. The number of larvae in each quadrant was counted, and those larvae that did not migrate more than 1cm from the middle of the plate were removed from the assay. A response index was calculated as the number of larvae in the stimulus quadrants minus the number of larvae in the control quadrants divided by the total number of larvae.

Phototaxis Assay

The design for the phototaxis assay was adapted from that used in Hassan et al. (2000) and Lilly and Carlson (1989). Petri dishes were used as described in the Contact Chemosensory Assay, except that each quadrant was filled with 1% agar until quadrant dividers were covered (approximately 55µl). To conduct the assay, a light box (containing a 20W Cool White Blub, Philips) was used which was fitted with a template exposing light only through a circle the size of the plate. A glass slide was then placed over the circle which had opposing quadrants covered with black tape to block light from

entering opposing quadrants. The plate was then placed on top of this glass slide and quadrant dividers were lined up with the black tape. The assay was conducted in a dark room under a red safelight. At the start of the assay, approximately 25-30 larvae were washed and placed in the center of the plate. Larvae were allowed to wander the plate for 15 minutes, with recordings taken every 5 minutes. The number of larvae in the light and dark quadrants was counted at each time interval and a response index was computed. The response index was calculated as the number of larvae in the dark quadrants minus the number of larvae in the light quadrants divided by the total number of larvae.

Food Dispersion Assay

Approximately 23- 30 larvae were picked from food plates and washed as described previously. Using a paintbrush, larvae were placed on a plate which contained a piece of filter paper which was moistened with ddH20. Larvae were left to starve for 2 hours. After the starvation period, larvae were transferred to a plate fitted with a piece of moistened filter paper and containing a dollop (approximately 1 cm in diameter) of blue yeast paste in the middle of the plate. Yeast paste is a blend of yeast, ddH30 and 2-3 drops of blue food colouring, and stored at 4°C for up to a week. Larvae were placed directly on top of the yeast paste and were left to feed for 1.5 hours. After the feeding period, larvae were scored as outside or inside the yeast paste, and measured for a presence or absence of a blue gut analyzed using a dissection scope.

2.7 Statistical Analysis

Microsoft Excel (2007) was used to analyze parameters obtained from locomotion and Response to light assays, and to calculate locomotion averages and response to light indices. It was also used to conduct Two-Sample T-tests to compare differences in Serotonin cell counts between larval genotypes. Comparisons of Response Indices and average locomotion, speed and direction changes between different larval genotypes were analyzed using One-way ANOVAs and Tukey's-pairwise comparisons in SPSS (Version 17). Non-parametric count data obtained from Olfaction Assays, Contact Chemosensory Assays, Phototaxis Assays and Food Dispersion Assays were analyzed using the SAS system (Version 9.2). A form of chi square analysis was conducted in SAS using a GENMOD procedure with a Binomial Distribution and a Link Function called 'Logit'. A significance level of α=0.05 was used in all statistical tests.

Chapter 3: Results

3.1 Impact of *dRanBPM* mutations on larval CNS Morphology

dRanBPM ^{k05201} mutants show a dramatic decrease in larval size at the 3rd instar stage. These mutants are approximately ¾ the size of wild-type controls and *dRanBPM* ^{revertant} controls (Scantlebury et al., 2010). This size difference is also seen in the larval CNS, where the overall volume of the larval CNS is smaller in *dRanBPM* ^{k05201} mutants compared to control larvae of the same age (unpublished results). Co-immunolabeling using an antibody specific to the long isoform of dRanBPM and the neuronal marker *elav*, revealed strong dRanBPM expression around the edges of the VNC in the larval CNS. dRanBPM^{long} was also found to co-localize to the KCs of the MB with MB specific *MB247:GFP* (Green Fluorescent Protein) expression (Scantlebury et al., 2010). Since dRanBPM^{long} shows strong expression within the larval CNS, we wanted to assess if the loss of *dRanBPM* gene function and the subsequent reduction in size of the larval CNS had an effect on the overall CNS morphology. To this end, I analyzed larval brains using different markers as a way to sample the nervous system and compare the morphology of 3rd instar *dRanBPM* mutants to control larvae of the same age. The markers that were used and the associated results are described in detail below.

MB

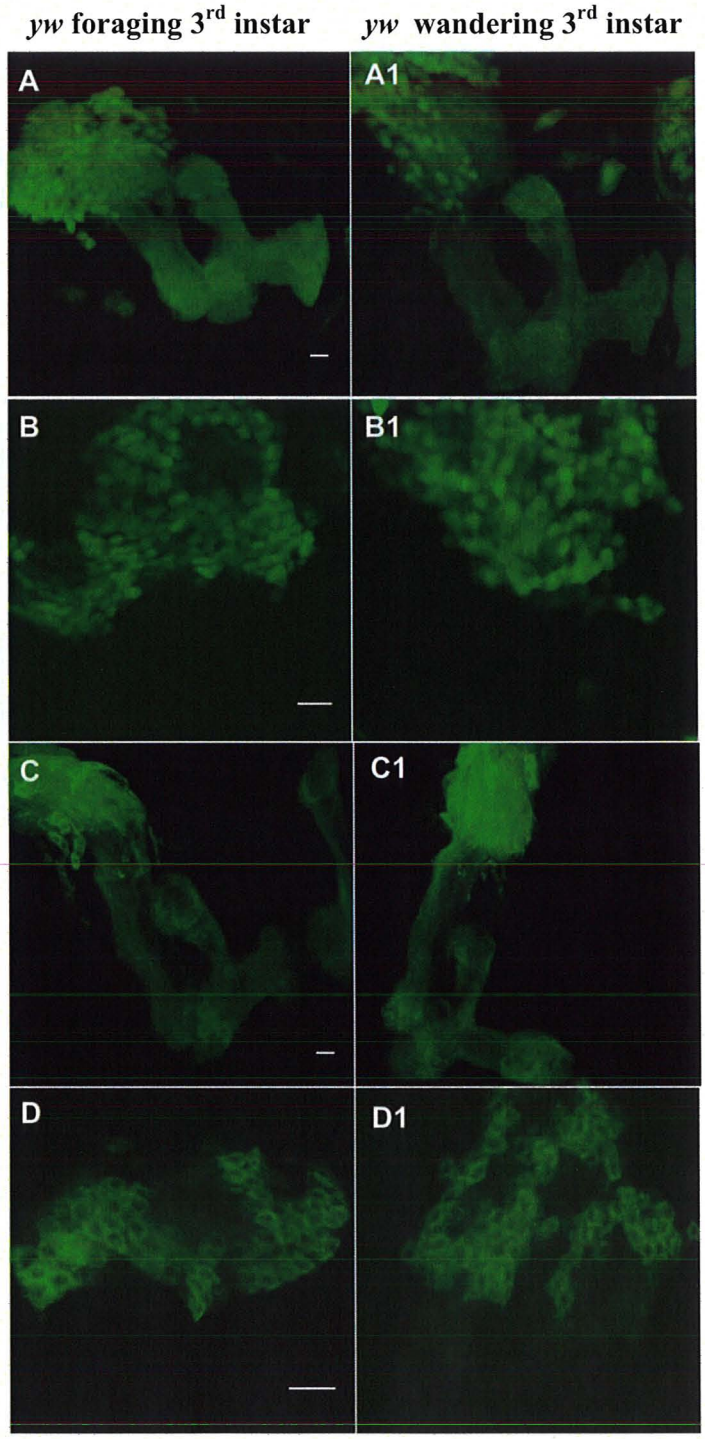
To investigate the effects of *dRanBPM* gene function on the structure of the MB, two different drivers were used with overlapping expression patterns in the MB. The *MB201Y*-GAL4 driver labels a larger subset of KCs and their axonal projections compared to those labeled by *MB247*-GAL4. *MB247*-GAL4 and *MB201Y*-GAL4 both

label all of the kenyon cells that project to the γ neurons and the calyces. *MB201Y*-GAL4 also labels all of the KCs that project to the surface, posterior, inner and outer core of the α/β neurons, while *MB247*-GAL4 only labels those KCs that project to the surface and posterior of the α/β neurons (Tanaka et al., 2008). As mentioned previously, MB γ neurons are formed first in the 1st instar larval stage, while the α'/β' neurons are formed during mid-3rd instar. The α'/β' neurons are not visualized in the 3rd instar larvae using the *MB201Y* and *MB247* drivers.

Before analyzing the mutant MBs, I wanted to know if morphological differences existed between 3rd instar foraging and wandering larvae when analyzing the γ neurons alone. Since the α'/β' neurons are not visualized using these two MB drivers, I expected the MB structure to be very similar. Both MB drivers were used to drive *UAS-CD8-GFP* at wandering and foraging stages, and 10 larval brains were examined per genotype (**Figure 4**). Overall, no major morphological differences in structure of the MB were present between wandering and 3rd instar larvae when expressing *UAS-CD8-GFP* using either GAL4 drivers. The MB neuropil structure was similar between foraging and wandering larvae using the *MB201Y*-GAL4 driver (compare A with A1) and between foraging and wandering larvae using the *MB247*-GAL4 driver (compare C with C1). The KCs also retained a similar morphology at these two developmental stages (compare B with B1 for *MB201Y*-GAL4 and D with D1 for *MB247*-GAL4).

Figure 4. No significant differences exist between wandering and foraging larvae when using *MB247*-GAL4 and *MB201Y*-GAL4 to examine the MB structure. *MB201Y*-GAL4 was used to drive expression of *UAS-CD8-GFP* in foraging larvae (A and B) and wandering (A1 and B1) larvae. No observable differences exists in the structure of the MB neuropil (row A) or in the kenyon cell bodies (row B). A second MB driver, *MB247*-GAL4, was used to drive expression of *UAS-CD8-GFP* in foraging (C and D) and wandering (C1 and D1) larvae. Again there were no observable differences between foraging and wandering larvae. White lines represent 10μm scale bars. [Row A and C: projections of 20 slices using 63x objective all at 1μm intervals. Row B and D: single slices using 63x objective].

Figure 4. No significant differences exist between wandering and foraging larvae when using *MB247*-GAL4 and *MB201Y*-GAL4 to examine the MB structure.

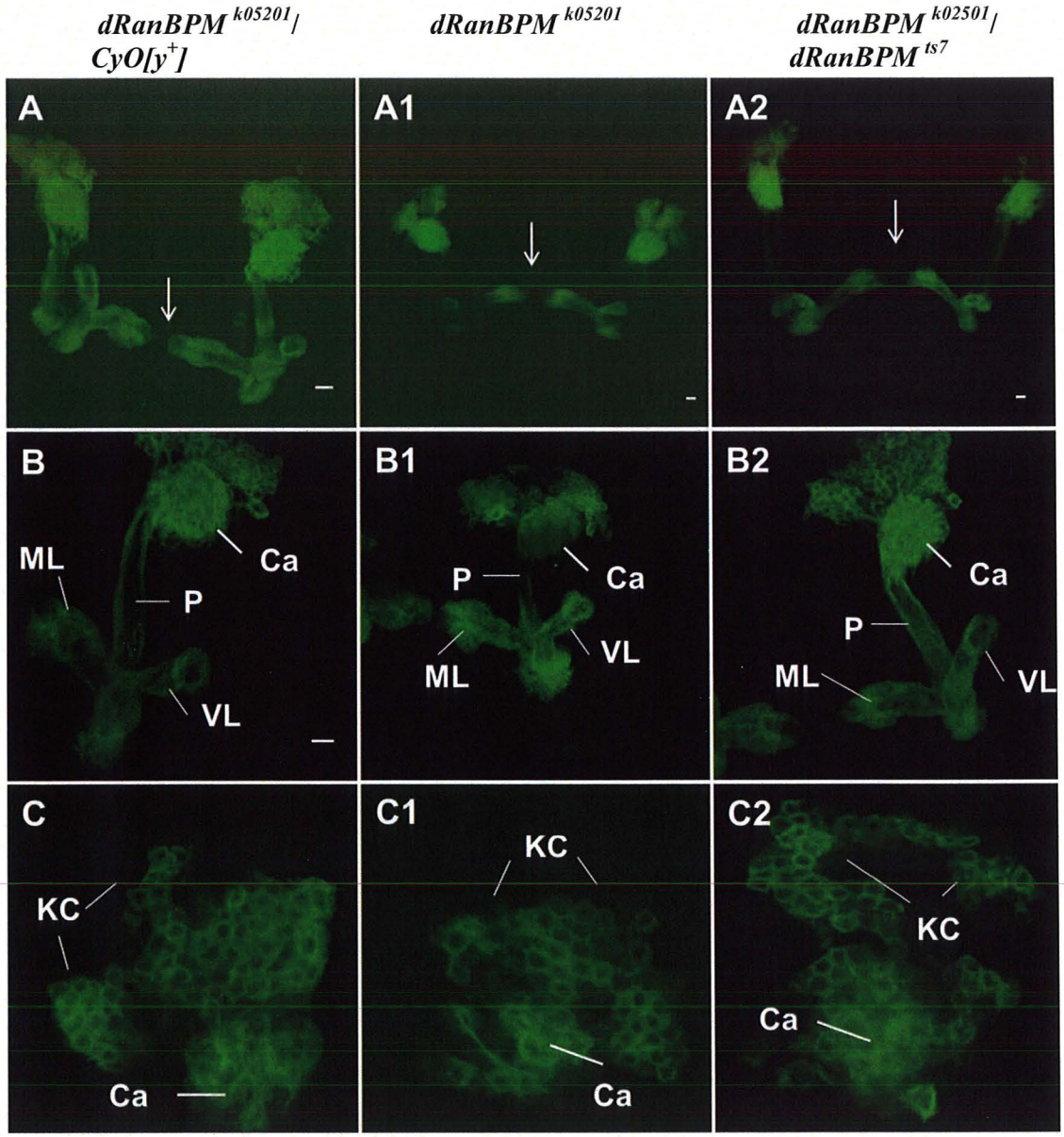


Using these MB specific GAL4 lines to drive *UAS-CD8-GFP*, the MB structure was examined in *dRanBPM*^{k05201} (**Figure 5**; A1, B1 and C1) and *dRanBPM*^{k05201}/ *dRanBPM*^{ts7} (A2, B2, and C2) mutant larvae and *dRanBPM*^{k05201}/*cy*(y^{+}) controls (A, B, and C). Larvae harbouring one copy of *dRanBPM*^{k05201} behave like wild- type controls. The major structural features of the MB are visible in all three genotypes examined. The calyx structure (Ca), the pedunculus (P), and the vertical (VL) and medial lobes (ML) are all illustrated in images B, B1 and B2.

Mutant phenotypes previously reported in the mushroom body have included; malformation of larval lobes in *fasciclin II* (*FasII*) mutants (Kurusu et al., 2002), abnormal β neuronal projections across the midline of the MB as in the case of *dfmr*^{1} mutant adults (Michel et al., 2004), stalled progression of all MB neurons over the course of development in mutants for the gene *unfulfilled* (Bates et al., 2010), and an overall reduction in the size of MB neurons in *eyeless* mutant larvae (Noveen et al., 2000). The MBs of *dRanBPM*^{k05201} mutant larvae did not display any of the above deformities and were phenotypically normal compared to controls. Absence of a midline crossing phenotype is highlighted in images A, A1 and A2 with arrows. The overall size of each MB neuropil was smaller in homozygous *dRanBPM*^{k05201} mutants compared controls and the heteroallelic *dRanBPM* combination (compare C1to C and C2). The Kenyon cells (KC) are shown in relation to the calyx structure (Ca). The reduction in size of the KC volume was not quantified.

Figure 5. d*RanBPM* mutants have reduced MB neuropils. MB specific driver, *MB247*-GAL4 was used to drive *UAS-CD8-GFP* in 3rd instar *dRanBPM*^{k05201} homozygous mutants (column 2), heteroallelic *dRanBPM* mutants of genotype *yw; dRanBPM*^{k02501}/ *dRanBPM*^{ts7} (column 3) and *yw; dRanBPM*^{k05201}/*CyO*[y^{+}] controls (column 1). Both MB neuropils are shown in row A for each larval genotype. Note the space between medial lobes of each MB neuropil at the midline (arrow). When single MB neuropils are compared (row B), the MB neuropil for *dRanBPM*^{k05201} homozygous mutants have a reduced volume compared to heteroallelic *dRanBPM* mutants and larvae of control genotype (Row B). MB neuropils in all larval genotypes have a long axonal stack emanating from the calyces called the Pedunculus (P). The pedunculus then bifurcates into the Medial Lobe (ML) and Vertical Lobe (VL). The reduction of neuropil volume of *dRanBPM*^{k05201} homozygous mutants can also be seen through examination of the Kenyon cell bodies (row C) which are shown in relation to the calyces (Ca). Volumes of Kenyon cell bodies were not quantified. White lines represent 10μm scale bars. [Row A: projections of 20 slices using 63x objective, intervals: A (0.65μm), A1 (0.9μm) and A2 (0.75μm); Row B: projections of 20 slices using 63x objective, intervals: B (1μm) and B1 (1μm) and B2 (0.55μm); Row C: single slices using 63x objective].

Figure 5. d*RanBPM* mutants have reduced MB neuropils.

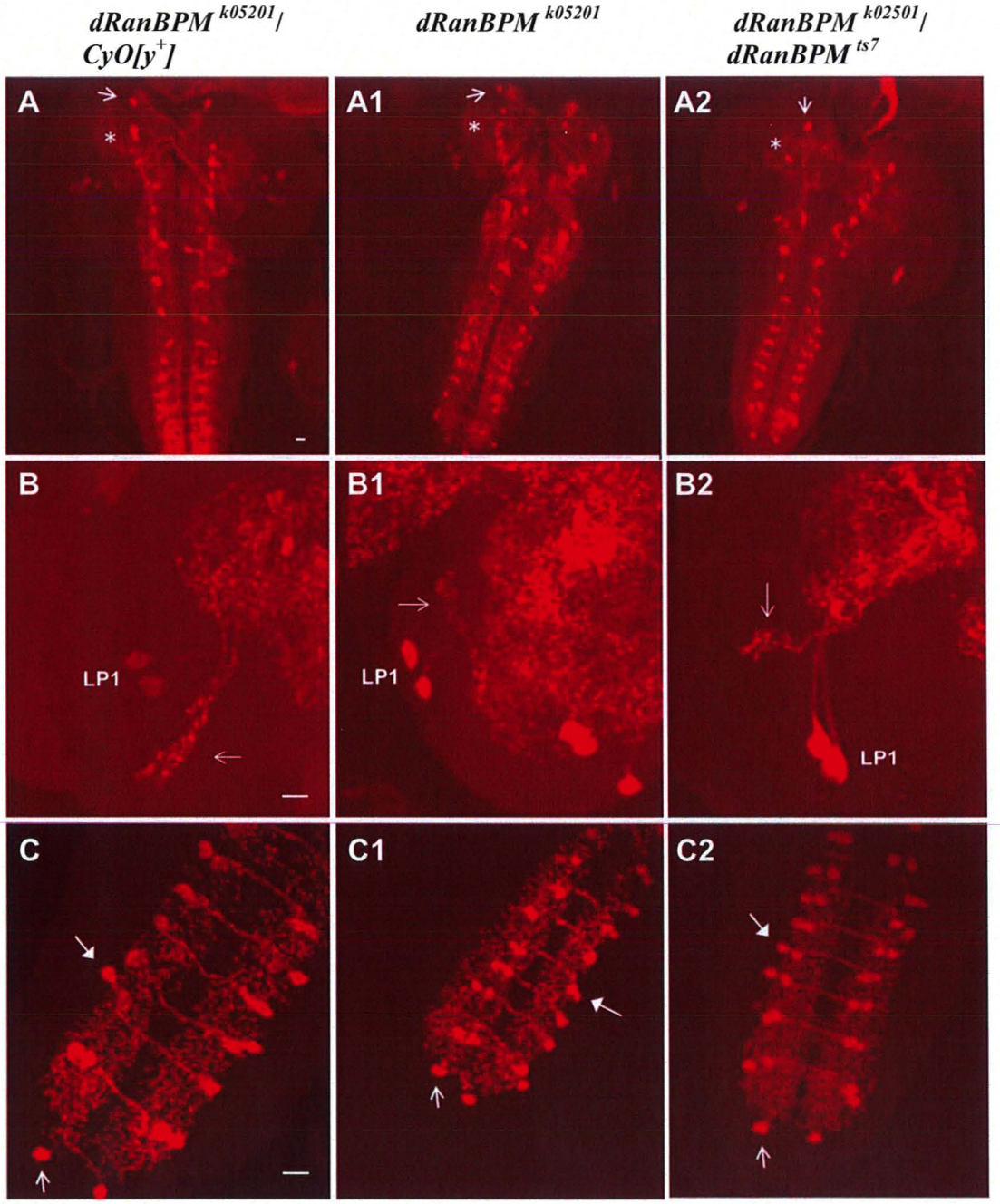


Serotonin

Previous research has suggested a role for serotonin in the regulation of photophobic behavior in larvae but not in the control of locomotion in response to light (Rodriguez Moncalvo and Campos, 2009). Suppression of serotonergic neurons in the larval CNS caused an increase in response to light as measured using the ON/OFF assay in the 3rd instar foraging stage. Further, mutants homozygous null for the *Tryptophan hydroxylase (TRH)* locus, whose gene product is an enzyme that is involved in the rate limiting step in 5-HT production, also had an increase in response to light as compared to wild type controls. Since, in contrast, $dRanBPM^{k05201}$ mutant larvae exhibit a reduction in response to light, I examined serotonin expression patterns in $dRanBPM^{k05201}$ (n= 15-20 for all genotypes, **Figure 6**: A1,B1,C1) and $dRanBPM^{k05201}/dRanBPM^{ts7}$ (A2,B2,C2) mutant larvae during the 3rd instar foraging period. Labeling of serotonergic neurons using anti-5HT revealed that they exhibited wild type (A,B,C) expression patterns similar to those seen by Valles and White (1988). Larval brain lobes contain clusters of serotonergic cells that form distinct patterns. The subesoesophageal ganglion SP cells sit close to the midline of the brain, with a single 5HT expressing cell, called SP1 (arrow in A- A2), that sits in the anterodorsal section of the larval brain. Below the SP1 cells are a cluster of SP2 cells (asterisk in A-A2) which are involved in the formation of the arborization in the larval optic neuropil (Rodriguez Moncalvo and Campos, 2005). This arborization is located close to a pair of LP1 cells (B-B2) which are present in each brain lobe. These serotonergic innervations in $dRanBPM^{k05201}$ mutant larvae also exhibit wild

Figure 6. *dRanBPM* mutants have wild type serotonin expression patterns in the 3rd instar larval CNS. 3rd instar larval brains were stained with rabbit anti-5HT followed by Texas Red-conjugated goat anti-rabbit IgG. Confocal z stack projections are shown for control larvae, *yw; dRanBPM^{k05201}/CyO[y^{+}]* (1st column A-C), the homozygous d*RanBPM^{k05201}* mutant larvae (2nd column A1-C1) and the heteroallelic mutant larvae, *yw; dRanBPM^{k02501}/ dRanBPM^{ts7}* (3rd column A2-C2). Whole mount brain projections are shown in row A for each genotype. The arrow points to one of the SP1 cells that is present in the anterodorsal section of each brain lobe. The asterisk (*) represents the location of the SP2 cell cluster that sits below the SP1 cells in each brain lobe. The images in row B illustrate a single larval brain lobe that each contains a pair of LP1 cells (as indicated). Serotonergic arborizations (indicated by the arrow) in all larval genotypes are phenotypically normal. The abdominal clusters of the ventral ganglion are shown in row C. Open arrows point to the single serotonergic cell at the tip of each side of the ventral ganglion. Closed arrows point to the paired serotonergic cell bodies that line each side of the ventral ganglion. White lines represent 10μm scale bars. [Row A: projections of 20 slices using 20x objective, intervals: A (1.5μm), A1 (1.5μm) and A2 (1μm); Row B: projections of 7-10 slices using 63x objective, intervals: B (0.95μm) and B1 (1.10μm) and B2 (1.35μm); Row C: projections of 20 slices using 63x objective, intervals: C (1.0μm) and C1 (1.45μm) and C2 (1.05μm)].

Figure 6. *dRanBPM* mutants have wildtype serotonin expression patterns in the 3rd instar larval CNS.



type characteristics compared to *dRanBPM*^{k05201}*/cy(y*$^{+}$*)* controls (Rodriguez Moncalvo and Campos, 2005). In the ventral ganglion, pairs of anti-5HT labeled cells are visible in 14 bilateral symmetrical clusters positioned along the midline resembling rungs of a ladder (closed arrow in C-C2). There are two exceptions to this pattern; three cell clusters contain three serotonergic cells instead of two and there is only one serotonergic cell at the tip of each side of the ventral ganglion (open arrow in C-C2). The last row of eight cell body clusters that make up the end of the ventral ganglion are referred to as abdominal clusters, A1 through A8. These patterns of ventral cord expression did not differ between *dRanBPM*^{k05201} mutants and wild type controls.

In order to assess the impact of *dRanBPM*^{k05201} mutant size on serotonergic expressing cells, I conducted cell counts to compare both mutants with controls (Table 1). In the whole larval CNS, *dRanBPM*^{k05201}*/ dRanBPM*^{ts7} mutants had an average of 71 ± 3.62 serotonin expressing cells. This number did not differ from the wild type controls which had an average of 74 ± 2.15 5HT labeled cells. *dRanBPM*^{k05201} mutant larvae had an average of 61 ± 2.26 serotonin expressing cells which was significantly fewer than *dRanBPM*^{k05201}*/ dRanBPM*^{ts7} mutants and wild-type controls, $F(2,30)= 6.123$, $p<0.006$.Further analysis would need to be conducted to determine if *dRanBPM*^{k05201} mutants have a slight reduction in serotonergic cell bodies throughout the entire larval CNS, or if this reduction is localized to a specific area of the CNS. Since many of the major patterns of serotonin expression are intact in *dRanBPM*^{k05201} mutants, it seems to suggest that there is a slight reduction in serotonin expressing cells that is distributed evenly throughout the whole CNS.

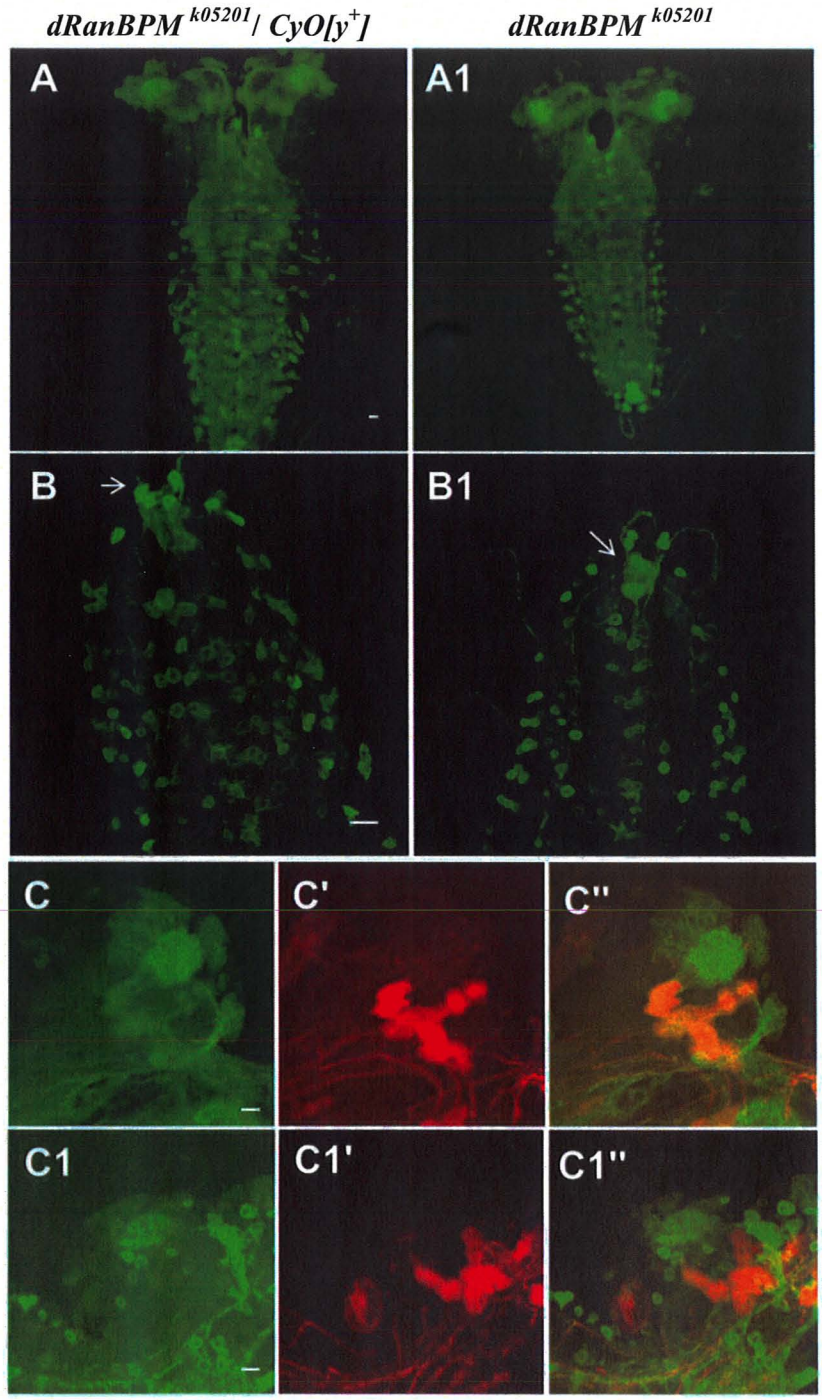
386-GFP

In *Drosophila*, 90% of neuropeptides undergo α-amidation, a form of post translational modification that is dependent on two enzymes, peptidylglycine alpha-hydroxylating monooxygenase (PHM) and peptidyl- alpha-hydroxyglycinealpha-amidating lyase (PAL). Taghert et al., (2001) generated different GAL4 drivers which had overlapping expression patterns with the PHM enzyme. Two of those lines created were *386*-GAL4 and *c929*-GAL4. *386*-GAL4 is located in the 3' end of the amontillado gene, which encodes the homolog of a vertebrate prohormone processing enzyme, PC2. In *Drosophila*, *amontillado* has high expression in the CNS and has been implicated as a prohormone processing enzyme within *Drosophila* neurons (Siekhaus and Fuller, 1999). *amontillado* mutants display reduced viability past the embryonic stage, have reduced growth during mid- second and mid- third larval instars, and possess double mouth hooks which indicate disrupted larval molting between 1st and 2nd instars (Rayburn et al., 2003).

It was found in previous studies in our laboratory that the short isoform of dRanBPM driven by *386*-GAL4 was sufficient to rescue the mutant *dRanBPM*^{k05201} response to light and feeding phenotypes (Scantlebury et al., 2010). *386*-GFP has a broad range of expression in the larval CNS, with many expressing cells located in the ventral ganglion and strong expression in the KCs of the MB (Taghert et al., 2001).Using *386*-GAL4 to drive UAS-CD8-GFP, I compared expression patterns of *dRanBPM*^{k05201} mutant larvae (**Figure 7**: A1,B1,C1) to controls of genotype *dRanBPM*^{k05201}/+ (A,B,C). *dRanBPM*^{k05201} mutant larvae had a similar expression pattern of 386-GFP compared to

Figure 7. *dRanBPM* mutants show wild-type *386-GFP* expression patterns. Panels A, B and C (C-C") are 3rd instar larval brain confocal z stack projections of control larvae of genotype *yw; dRanBPM*^{k05201}*/ CyO[y*$^{+}$*]; 386-GAL4/ UAS-CD8-GFP*. Panels A1, B1 and C1(C1-C1") show larvae of genotype *yw; dRanBPM*^{k05201}*/ dRanBPM*^{k05201}*; 386-GAL4/ UAS-CD8-GFP*. Row A shows projections (20 slices) of whole mount brains of both larval genotypes. Many cells in the ventral ganglion express *386-GFP* including a bright mass of cells at the very tip (arrows in B and B1) likely the PDF cells. *386-GFP* labels the MB as shown through double labeling in panels C and C prime (') for both larval genotypes. C and C1 show *386-GFP* expression of a single MB neuropil. Panels C and C1 prime show labeling with anti-FasII primary and Alex-594 secondary. Anti-FasII labels axons which include the lobes of the MB. Panels C and C1 double prime (") show a merged image of these two markers. White lines represent 10μm scale bars.[Row A: projections of 20 slices using 20x objective, intervals: A (1.55μm) and A1 (1.75μm); Row B: projections of 20 slices using 63x objective, intervals: B (1μm) and B1 (1.4μm); Row C: projections of 10-15 slices using 63x objective, intervals: C (1.7μm) and C1 (2μm)].

Figure 7. *dRanBPM* mutants show wild-type *386-GFP* expression patterns.



had a bright mass of cells at the very of the ventral ganglion (arrow in B and B1). This bright mass is likely the PDF neurons which are thought to require α-amidation (Park et al., 2008). KC expression was visible in both mutant and control larvae, and was verified by co- immunolabeling with FasII (C', C1') which stains all neurons of the CNS including the MB (C'' and C1'').

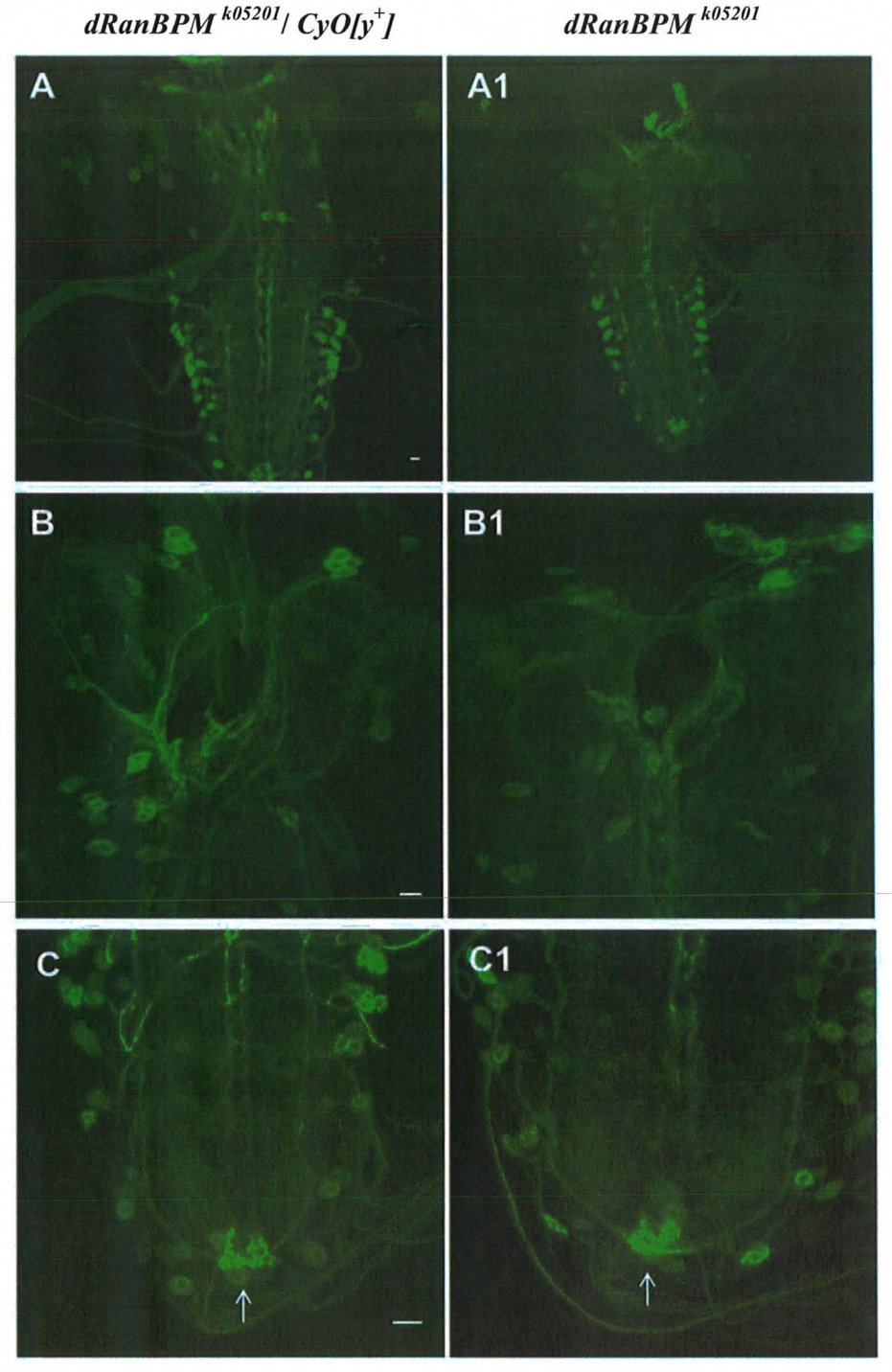
c929-GFP

As mentioned previously *c929*-GAL4 has overlapping expression patterns with the PHM enzyme involved in neuropeptide α-amidation (Taghert et al., 2001). *c929*-GAL4 is located in the *crytocephal* gene (Hewes et al., 2000), however its pattern of expression has been shown to more closely mirror that of Dimmed (DIMM) (Park et al., 2008). DIMM is a bHLH transcription factor which functions to promote the differentiation of neurosecretory cells (Taghert et al., 2001). Physical mapping of DIMM positive cells revealed that almost all DIMM cells are peptidergic, yet not all peptidergic cell bodies express DIMM (Park et al., 2008).

Both isoforms of dRanBPM driven by *c929* were sufficient to rescue response to light, locomotion, feeding and viability (Scantlebury et al., 2010). Using *c929*-GAL4 to drive UAS-CD8-GFP, I compared expression patterns of $dRanBPM^{k05201}$ mutant larvae with $dRanBPM^{k05201}/cy(y+)$ controls (n=10 for each genotype). There is strong staining of *c929-GFP* in the ventral ganglion, with fewer GFP expressing cells in the brain lobes compared to expression of *386-GFP*. *c929-GFP* also does not label the KCs of the MB

Figure 8. *dRanBPM* mutants show wild type *c929*-GFP expression patterns. *c929*-GAL4 was used to drive expression of *UAS-CD8-GFP* in control lines of genotype *dRanBPM*^{k05201}/ *CyO*[y^+] (Panels A,B, and C) and in *dRanBPM*^{k05201} homozygous mutants (Panels, A1, B1 and C1). Row A shows whole mount brain projections for both mutant and control lines. Row B shows projections for the region between the two lobes. Note the absence of MB expression compared to patterns of *386-GFP* larvae. Many cells in the ventral ganglion (C and C1) express *c929-GFP* in both larval genotypes including the PDF cells (arrows in C and C1). White lines represent 10μm scale bars. [Row A: projections of 20 slices using 20x objective, intervals: A (2μm) and A1 (2.60μm); Row B: projections of 20 slices using 63x objective, intervals: B (2.45μm) and B1 (2.6μm); Row C: projections of 20 slices using 63x objective, intervals: C (2.00μm) and C1 (2.35μm)]

Figure 8. *dRanBPM* mutants show wild type *c929*-GFP expression patterns.



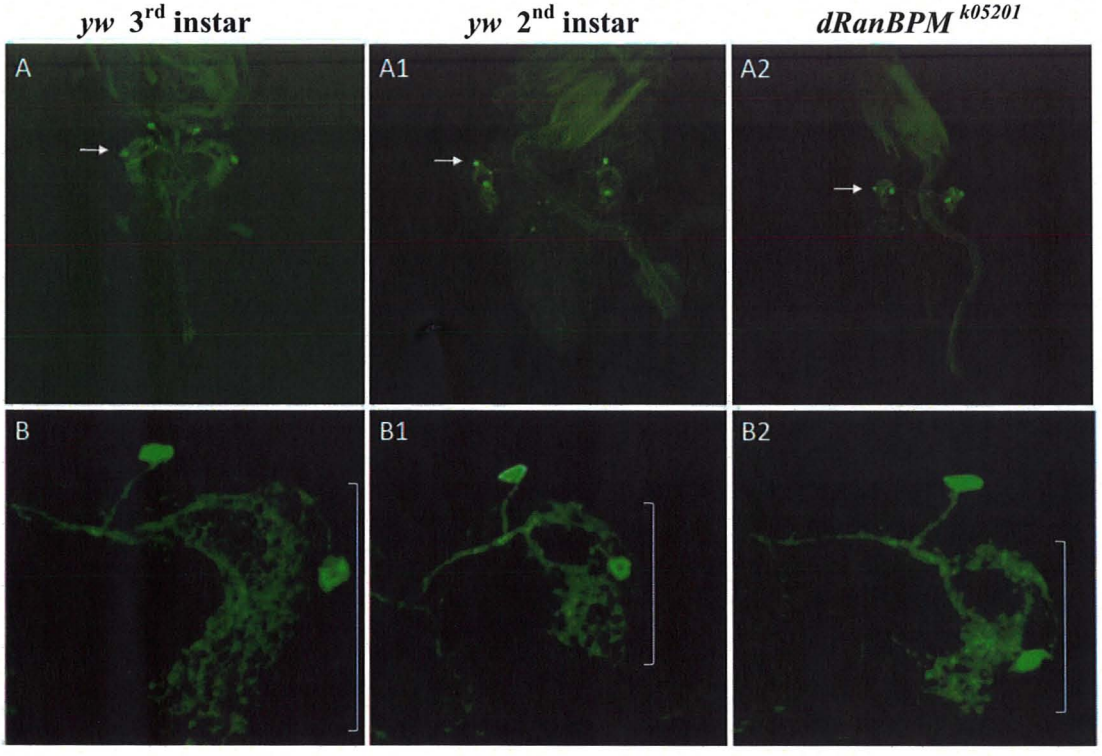
(Park et al., 2008). *dRanBPM*^{k05201} mutant larvae exhibit wild type patterns of *c929-GFP* expression throughout the whole larval CNS (**Figure 8**: A, A1). Both mutant and control larvae have many GFP expressing cells in the ventral ganglion (C and C1), and like *386-GFP* expression, there is strong staining in the PDF neurons of both genotypes (arrows in C and C1). The PDF neurons colocalize in the ventral cord with DIMM expression patterns (Park et al., 2008). There are few GFP labeled cells in the brain lobes of both mutants and controls (B and B1), and as mentioned there is no expression of GFP in the MB.

dNPF and sNPF

dnpf has been implicated in the control and regulation of feeding behaviours in *Drosophila* larvae (reviewed by Nassel and Homberg, 2006). Larvae with ablated *dnpf* neurons display food aversion behaviours and reduced eating which is similar to behaviours displayed by wandering larvae. In addition, *dnpf* expression is heightened in feeding 3rd instar larvae compared to wandering larvae. Ectopic expression of *dnpf* in the 3rd instar larval stage causes heightened attraction to food and lengthened feeding periods (Wu et al., 2003). dNPF is expressed in the larval CNS in four to six neurons which extend extensive axonal processes in each brain lobe (Wu et al., 2003). Since deficiencies of dNPF cause food aversion behaviours similar to *dRanBPM*^{k05201} mutants it was important to assess its expression in the larval CNS. *dnpf*-GAL4 was used to drive *UAS-CD8-GFP* in *dRanBPM*^{k05201} mutants and *yw* controls in the 3rd instar foraging stage (n= 10 for all genotypes). Both mutants and controls showed expression of *dnpf* in 4-6

Figure 9. *dRanBPM* mutants have a reduction in *dnpf* neuronal networks. *dnpf*-GAL4 was used to drive expression of UAS-CD8-GFP in 2nd instar (A1 and B1) and 3rd instar (A and B) yw controls, and in homozygous *dRanBPM*^{k05201} mutant (A2 and B2) larvae. Confocal images of whole mount brains are seen in row A for all genotypes. Each brain hemisphere contains two bright cells in each brain lobe, arrows in A-A2 points to one of these cells. The *dnpf* neuronal network of one brain lobe is magnified in B-B2 for each genotype. The brackets illustrate the size difference that exists between these *dnpf* projections. Notice that the *dRanBPM*^{k05201} mutants have a *dnpf* volume that is comparable to the 2nd instar yw controls (compare B2 to B1) and is much smaller than their same age controls (compare B2 to B). [row A: projection of 20 slices using a 10x objective. Row B: projection of 20 slices using a 63x objective]

Figure 9. *dRanBPM* mutants have a reduction in *dnpf* neuronal networks



neurons in the larval brain lobes (See arrows in Row A of **Figure 9**). The volume of the axonal projections emanating from the *dnpf* expressing cells was appeared reduced in *dRanBPM*^{k05201} mutants compared to controls (compare B2 with B). Their volume was, however, similar to younger yw controls expressing *dnpf-GFP* (compare B2 with B1).

Antibody staining with FMRF amide cross-reacts with 5 neuropeptides including dNPF, all of which contain an RFamide carboxy termini. The other C-termini RLRF amides that are included in this reaction are peptide products for the genes of *fmrf*, *dsk*, *dms*, and *snpf* (reviewed by Johard et al., 2008). Similar to the function described for *dnpf*, sNPF has also been shown to play a role in larval feeding behaviours. RNAi knockdown of *sNPF* leads to suppression of food intake in foraging larvae, and overexpression of *sNPF* causes an increase in food intake. In contrast to *dnpf*, *sNPF* overexpression does not lead to a prolonged feeding period (Lee et al., 2004). The gene for *dnpf* encodes an extended RFamide peptide in comparison to the peptide encoded by *sNPF* (reviewed by Johard et al., 2008). These two peptides also differ in their localization in the larval CNS. Unlike dNPF, sNPF is expressed in a large number of neurons of the CNS including the anterior dorsal neurons of the brain lobes and in the subesophagus region, the ventral midline and the thoracic and abdominal regions of the ventral ganglion (Lee et al., 2004). In addition, it has been suggested that sNPF is the only neurotransmitter to be expressed in the KCs of the MB (Johard et al., 2008). Using the FMRF antibody I stained 3rd instar foraging larvae of *dRanBPM*^{k05201} mutants (**Figure 10A**: A1-D1) and *dRanBPM*^{k05201}*/cy(y*$^{+}$*)* controls (A-D) of the same age (n= 10

Figure 10. *dRanBPM* mutants have wild type FMRF amide expression patterns in the 3rd instar larval CNS. Part A. 3rd instar larval brains were stained with rabbit anti-FMRF amide and Cy3-conjugated goat anti-rabbit IgG. Confocal images of control larvae of genotype *yw; dRanBPM*^{k05201}*/CyO[y*$^{+}$*]* are shown in the first column (A-D) and homozygous *dRanBPM*^{k05201} mutant larvae are shown in the second column (A1-D1). Confocal projections of whole mount larval brains are shown in row A. FMRF amide is expressed in the γ lobes of the MB, which bifurcate into medial and vertical lobe, shown as ML and VL respectively. The MB Peduncle (P) is also shown. The MB is shown in relation to FMRF amide innervations into the Aorta (Ao) and the Corpora Cardiaca portion of the ring gland (CC). Row C is a single slice of one larval brain lobe. The Peduncle (P) and Medial lobe (ML) of the MB are shown positioned above the subesophageal ganglion (SEG). FMRF amide is expressed in many cells of the ventral ganglion, specifically it is expressed in a pair of dorsal neurons positioned along the midline of the first abdominal neuromere denoted with arrows in row D. [Row A: projections of 20 slices using 20x objective, intervals: A (1.60μm) and A1 (2.50μm); Row B: projections of 20 slices using 63x objective, intervals: B (2.5μm) and B1 (2.35μm); Row C: projections of 2-3 slices using 63x objective, intervals: C (2.25μm) and C1 (2.75μm); Row D: single slices using 63x objective]. ***Part B***. Double labeling of anti-FMRF amide with *MB247*-GFP which is specific to the mushroom bodies. Kenyon cell bodies are shown with FMRF amide alone (A,A1), 247-GFP alone (B,B1) and both markers merged (C,C1) for both genotypes as described in part A. White lines represent 10μm scale bars. [Single slices using 63x objective].

Figure 10 (Part A). *dRanBPM* mutants have wild type FMRF amide expression patterns in the 3rd instar larval CNS.

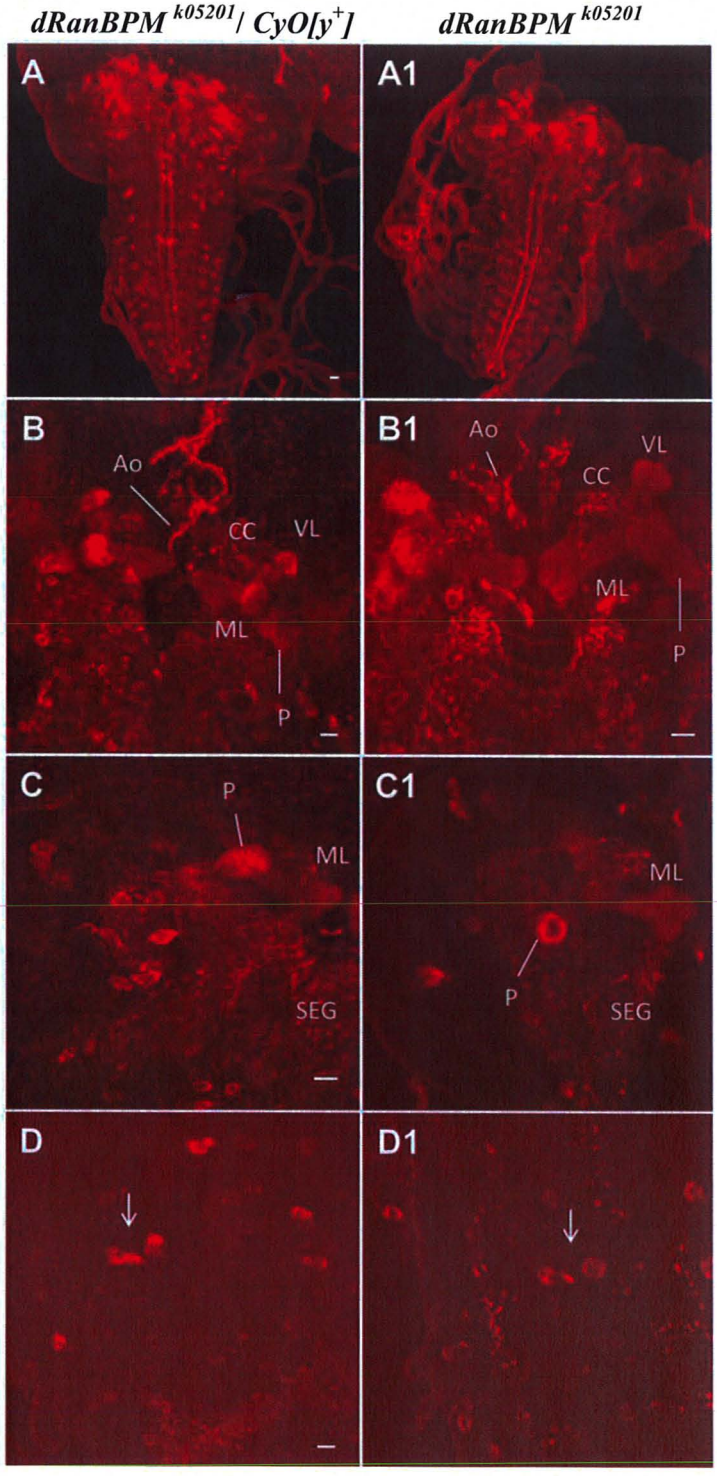
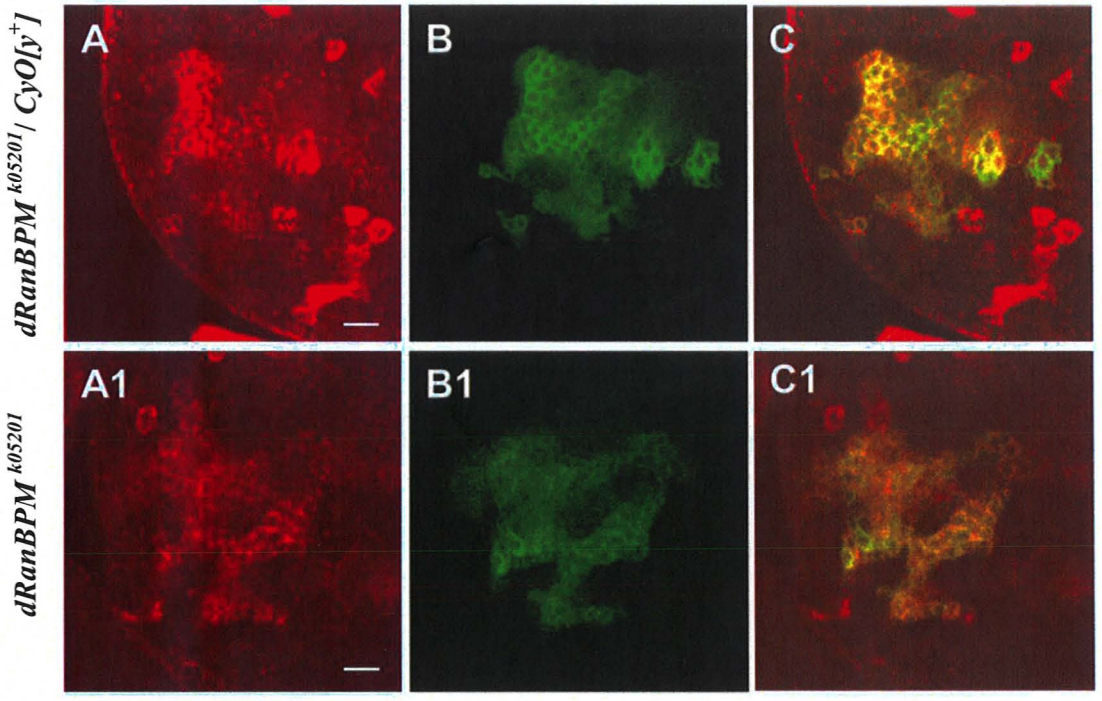


Figure 10 (Part B). *dRanBPM* mutants have wild-type RFamide expression patterns in the 3rd instar larval CNS.



for all genotypes). FMRF expression patterns did not differ between *dRanBPM*^{k05201} mutants and controls. Both mutants and controls exhibited FMRF innervations into the aorta (Ao) and the Corpora Cardiaca portion of the ring gland (CC), B and B1. Positioned above the subesophageal ganglion (SEG), the vertical (VL) and medial lobes (ML) of the MB as well as the peduncle (P) show strong FMRF expression in both *dRanBPM*^{k05201} mutants and controls (B,B1 and C,C1). The FMRF amide is also expressed in many cells in the ventral cord, including a pair of dorsal neurons which are positioned along the midline (arrow in D, D1).

It has been reported that almost all of the 600 KCs present in each larval brain lobe express *sNPF* (Nassel et al., 2008). Additionally, the other RFamides that bind the FMRF antibody are not expressed in the MB (reviewed by Lee et al., 2004). To confirm that the cluster of cells in each brain lobe visualized using the FMRF antibody were the KCs of the MB, I drove expression of GFP using *MB247* (**Figure 10B**: B and B1) in control and mutant backgrounds. The FMRF amide stains for the MB KCs to a similar degree in both *dRanBPM*^{k05201} mutants and controls (A, A1 and merged in C,C1) .

3.2 *dRanBPM* affects the degree of attraction and repulsion to olfactory substances

At the 3rd larval instar, *dRanBPM*^{k05201} mutants do not feed when directly placed into a food source. *dRanBPM*^{k05201}mutants also fail to remain in the food source when placed inside it, and do not travel towards the food source when placed outside of it (Scantlebury et al., 2010). Mutants deficient in olfactory response also fail to wander

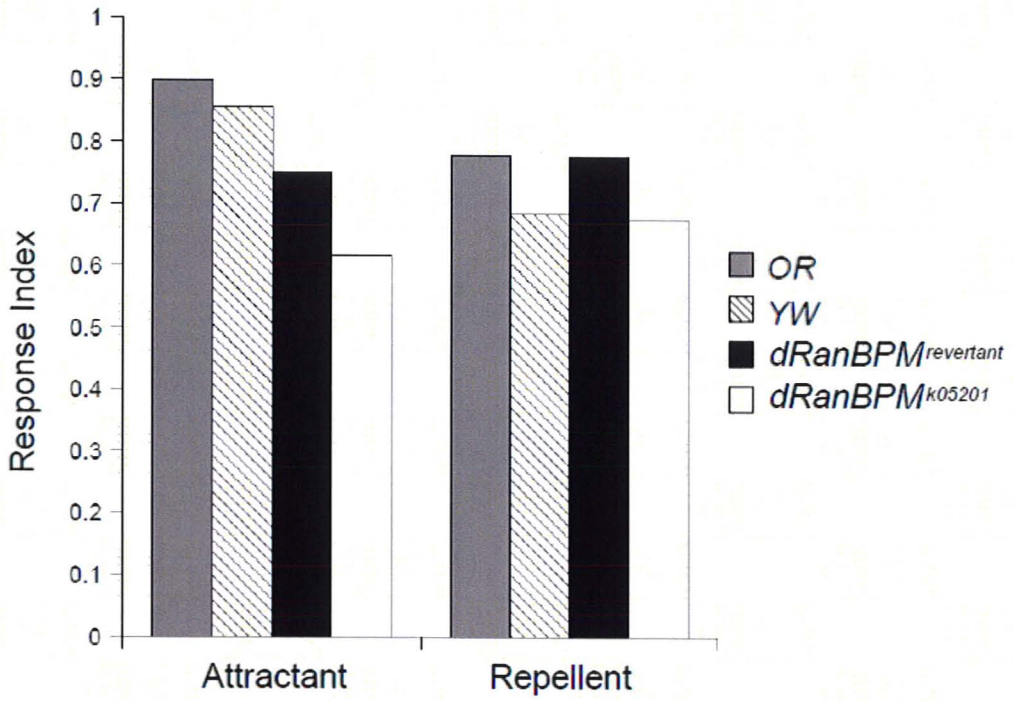
towards an odor cue, such as a food source, when placed at a distance from it (Aceves-Pina et al., 1979). Aceves-Pina et al., (1979) compared the amount of time it took larvae that were unable to smell to travel towards an odor cue to response times elicited from wild-type *Canton S* control larvae. Results showed that *Canton S* larvae travelled towards the odor source within 3 minutes, whereas mutants deficient in olfactory response failed to migrate towards the source by the end of the assay. To test the possibility that *dRanBPM* ^{k05201} mutants are less attracted to food sources due to a disruption in olfaction, their response times to attractive and repulsive substances were measured in olfaction assays. Larvae show attraction to many volatile compounds, in particular, ethyl acetate, isoamyl acetate and propionic acid have consistently elicited a high response from wild type larval populations (Monte et al., 1989). For our purposes, propionic acid was chosen due to its ability to function as both an attractive substance at a dilution of 1^{-10}, and as a repulsive substance at a regular stock concentration of 13.4 mol/L (Montel et al., 1989). When presented with diluted concentrations of propionic acid, wild type larvae wander onto the odor source and remain within a 13mm radius from the center of the odor (Monte et al., 1989). Regular concentrations of propionic acid also elicit some attractive behaviours, and are not completely repulsive. When presented with 13.4mol/L of propionic acid, larvae initially show high levels of attraction and will move towards it. Once they get close to the source of the odor they pause and remain at around a 22mm radius from the center of the source. The concentration of larvae sitting at this distance from the odor is termed the "ring of repulsion" (Rodrigues, 1980; Monte et al., 1989).

The olfaction assays were designed in a similar manner to those described in Monte et al. (1989). Pieces of filter paper were pre-cut to ¼ inch diameter and placed at opposing ends of an agar filled petri dish that measured approximately 5cm in diameter. Half of the plate containing one piece of filter paper was designated as the control half of the plate and the other half was designated as the stimulus side of the plate. Approximately 25 larvae were placed in the center of a plate. Immediately following, distilled water was added to the filter paper on the control side of the plate and either diluted propionic acid or regular propionic acid was added to the filter paper on the stimulus side of the plate, to measure larval responses to attractive or repulsive substances respectively. Larvae were left to move around the plate in complete darkness for 10 minutes. After this time period a response index was calculated as the number of larvae on the stimulus side of the plate minus the number of larvae on the control side of the plate, divided by the total number of larvae used in the assay (**Figure 11**). Thus a response index of 1 would mean that all of the larvae were on the stimulus side of the plate after the 10 minute period, when either the attractive or repulsive substance was used. $dRanBPM^{k05201}$ mutants along with control genotypes of *yw*, *OR*, and $dRanBPM^{revertant}$ were found preferentially on the side of the plate that contained either diluted or regular concentrations of propionic acid, $p<0.0001$.

The calculation of response indices for our purposes fails to distinguish between the degree of attraction between the two concentrations of propionic acid. Although it was expected that when presented with these two different odors larvae would move to

Figure 11. *dRanBPM*^{k05201} mutants respond normally to attractant and repulsive levels of propionic acid. Larvae were exposed to repulsive (13.4mol/L) and attractive (10^{-10} dilution) concentrations of propionic acid. Larvae were left to wander for 10 minutes between filter paper containing water, used as a control, and a stimulus piece of filter paper containing either concentration of propionic acid. After the allotted time period, a response index was calculated as the number of larvae on the stimulus side of the plate minus the number of larvae on the control side of the plate, divided by the total number of larvae used in the assay. All genotypes equally preferred both stimuli concentrations of propionic acid over the control stimuli $p<0.0001$. Larvae were tested in groups of 25 for each trial, and 4-5 trials were conducted per genotype.

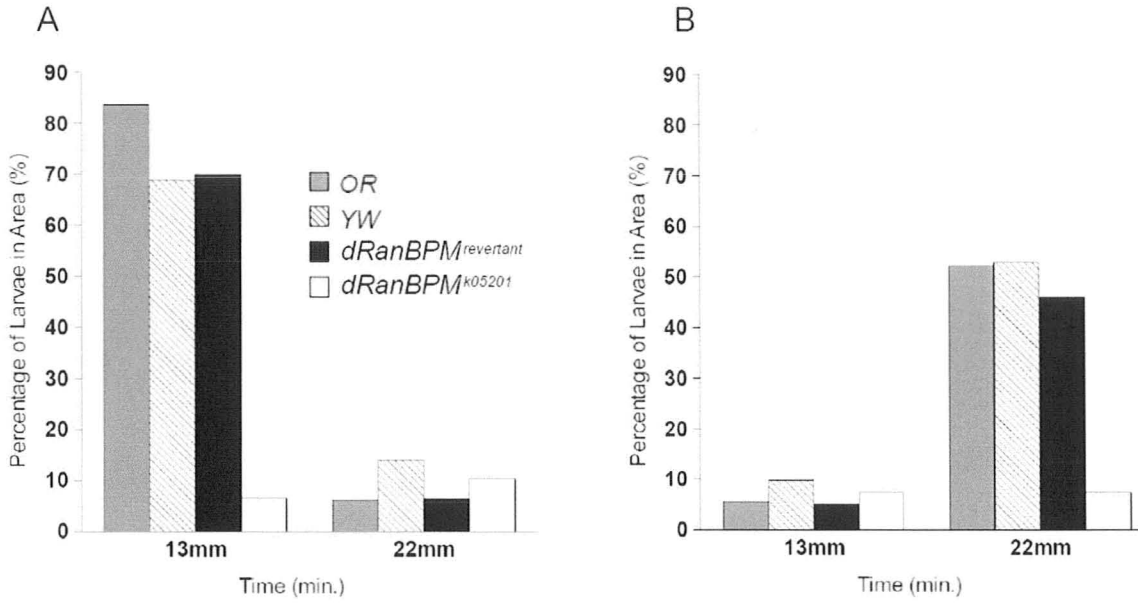
Figure 11. *dRanBPM* ^{k05201} mutants respond normally to attractant and repulsive levels of propionic acid.



the stimulus side of the plate, it was also expected that larvae wouldn't move as close to the filter paper when presented with regular concentrations of propionic acid as they would when presented with diluted propionic acid. To measure this degree of attraction, the position of larvae on the stimulus side of the plate, which contained either concentration of propionic acid, was noted. The position of larvae was measured as being within a 13mm radius of the stimulus filter paper, around a 22mm radius from the filter paper, or outside of the 22mm border yet still on the stimulus side of the plate. When presented with diluted concentrations of propionic acid, larvae of the three control genotypes were preferentially located within the 13mm radius of the filter paper, than in the other two regions on the stimulus side of the plate. *dRanBPM*^{k05201} larvae, did not show a high degree of attraction to the filter paper and were not located in high percentages at the 13mm (6.41%) or 22mm (10.3%) boundary, $p<0.001$, $\chi 2= 9.65$, $df= 3$, $p<0.05$ (**Figure 12A**). When presented with regular concentrations of propionic acid, larvae of control genotypes were preferentially located at the 22mm (45.9% - 52.9%) boundary compared to the 13mm (5.10% - 9.80%) boundary. In contrast, the number of *dRanBPM*^{k05201} mutants located at the 22mm (7.35%) boundary was significantly lower than larvae of control genotypes, $p<0.05$, $\chi 2= 30.60$, $df= 3$, $p<0.001$ (**Figure 12B**).

Figure 12. *dRanBPM*^{k05201} mutants are not attracted or repelled to the same degree as wild type controls. Using the results from the olfaction assay described in *Figure 8*, larvae on the stimuli side of the plate in both the repellent and the attractant assays were scored as to whether they occupied the 13mm or the 22mm diameter around the stimulus piece of filter paper. ***Part A.*** *dRanBPM*^{k05201} mutants did not approach the 13mm boundary in the same percentage as control larvae when presented with an attractive concentration of propionic acid, $p<0.05$. ***Part B.*** *dRanBPM*^{k05201} mutants did not approach the 22mm boundary in the same percentage as control larvae when presented with a repulsive substance, $p<0.001$.

Figure 12. *dRanBPM* ^{k05201} mutants are not attracted or repelled to the same degree as wild type controls.

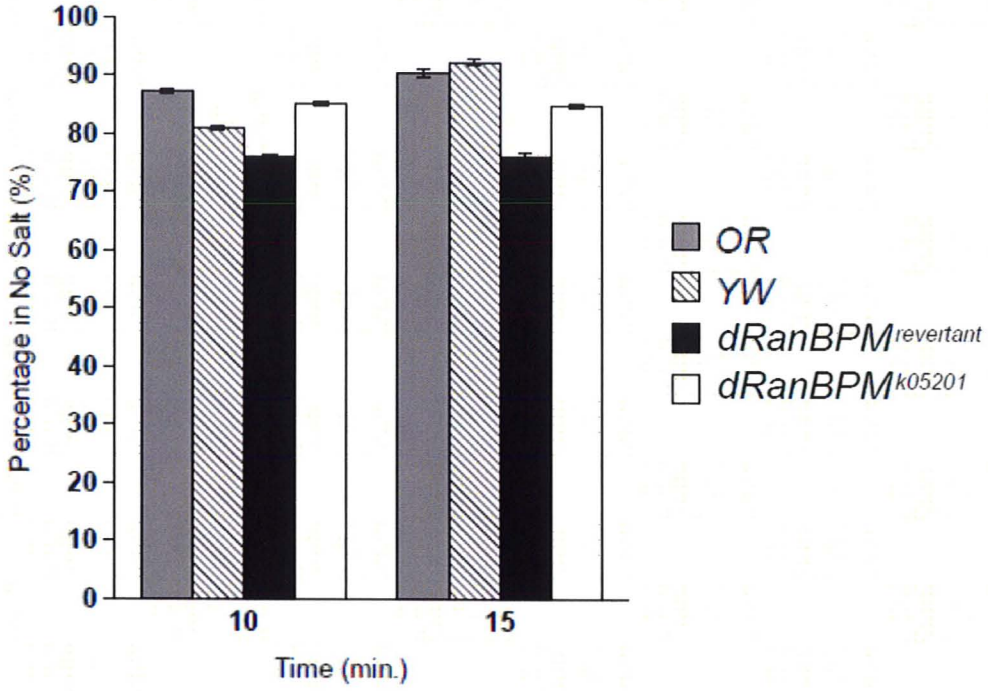


3.3 Mutations in *dRanBPM* do not affect gustatory avoidance behaviour to high salt concentrations.

Larvae exhibit high attraction to all types of sugar and to very low concentrations of salt. High salt concentrations, however, elicit an avoidance response (reviewed by Heimbeck et al., 1999). Gustatory responses are an essential part of larval foraging behaviour in that they allow the larvae to distinguish between edible and non-edible food sources. To determine if the food avoidance behaviours exhibited by *dRanBPM*^{k05201} mutants could be explained by an inability to sense gustatory stimuli, a chemosensory assay was conducted (**Figure 13**). 25 larvae were placed in the center of a plate which was divided into 4 quadrants. Opposing quadrants contained 1% agar solution and the remaining quadrants contained 1M NaCl in 1% agar solution. Larvae were left on the plate and after 10 and 15 minutes, their position was noted. Response indices were calculated as the number of larvae in the salted quadrants minus the number of larvae in the control quadrants divided by the total number of larvae used. A 1M concentration of NaCl has been shown produce the maximal amount of avoidance behaviour (Heimbeck et al., 1999). *dRanBPM*^{k05201} mutants exhibited normal avoidance to salted quadrants and did not differ significantly from controls after 10 (χ^2=2.70, df=3, p=0.44) or 15 (χ^2=3.84, df=3, p=0.28) minutes.

Figure 13. Mutations in *dRanBPM* do not affect gustatory avoidance response as measured in a chemosensory salt assay. Larvae were placed on plates containing opposing quadrants of salted and unsalted agar. After 10 and 15 minute intervals their position on the plate was measured. After both time intervals, there was no difference among genotypes in their preference for no salt quadrants over salted quadrants (10 minutes, χ^2=2.70, df=3, p=0.44; 15 minutes, χ^2=3.84, df=3, p=0.28). Larvae were tested in groups of 25 for each trial, and 4-5 trials were conducted per genotype. (10 minutes, χ^2=2.70, df=3, p=0.44; 15 minutes, χ^2=3.84, df=3, p=0.28.)

Figure 13. Mutations in *dRanBPM* do not affect gustatory avoidance response as measured in a chemosensory salt assay.



3.4 *dRanBPM* mutants have a reduced preference for dark quadrants

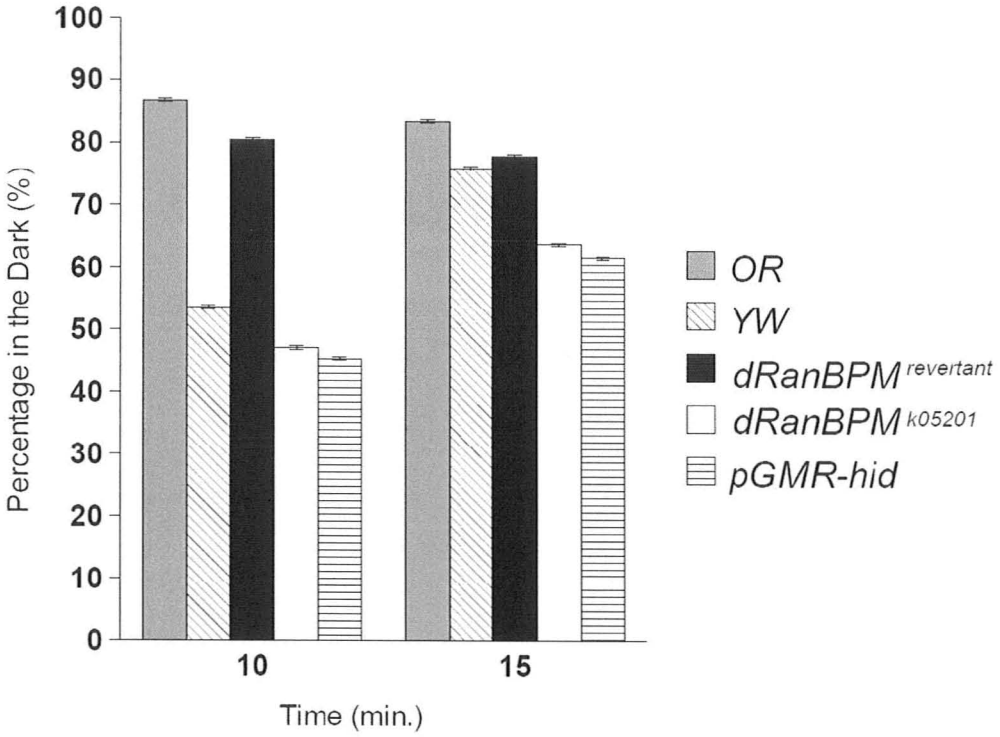
Mutations in *dRanBPM* have been shown to affect larval locomotion mediated by response to light, however its preference for dark environments has not been assessed. Previous assays have assessed phototaxis behaviour through quadrant assays in which two opposing quadrants are blocked from a light source and two quadrants are left exposed (Lilly and Carlson, 1989). This assay presents larvae with a choice between light and dark environments simultaneously, in contrast to the ON/OFF assay in which larvae are presented with light and dark environments sequentially.

$dRanBPM^{k05201}$ mutants and controls including, *OR*, *yw* and $dRanBPM^{revertant}$, were assessed for their preference for dark quadrants using the phototaxis quadrant assay which was modified from that used in studies by Lilly and Carlson (1989) and Sawin-McCormack et al. (1995). In this assay, 25 larvae were placed in the center of a plate which was divided into 4 quadrants and filled with 1% agar. The plate was placed on top of a slide which further divided the plate into light and dark quadrants using black tape. The slide and the plate were placed on top of a light box. Larvae were left to wander for 10 and 15 minutes and their quadrant location was noted at each time point. Response indices were calculated as the number of larvae in the dark quadrants minus the number of larvae in the light quadrants divided by the total number of larvae used (**Figure 14**).

Since this assay was carried out on top of a light box, the temperature of the plate has the potential to become a confounding variable. It has been shown that larvae exhibit thermotaxis behaviours, preferring temperatures between 25°C and 30°C. At temperatures

Figure 14. *dRanBPM*^{k05201} larvae have no preference for light or dark quadrants as measured using a phototaxis assay. Larvae were place in the center of a plate where opposing quadrants were exposed to light using a light box, and the remaining quadrants were left in darkness. After 10 and 15 minute intervals, the position of larvae was measured. After 10 minutes, *dRanBPM*^{k05201} mutants and *pGMR- hid* larvae were equally distributed in both light and dark quadrants, and control larvae showed strong preference for dark quadrants, χ^2=39.45, df=4,p<0.0001. After 15 minutes, all genotypes preferred dark quadrants over light quadrants, however control genotypes preferred dark quadrants to a greater degree than *dRanBPM*^{k05201} mutants and *pGMR- hid* larvae, χ^2=14.40, df=4,p<0.006.

Figure 14. *dRanBPM*^{k05201} larvae have no preference for light or dark quadrants as measured using a phototaxis assay.



higher than 30°C, larvae move towards cooler environments (reviewed by Luo et al., 2010). To control for possible temperature effects, *pGMR-hid* larvae were included in this assay. In *pGMR-hid* larvae, programmed cell death induced by the hid (head involution defective) protein is driven specifically in the eye disks (Grether et al., 1995). These larvae lack photoreceptors, and would thus govern their movements based on temperature in the photoaxis assay. After 10 minutes the *dRanBPM*^{k05201} mutants and the *pGMR-hid* larva exhibited preferences between the light and dark quadrants that were not significantly different from chance, p=0.06 and p=0.14, respectively. All control genotypes significantly preferred dark quadrants over light quadrants after 10 minutes (for yw p<0.02, for *dRanBPM*^{revertant} and *OR* p<0.0001; χ^2=39.45, df=4,p<0.0001). *OR* and *dRanBPM*^{revertant} had the highest preference for dark quadrants, and were both significantly different from all other genotypes including yw, p<0.004. After 15 minutes all genotypes preferentially preferred dark quadrants, p<0.03 (χ^2=14.40, df=4,p<0.006). Controls of *yw*, *OR* and *dRanBPM*^{revertant} showed the highest preference for dark quadrants and were not significantly different from one another, p=0.62. *dRanBPM*^{k05201} mutants were not significantly different from *pGMR-hid* larvae or from *yw* controls, p=0.42. Percentages and n values are shown in Table 5.

3.5 MB suppression causes a reduction in larval feeding

Tetanus toxin (TNT) is a neurotoxin that prevents neurotransmitter vesicle release into the synapse. TNT contains a heavy chain and a light chain. The heavy chain is

important for edocytosis through the membrane while the light chain contains a proteolytic domain which functions to cleave components of the SNARE complex (reviewed by Humeau et al., 2000). The SNARE complex, composed of synaptobrevin, syntaxin 1A and SNAP-25, is an essential component of neurotransmitter vesicle fusion on the presynaptic membrane (reviewed by Carr and Munson, 2007; reviewed by Rizo and Rosenmund, 2008). When components of the SNARE complex are cleaved by TNT, neurotransmitter release is prevented and synaptic transmission does not occur. In *Drosophila*, expression of the gene for *tetanus toxin light chain* (*TNT-G*) can be activated using the GAL4/UAS system to inhibit synaptic transmission in a tissue specific manner (Sweeney et al., 1995). Previous studies in our laboratory have investigated the effects of MB suppression on larval response to light. Responses of larvae carrying *UAS-TNT-G* driven by MB specific drivers of either *MB247*-GAL4 or *MB201Y*-GAL4, were compared to control larvae carrying *UAS-TNT-VIF*, an inactive form of tetanus toxin, driven by a MB specific driver. Neuronal silencing of the MB in 3rd instar larvae reared at 25°C, caused a reduction in their response to light compared to larvae expressing *MB247-TNT-VIF* or *MB201Y-TNT-VIF* as measured using the ON/OFF assay. Rearing *MB247-TNTG* or *MB201Y-TNT-G* larvae at 29°C caused a further reduction in response to light. MB silencing had no effect on locomotion as measured by the total distance travelled during the assay. However MB silencing did cause the larvae to change direction less frequently than controls.

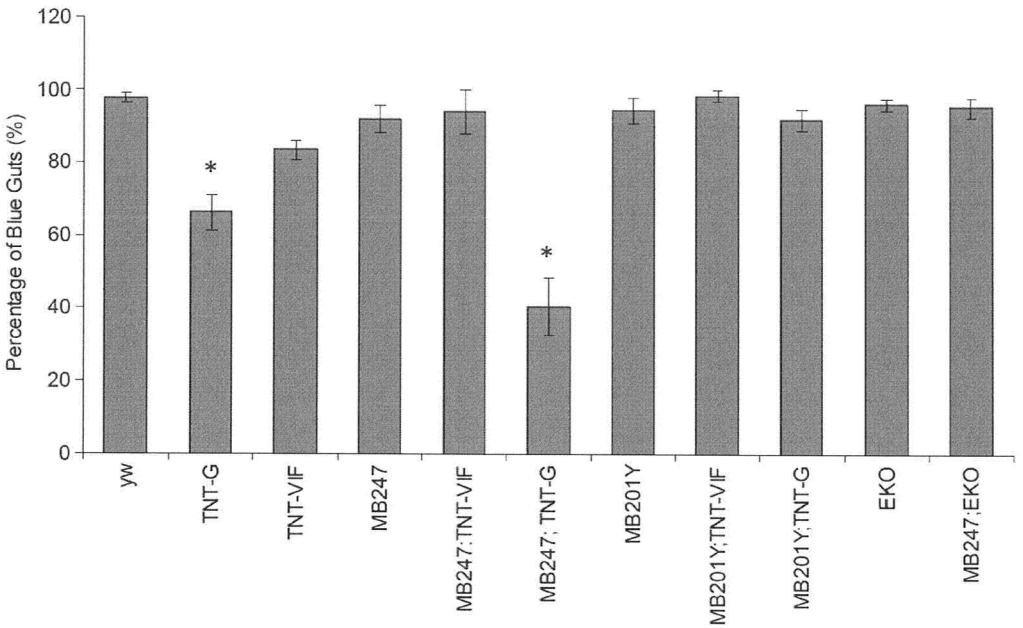
MB silenced 3rd instar larvae were further tested for feeding behaviours. Larvae were starved for 2 hours and then left to feed on food plates laced with blue food dye for

30 min. After this period the percentage of larvae with blue guts was counted. It was found that MB silencing had no effect on feeding since MB silenced larvae fed the same amount as controls (Dineen, 2009).

Since it was reported that in the ON/OFF assay, MB silenced larvae exhibited a reduction in response to light and in the average amount of direction change, it was suggested that the MB may be involved in food searching behaviours (Dineen, 2009). To further assess this possibility, I conducted an additional feeding assay that measured the percentage of MB silenced larvae that remained in a food source to feed over a period of time. In this "food dispersal assay", larvae are washed and starved for 2 hours. After this period, approximately 25 3rd instar larvae are placed on a dollop of blue yeast paste that is in the center of an empty petri dish. Larvae are left to feed for 1.5 hours and their position on the plate relative to the yeast paste is noted, as well as the percentage of larvae that contain blue guts (**Figure 15**). On average, 40% of larvae that expressed *UAS-TNT-G* driven by *MB247*-GAL4 contained blue guts (*). This is significantly lower than larvae of genotype *w; UAS-TNT-VIF/+; MB247/+* where 96% ate, $p<0.0001$. Additional controls included larvae that contained a single copy of *UAS-TNTG*, *UAS-TNT-VIF* or 247-GAL4 to ensure that the transgene insertion itself had no effect on feeding behaviours. A high percentage of larvae of genotype *w; UAS-TNT-VIF* and *w; MB247*-GAL4 had blue guts, 81% and 95% respectively. Larvae of genotype *w; UAS-TNT-G* fed significantly less than all other control genotypes where it was observed that 61% of larvae had eaten (*), $p<0.0001$. This was still significantly higher than larvae of genotype *w; UAS-TNT-G/+; 247*-GAL4/+, $p<0.0001$.

Figure 15. Larval MB suppression causes a reduction in larval feeding. Larvae of all genotypes were assessed for the presence of blue guts after being starved for 2 hours, then fed for 30 minutes in a food dispersal assay. MB suppression was achieved by driving the active form of *tetanus toxin* (*TNT-G*) using MB drivers, *MB247* and *MB201Y*. The inactive form of *tetanus toxin* (*TNT-VIF*) was also driven using these MB drivers as controls. MB silencing achieved using *MB247* resulted in the lowest percentage of blue guts, $p< 0.0001$. A single copy of *TNT-G* also had a significantly lower percentage of blue guts, $p<0.0001$. An alternative approach to silencing was achieved by driving *electrical knockout* (*EKO*) using *MB247*. This genotype did not differ in the percentage of blue guts compared to the other genotypes.

Figure 15. Larval MB suppression causes a reduction in larval feeding.



Food dispersion assays were conducted with an additional MB driver, *MB201Y*-GAL4. As discussed previously, *MB201Y*-GAL4 is expressed in a larger subset of KCs compared to *MB247*-GAL4 (Tanaka et al., 2008). There was no significant difference between larvae of genotypes *w; UAS-TNT-VIF/+; MB247/+* and *w; UAS-TNT-G/+; MB247*-GAL4/+ in the percentage of larvae with blue guts, 97% and 94% respectively. Additionally these percentages did not differ from control lines of *w; MB201Y*-GAL4 and *w;UAS-TNT-VIF*, and were significantly higher than *w; UAS-TNT-G*. At the end of the assay the majority of larvae of all genotypes were found within the food source (90-100%), and did not show food avoidance behaviours in the sense that they did not wander away from the yeast pellet. It was also observed that larvae of all genotypes were found equally dispersed in the food pellet, whereas larvae of genotype *w; UAS-TNT-G/+; MB247*-GAL4/+ were found clustered underneath the pellet. This effect was not directly measured and should be explored further.

It was expected that MB silencing effects on feeding could be recapitulated by using an alternative silencer, *UAS-EKO* (*electrical knockout*) driven by *MB247*-GAL4 at 29°C. *EKO* is modified from the *Drosophila* Shaker channel and when combined with the GAL4/UAS system it is able to suppress neuronal excitability by shunting the potassium current in targeted cells (White et al., 2001). Larvae of genotype *w; UAS-EKO/+; MB247*-GAL4 had the same percentage of blue guts compared to controls of both transgenes alone. It was observed however that MB silenced larvae using this method had fainter blue guts compared to controls indicating that these silenced larvae may feed at a reduced level. This variation in degree of feeding may also explain why the original

feeding results reported for MB silenced larvae by Dineen (2009), differ from my results with the same larvae using the food dispersion assay.

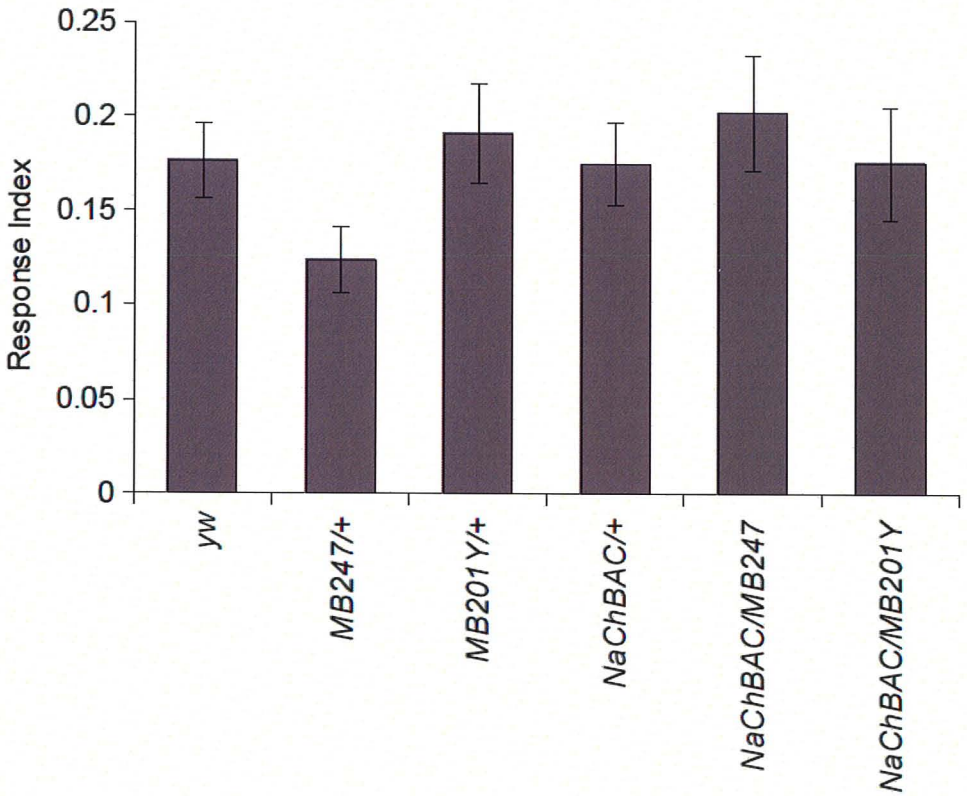
3.6 Hyperexcitability of Larval MB neurons does not affect Response to Light or Feeding Behaviours

Neuronal stimulation is a beneficial tool to study the function of individual or groups of neurons. Different stimulation techniques have been used in adult *Drosophila* to study processes such as sleep (Joiner et al., 2006), circadian rhythmicity, and flight (Nitabach et al., 2006). Initially developed for *Caenorhabditis elegans* (Tobin et al., 2002), electrical activity can be enhanced by targeted expression of a cloned *vanilloid receptor subtype 1* (*VR1*), a member of the TRP receptor family and a component of the mammalian pain pathway (Caterina et al., 1997). VRI is activated by capcaisin, the component in chilli peppers that gives them their heat. Marella et al., 2006 used the GAL4/ UAS system to drive expression of *VR1E600K*, a variant of *VR1* that shows more robust stimulation by capsaisin, in cells that contain different taste receptors. When flies were presented with food containing increasing concentrations of capsaisin, they became more and more attracted to the food source. This suggested that activation of taste receptors increases attractive feeding behaviours. The impact of neuronal stimulation on *Drosophila* behaviour has also been assessed through photostimulation techniques. Purinergic receptor, ($P2X_2$) is a light activated ligand gated ion channel that causes neuronal depolarization in the presence of UV laser light (Lima and Miesenbock, 2005).

Expression of $P2X_2$ was targeted to *Drosophila* dopaminergic neurons using the GAL4/UAS system to examine the effect of dopaminergic neuronal stimulation on avoidance behaviours, such as jumping and initiation of flight. When *Drosophila* adults were exposed to sets of UV light pulses, they spontaneously initiated jumping, wing opening, and wing flapping movements, suggesting that activation of dopaminergic neurons may be important in *Drosophila* escape behaviours (Lima and Miesenbock, 2005; reviewed by Herlitze and Landmesser, 2007). Although these two methods of neuronal stimulation have benefited studies of *Drosophila* behaviour, they require the presentation of either a capcaisin food or light stimulus to trigger activation. An alternative method that combined the *bacterial sodium channel*, *NaChBAC* with the GAL4/UAS system does not require ligand binding for activation. The *NaChBAC* gene was original isolated from *bacillus halodurans* and the channel was found to display slower activation leading to action potentials, slower inactivation following action potentials and slower recovery from inactivation (Ren et al., 2001). Slowed deactivation has been demonstrated to oppose neuronal repolarization following an action potential causing additional channel reopenings and bursts characteristic of neuronal hyperexcitability (Featherstone, et al., 1998; Nitabach et al., 2006; Luan et al., 2006; reviewed by Lehmann-Horn and Jurkat-Rott, 1999). To analyze the importance of the MB in *Drosophila* sleep behaviour, *UAS-NaChBAC* was driven by the MB specific GAL4 driver, *MB201Y*. When *Drosophila* adults were analyzed for 5 min bouts of inactivity, characteristic of sleep, Joiner et al. (2006) found that increasing MB excitation causes a decrease in sleep.

Figure 16. Excitation of larval MB neurons does not affect response to light. Movies of ON/OFF assays were assessed using DIAS and response to light was calculated for each genotype. Response to light is calculated as the distance travelled in the dark minus the distance travelled in the light, divided by the total distance travelled over the course of the assay. MB excitation was achieved by driving the bacterial sodium channel (NaChBAC) using drivers *MB247* and *MB201Y*. There were no significant differences in response indices among larvae of each genotype, $F(5,40) = 1.080$, $p= 0.386$.

Figure 16. Excitation of larval MB neurons does not affect response to light.

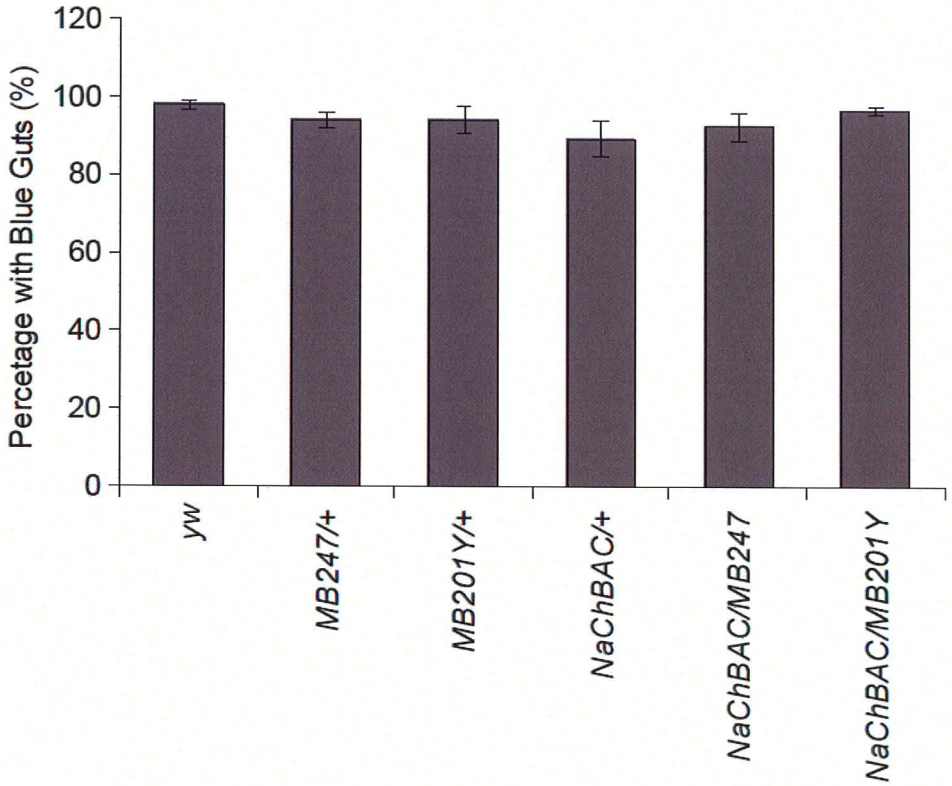


Studies in our laboratory to date have focused on the role of the MB in response to light, locomotion and feeding behaviours. As discussed previously, MB suppression was found to cause uncoordinated locomotion in 3rd instar *Drosophila* larvae. I have also found that MB suppression causes an increase in food dispersal behaviours when TNT-G is driven with *MB247*-GAL4 only. To further elucidate the role of the MB in larval response to light and feeding behaviours, *MB247*-GAL4 and *MB201Y*-GAL4 were used to drive *UAS-NaChBAC* in 3rd instar foraging larvae. The *UAS-NaChBAC* line used in this study was described as functioning at medium efficiency and was the strongest line available from the BSC. All larvae were reared at 29°C to allow for optimal functioning of the GAL4/UAS system.

Movies of the ON/OFF assay were assessed using DIAS to calculate the response indices of 3rd instar foraging larvae of genotypes *yw; MB247*-GAL4/+; *UAS-NaChBAC* and *yw; MB201Y*-GAL4/+; *UAS-NaChBAC* against controls carrying one copy of each transgene (**Figure 16**). There were no significant differences in response indices among larvae of each genotype, $F(5,40) = 1.080$, $p=0.386$. To examine the effect of MB hyperexcitability on larval feeding behaviours, the food dispersion assay was conducted as described previously. Larvae were assessed for the presence or absence of a blue gut after a 30 min period (**Figure 17**). Larvae of genotypes *yw; MB247*-GAL4/+; *UAS-NaChBAC* and *yw; MB201Y*-GAL4/+; *UAS-NaChBAC* and controls carrying one copy of each transgene, all exhibited a high presence of blue guts ($\chi2=8.24$, $df=5$, $p<0.004$) and there were no significant differences between these genotypes and controls. These results

Figure 17. Excitation of larval MB neurons does not affect larval feeding. Larvae of all genotypes were tested using the food dispersal assay as described. Larvae were assessed for the presence of blue guts after a 30 minute feeding period, which followed 2 hours of starvation. All larvae exhibited a high percentage of blue guts, $\chi 2=8.24$, df=5, $p<0.004$.

Figure 17. Excitation of larval MB neurons does not affect larval feeding.



indicate that the excitation of the larval MB γ neurons does not impact response to light behaviour or feeding dispersion behaviours.

Chapter 4: Discussion

4.1 *dRanBPM* may function in embryogenesis in the development of *dnpf* and serotonergic neurons, and in neurons of the MB.

dRanBPM^{k05201} mutants show a severe CNS size deficit when compared to wild type controls of the same age. The long isoform of dRanBPM has also been detected in dissected larval brains, more specifically in the VNC and in the MB KCs (Scantlebury et al., 2010). *dRanBPM* has been shown to be important in behaviours related to larval locomotion in response to light, feeding and overall larval viability. Thus it was important to assess if loss of *dRanBPM* gene function affected the overall morphology of the larval CNS. Of the markers that were used, antibodies labeling serotonergic neurons, and GFP labeling of *dnpf* neurons and the MB structure revealed differences in *dRanBPM* mutants compared to controls.

Early *Drosophila* neurogenesis can be described as occurring in two waves. During embryogenesis, neuroblasts proliferate, generating primary neurons which are necessary for the wiring of the larval brain (Truman and Bate, 1988; Ito and Hotta, 1992). The second wave of neuronal development occurs when neuroblasts re-enter mitosis after a period of cell cycle arrest during the early larval stages. This period of proliferation is responsible for the production of a larger set of neurons called the secondary neurons which will function in adult specific processes (Truman and Bate, 1988). These neurons will not differentiate before metamorphosis, and are visible as unbranched neurons referred to as cell body fibres (Hertweck, 1931; Dumstrei et al., 2003). Neuroblasts responsible for the development of the MB structure are unique in that they continue to

proliferate throughout embryogenesis and early larval stages. In embryogenesis these neurons give rise to 250 KCs, and this number increases to around 2000 KCs by the end of the third larval instar (Technau and Heisenberg, 1982).

dRanBPM mutants had a visible reduction in the neuronal networks that expressed serotonin, *dnpf* and in the KCs of the mushroom body. Since the patterning of these neuronal networks is predetermined in embryogenesis, it seems likely that dRanBPM functions during this stage in the development of these neurons.

Staining of 3rd instar larval brains with an anti-5HT antibody revealed that *dRanBPM*^{k05201} mutants had significantly fewer serotonin expressing cells (61 ± 2.26) compared to wild-type controls (73.67 ± 2.15). *dRanBPM*^{k05201}/*dRanBPM*^{ts7} had 71 ± 3.62 serotonin expressing cells which was not statistically different from larval controls. Serotonergic neurons have been shown to be involved in the modulation of locomotion (Dasari and Cooper, 2004) and in larval response to light (Rodriguez Moncalvo and Campos, 2009). More specifically, larval response to light appears to be governed by 5HT neurons located in the brain hemispheres. When serotonergic neurons were ablated using a driver that is responsible for the development of 5HT neurons located only in the VNC, there was no affect on the larval response to light (Rodriguez Moncalvo and Campos, 2009). It would be interesting to examine where the serotonergic cell bodies are reduced in *dRanBPM* mutants, since they are deficient in larval response to light it seems plausible that they have altered serotonergic expression in the brain lobes. However, no gross differences between patterns of sertonergic expression were detected between

dRanBPM^{k05201} mutants and controls. *dRanBPM*^{k05201} had wild type patterns of sertonergic expression in the brain lobes, where serotonin is expressed in the SP1 and SP2 cells which aid in the formation of a dendritic arborization. Serotonin expressing cells are also intact in the ventral cord of *dRanBPM*^{k05201} mutants where cells align down the midline in pairs. The heteroallelic combination of *dRanBPM*^{k05201}/ *dRanBPM*^{ts7} which have a response to light phenotype that is not as severe as that elicited by *dRanBPM*^{k05201} mutants, had a normal number of serotonin expressing cells.

Reduction in arborization was detected in the *dnpf* neurons of *dRanBPM*^{k05201} mutants, when *dnpf* neurons were visualized using UAS- CD8:GFP driven by *dnpf*-GAL4. It was also observed that *dRanBPM*^{k05201} mutants and controls had similar numbers of *dnpf* expressing cells. 3rd instar *dRanBPM*^{k05201} mutants, however, had a visible reduction in volume of the dendritic arborizations that emanate from the *dnpf* expressing cells. The volume of these projections was more comparable to younger wild-type 2nd instar controls, than to controls of the same age. *dnpf* has been proposed to play a role in a variety of behaviours including foraging (DiBona, 2002), stress tolerance (Thorsell et al., 2000) and responsiveness to aversive stimuli (Wu et al., 2003). It has further been demonstrated that levels of *dnpf* RNA, fluctuates in the presence of sugar. *dnpf* expression is stronger in feeding larvae than in non-feeding larvae (Wu et al., 2003). Although immunohistochemical analysis revealed a slight deficit in volume of the neuronal projections emanating from the *dnpf* expressing cells, the levels of *dnpf* expression have not been examined. Since *dRanBPM*^{k05201} mutants exhibit a reduction in

feeding behaviours, it would be interesting to see if they also have altered levels of *dnpf* expression.

As mentioned previously, the volume of the MB neuropil was visibly reduced in *dRanBPM*^{k05201} mutants compared to controls, but this difference was not quantified. The MB kenyon cell bodies project their axons forming a stalk like structure called the peduncle, then these axons bifurcate forming vertical and medial lobes (Lee, Lee and Luo, 1999). A reduction in KC number or cell size could lead to the reduction of overall MB volume, as was the case with adult *mushroom body miniature*^{1} *(mbm*^{1}*)* mutants. The MB axons of these mutants begin to degenerate in the 3rd larval instar and fail to regenerate in pupal stages, resulting in an overall reduction in MB volume (Raabe et al., 2004). Thus the difference in volume of the MB neuropil in *dRanBPM*^{k05201} mutants compared to controls is likely caused by a reduction in KC number, however this remains to be quantified. Even though the MB KC neurons divide throughout larval life, similar to secondary neurons, the KC bodies that proliferate in larval stages may not be required for larval specific behaviours. A recent study on larval ability to form appetitive odor associations suggested that only embryonically born KCs are necessary for this behaviour (Pauls et al., 2010). In this study, newly hatched larvae were treated with Hydroxy Urea (HU) to prevent proliferation of MB Nb into larval stages, and then were tested in second or third instars on an appetitive olfactory learning paradigm. There were no differences in performance between larval groups treated and not treated with HU, suggesting that larval born MB neurons are not essential for performance in this paradigm. Similar results were obtained when *shibire*, used to block synaptic transmission, was driven in

larval born MB KCs under the control of a heat shock promoter (Pauls et al., 2010). These results suggest that like the majority of neurons generated throughout larval life, only those produced during embryogenesis are functional during larval stages.

Further support for a role of *dRanBPM* during embryogenesis comes from studies of the vertebrate system. Through co-immunoprecipitation experiments, it has been shown that dRanBPM interacts with Tropomyosin-Related Kinase Receptor B (TrkB), which is a highly expressed neurotrophin receptor in the mammalian CNS (Yin et al., 2010). Neurotrophins are important in regulating neuronal growth, mainly through the prevention of apoptosis. They also function in neuronal differentiation and maturation (Huang and Reichardt, 2001). In vertebrates, RanBPM is thought to participate in the brain-derived neurotrophic factor (BDNF) – induced MAPK signalling pathway. Over expression of RanBPM has been shown to increase BDNF- induced neuronal differentiation, while knock-out of RanBPM decreases dendritic branching in tissues of the hippocampus (Yin et al., 2010). There is limited research on the presence and function of neurotrophins in *Drosophila* and only one neurotrophin, *Drosophila* Neurotrophin 1 (DNT1), has been identified (Zhu et al., 2008). Because of this, it is hard to draw correlations between the role of vertebrate RanBPM pertaining to neurotrophins and its *Drosophila* ortholog.

Neuron development during embryogenesis is essential to the morphological makeup of the larval CNS and to the behaviours that characterize larvae during this period. From my analysis of larval CNS markers, I have identified that dRanBPM may be

involved in the pathway leading to neuronal differentiation of serotonergic, *dnpf* and MB neurons in embryogenesis. It is unclear however how the altered morphologies of these neurons contribute to *dRanBPM* mutant behaviours.

4.2 *dRanBPM* mutants show slightly altered olfaction responses.

Behavioural analysis of *dRanBPM*^{k05201} mutants have revealed that these larvae fail to consume wild-type amounts of food, and appear disinterested in moving towards a food source (Scantlebury et al., 2010). Since failure to be attracted to a food source could be the result of reduced ability to smell or taste the food medium, I wanted to see if loss of dRanBPM function affected these larval sensory behaviours. In chemosensory paradigms involving simultaneous presentation of salted and unsalted quadrants, *dRanBPM*^{k05201} mutants exhibited wild type response patterns suggesting that loss of *dRanBPM* gene function did not affect pathways responsible for the ability to smell. In addition, *dRanBPM*^{k05201} mutants showed normal preferences for propionic acid at both high and low concentrations compared to larval controls. As discussed previously, 25 larvae were placed in the center of a plate between a stimulus, propionic acid and a control, ddH_2O. Response indices were calculated based on the number of larvae that had moved to the stimulus side of the plate containing the odor, rather than the control side of the plate containing distilled water.

Lilly and Carlson (1989) and Aceves –Pina and Quinn (1979) used this olfaction assay to isolate mutant larvae that were defective in olfactory response. The assay they

used was similar in design to the one used in my experiments, where 3rd instar larvae chose between different concentrations of propionic acid and a ddH_2O control. Through this assay they identified that the larval *smellblind* (*sbl*) mutant, previously known to have decreased responsiveness in an olfactory-driven learning paradigm (Aceves-Pina and Quinn, 1979), also exhibited reduced responses to propionic acid (Lilly and Carlson, 1989). When presented with a low concentration of propionic acid, a 10^{-1} dilution, control larval strains of *Canton-S* (*CS*) respond with a response index of 0.85 while *sbl* mutants have a low response of 0.2 after 5 minutes (Lilly and Carlson, 1989). *dRanBPM*^{k05201} mutants had a wild type response of 0.95 to this low concentration of propionic acid suggesting that they have retained more olfactory functioning compared to the *sbl* mutants.

A major difference in the olfactory assay used in these studies compared to that used for the *sbl* mutants is the duration of the assay. Due to the reduced locomotor movement of *dRanBPM*^{k05201} mutants, the length of the assay was increased from 5 min to 10 min to allow them time to move around the entire plate. In a 5 min assay, most of the *dRanBPM* mutants remain in the center of the plate and thus are removed from the assay (data not shown). In order to measure the fast olfactory responses of a 5 min assay, but still allow for reduced movement it would be beneficial to perform this olfaction assay in a smaller plate. A smaller arena would allow mutants the same degree of mobility as control larva and the assay could be administered in a shorter amount of time which would allow for a shortened exposure to the stimulus.

3rd instar *dRanBPM*^{k05201} mutants show a reduction in the degree of attraction to diluted concentrations of propionic acid, a concentration that elicits strong attraction behaviours in control larva. Their degree of attraction was measured by the number of larva positioned within 13mm from the center of the filter paper containing the propionic acid compared to larvae on the rest of the stimulus side of the plate. Similarly, 3rd instar *dRanBPM*^{k05201} mutants also show a reduction in the degree of attraction to high concentrations of propionic acid. High concentrations of propionic acid elicit a strong attractive response up until 20mm from the center of the odor, after this point the larvae are repelled. This larval formation is termed the "ring of repulsion" (Monte et al., 1989). In this assay, control larvae are positioned along this 20mm border region after 10 min. *dRanBPM*^{k05201} mutants fail to approach this ring of repulsion and are mostly positioned outside this region yet still on the stimulus side of the plate. These results suggest that at both high and low concentrations of propionic acid, *dRanBPM*^{k05201} mutants show reduced attraction compared to control larvae.

Mutant larvae exhibiting similar behavior to *dRanBPM* mutants were isolated in a study by Ryuda et al., 2008. Mutants from the Gene Search collection of P-element insertions, were screened in a food preference assay to identify mutants that failed to display the wild type response to soybean and flour based foods. *yw* larvae show a high degree of preference to soybean foods over those composed of cornmeal, as measured by the presence of a colored abdomen that matches the food source after a 2 hour period (Ryuda et al., 2008). *GS1189* larval mutants, containing a P-element insertion in the open reading frame of the *CG33071* gene which is of unknown function, showed no preference

for soybean food over food containing other flours (Ryuda et al., 2008). When presented with two diametrically opposed food sources, one high in yeast and the other a control agar mixture, *GS1189* mutants took a longer time to access the high yeast food compared to controls. After 20 minutes, 50% of control *yw* larvae had accessed the high yeast food, and after 60 minutes it had been accessed by all control *yw* larvae. In contrast, it took 120 minutes for 50% of *GS1189* mutants to access the high yeast food (Ryuda et al., 2008). These *GS1189* mutants were further tested in an olfaction screen of a similar design to that used with $dRanBPM^{k05201}$ mutants, where they were presented with low concentrations of propionic acid in yeast. At the end of this 5 min assay, *GS1189* mutants were found in a higher percentage on the control side of the plate, as opposed to the stimulus side of the plate (Ryuda et al., 2008). *GS1189* mutants take a longer amount of time to access the presented stimulus and after 5 minutes they show no attraction to low concentrations of propionic acid.

It is possible that $dRanBPM^{k05201}$ mutants did not elicit a strong attraction response to diluted and high concentrations of propionic acid because they were not given enough time in the assay to do so. Another assay should be conducted similar to the food assay described for *GS1189* mutants in which larvae are given unlimited time to travel towards the odor source. The percentage of $dRanBPM^{k05201}$ mutants at the 20mm or 13mm border region at different time intervals could then be measured and compared to controls. If larvae are positioned at these border regions in numbers similar to controls but after a longer time period, this would suggest that reduced response indices are a result of reduced larval locomotion and not reduced olfaction. If however, as in the case

of the *GS1189* mutants, larvae never reach high numbers near the odor source, it would be more supportive evidence of a disrupted olfactory system.

It is also important to note that different *Drosophila* larval strains elicit varying responses to the presence of different odorants (Monte et al., 1980). When tested in a similar olfaction assay and using the same high propionic acid concentration used to test *dRanBPM*^{k05201} mutants, *OR* and *CS* larvae exhibited very repulsive behaviours. When presented with a low concentration of propionic acid, 35% of *CS* larvae were positioned at the 20 mm border whereas roughly 10% of *OR* larvae were positioned at this region. In the olfaction assay I conducted, 40% of *OR* larvae were positioned at this region when presented with undiluted propionic acid. However the assay I conducted also lasted for 5 minutes longer than this assay conducted by Monte et al. (1980). Although it is important to consider different backgrounds as a factor in analyzing olfactory avoidance behaviours, *dRanBPM*^{k05201} larvae were of the same background as the *dRanBPM*^{revertant} and *yw* control larva that elicited wild type responses.

Changes in the design of this olfactory assay to accommodate the reduced locomotor behavior evident in *dRanBPM*^{k05201} mutants may be necessary to better evaluate their suspected odor impairment. Reducing the size of the arena used for the assay or measuring the time it takes the larvae to reach the odor source instead of measuring their position after a defined amount of time may allow for a better comparison between *dRanBPM*^{k05201} mutants and controls.

For future experiments it may be interesting to investigate the role of *dRanBPM* within the MB and how that impacts olfactory behaviours. *Drosophila* larvae sense odors through a larval dorsal organ positioned at the larva's anterior which is innervated by the dendrites of 21 olfactory receptor neurons (ORNs) (reviewed by Stocker, 2001). Odor signals received by the receptors of the ORNs are then transmitted to the larval antennal lobe golmeruli (Python and Stocker, 2002a). Projection neurons (PNs) that innervate these glomeruli send axons to other golumeruli in the MB calyx (Ramaekers et al., 2005). Mapping of the projections of larval MB KCs to the calyx golumeruli have shown that each KC receives olfactory input from 6 calyx glomeruli. Olfactory processing is achieved through the integration of these inputs into the KCs (Masuda-Nakagawa et al., 2005). Since the MB is required for higher order olfactory processing, loss of *dRanBPM* gene function in the MB could result in disrupted olfactory responses. It would be interesting to try and rescue the olfactory phenotype of $dRanBPM^{k05201}$ mutants by driving each isoform of dRanBPM in the MB using a MB specific driver such as *MB247*.

4.3 Loss of *dRanBPM* gene function causes a reduction in phototaxis response

It has been demonstrated that loss of *dRanBPM* gene function affects larval locomotion in response to light, as measured using an ON/OFF assay (Scantlebury et al., 2010). This assay is useful in analyzing the larvae locomoter changes that accompany a switch from a dark to light environment, however it does not address larval preferences for light or dark. 3rd instar foraging larvae have been shown to prefer dark environment

over light ones (Lilly and Carlson, 1989). Since loss of *dRanBPM* gene function disrupts the response to light response, it was important to assess if these larvae retained their preference for dark environments.

dRanBPM^{k05201} mutants show no preference between dark and light environments after a 10 minute period, as measured in a phototaxis plate assay. After 15 minutes, *dRanBPM*^{k05201} mutants did show a preference for dark quadrants, however *pGMR-hid* larvae which lack photoreceptors also preferred dark quadrants after this time period. Thus, it seems that after 15 minutes in this assay, heat was the be the guiding factor causing larvae to prefer cooler dark quadrants over hotter lit ones. These results are consistent with the performance of *dRanBPM*^{k05201} mutants in the ON/OFF assay. When mutants are exposed to intermittent pulses of light and dark they move at a reduced rate in both the dark and light pulses of the assay. Control larvae in contrast, move significantly slower in light pulses and exhibit increased amounts of direction change and turning behaviours. *dRanBPM*^{k05201} mutant larvae exhibit high direction change in both pulses of the assay and in addition show very uncoordinated and slower locomotion (Scantlebury et al., 2010). These results suggest that loss of *dRanBPM* gene function reduces larval preference for dark environments at the 3rd instar stage, and also hinders their locomotor response to light environments.

The phototaxis assay used here has three main differences from the "ON/OFF" response to light assay that has been discussed previously. Firstly, the phototaxis assay does not assess characteristic changes in larval locomotion, such as head swinging,

turning frequency and speed of linear locomotion, in light and dark environments. It simply measures the position of the larvae with respect to dark and lit quadrants after 10 and 15 minute time intervals. Secondly, the larvae are presented with light and dark stimuli at the same time, in contrast to the ON/OFF assay where light and dark environments are created in sequence. The presentation of light and dark environments simultaneously allows for the assessment of preference between these two environments, something that isn't offered in the ON/OFF assay. Third, the phototaxis assay is susceptible to population effects where 25 larvae are tested together. This is not an issue in the ON/OFF assay as only one larva is tested at a time. A different version of the phototaxis assay called the "immediate light/dark choice assay" assesses larval preference for light and dark while testing one larva at a time (Gong, 2009). In this assay, the larvae are placed at a 1cm distance from a midline boundary that divides the plate into dark and light halves. The larva would be placed 1cm away from the midline in the dark quadrant and 1cm away from the midline in the light quadrant in equal trials. Larvae are positioned such that their head faces the midline and the tip of their head lines up with the 1cm boundary. If after 2 seconds the whole body of the larva passes the midline onto either the light or dark half of the plate, then the test is scored as a pass. If the larva turns back when the head touches the midline, or if it returns to the side it was originally on within the 2 seconds, then the test is scored as a fail (Gong, 2009). Since the *dRanBPM*^{k05201} mutants have reduced mobility, the test should be increased to 3 or 4 seconds. It would be expected that wild-type larvae would have more passing tests when travelling from the light half into the dark half of the plate than from the dark half into the light half. Since

results from the phototaxis assay I conducted show that *dRanBPM*^{k05201} mutants show no preference for light or dark environments, I would expected that there would be no difference in the amount of passing tests when larvae are positioned in either half of the plate facing the midline. Using an assay that is as short as this one also prevents the larvae from feeling the heat affects associated with the use of a light source. However, the *pGMR-hid* larvae should still be used as controls to ensure that heat is not a confounding factor.

4.4 Decreasing but not increasing excitability in the γ neurons of the MB effect Response to Light and Feeding behaviours

There is little known about the function of the MB in larval behaviours, even less is known about how the MB functions at this stage to control response to light and feeding behaviours. Classical experiments used to investigate MB function and its subsequent morphological and phenotypic outcomes usually involve MB ablation using HU (Heisenberg, 1989; Osborne et al., 2001). Feeding newly hatched larvae HU, only prevents the formation of larval born neurons and fails to inhibit the embryonically born γ neurons from developing. As mentioned previously, it has recently been suggested that embryonically born MB KCs and their associated projections may be the only MB neurons that function during larval stages (Pauls et al., 2010). In order to assess the function of the MB in larval response to light and feeding behaviours, I used drivers to

inhibit (using *TNT-G*) and excite the (using NaChBAC) the γ neurons during all of larval development.

TNT-G blocks neurotransmitter release when expressed in the pre-synaptic neuron (Sweeney et al., 1995). It exerts its effect by cleaving synaptobrevin, an essential component of the SNARE complex which is responsible for neurotransmitter vesicle release into the pre-synaptic synapse (reviewed by Carr and Munson, 2007). Disrupted neurotransmitter release blocks both inhibitory and excitatory signals from reaching the post-synaptic neuron. When *TNT-G* was driven in the MB, using *MB247*-GAL4 and *MB201Y*-GAL4, foraging third instar larvae had reduced response to light when measured using the ON/OFF assay. When raised at temperatures of 25°C and 29°C, MB silenced larvae had significantly lower response indices compared to wt controls and to larvae expressing MB driven, *UAS-TNT-VIF* (Dineen, 2009). In the food dispersal assay, I found that MB silenced larvae of genotype *MB247*-GAL4*; UAS-TNTG* raised at 29°C had a significant reduction in feeding, as measured through the presence of a blue gut. This result was not observed with the other MB driver *MB201Y*-GAL4. Taken together these results suggest that when neurotransmitter release is prevented in the MB and signal transduction is blocked, larva's ability to respond appropriately to light and to consume wild type amounts of food are disrupted.

Consistent with the results presented here, previous studies involving larvae MB ablation, have found that larvae that are fed HU after hatching, are fully able to move towards an attractive food source (Osborne et al., 2001). MB silencing using *TNT-G* did

not affect the time larvae spend in linear locomotion (Dineen 2009), nor did it cause larvae to wander away from the food source (data not shown). This suggests that MB silenced larvae are able to move towards and are attracted to food sources, but simply fail to eat the food presented.

While *TNT-G* acts to suppress action potentials (Sweeney et al., 1995), the transgene *NachBAC* depolarizes neurons allowing for increased action potentials (Ren et al., 2001). Rendering the γ neurons hyperexcitable through the use of *NaChBAC* had no effect on response indices of third instar foraging larvae or on feeding behaviours as measured through the presence of blue guts in the food dispersal assay. These results suggest that decreasing but not increasing excitability of the MB γ neurons have an effect on feeding and response to light behaviours. The failure of hyperexcitability of MB γ neurons to result in behavioural changes, could be explained by the presence of compensatory mechanisms which function to balance the increased excitability of these neurons (reviewed by Hodge, 2009). Examples of such mechanisms could include an increased synaptic inhibition or alterations in the regulation of ion channels (Kelsch et al., 2009). An important caveat of this experiment is that electrophysiological recordings were not conducted to measure the changes in electrical activity suspected to result while driving these various transgenes in the MB γ neurons. Therefore, the degree of suppression and hyperexcitability in these neurons is not known.

My results suggest that the suppression of larval λ neurons within the MB are important in larval feeding behaviours and response to light behaviours as previously

documented (Dineen, 2009). In contrast, hyperexcitation of these neurons has no effect on these behaviours.

4.5 Conclusions

Taken together, my results combined with those previously published in my laboratory (Dineen, 2009; Scantlebury et al., 2010) provide evidence that *dRanBPM* is a pleiotropic gene required for different aspects of larval behaviour. The mechanism with which *dRanBPM* acts to influence behaviour requires further investigation. My results suggest that *dRanBPM* may function to regulate the development of distinct classes of neurons, including those that comprise the MB. Since only embryonically born neurons are required for larval behaviours (Ito and Hotta, 1992), *dRanBPM* may function during the embryonic period to regulate neuronal proliferation. A single report suggests that the neurons of the MB may also follow this developmental pattern, with larval born KCs not retaining full function until adult stages (Pauls et al., 2010). This, however, requires further investigation.

The resulting disruption of neuronal development at this stage with the loss of *dRanBPM* gene function, may interfere with behaviours including response to light, locomotion, feeding and additional larval sensory behaviours, of olfaction and phototaxis. For instance, it has been previously demonstrated that serotonin and *dnpf* neuronal pathways influence response to light and feeding, respectively (Rodriguez-Moncalvo and Campos, 2009; Wu et al., 2003).

Embryonically born γ neurons of the MB may also play a role in behaviours of response to light and locomotion, as it is well known that this structure functions in a variety of behaviours at almost every stage of *Drosophila* development (reviewed by Heisenberg, 2003).

Evidence presented here and presented previously (Scantlebury et al., 2010), suggests that *dRanBPM* plays a role in MB development and/or is involved in the regulation of MB behavioural control. Additional studies are needed, however, to provide more direct evidence of how dRanBPM function involves the MB.

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Appendix

Appendix A: Genetic Schemes

(A) Immunohistochemistry of d*RanBPM*^{k05201} mutants

i) Driving GFP with MB specific drivers

$$\frac{yw}{Y};\frac{dRanBPMk05201}{cy(y+)};\frac{247-GAL4}{247-Gal4}\ \text{x}\ \frac{yw}{yw};\frac{dRanBPMk05201}{Cy(y+)};\frac{UAS-CD8-GFP}{UAS-CD8-GFP}$$

$$\frac{yw}{yw};\frac{dRanBPMk05201}{dRanBPMk05201};\frac{247-GAL4}{UAS-CD8-GFP}$$

and

$$\frac{yw}{Y};\frac{dRanBPMk05201}{cy(y+)};\frac{201Y-GAL4}{201Y-Gal4}\ \text{x}\ \frac{yw}{yw};\frac{dRanBPMk05201}{Cy(y+)};\frac{UAS-CD8-GFP}{UAS-CD8-GFP}$$

$$\frac{yw}{yw};\frac{dRanBPMk05201}{dRanBPMk05201};\frac{201Y-GAL4}{UAS-CD8-GFP}$$

iii) Driving GFP in Peptidergic neurons

$$\frac{yw}{Y};\frac{dRanBPMk05201}{cy(y+)};\frac{386-GAL4}{386-Gal4}\ \text{x}\ \frac{yw}{yw};\frac{dRanBPMk05201}{Cy(y+)};\frac{UAS-CD8-GFP}{UAS-CD8-GFP}$$

$$\frac{yw}{yw};\frac{RanBPMk05201}{RanBPMk05201};\frac{386-GAL4}{UAS-CD8-GFP}$$

iv) Driving GFP in DIMM expressing cells

$$\frac{yw}{Y};\frac{dRanBPMk05201}{cy(y+)};\frac{c929-GAL4}{c929-Gal4}\ \text{x}\ \frac{yw}{yw};\frac{dRanBPMk05201}{Cy(y+)};\frac{UAS-CD8-GFP}{UAS-CD8-GFP}$$

$$\frac{yw}{yw};\frac{RanBPMk05201}{RanBPMk05201};\frac{c929-GAL4}{UAS-CD8-GFP}$$

v) Driving GFP in *dnpf* expressing cells

$$\frac{yw}{Y};\frac{dRanBPMk05201}{cy(y+)};\frac{dnpf-GAL4}{dnpf-Gal4}\ \text{x}\ \frac{yw}{yw};\frac{dRanBPMk05201}{Cy(y+)};\frac{UAS-CD8-GFP}{UAS-CD8-GFP}$$

$$\frac{yw}{yw};\frac{dRanBPMk05201}{dRanBPMk05201};\frac{dnpf-Gal4}{UAS-CD8-GFP}$$

(B) Silencing of the MBs using active *tetanus toxin light chain*

$$\frac{yw}{Y};\frac{+}{+};\frac{247-GAL4}{247-Gal4}\ \text{X}\ \frac{w}{w};\frac{UAS-TNT-G}{UAS-TNT-G};\frac{+}{+}$$

$$\frac{yw}{w};\frac{UAS-TNT-G}{+};\frac{247-GAL4}{+}$$

and

$$\frac{yw}{Y};\frac{+}{+};\frac{201Y-GAL4}{201Y-Gal4}\ \text{X}\ \frac{w}{w};\frac{UAS-TNT-G}{UAS-TNT-G};\frac{+}{+}$$

$$\frac{yw}{w};\frac{UAS-TNT-G}{+};\frac{201Y-GAL4}{+}$$

(C) Silencing of the MBs using inactive *tetanus toxin light chain*

$$\frac{yw}{Y};\frac{+}{+};\frac{247-GAL4}{247-Gal4}\ \text{X}\ \frac{w}{w};\frac{UAS-TNT-VIF}{UAS-TNT-VIF};\frac{+}{+}$$

$$\frac{yw}{w};\frac{UAS-TNT-VIF}{+};\frac{247-GAL4}{+}$$

and

$$\frac{yw}{Y};\frac{+}{+};\frac{201Y-GAL4}{201Y-Gal4}\ \text{X}\ \frac{w}{w};\frac{UAS-TNT-VIF}{UAS-TNT-VIF};\frac{+}{+}$$

$$\frac{yw}{w};\frac{UAS-TNT-VIF}{+};\frac{201Y-GAL4}{+}$$

(D) Silencing of the MBs using *electrical knockout*

$$\frac{yw}{Y};\frac{+}{+};\frac{247-GAL4}{247-Gal4}\ \text{X}\ \frac{w}{w};\frac{UAS-EKO}{UAS-EKO};\frac{+}{+}$$

$$\frac{yw}{w};\frac{UAS-EKO}{+};\frac{247-GAL4}{+}$$

(E) Hyperexcitation of the MBs using the *Bacterial Sodium Channel*

$$\frac{yw}{Y};\frac{+}{+};\frac{247-GAL4}{247-Gal4}\ \text{X}\ \frac{w}{w};\frac{UAS-\text{NaChBAC}}{UAS-\text{NaChBAC}};\frac{+}{+}$$

$$\frac{yw}{w};\frac{UAS-\text{NaChBAC}}{+};\frac{247-GAL4}{+}$$

and

$$\frac{yw}{Y};\frac{+}{+};\frac{201Y-GAL4}{201Y-Gal4}\ \text{X}\ \frac{w}{w};\frac{UAS-\text{NaChBAC}}{UAS-\text{NaChBAC}};\frac{+}{+}$$

$$\frac{yw}{w};\frac{UAS-\text{NaChBAC}}{+};\frac{201Y-GAL4}{+}$$

Appendix B: Tables

Table 1. Number of serotonin expressing cells per larval brain of each genotype

	Number of serotonin cells per larval brain																
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	$\bar{X} \pm$ SE	n
dRanBPM^{k05201}	72	52	57	61	61	70	58	66	55							61.33 ± 2.26	9
dRanBPM^{k05201}/ *dRanBPM*^{ts7}	82	71	82	52	85	73	68	64	62							71 ± 3.62	9
dRanBPM^{k05201}/ *cy(y+)*	68	77	80	70	52	71	72	75	79	76	82	73	70	74	86	73.67 ± 2.15	15

Table 2. Number of larvae (and percentages %) located in the different regions of the plate during the Olfaction Attraction Assay after 10 minutes and across all trials.

Genotype	*yw*	*dRanBPM*^{k05201}	*dRanBPM*^{revertant}	*OR*
Control	5 (7.25%)	15 (19.2%)	10 (12.5%)	5 (5.10%)
Stimulus	64 (92.7%)	63 (80.7%)	70 (87.5%)	93 (94.9%)
22mm	10 (14%)	8 (10.3%)	5 (6.25%)	6 (6.12%)
13mm	48 (69%)	5 (6.41%)	56 (70.0%)	82 (83.7%)
N	69	78	80	98
# Remained in center of plate (% of Total)	5 (6%)	17 (18%)	8 (9%)	5 (4%)

Table 3. Number of larvae (and percentages %) located in the different regions of the plate during the Olfaction Repellent Assay after 10 minutes and across all trials.

Genotype	*yw*	*dRanBPM*^{k05201}	*dRanBPM*^{revertant}	*OR*
Control	8 (15.7%)	11 (16.2%)	11 (11.2%)	10 (11.1%)
Stimulus	43 (84.3%)	57 (83.8%)	87 (88.7%)	80 (88.9%)
22mm	27 (52.9%)	5 (7.35%)	45 (45.9%)	47 (52.2%)
13mm	5 (9.80%)	5 (7.35%)	5 (5.10%)	5 (5.56%)
N	51	68	98	90
# Remained in center of plate (% of Total)	6 (11%)	20 (23%)	2 (2%)	4 (4%)

Table 4. Number of larvae (and percentages %) located in Salt or No Salt quadrants after 10 and 15 minutes across all trials.

	10 minutes		15 minutes		
Genotype	Salt	No Salt	Salt	No Salt	N
OR	12 (13.04%)	80 (86.96%)	9 (9.78%)	83 (90.22%)	92
yw	15 (18.52%)	66 (81.48%)	6 (7.41%)	75 (92.59%)	81
dRanBP ^{revertant}	19 (24.05%)	60 (75.95%)	19 (24.05%)	60 (75.95%)	79
dRanBPM ^{k05201}	10 (14.29%)	60 (85.71%)	11 (15.71%)	59 (84.29%)	70

Table 5. Number of larvae (and percentages %) located in light or dark quadrants after 10 and 15 minutes and across all trials.

	10 minutes		15 minutes		
	Dark	Light	Dark	Light	N
OR	79 (86.8%)	15 (16.5%)	76 (83.5%)	15 (16.5%)	91
yw	63 (53.6%)	38 (33.9%)	85 (75.9%)	27 (24.1%)	112
dRanBPM ^{revertant}	87 (80.5%)	22 (20.4%)	84 (77.8%)	24 (22.2%)	108
dRanBPM ^{k05201}	48 (47.1%)	32 (31.4%)	65 (63.7%)	37 (36.3%)	102
pGMR-hid	39 (45.3%)	47 (54.7%)	53 (61.6%)	33 (38.4%)	86

Table 6. Percentage of larvae that ate during the MB silencing Feeding assay. Percentages are shown individually per trial and then averaged across trials.

	Percentage Fed Per Trial						
Genotype	1	2	3	4	5	Average ± SE	Total N
yw	100	96	100	95		97.7 ± 1.3	89
TNT-G	80	61	66	58		69.5 ± 4.9	93
TNT-VIF	100	100	100	100		80.8 ± 3.4	67
MB247	100	94	92	82		92 ± 3.7	69
MB247; TNT-VIF	88	76	85	85	70	80.1 ± 6	111
MB247; TNT-G	28.8	59.2	56.6	40	17.64	46.2 ± 7.1	134
MB201Y	85.7	100	92	100		94.4 ± 3.5	87
MB201Y; TNT-VIF	100	100	100	94		98.5 ± 1.5	94
MB201Y; TNT-G	86.6	91	90	100		91.9 ± 2.9	73
EKO	96	100	92.6	95.7		96.1 ± 1.5	98
MB247; EKO	89.3	92.3	100	100		95.4 ± 2.7	104

Table 7. Percentage of larvae that ate in the MB hyperexcitability assay. Percentages are shown individually per trial and then averaged across trials.

	Percentage Fed Per Trial						
Genotype	1	2	3	4	5	Average ± SE	Total N
MB247	94.11	90	100	93.1		94 ± 2,09	81
MB201Y	85.71	100	92	100		94.25 ± 3.46	87
MB247; NaChBAC	100	86.36	100	86.95	86.95	92 ± 3.85	118
MB201Y; NaChBAC	96	94.73	95.45	100		96.5 ± 1.18	86
NaChBAC	95.83	79	86.36	96.8	85.7	89.38 ± 4.81	113

Appendix C: Using Mosaic Analysis with a Repressible Marker (MARCM) to study the effect of *dRanBPM* on Larval Size

Using Mosaic Analysis with a Repressible Marker (MARCM) to study the effect of *dRanBPM* on Larval Size

dRanBPM^{k05201} homozygous mutant larvae are approximately 3/4 the size of wild type larvae in the 3rd instar foraging stage. To investigate the degree to which *dRanBPM* expression is responsible for larval size maintenance, I constructed MARCM (Mosaic Analysis with a Repressible Cell Marker; Lee and Lou, 1999) ready flies to be used for this purpose in future experiments. This type of mosaic analysis allows for the study of cell lineage analysis, neuronal circuit tracing as well as the investigation of gene function in a small population of cells (Lee and Lou, 2001). MARCM combines the properties of the GAL80 repressor with the FLP/FRT system to positively label homozygous mutant clones with GFP. The final MARCM ready fly will harbor cells which contain one mutant copy of the *dRanBPM*^{k05201} allele, downstream of a pre-selected FRT (*FLP recognition target*) site, FRT 42D. It will also contain one copy of the GAL80 transgene driven by the *tub*-GAL4 (*tubulin*) downstream of the FRT 42D site, and FLP recombinase under the control of a heat shock (hs) promoter. Once FLP is activated through heat shock, a recombination event will occur in cells undergoing mitosis. FLP will act on the FRT sites to recombine the chromosomal segment containing the *dRanBPM*^{k05201} mutation with the segment containing the *tub*-GAL80 transgene. Upon subsequent divisions, cells will either contain, two copies of *tub*-GAL80, two copies of the *dRanBPM*^{k05201} deletion, or have the genotype of the original parental cell. In cells with two copies of *dRanBPM*^{k05201}, the absence of GAL80 will permit the expression of GFP which is driven by *elav*. Thus, all mutant clones will express GFP. The crosses that I performed that will lead to this final MARCM ready fly are depicted below. A caveat of this experiment is that not every mutant clone will carry GFP, thus flies carrying rescue constructs were also created. These lines are to be used in parallel with the experimental lines to ensure that any visible phenotypic effects are caused by loss of *dRanBPM.* These rescue experiments will also confirm the cell autonomousity of dRanBPM function.

For our purposes, it would be beneficial to expose newly hatched larvae to various heat shock times and durations. Long periods of heat shock are known to produce larger clones, while shorter heat shock exposures produce smaller clones. In addition, since the mutant phenotype is normally visible in the 3rd instar, heat shocks should also be administered at different larvae stages (ie.., early 1st instar, early 2nd instar, late 2nd instar). These initial experiments are important in order to establish the amount of heat exposure and the developmental timing that is necessary to cause a significant reduction in larval size similar to that seen in *dRanBPM*^{k05201} mutants. Once this is established, brains of both experimental and control strains, could be stained with a neuronal marker like FasII, which also labels the MB. GFP labeled clones could then be scored based on their

location within the larval CNS, and these results could be statistically correlated to the presence or absence of the larval size phenotype.

A) *dRanBPM*^{k05201} Recombination Cross

Step1: Create fly containing all transgenes

$$\frac{yw}{Y};\frac{dRanBPMk05201}{cy(y+)};\frac{+}{+} \quad \text{x} \quad \frac{w}{w};\frac{NeoFRT(42D),UbiGFP}{Cy};\frac{+}{+}$$

$$\frac{yw}{Y};\frac{dRanBPMk05201}{NeoFRT(42D),UbiGFP};\frac{+}{+}$$

Step 2: Recombination

$$\frac{yw}{Y};\frac{dRanBPMk05201}{NeoFRT(42D),UbiGFP};\frac{+}{+} \quad \text{x} \quad \frac{yw}{yw};\frac{S}{cy};\frac{+}{+}$$

Screen for virgins with Neo resistance and lacking GFP

$$\frac{yw}{yw};\frac{NeoFRT(42D),\ dRanBPMk05201}{Cy};\frac{+}{+}$$

Step 3: Generate Stock

$$\frac{yw}{yw};\frac{NeoFRT(42D),\ dRanBPMk05201}{Cy};\frac{+}{+} \quad \text{x} \quad \frac{yw}{Y};\frac{S}{cy(y+)};\frac{+}{+}$$

$$\frac{yw}{yw};\frac{NeoFRT(42D),\ dRanBPMk05201}{Cy(y+)};\frac{+}{+} \quad \text{x} \quad \frac{yw}{Y};\frac{NeoFRT(42D),\ dRanBPMk05201}{Cy(y+)};\frac{+}{+}$$

B) Balancing all MARCM stocks

Step 1: Adding a first chromosome balancer

$$\frac{yw}{Y};\frac{NeoFRT(42D),tub-GAL80}{Cy(y+)};\frac{+}{+} \quad \text{x} \quad \frac{+}{FM7};\frac{Star}{cy(y+)};\frac{+}{+}$$

$$\frac{FM7}{Y};\frac{NeoFRT(42D),tub-GAL80}{Cy(y+)};\frac{+}{+}$$

Step 2: Adding a second and third chromosome balancer

$$\frac{elav-GAL4,UAS-CD8-GFP,hs-FLP}{FM7};\frac{+}{+};\frac{+}{+} \quad \text{x} \quad \frac{yw}{Y};\frac{Sp}{TS};\frac{Ly}{TL}$$

$$\frac{elav-GAL4,UAS-CD8-GFP,hs-FLP}{Y};\frac{+}{TS};\frac{+}{TL} \quad \text{X} \quad \frac{yw}{FM7};\frac{Sp}{+};\frac{Ly}{+}$$

$$\frac{elav-GAL4,UAS-CD8-GFP,hs-FLP}{FM7};\frac{Sp}{TS};\frac{Ly}{TL}$$

C) Final Crosses

Step 1: Create a fly stock harboring transgenes from both balanced stocks

$$\frac{elav-GAL4,UAS-CD8-GFP,hs-FLP}{FM7};\frac{Sp}{TS};\frac{Ly}{TL} \quad \text{X} \quad \frac{FM7}{Y};\frac{NeoFRT(42D),tub-GAL80}{Cy(y+)};\frac{+}{+}$$

$$\frac{elav-GAL4,UAS-CD8-GFP,hs-FLP}{FM7};\frac{NeoFRT(42D),tub-GAL80}{TS};\frac{+}{TL}$$

Step 2: Create experimental Line

$$\frac{elav-GAL4,UAS-CD8-GFP,hs-FLP}{FM7};\frac{NeoFRT(42D),tub-GAL80}{TS};\frac{+}{TL} \quad \text{X} \quad \frac{yw}{Y};$$
$$\frac{NeoFRT(42D),\ dRanBPMk05201}{Cy(y+)};\frac{+}{+}$$

$$\frac{elav-GAL4,UAS-CD8-GFP,hs-FLP}{FM7};\frac{NeoFRT(42D),tub-GAL80}{NeoFRT(42D),\ dRanBPMk05201};\frac{+}{+}$$

D) MARCM ready lines with rescue constructs

Step 1: Adding rescue construct to the third chromosome

$$\frac{elav-GAL4,UAS-CD8-GFP,hs-FLP}{FM7};\frac{NeoFRT(42D),tub-GAL80}{TS};\frac{+}{TL} \quad \text{X} \quad \frac{yw}{yw};\frac{Star}{Cy(y+)};\frac{dRanBPM\ Long}{dRanBPM\ Long}$$

$$\frac{elav-GAL4,UAS-CD8-GFP,hs-FLP}{FM7};\frac{NeoFRT(42D),tub-GAL80}{TS};\frac{dRanBPM\ Long}{TL}$$

Step 2: Create MARCM flies with rescue construct

$$\frac{elav-GAL4,UAS-CD8-GFP,hs-FLP}{FM7};\frac{NeoFRT(42D),tub-GAL80}{TS};\frac{dRanBPM\ Long}{TL} \quad \text{X} \quad \frac{yw}{Y};$$
$$\frac{NeoFRT(42D),\ dRanBPMk05201}{Cy(y+)};\frac{+}{+}$$

$$\frac{elav-GAL4,UAS-CD8-GFP,hs-FLP}{FM7};\frac{NeoFRT(42D),tub-GAL80}{NeoFRT(42D),\ dRanBPMk05201};\frac{dRanBPM\ Long}{+}$$