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ANALYSIS OF THE CRMP GENE IN DROSOPHILA: DETERMINING THE REGULATORY ROLE OF CRMP IN SIGNALING AND BEHAVIOR

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ABSTRACT OF DISSERTATION

Deanna Hardt Morris

The Graduate School
University of Kentucky
2010
ANALYSIS OF THE CRMP GENE IN DROSOPHILA: DETERMINING THE REGULATORY ROLE OF CRMP IN SIGNALING AND BEHAVIOR

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By
Deanna Hardt Morris
Lexington, Kentucky

Director: Dr. John M. Rawls, Professor of Biology
Lexington, Kentucky
2010

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ABSTRACT OF DISSERTATION

ANALYSIS OF THE CRMP GENE IN DROSOPHILA: DETERMINING THE REGULATORY ROLE OF CRMP IN SIGNALING AND BEHAVIOR

The mammalian genome encodes five collapsin response mediator protein (CRMP) isoforms. Cell culture studies have shown that the CRMPs mediate growth cone dynamics and neuron polarity through associations with a variety of signal transduction components and cytoskeletal elements. CRMP is also a member of a protein family including the presumably ancestral dihydropyrimidinase (DHP) protein that catalyzes the second step in pyrimidine degradation. In Drosophila, CRMP and DHP proteins are produced by alternatively spliced transcripts of the CRMP gene. The alternative protein forms have a 91% sequence identity, but unique expression patterns. CRMP is found exclusively in neuronal tissues and DHP is ubiquitously expressed in non-neuronal tissues. Comparative analysis of CRMP homologous sequences from insect taxa show CRMP alternative splicing is a common feature and probably represents the ancestral state of this gene family.

To investigate the regulatory role of CRMP, loss-of-function mutations of CRMP that lack both proteins were isolated; homozygous animals display DHP-null phenotypes but exhibit no overt developmental or neurological defects. To determine possible interactions of Drosophila CRMP with signaling pathways in which mammalian CRMP has been shown to act, the UAS-GAL4 system was utilized. Phenotypes produced by misexpression of a variety of UAS signal transduction mediator responders were modified in a CRMP mutant background. The modification entails enhancement or suppression of a specific phenotype in a direction that corresponds to the hypothesized involvement of mammalian CRMP in signaling pathways that regulate growth cone dynamics. These data suggest that Drosophila CRMP has a role in cell signaling pathways similar to the role of the mammalian CRMPs.

Furthermore, recent findings demonstrate that CRMP plays an important role in learning and memory of mice, leading to the assessment of new phenotypes in the Drosophila CRMP mutants. Tests utilizing the Pavlovian olfactory conditioning assay reveal that loss of CRMP function leads to significant learning, 3 hour memory, and long term memory deficits. Preliminary data also suggest that Drosophila CRMP may be required for normal circadian locomotor rhythms. Collectively, the data presented here demonstrate CRMP’s role in adult behavioral processes and regulating signaling events comparable to mammalian CRMP signaling.
KEYWORDS: CRMP, *Drosophila*, UAS-GAL4 system, Pavlovian olfactory learning and memory, circadian rhythms

Deanna Hardt Morris

February 26, 2010
ANALYSIS OF THE CRMP GENE IN DROSOPHILA: DETERMINING THE REGULATORY ROLE OF CRMP IN SIGNALING AND BEHAVIOR

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DISSERTATION

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This work is dedicated to my late grandfather Roy Forsythe who shared the same love for science.
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Semaphorin Signaling Pathway

Semaphorins are a large family of secreted, transmembrane, and GPI-linked molecules that play a central role in cell-cell signaling events during axon guidance as well as in other non-neuronal functions (Yazdani and Terman 2006). There are twenty human semaphorin members and Drosophila has five characterized semaphorins, all of which are grouped into eight classes based on phylogenetic analysis and structural elements (Semaphorin Nomenclature Committee 1999; Yazdani and Terman 2006). Drosophila semaphorin gene family members are found in only class 1 and 2, which are unique to invertebrates (Semaphorin Nomenclature Committee 1999; Yazdani and Terman 2006). Due to the characterization of a large number of semaphorins and their complex functionality a common pathway has yet to be identified.

Although the molecular mechanisms of semaphorin signaling remain poorly understood, the most characterized member of the semaphorins is semaphorin 3A (Sema3A). The function of Sema3A in the nervous system is mediated by neuropilin and plexin transmembrane receptor families (Yazdani and Terman 2006). This signaling cascade begins by binding of the Sema3A ligand to the neuropilin receptor. Neuropilin, once bound to Sema3A, interacts at the cell membrane with the plexin receptor which transduces the signal into the cell through its cytoplasmic domain. The semaphorins function through these receptors to alter intracellular cytoskeleton dynamics at the growing tips of axons. The steps linking the activated receptor complex to the downstream cytoskeleton targets remains unclear.

In vitro studies have provided some insight into the signal transducers that mediate the Sema3A receptor complex response. A variety of molecules have been shown to be involved in the intracellular signaling pathway for the actions of Sema3A, including collapsin response mediator protein (CRMP) (Figure 1.1) (Brown et al. 2004; Castellani and Rougon 2002; Kruger et al. 2005; Mann and Rougon 2007; reviewed in Schmidt and Strittmatter...
In vivo studies that focus on revealing the role of CRMP should help refine the semaphorin intracellular signaling pathway that plays an important part in the action of a growing axon during nervous system development and potentially a role in neuronal connectivity in the adult.

**Collapsin Response Mediator Proteins**

In mammals, CRMPs have been shown to play important roles in neurogenesis. The CRMPs have been implicated as mediators of growth cone dynamics in cultured mammalian neurons and biochemical studies have shown associations with a variety of signal transduction components and cytoskeletal elements (Goshima *et al.* 1995; Deo *et al.* 2004; Hall *et al.* 2001; Fukata *et al.* 2002; Brown *et al.* 2004; Eickholt *et al.* 2002; Yoshimura *et al.* 2006). The mammalian genome encodes five CRMP isoforms (Figure 1.2), with similar molecular sizes of 60-66 kDa (Deo *et al.* 2004; Fukada *et al.* 2000; Goshima *et al.* 1995; Wang and Strittmatter, 1996; Quinn *et al.* 1999). The CRMP-1, CRMP-2, CRMP-3, and CRMP-4 family members have a 68%-75% identity in protein sequence (Deo *et al.* 2004). CRMP-5 only shares a 50% protein sequence identity with other CRMPs and a 51% sequence identity with the apparently ancestral protein, dihydropyrimidinase (DHP) (Fukada *et al.* 2000). DHP, encoded by a separate mammalian gene, is an enzyme that is important to the maintenance of proper pyrimidine levels in the cell by catalyzing the second step in the degradation of uracil into β-alanine (Rawls 2006). Although the sequence similarity between DHP and CRMP suggests an evolutionary relationship, the DHP key active site residues involved in zinc ion interactions and ligand binding are missing in CRMP; therefore, CRMPs are not amidohydrolases like their ancestral relative DHP (Schnackerz and Dobritzsch, 2008).

CRMPs expression in rodents encompasses post-mitotic neuronal cells at the start of embryonic life, to strongest levels one week postnatal, to lowest levels in areas of the adult nervous system that retain neurogenesis (Minturn *et al.* 1995; Minturn *et al.* 1995; Wang and Strittmatter, 1996; Fukada *et al.* 2000; Quach *et al.* 1997; Kamata *et al.* 1998). This expression pattern suggests that CRMPs play important roles in neuronal development as well as in adult neuronal plasticity (Charrier *et al.* 2003). Regions of the adult brain that express CRMP include the hippocampus (Minturn *et al.* 1995; Wang and Strittmatter, 1996;
Quach et al. 2000; Ricard et al. 2000), cerebellum (Wang and Strittmatter, 1996; Ricard et al. 2000), and olfactory bulbs (Wang and Strittmatter, 1996; Pasterkamp et al. 1998; Veyrac et al. 2005), all of which are anatomical sites that continuously undergo neurogenesis. Therefore, much is to be learned about the role CRMPs have in neural connectivity/synaptic plasticity as it pertains to learning, memory, emotion, sleep and other adult behaviors. In this dissertation, the question of whether CRMP impacts the adult behaviors of learning, memory and sleep will all be addressed.

As previously mentioned, the CRMPs are cytosolic phosphoproteins involved in processes like neuronal differentiation and axonal guidance. CRMP isoforms were initially identified as mediators of the Sema3A pathway in cultured mammalian neurons, where antibodies against CRMPs block Sema3A-induced growth cone collapse (Goshima et al. 1995). Other signal transduction events in which CRMP has been shown to participate include lysophosphatidic acid (LPA) signaling in growth cone collapse (Arimura et al. 2000), Reelin signaling in cortical neuronal migration (Yamashita et al. 2006), Ephrin signaling in growth cone guidance (Arimura et al. 2005), and neurotrophin-3 (NT3) signaling to promote axon outgrowth (Yoshimura et al. 2005). Furthermore, studies have identified CRMP-2 as a regulator of neuronal polarity due to its role in specifying axon/dendrite fate (Arimura et al. 2004). Overexpression of CRMP-2 has been shown to induce the growth of numerous axons, and it is also involved in the maturation of neurites and pre-existing dendrites to axons (Yoshimura et al. 2005). Evidence suggests that CRMP-2 associates with tubulin dimers to promote microtubule assembly for neurite elongation (Gu & Ihara 2000; Fukata et al. 2002; Mimura et al. 2006). Data also suggest the important roles of CRMP-2 and microtubules in the inhibition of the axon regeneration by the myelin-derived inhibitors (Mimura et al. 2006). CRMP-2 has also been shown to bind to Numb and through this interaction contribute to endocytosis at the growing tip of the axon (Nishimura et al. 2003). More recent studies report CRMP-2 binds to and interferes with dynein function and binds to kinesin-1 to regulate protein transport to the growth cone, perhaps transport of tubulin (Kimura et al. 2005; Arimura et al. 2009). In summary, Schmidt and Strittmatter have proposed a model demonstrating that all of these events mediated by CRMP work together in the growing tips of axons to regulate cytoskeletal dynamics (Figure 1.1).
The activity of CRMP family proteins are regulated by several post-translational modification steps. CRMP-2 has been found to be O-glycosylated (Cole and Hart 2001). Rho kinase (ROCK) physically interacts with and phosphorylates CRMP-2 at Thr555 during LPA signaling (Hall et al. 2001; Arimura et al. 2000; Arimura et al. 2005). CRMP-2 is also phosphorylated during Sema3A signaling by cyclin-dependent kinase 5(Cdk5) at Ser522 (Brown et al. 2004) and glycogen synthase kinase 3β (GSK-3β) at Thr509, Thr514, and Ser518 (Cole et al. 2004). The phosphorylation by GSK-3β is prompted by phosphorylation of CRMP-2 by Cdk5 and in this state CRMP-2 interaction with tubulin or Numb is blocked (Cole et al. 2004; Cole et al. 2006; Uchida et al. 2005; Arimura et al. 2005). Most recent data reports that CRMP-2 is also modified by phosphorylation at Tyr32 by Fyn in Fyn-mediated Sema3A signaling (Uchida et al. 2009). All of the kinases mentioned provide excellent candidates to test for similar interactions with Drosophila CRMP.

When digesting the data that has accumulated for CRMP isoforms, it is clear that CRMPs exist in two states within the cell, an active or inactive state. During growth cone collapse triggered by LPA or Sema signaling, CRMP is found in a phosphorylated inactive state. In this phosphorylated state CRMP undergoes a conformational change that perturbs its interaction with proteins like Numb, Sra, and tubulin; thus preventing actin and microtubule polymerization (Figure 1.1). On the other hand, during growth cone extension and branching, the growing neurite encounters adhesion molecules and positive guidance cues that promote a CRMP active conformation, which allows for actin and microtubule stability through CRMP’s interactions with Numb, Sra, and tubulin. It has been hypothesized that the CRMP N-terminal and C-terminal regions that are modified may collaborate to control the activation state; and therefore, the associations CRMP has with proteins that directly regulate the cytoskeleton (Uchida et al. 2009).

The crystal structure of human CRMP-2 has been resolved (Ogg et al. 2006; Stenmark et al. 2007). Catalytic residues of the DHP active site are not conserved in CRMP-2. The catalytic lysine and two of the Zn-coordinating histidine residues are not conserved in CRMP-2 (Stenmark et al. 2007). However, this region of the CRMP protein still has potential to bind small molecules (Stenmark et al. 2007). Unfortunately, the C-terminal tail of the protein remains unresolved (Stenmark et al. 2007). It is the residues found in the C-terminal region of the protein that undergo the phosphorylation events
mentioned before. Furthermore, the X-ray structure of murine CRMP-1 has also been determined (Deo et al. 2004). Experimental mutagenesis on surface-exposed residues of CRMP-1 has shown that alanine substitutions in one domain (S4 & S5 or S5 & S6 linker, residues 46-57) of CRMP-1 caused Sema3A-independent COS-7 cell contraction (Deo et al. 2004). The sequence of this region is also resolved in the CRMP-2 crystal structure (Stenmark et. al. 2007). Similar to the CRMP-2 protein, the C-terminal segment of the CRMP-1 protein was not resolved due to possible proteolytic cleavage (Deo et al. 2004) or non-specific location in the crystal structure.

CRMP significance

Providing insight into the mechanism(s) that control CRMP expression and function is important due to it being implicated in the involvement of multiple cellular and molecular signaling events, which are involved in neuronal apoptosis/proliferation, cell migration, and differentiation (Shirvan et al. 1999; Inagaki et al. 2001; Charrier et al. 2003). In addition, up-regulation of CRMP1, 2, & 5 is essential in axon growth and regeneration in response to nervous system injury (Suzuki, et al. 2003). Furthermore, many neurodegenerative disorders are also associated with altered forms of CRMP. CRMP-2 expression has been shown to be higher in patients with Alzheimer’s disease and is associated with neurofibrillary tangles in a hyper-phosphorylated state (Yoshida et al. 1998; Gu et al. 2000; Castegna et al. 2002; Uchida et al. 2005). Transgenic mice that overexpress the amyloid precursor protein (APP) intracellular domain also exhibit high levels of phosphorylated CRMP-2 (Ryan and Pimplikar 2005). In Parkinson’s disease, CRMP-2 has been showed to be involved in neuronal apoptosis (Barzilai et al. 2000). In addition, CRMPs have been shown to be the targets of auto-antibodies produced during paraneoplastic neurological diseases (Honnorat et al. 1999). Paraneoplastic neurological diseases are linked to cancer, and studies have shown that lung cancer tumor cells express CRMPs (Yu et al. 2001). Most recent studies have linked CRMP to deficiencies in learning and memory. In this case, CRMP-1 knockout mice exhibited a reduction in long-term potentiation and impaired spatial learning and memory (Su et al. 2007). Thus, CRMP isoforms have a key importance in the physiopathology of the adult nervous system and characterizing how their function is controlled and regulated should provide significant clinical insights.
**Drosophila CRMP: A model system**

Multiplicity of vertebrate CRMPs, their heteromultimeric potential, and high sequence and expression similarities (Deo et al. 2004; Stenmark et. al. 2007) complicates the study of their individual biological functions *in vivo*; however, *Drosophila melanogaster* offers a simpler model system for resolving biological roles of CRMP. The *Drosophila* genome only contains one gene, *CRMP*, which encodes both DHP and CRMP proteins (Figure 1.2) (Rawls 2006). The *CRMP* gene is located on the right arm of the third chromosome and is ~8084 bp in length. Thus, it will be interesting to determine specifically how this gene encodes both DHP and CRMP, two structurally similar yet functionally different proteins. Furthermore, these proteins have a 91% sequence identity, suggesting that the distinct functions of the two proteins are derived from differences in a small region of the protein encoded by exon E9 (Figure 1.3).

Full-length sequencing of EST clones from the Berkeley Drosophila Genome Project (BDGP) revealed two distinct classes of *CRMP* RNAs, differing in alternative inclusion of one of two exons near the center of the gene (Rawls and Morris, in preparation). These mutually exclusive exons encode paralogous peptide segments. Thus, the *Drosophila CRMP* gene generates the CRMP protein by alternative splicing; however, the players that mediate this process have yet to be disclosed (Rawls and Morris, in preparation). As previously mentioned, the crystal structure of human CRMP2 has been resolved (Ogg et al. 2006; Stenmark et. al. 2007). Interestingly, the divergent region of the *Drosophila* CRMP and DHP proteins, exon E9, defines a core within the CRMP homotetramer (Figure 1.4).

Upon the discovery of the alternatively spliced *CRMP* transcript, the Rawls lab created transgenic *Drosophila* lines. The transgenic animals included a line that contained the *P{PYD2}* construct, which contains a genomic DNA segment that spans the *CRMP* gene (Rawls 2006). The transgene is also functional in that it rescues the DHP-null phenotypes, (suppress pyrimidine metabolism mutant phenotypes and enhancing sensitivity to pyrimidine analog toxicity), of *CRMP*sup mutations (Rawls 2006). The original transgene was modified to create the *P{PYD2GFP}* transgene by inserting a GFP cassette into the 3′ end of the *CRMP*
gene, such that all protein forms should contain a C-terminal GFP tag (Figure 1.5) (Rawls and Morris, in preparation). The transgenic animals that possess this construct express tagged-protein in most larval tissues, especially strong in neural tissue (Figure 1.5A). Introduction of a frameshift mutation within E9a abolishes all non-neural expression and blocks rescue of the DHP-null phenotype (Figure 1.5B). A frameshift mutation within E9b abolishes only neural expression, but retains DHP function (Figure 1.5C). In conclusion, E9a-containing RNA encodes DHP and is expressed ubiquitously in non-neural tissues, and E9b-containing RNA encodes the *Drosophila* ortholog of vertebrate CRMP, and this RNA is expressed exclusively in neural tissues (Rawls and Morris, in preparation).

By comparing sequences of *CRMP* in other Drosophilids, the *CRMP* gene family was identified in all twelve *Drosophila* species whose genome sequences have been revealed (Clark *et al.* 2007; Stark *et al.* 2007; http://flybase.bio.indiana.edu/). In addition, the same alternative exons located at the same genomic site (Muller chromosome element E) exist in all of the other species within the Sophophora subgenus, as seen in *D. melanogaster*. Preliminary sequence analysis revealed that *D. virilis*, *D. grimshawi*, and *D. mojavensis* lack the exon sequence that encodes for DHP in this chromosomal location. The divergence in the *CRMP* gene region of these three species is not surprising considering that they are the most distant Drosophilidae relatives to *D. melanogaster*, with all three falling under the subgenus *Drosophila* and the remaining eight species belonging to the Sophophora subgenus. Upon further sequence analysis of the *D. virilis*, *D. grimshawi*, and *D. mojavensis* genomes, BLAST searches identified a DHP gene at a completely different cytological map location (Muller chromosome element B). Therefore, these three species have undergone changes in the native *CRMP* gene region (presumably gene duplication events), which has culminated in different genes found on different chromosomes that encode for DHP and CRMP proteins. The neighboring genes that are found upstream and downstream of the new sequences are scrambled in comparison to the *D. melanogaster CRMP* gene region. The lack of annotation of both genes on separate chromosomes in these three species could be attributed to the varying degree of sequencing and assembly accuracy (Clark *et al.* 2007; Stark *et al.* 2007). Furthermore, the emergence of paralogous CRMP and DHP genes from once an ancestral single gene in these invertebrate species exemplifies how vertebrates could have evolved to encode these proteins from paralogous genes.
Comparative analysis of CRMP homologous sequences from a variety of insect taxa, including *Apis mellifera* (honey bee) and *Tribolium castaneum* (red flour beetle), showed that the CRMP/DHP alternative exons are a common feature of the insect genes and nucleotide sequence analysis gave E-values (number of hits one can ‘expect’ to see by chance when searching a database of a particular size) ranging from 0.0 to 0.0004 (Figure 1.6). This conservation over millions of years of evolution possibly represents the ancestral state of this gene family. It also suggests that duplication events have given rise to different genes that independently encode CRMPs and DHP in vertebrate genomes.

Previous work in the lab also involved the isolation of a variety of deletion and nucleotide substitution CRMP mutations (Rawls 2006). Further analysis showed that homozygous mutant animals are fully viable, fertile, and display no gross abnormality (Rawls unpublished). All produce DHP-null phenotypes, but no obvious morphological or behavioral phenotypes have been observed (Rawls 2006). The CRMPsupK1 loss-of-function mutant was used extensively in the dissertation research (Figure 1.7). The supK1 mutation is a 153 nucleotide deletion that occurs at the highly conserved exon4/intron4 junction and was created by HMPA mutagenesis (Rawls and Morris, in preparation). This large deletion creates a nonsense termination early in the protein sequence.

To search for gain-of-function CRMP phenotypes, a CRMP-specific cDNA was used to create *P{UAS-CRMP}* transgenic animals (Brand and Perrimon 1993) and two mis-expression phenotypes have been discovered: ubiquitous expression of CRMP (*P{act5C-GAL};P{UAS-CRMP}* animals) arrests development in an extended first larval stage producing the “peter pan” effect; *P{ap-GALMD544};P{UAS-CRMP}* animals, which have wing-targeted expression, produce curled, mis-shapened wings. Both drivers produce similar phenotypes using *P{UAS-DHP}* which contains a DHP-specific cDNA. This suggests that these mis-expression phenotypes derive from the common structures of the DHP and CRMP proteins. Other GAL4 drivers were tested including *P{dpp-GAL4}, P{ptc-GAL4}, P{elav-GAL4}, P{ey-H-GAL4}, P{GMR-GAL4} and *P{GAL4-e16E}(en)*, but normal development resulted in combination with both *P{UAS-DHP}* and *P{UAS-CRMP}*. 

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Figure 1.1. Semaphorin signaling pathway. Previous research suggests a CRMP-dependent pathway linking Sema-3A and growth cone dynamics. This figure shows the Sema3A signaling pathway indicating players, including CRMP, that mediate this cascade. A) In the absence of ligand, neuropilin-1 (NP1) attenuates the interaction between plexin-A (PlexA) and CRMP. B) In the presence of ligand, the PlexA/NP1 Sema3A receptor complex is formed allowing plexin and CRMP to interact. The CRMP tetramer is then phosphorylated by a variety of kinases (Cdk5, GSK3β, and Fes) leading to a change in conformation and activation state. C) Demonstrates a model for modification of the cytoskeleton dynamics underlying growth cone repulsion by secreted semaphorins. CRMP, in its phosphorylated state, can no longer promote microtubule assembly and actin polymerization (red Xs). (Modified from Schmidt & Strittmatter, 2007).
Figure 1.2. The DHP/CRMP Gene Family. Phylogenetic tree of DHPs and CRMPs. *Drosophila* has one CRMP gene that encodes for both DHP and CRMP (pink asterisk). Gene duplication has given rise to multiple vertebrate genes that have divergent DHP/CRMP functions. (Takemoto, *et al.* 2000)
Figure 1.3. CRMP/DHP protein alignment. Alignment analysis of predicted amino acid sequences of *D. melanogaster* CRMP (first line) and *D. melanogaster* DHP (second line). The third line shows the consensus between the two protein sequences. The blocks of conserved amino acids are highlighted in yellow. The residues highlighted in green indicate similar amino acids between the two proteins. The two sequences only differ in the Exon 9 region (blue underline), otherwise the sequences share 91% homology. The putative carboxyl terminal ‘tail’ is underlined in purple. Alignment was performed with AlignX, a component of Vector NTI Advance 11.0, Invitrogen Corporation 2008.
Figure 1.4. Crystal Structure of human CRMP2 (Stenmark et al. 2007). Yellow highlighted region indicates the divergent region of the two *Drosophila CRMP* isoforms. The structure was viewed and modified using Cn3D version 4.1 a component of the National Center for Biotechnology Information and National Library of Medicine.
Figure 1.5. Identifying roles of the E9 paralogs. The \(P\{PYD2GFP\}\) transgene is a genomic DNA fragment encoding a C-terminal GFP fusion; it rescues DHP-null phenotypes of \(CRMP^{sup}\) mutations (Rawls 2006).

A: \(P\{PYD2GFP\}\) animals express GFP in most larval tissues, with copious expression in neural tissue.

B: A frameshift mutation within E9a blocks non-neural GFP expression; fails to rescue DHP-null phenotypes.

C: A frameshift mutation within E9b results blocks neural GFP expression; rescues DHP-null phenotypes.
Figure 1.6. Conservation of CRMP/DHP gene alternative splicing among insects. The alternative exons found in *Drosophila* are also preserved in the genomes of honeybee, flour beetle and other insects.
Figure 1.7. \textit{CRMP^{supK1}} loss-of-function mutation. The mutation was selected by DHP-null phenotype. \textit{supK1} is a 153 nucleotide deletion that removes the E4-I4 junction, apparently resulting in a premature nonsense termination in the protein sequence.
Chapter Two
Regulatory Roles of CRMP in *Drosophila* Signaling Cascades

Introduction

CRMP/DHP Involvement in Signaling

As previously mentioned, CRMPs and DHP constitute a protein family that exhibit highly conserved structures, but have highly divergent roles. DHP, an amidohydrolase, has been well studied in a variety of organisms from prokaryotes to eukaryotes and is known to catalyze the second step of pyrimidine catabolism (Schnackerz and Dobritzsch, 2008). CRMPs production is limited to the nervous system and adult testis of metazoans, where the various isoforms have been shown to mediate a variety of processes including neuronal signal transduction, cytoskeletal dynamics, and neuronal polarity (Taketo *et al.* 1997; Charrier *et al.* 2003; Schmidt and Strittmatter 2007). The catalytic function of CRMPs is unknown and the key active site residues found in DHP are missing; therefore, CRMPs are not amidohydrolases like their ancestral relative DHP (Schnackerz and Dobritzsch, 2008). Could it be that CRMPs have evolved into enzymes that lack catalytic ability and only play a regulatory role in signaling pathways? If so, in these signaling cascades what are the downstream and upstream members in relation to CRMP? Considering that CRMPs are phosphorylated, could these proteins possess multiple regulatory roles depending on their conformational state? Furthermore, how do the different signaling cascades modify CRMPs physical state to mediate growth cone dynamics? To address these questions the loss of the sole CRMP protein in *Drosophila* and its misexpression will be investigated in this chapter.

Since it is possible that CRMPs have lost their amidohydrolase function, trying to resolve their role(s) in vertebrates would prove to be challenging due to the complexity of having multiple isoforms, many of which are products of alternatively spliced transcripts. In addition, the high sequence homology between the five isoforms provides the potential for functional redundancy and heterodimerization amongst the proteins (Wang and Strittmatter 1997; Deo *et al.* 2004). Since generating a mammalian knock-out for all CRMP isoforms has yet to be done, very little *in vivo* analysis has been done on this class of proteins. Individual
mouse lines deficient in CRMP-1 or CRMP-2 have been created, but neither exhibits any obvious mutant phenotypes and both are fully viable and fertile, possibly due to functional redundancy (Charrier et al. 2006; Su et al. 2007). Therefore, *Drosophila*, with only a single CRMP protein, is an excellent model to begin to deduce the general role of such a physiologically important protein.
Results

The CRMP mutants display no obvious aberrant phenotypes

Previous research has revealed that vertebrate CRMP isoforms potentially play important roles in signaling events that regulate development and adult health. To further investigate the necessity of CRMP, new loss-of-function mutations of DHP and CRMP were produced in Drosophila. Intragenic deletion mutants of CRMP were generated by hexamethylphosphonamide (HMPA) mutagenesis (Nairz et al 2004) and P-element mobilization mutagenesis. The chemical mutagen, HMPA, was initially used to produce mutations in the CRMP gene. HMPA is a DNA cross-linking reagent that results in microlesions ranging from approximately 2 bp to 315 bp. Imprecise excision of the P{EP} transposon inserted in the 5′ UTR of exon one of the P{EP}CRMP EP3238 fly line was used to create additional, unambiguous CRMP deletion mutations (Figure 2.1). The P{EP}CRMP EP3238 strain has orange eyes due to the presence of the mini white gene on the P-element vector. The P-element was mobilized from the original insertion site by a providing a source of transposase, which sometimes results in excision events. CRMP excision mutants were created when removal of the P-element takes flanking genomic DNA with it and does not reinsert elsewhere in the genome. The mutants of this type were identified by white eyes due to the loss of the mini white gene. To determine if a CRMP mutation event occurred, subsequent crosses in the screen checked for the DHP null phenotype of normalized wings (Rawls 2006). To determine the nature of the transposon excision lesions and chemical mutagen lesions, PCR analysis was carried out on prepared mutant fly genomic DNA. The primers used in the PCR reactions flank the original insertion site of the transgene or provided genomic fragments of known normal size. After conducting a series of PCR reactions using primer combinations that cover the entire CRMP gene, results showing bands of abnormal size were gel purified and sequenced (DNA Sequencing Facility of Cincinnati Children’s Hospital). The mutagenesis screens resulted in isolation of two null alleles of CRMP (Figure 2.2).

Sequencing of CRMP supla1 showed a 5380 bp deletion of the CRMP gene region. The deletion includes all of exons E1 through E9a, which comprise 42% of the protein open reading frame, including the native start codon and regions indicated in the function of the
CRMP protein *in vitro* (Schmidt and Strittmatter 2007). The sequencing results for the independently derived CRMPsupK1 mutant identified a 153 bp deletion that removes 152 bases at the 3′ end of exon 4 and one base at the start of intron 4. This deletion results in the loss of the 5′ splice site at the junction of E4/I4 and predicted production of a processed transcript with a fusion of the remaining E4/I4 region. The predicted open reading frame created by the E4/I4 fusion leads to a pre-mature stop codon within I4, which would result in a protein product consisting of only the first 10% of the N-terminus or nonsense mediated decay of the transcript and no protein product. Thus, the 50 amino acid deletion is predicted to abolish CRMP protein function. To test whether novel splicing of the mutant transcript might produce a translatable mRNA product, RT-PCR of RNA isolated from CRMPsupK1 adults was conducted. Sequencing of the cDNA detected only sequence containing the E4/I4 fusion (Figure 2.3). In addition, two independent insertion P{PYD2GFPsupK1} transgenic lines, in which the CRMPsupK1 deletion was substituted for the corresponding normal fragment of the P{PYD2GFP} transgene, exhibited no GFP fluorescence (Rawls unpublished). Collectively, these results show that novel splicing events fail to restore the native translational reading frame downstream from the deletion site.

Pure lines for both of the mutations were generated and homozygous adults exhibited no overt abnormal morphology and were fully viable and fertile. Therefore, similar to previously reported mutations that lack DHP activity, loss of CRMP results in no obvious mutant defects in adults (Rawls 2006). Even though no obvious defects in development, morphology or fertility were detected in the adult mutants, data will be presented later in this chapter that suggests these mutations modify signal transduction cascades similar to those demonstrated for mammalian CRMPs.

*Homozygous CRMP mutant embryos exhibit no obvious defects in ventral nerve cord development*

As mentioned before, semaphorins act as chemorepellents through the plexin/neuropilin receptor complex to help guide axonal projections towards their synaptic targets. Previous studies have shown CRMP to play a part in this signaling pathway (reviewed in Schmidt and Strittmatter 2007). CRMPsupK1 homozygous flies exhibit no detectable mutant phenotype in
adults, perhaps due to compensation by activity of other neuronal signaling pathways that do not require CRMP activity. However, it remains possible that CRMP mutants possess delays or defects early in embryogenesis when the nervous system is being developed that are masked in the adult animal. Therefore, the objective of this approach was to determine whether CRMP function contributes to embryonic development of the CNS in Drosophila.

The development of the ventral nervous system during Drosophila embryogenesis was examined in fly embryos that cannot produce a functional form of the CRMP protein. CRMPsupK1 embryos were examined at stages 13 (10 hours 30 min. to 11 hours 30 min. after fertilization) and 16 (15 hours after fertilization) during development. To provide an internal control, the CRMPsupK1 homozygous animals were crossed to animals that are heterozygous for the CRMP deletion mutation (Df(3R)noi-B) and have the genotype w;+; Df(3R)noi-B/TM3, Ser (twi-GFP). From this cross, the embryos that possess GFP fluorescence carry a wild-type copy of the CRMP gene (CRMPsupK1/TM3,CRMP+ Ser P{twi-GFP}) and were easily sorted from the homozygous mutant embryos (CRMPsupK1/Df(3R)noi-B). The embryos at proper stages were stained using an antibody against the CNS proteins (BP102) followed by secondary staining with horseradish peroxidase-coupled secondary antibody. Finally, treatment with 3,3′-diaminobenzidine (DAB) allowed for visualization of the developing ventral nerve cord during embryogenesis. The BP102 primary antibody binds ligands in the anterior commissure, the posterior commissure, and the longitudinal connectives. The embryos were examined for misconnecting or missing commissures that might occur across the midline and for distance discrepancies among connections, which have been detected in various mutants of other neuronal pathway studies (Bhat 2005; Keleman and Dickson 2001). Comparison to 90 wild-type embryos revealed that none of the 71 homozygous CRMP mutant embryos exhibited obvious defects. The ventral nerve cord of homozygous CRMP mutants at stages 13 and 16 during development appears comparable to that of the wild-type siblings at the same stages (Figure 2.4, A-C). These data support the conclusion that CRMP is not necessary for anatomically normal nerve cord development in the embryo.

Drosophila CRMP behaves similar to mammalian CRMP isoforms in signaling cascades
As previously mentioned, many studies have provided results that are consistent with CRMP isoforms playing roles in many pathways, diseases and cellular processes, but the exact function of CRMP remains ill-defined. Guided by biochemical studies in mammals that have revealed associations of CRMP with signal transduction components, this section of the dissertation is directed toward pinpointing CRMP’s role in *D. melanogaster* signaling events. Mammalian cell culture work has identified many pathways and molecules in which vertebrate CRMP isoforms interact *in vitro* (Figure 2.5), and it is members of these pathways that will be targeted to investigate the function of fly CRMP *in vivo*.

The UAS-GAL4 transgenic system (Brand and Perrimon 1993; Duffy 2002) was utilized in experiments to investigate possible interactions of *Drosophila* CRMP with various signal transduction components in which vertebrate CRMP isoforms have been shown to interact. Misexpression of a variety of signal transduction components as UAS responders with several GAL4 drivers was examined in either a CRMP⁺ or CRMPˢᵘᵖᴷ¹ background. The various UAS constructs express homologues of mammalian CRMP interactors, and consist of *P{EP}Rac²Ep3118*, *P{UAS-Rac1.N17}*; *P{UAS-Rac1.V12}*, *P{UAS-Ras}5-1*, *P{UAS-Ras.N17}*, *P{UAS-Ras64B.V14}*, *P{UAS-sggB}MB14*, *P{UAS-sgg.S9A}MB14*, *P{UAS-Rho1.V14}2.1*, *P{UAS-Rho1.N19}*, *P{UAS-Akt1.Exel}*, *P{UAS-Pi3K92E.Exel}*, *P{UAS-Pi3K92E.CAAX}*, and *P{UAS-Pi3K92E.A2860C}*. and were under the control of eye specific neuronal GAL4 drivers, *P{sevEP-GAL4}*, *P{elav-GAL4}*, *P{ey-H-GAL4}*, and *P{GMR-GAL4}*. (stocks obtained through Bloomington Drosophila Stock Center at Indiana University). Transgenic flies with the following genotypes were created and scored: {GAL4 driver} {UAS-X} in CRMP⁺/CRMPˢᵘᵖᴷ¹ heterozygous or CRMPˢᵘᵖᴷ¹/CRMPˢᵘᵖᴷ¹ homozygous backgrounds. If *Drosophila* CRMP is a mediator of the same signaling pathway or interacts with one of the chosen signal transduction proteins tested, then a CRMPˢᵘᵖᴷ¹ effect, either suppression or enhancement of the misexpression phenotype, might result. Crosses showing a strong mutant phenotype at 25°C were retested at 19°C and 29°C to test effects at different growth conditions (Brand *et al.* 1994).

The GAL4 drivers chosen to misexpress the signal transduction proteins have unique expression patterns. The *elav-GAL4* driver is a pan-neuronal GAL4 driver (Zhang *et al.* 2002). The expression pattern includes all post-mitotic neurons, a subset of motor neurons, the ventral nerve cord, and presumptive photoreceptor cells in the developing fly and
mushroom body in the adult fly. The **eyeless-GAL4** driver directs expression of GAL4 in the eye primordia (Hazelett *et al.* 1998). The eye-specific **sevenless-GAL4** driver was also utilized for misexpression in a subset of the photoreceptor cells, the mystery cells and the cone cells during fly eye development (Wan *et al.* 2000, Zhang *et al.* 2001). **GMR-GAL4** drives high level expression in the eye imaginal discs in cells posterior to the morphogenetic furrow (Freeman 1996). These GAL4 drivers were chosen due to their primarily neuronal expression and, with the exception of **elav-GAL4**, malformations created by them are largely limited to the eye, which is a dispensable organ for fly survival.

*CRMPsupK1* mutants regulate Rac signaling during eye development

In mammals, CRMP-2 interacts with proteins regulated by members of the Rho GTPase family to mediate neuronal growth cone collapse (Hall *et al.* 2001; Arimura *et al.* 2005). In the steps of this cascade, the Rho, Rac1, and Rac2 proteins in the GTP-bound state bind to and activate effector molecules. GTP-bound Rac1 and Rac2 promote growth cone extension and neurite outgrowth, while Rho activation leads to growth cone collapse (Kozma *et al.* 1997). Previous research utilizing the neuroblastoma N1E-115 cell line has shown that co-expression of CRMP-2 and dominant active Rac1 leads to peripheral cell collapse, a result opposite the normal Rac morphology (Hall *et al.* 2001). The cell culture data also suggested that CRMP-2 acts downstream of Rac1 (Hall *et al.* 2001). To test whether *Drosophila* CRMP participates downstream of these Rac-mediated signaling events, two Rac homologues in *Drosophila* (Hariharan *et al.* 1995) were misexpressed using a variety of GAL4 drivers. The *Drosophila* Rac1 and Rac2 GTPases share a 92% sequence identity and most differences between the proteins occur in the C-terminal region (Hariharan *et al.* 1995). The Rac1 containing transgenes employed in the misexpression assay include **P{UAS-Rac1.N17}** and **P{UAS-Rac1.V12}**. The **{Rac1.N17}** construct expresses a dominant-negative form of Rac1, whereas the **{Rac1.V12}** construct expresses a constitutively active Rac1. The misexpression phenotypes produced were compared in homozygous *CRMPsupK1* and heterozygous *CRMPsupK1/CRMP+* siblings.

Both Rac1 transgenes, when expressed under control of drivers **elav-GAL4**, **ey-GAL4** or **GMR-GAL4**, were lethal in animals lacking CRMP and in animals that had a wild-type copy
of CRMP. The lethality of these two transgenes when paired with elav-GAL4 had previously been observed in a wild-type genetic background (Fritz and VanBerkum 2002). Since these animals could not escape lethality, a CRMP effect on Rac1 signaling could not be assessed using these drivers. A CRMP effect was only noticeable in animals where the constitutively active form of Rac1 was expressed under the control of the eye-limited driver {sev-EP-GAL4}. In this case, animals heterozygous for CRMPsupK1 exhibited a reduced rough eye phenotype and the phenotype was exaggerated in animals homozygous for CRMPsupK1 (Figure 2.6, C-D). To rule out the possibility that the TM3 balancer in heterozygous animals caused the difference in eye phenotypes, the {sev-EP-GAL4} driver line was crossed to {UAS-Rac1.V12} animals in an otherwise wild-type genetic background. These animals exhibited eye phenotypes similar to w;{sev-EP-GAL4}/+;{UAS-Rac1.V12} CRMPsupK1/TM3, CRMP+ animals. To see if the changes in temperature could influence this phenotype, the same crosses were reset at 19°C and 29°C (Figure 2.6, A-B and E-F). Similar results were observed at these temperatures (Figure 2.6, A-B and E-F); however, a CRMP effect was more noticeable at 19°C (Figure 2.6, A-B). Animals bearing a wild-type copy of CRMP exhibited a reduced eye phenotype and this phenotype was enhanced in animals that completely lacked functional CRMP protein. Furthermore, misexpressing a dominant negative form of Rac1 revealed no CRMP effect: w;{sev-GAL4};{UAS-Rac1.N17} animals exhibited the same reduced rough eye phenotype regardless of the CRMP genetic background (Figure 2.7 A-F) (Fanto et al. 2000). The temperature at which these animals were raised had no influence on the phenotypic outcome.

Misexpression of Rac2 was examined using the enhancer trap {Rac2}EP3118 that expresses a normal form of the protein. In combination with the eye driver {ey-GAL4}, {Rac2}EP3118 produces reduced rough eyes in CRMPsupK1/CRMP+ animals (Figure 2.8, C) (Tseng and Hariharan 2002), yet severely reduced rough eyes in CRMPsupK1/ CRMPsupK1 animals at 25°C (Figure 2.8, D). The same phenotypic trend was observed in these flies at both 19°C and 29°C (Figure 2.8, A-B and E-F). {GMR-GAL4}/+;{UAS- Rac2}EP3118 CRMPsupK1/TM3, CRMP+ animals exhibit a glassy, rough eye phenotype; whereas {GMR-GAL4}/+;{UAS-Rac2}EP3118 CRMP supK1/CRMPsupK1 adults display a substantially more reduced rough eye defect at 25°C (Figure 2.9, C-D). The rough eye phenotype observed in animals of these genotypes was similar when raised at 19°C (Figure 2.9, A-B). Misexpressing Rac2EP3118 using the GMR-GAL4 driver at 29°C resulted in lethality in
homozygous CRMP<sup>supK1</sup> flies; CRMP<sup>supK1</sup>/CRMP<sup>+</sup> siblings survived and displayed severely roughened eyes (Muller et al. 2005) (Figure 2.9 E). w; {GMR-GAL4}/+; {UAS-Rac2}<sup>EP3118</sup> CRMP<sup>supK1</sup>/CRMP<sup>supK1</sup> animals raised at 29°C die at the pupal and late third instar larval stage. Misexpression of Rac2<sup>EP3118</sup> with the pan-neuronal elav-GAL4 driver was also tested, resulting in CRMP<sup>supK1</sup>/CRMP<sup>supK1</sup> animals with slightly rough eyes and normal eye development in CRMP<sup>supK1</sup>/CRMP<sup>+</sup> animals, which was more detectable at 29°C (Figure 2.10 A-F). Thus, the CRMP loss-of-function mutation enhances the misexpression phenotypes of Drosophila Rac2. These data suggest that CRMP functions upstream of Rac2 in semaphorin signaling. Flies misexpressing Rac2<sup>EP3118</sup> via sev-GAL4 had normal eye development regardless of the CRMP genetic background.

The final Rho GTPase to be studied in the misexpression assay was Drosophila Rho1. This GTPase shares an 86% protein sequence identity with the three human Rho GTPases, RhoA, B and C (Hariharan et al. 1995). Mammalian RhoA GTPase has been shown to function upstream of CRMP during LPA signaling, which leads to growth cone collapse in the absence of Sema3A (Arimura et al. 2000). RhoA GTPase activates ROCK, which once activated phosphorylates CRMP at Thr555 causing a conformational change that puts CRMP in an inactive state (Figure 2.5, A). The two Rho transgenes utilized include P<sup>{UAS-Rho1.V14}</sup> and P<sup>{UAS-Rho1.N19}</sup>. The {Rho1.V14} construct misexpresses a constitutively active form of the protein, whereas {Rho1.N19} misexpresses a dominant negative form of the protein. When either transgene was expressed using the elav-GAL4, ey-GAL4 or GMR-GAL4 driver lethality resulted regardless of the CRMP genetic background (Fritz and VanBerkum, 2002). Another unrevealing phenotype was observed when misexpressing both constructs using the sev-GAL4 driver. These animals produced a rough eye phenotype in both a CRMP<sup>+</sup> (Fanto et al. 2000) and CRMP<sup>-</sup> background (Figure 2.11, A-B). These data show that loss of CRMP function has no readily detectable influence on the activity of Rho1 in D. melanogaster upon misexpression with {sev-EP-GAL4}.

*Drosophila* CRMP mediates the Ras/PI3-kinase/Akt/GSK-3β pathway

Previous research has shown that mammalian CRMP-2 plays a role in the PI3-kinase/Akt/GSK-3β signaling cascade that determines neuronal polarity (Yoshimura et al.
Additional studies by the same group led to the discovery that overexpressing the small GTPase Ras in cultured hippocampal neurons resulted in the formation of multiple axons (Yoshimura et al. 2006). Furthermore, they demonstrated that this phenotype was attenuated upon inhibition of PI3-kinase, which functions downstream of Ras. This overexpression of Ras also blocked GSK-3β phosphorylation of CRMP-2. Together, these data point to CRMP-2 regulating cytoskeletal dynamics downstream of Ras signaling to establish axon/dendrite fate in a developing neurite.

To determine if Drosophila CRMP plays a similar role in the same pathway, misexpression studies were conducted. D. melanogaster has a single homologue of mammalian N-ras, H-ras and K-ras genes called Ras1 (Simon et al. 1991). Upon misexpressing wild-type Ras1 in the fly eye using sev-GAL4, elav-GAL4 and ey-GAL4, normal eye development was observed in both CRMPsupK1/CRMP+ and CRMPsupK1/CRMPsupK1 animals. When the {UAS-Ras}5-1 transgene was misexpressed using the {GMR-GAL4} driver in a CRMPsupK1 heterozygous genetic background at 25°C, a rough eye phenotype resulted (Figure 2.12, C); whereas, misexpression in a homozygous CRMPsupK1 background at 25°C resulted in essentially normal development (Figure 2.12, D). A normal eye phenotype was observed in both CRMP+ and CRMP+ animals when the {UAS-Ras}5-1 transgene was misexpressed using the same driver at 19°C (Figure 2.12, A-B). The phenotypic outcome at 29°C was more severe than the result reported for the same crosses at 25°C; however, there was still a noticeable suppression of the rough eye phenotype in CRMP- animals (Figure 2.12, E-F). The data are consistent with previous results that implicate CRMP in Ras signaling, and the data also imply that CRMP functions downstream of the Ras protein to regulate cytoskeleton dynamics.

Further analysis of the interaction between CRMP and Ras was conducted by misexpressing a dominant negative form of Ras1 using the same GAL4 drivers. The {UAS-Ras1.N17} transgene under the control of the elav-GAL4, ey-GAL4 or GMR-GAL4 driver resulted in a rough eye phenotype regardless of the CRMP genetic background (Figure 2.13, A-F). Due to no detectable phenotypic difference in eye morphology, the crosses were not reset at 19°C or 29°C. When the dominant negative form of Ras1 was misexpressed using the sev-GAL4 driver, a CRMPsupK1 effect was not observed and a normal eye was produced. Since no revealing data was obtained using a dominant negative form of Ras1, next a
constitutively active form of Ras2 was utilized. The D. melanogaster Ras2 gene is homologous to the mammalian R-ras gene (Lowe et al. 1987). Upon misexpressing {UAS-Ras64B.V14} with elav-GAL4 or GMR-GAL4 at 25°C, lethality resulted in both CRMP⁺ and CRMP⁻ animals. Previous data report that misexpressing a constitutively active form of Ras2 using the GMR-GAL4 driver results in a reduced rough eye phenotype (Kramer et al. 2003), which is contrary to the results reported here. The researchers did not report the temperature at which the crosses were kept, and a lower temperature could permit rescue from the reported lethality seen at 25°C in this work. In addition, the {Ras64B.V14} transgene was placed under the control of the sev-GAL4 driver and in both CRMP⁺ and CRMP⁻ animals a rough eye phenotype was observed at all three temperatures (Figure 2.14, A-F) (Brand and Perrimon, 1993). Lastly, the same constitutively active form of Ras2 under control of the ey-GAL4 driver resulted in a rough eye phenotype at 19°C (Figure 2.15, A-B), a rough, overgrown eye phenotype at 25°C (Figure 2.15, C-D), and lethality at 29°C in both CRMP wild-type and mutant animals. Therefore, regardless of the driver used to express either a dominant negative or constitutively active form of Ras, a CRMP⁻⁹⁸ effect was not detectable.

Since a CRMP⁻⁹⁸ effect was seen when misexpressing wild-type Drosophila Ras1 in the eye imaginal disc during development in a direction that agrees with CRMP’s role in mammalian Ras signaling, then it is hypothesized that similar results should be obtained when misexpressing other members of this pathway using the same drivers. To test this hypothesis the next molecule immediately downstream of Ras, PI3-kinase, was utilized in the same type of experimentation. In the Ras/PI3-kinase/Akt/GSK-3β pathway GTP bound Ras activates PI3-kinase, PI3-kinase in return activates Akt, which inhibits an inhibitor of CRMP. There are four different PI3-kinase proteins in D. melanogaster; however, only one was available for testing. Three different PI3-kinase92E transgenes were misexpressed using the same neuronal specific GAL4 drivers. P{UAS-Pi3K92E.Exel} transgene expresses a wild-type copy of PI3-kinase, P{UAS-Pi3K92E.CAAX} transgene expresses a constitutively active form of PI3-kinase, and P{UAS-Pi3K92E.A2860C} expresses a dominant negative form of PI3-kinase. Misexpression of all three transgenes with the sev-GAL4 or elav-GAL4 driver results in normal eye development in both wild-type and mutant CRMP genetic backgrounds. The wild-type construct, when misexpressed with either ey-GAL4 or GMR-GAL4 results in a reduced rough eye phenotype regardless of the CRMP state at 25°C (Figure 2.16, C-D and
Figure 2.17, C-D, respectively). The rough eye phenotype was also comparable in both wild-type and mutants at 19°C and 29°C (Figure 2.16, A-B and E-F and Figure 2.17, A-B and E-F). Misexpression of the constitutively active and dominant negative forms of PI3-kinase with ey-GAL4 or GMR-GAL4 results in a rough eye phenotype in both homozygous CRMPsupK1 and heterozygous CRMPsupK1 animals at 25°C (Figure 2.18, A-D and Figure 2.19, A-D). Collectively, these data fail to detect a role for CRMP in PI3-kinase 92E signaling in Drosophila. Very similar CRMP independent phenotypes were also seen using a {UAS-Akt1.Exel} construct, which encodes for a wild-type copy of the only Akt1 protein in D. melanogaster, using the same drivers.

The Drosophila homologue of mammalian GSK-3β is Shaggy (Sgg). Two shaggy containing UAS-constructs were used to detect a CRMPsupK1 effect. The {UAS-sggB} construct contains a wild-type copy of the sgg gene. Misexpression of wild-type sgg using the sev-GAL4 driver results in flies with normal eyes in both CRMP+ and CRMP− animals. When the same sgg construct is under control of the elav-GAL4 driver, a mild rough eye phenotype is detected in both CRMP+ and CRMP− animals at 19°C, 25°C, and 29°C (Figure 2.20, A-F). The rough eye phenotype is most noticeable at 29°C. A CRMPsupK1 effect is only detectable when the sgg construct is expressed using the ey-GAL4 and GMR-GAL4 driver lines. In combination with wild-type CRMP, both crosses result in lethality at 25°C. The animals die as pharate adults or pupae lacking head or significant eye structures (Figure 2.21, A and Figure 2.22, C). In combination with mutant CRMP, both crosses result in adult flies with reduced rough eyes at 25°C (Figure 2.21, B and Figure 2.22, D). The number of adults in a CRMP mutant background that survive is small, demonstrating partial lethality of these genotypes. The {UAS-sggB}/[ey-GAL4]; CRMPsupK1/CRMPsupK1 animals that die prior to adulthood, die as pharate adults with detectable eye structures. When the {UAS-sggB}/[ey-GAL4] animals in either a CRMPsupK1/CRMPsupK1 or CRMPsupK1/CRMP+ genotypic background were raised at 19°C or 29°C no adults eclosed. When the {UAS-sggB}/[GMR-GAL4] animals in either a CRMPsupK1/CRMPsupK1 or CRMPsupK1/CRMP+ genotypic background were raised at 19°C a rough eye was produced (Figure 2.22 A-B) and at 29°C lethality resulted. Collectively, these results are consistent with CRMP functioning downstream of Sgg in the Ras/PI3-kinase/Akt/GSK-3β pathway.
The \{UAS-sgg.S9A\} construct encodes a constitutively active form of the Sgg protein. In combination with the elav-GAL4, ey-GAL4 or GMR-GAL4 driver lethality resulted in both a CRMP\textsuperscript{+} and CRMP\textsuperscript{-} background. When the \{UAS-sgg.S9A\} construct was paired with sev-GAL4 a very mild rough eye phenotype resulted in both wild-type and homozygous CRMP mutant animals (Figure 2.23, A-B). The absence of a CRMP\textsuperscript{supK1} effect on this very mild rough eye phenotype could be due to the abundance of active Sgg having an influence on other signaling pathways that do not require CRMP. In summary, no CRMP effect on sgg.S9A misexpression was detected.

New gain-of-function phenotypes using in vitro mutagenized CRMP

A second approach to produce gain-of-function CRMP phenotypes was carried out to investigate CRMP’s role in Drosophila. X-ray crystallography resolved the structure of human CRMP-2 (Ogg et al. 2006; Stenmark et al. 2007). Interestingly, the divergent region of the Drosophila CRMP and DHP proteins, exon 9, defines a core within the CRMP homotetramer. Features of the CRMP C-terminal region resemble a “gate” regulating access to the core of the protein or the C-terminal region may act as an appendage that potentially interacts with other proteins. Furthermore, serine and threonine residues found in the C-terminal ends of mammalian CRMP isoforms undergo highly conserved phosphorylation events, which have been shown to regulate CRMP activity (Arimura et al. 2000; Brown et al. 2004; Cole et al. 2004).

To determine if the CRMP C-terminal region is functionally important in Drosophila, large deletions within the C-terminal region, amino acids 507-587, were created by in vitro mutagenesis of CRMP cloned in vector pUAST (Brand and Perrimon, 1993) (Figure. 2.24). The transgenic lines containing the mutant CRMP derivatives were crossed to a variety of neuronal GAL4 drivers in either a CRMP\textsuperscript{supK1}/CRMP\textsuperscript{+} or CRMP\textsuperscript{supK1}/CRMP\textsuperscript{supK1} background. In addition to P\{sevEP-GAL4\}, P\{ey-H-GAL4\}, P\{elav-GAL4\}, and P\{GMR-GAL4\}, expression in different regions of the CNS were tested using additional GAL4 drivers: CNS (P\{GawB-GAL4\}389), cholinergic neurons (P\{Cha-GAL4\}), dopaminergic and serotonergic neurons (P\{Ddc-GAL4.L\}), R7 photoreceptor cells (P\{Pan-R7-GAL4\}), RP2, aCC, pCC
neurons ($P\{RN2-GAL4\}$), and pan-neuronal ($P\{GawB-GAL4\}1407$) (Stocks obtained from the Bloomington Drosophila Stock Center at Indiana University).

Among the GAL4 transactivators used to misexpress the CRMP C-terminal deletion constructs, only GMR-GAL4 produced a CRMP-responsive phenotype. $\{UAS-CRMP^{mutA1}\}$ misexpression using the $\{GMR-GAL4\}$ driver resulted in a severe rough eye phenotype in CRMP$^{supK1}$/CRMP$^+$ animals (Figure 2.25, E); this phenotype was normalized in CRMP$^{supK1}$/CRMP$^{supK1}$ animals (Figure 2.25, F). The GMR-GAL4 induced misexpression phenotype was also seen in $\{UAS-CRMP^{mutA2}\}$ and $\{UAS-CRMP^{mutA3}\}$ lines in a CRMP$^{supK1}$ heterozygous background (Figure 2.25, G and I). The eye phenotype reverted to normal in CRMP$^{supK1}$ homozygous animals (Figure 2.25, H and J), as seen in $w;\{UAS-CRMP^{mutA1}\}/\{GMR-GAL4\};$ CRMP$^{supK1}$/CRMP$^{supK1}$ animals. $\{UAS-CRMP\}$ and $\{UAS-DHP\}$ lines; however, produced a mild rough eye phenotype when crossed to the $\{GMR-GAL4\}$ line (Figure 2.25, A and C). In addition, loss of wild-type CRMP did not rescue the phenotype in $\{UAS-CRMP\}$ and $\{UAS-DHP\}$ animals (Figure 2.25, B and D). This mild rough eye phenotype is also seen in animals that only possess the $\{GMR-GAL4\}$ construct (data not shown).
Discussion

A signaling model for *Drosophila* CRMP

The multiple CRMP isoforms in mammals complicate the analysis and interpretation of their individual roles, due to the potential for functional redundancy and interactions (e.g., heteromultimerization). Results from *in vitro* studies focusing on individual CRMP isoforms show that CRMP can function in mammalian signaling pathways that regulate neuronal maturation (Figure 2.5). Does CRMP’s function in these proposed signaling pathways hold true in a native environment? If so, do the *in vivo* experimental outcomes of such studies agree or disagree with what is already known *in vitro*? The single CRMP protein of *Drosophila* offers a model system to elucidate the role of CRMP *in vivo*. In this work, the role that CRMP plays in regulating signaling pathways in which mammalian CRMPs have been shown to act is addressed and a summary of the results can be found in Table 2.1.

The UAS-GAL4 system was employed in these studies to produce dominant phenotypes in adults. Severe developmental defects can be studied, because the eye is a relatively dispensable organ. CRMP mutant suppression or enhancement of the misexpression phenotypes was used to identify CRMP interacting gene products or to establish epistatic relationships amongst the proteins. Many of the misexpression genotypes studied resulted in lethality, which precluded interpretation of CRMP effects. The phenotypes of other misexpression genotypes were unaffected by CRMP, negative results that suggest no role for CRMP in the studied signal transduction system within the tissues in which the GAL4 driver targets. In contrast, four interactions showed CRMP<sup>K1</sup> effects and provide evidence for CRMP protein function in eye development:

- The rough eye phenotype observed when misexpressing wild-type Ras1 using GMR-GAL4 is suppressed in homozygous CRMP<sup>K1</sup> mutants.
- The lethality produced by misexpressing wild-type Sgg using both GMR and eyeless GAL4 drivers is partially rescued in homozygous CRMP<sup>K1</sup> mutants.
- Rough eye phenotypes produced by misexpressing wild-type Rac2 using GMR, eyeless, and elav GAL4 drivers are enhanced in homozygous CRMP<sup>K1</sup> mutants.
• The rough, reduced eye phenotype produced by misexpressing constitutively active Rac1 using sev-GAL4 is enhanced in homozygous CRMPsupK1 mutants.

Fly CRMP appears to play a role in Ras/PI3-kinase/Akt/GSK-3β pathway signaling that is similar to the role that mammalian CRMPs play in axon extension (supported by the first two modifications listed above). In mammals, activated Ras signals to promote a CRMP active state in the cell leading to axon extension and branching (Yoshimura et al. 2006). Misexpression of wild-type Ras using the GMR-GAL4 driver resulted in a rough, overgrown eye phenotype (Figure 2.12), perhaps due to promotion of ectopic axonal projections and the lack of axonal pruning. The rough eye phenotype is converted to a normal eye phenotype in the absence of CRMP. These data are consistent with CRMP mediating Ras signaling through this pathway and with CRMP being a downstream target of Ras. On the other hand, GSK-3β, the Shaggy ortholog in mammals, phosphorylates CRMP rendering it inactive, resulting in growth cone collapse in mammals (Yoshimura et al. 2005). Misexpression of wild-type Shaggy in the eye of CRMP+ flies using either GMR-GAL4 or ey-GAL4 leads to lethality. Loss of a functional CRMP protein rescues the lethality of these animals perhaps by blocking the misexpression signal, a result that is consistent with CRMP functioning downstream of Sgg. In conclusion, these results suggest that insect CRMP regulates signaling through the Ras/GSK-3β pathway in a manner similar to that of mammalian CRMPs.

The enhancement of Rac misexpression phenotypes in animals that lack CRMP (the last two modifications listed above) suggests that CRMP may influence Rac signaling in the fly eye and in a direction that agrees with the proposed model of mammalian CRMP function upstream of Rac in the semaphorin signaling pathway (Figure 2.5). In the absence of Sema3A, Rac proteins have been shown to directly interact with actin to promote polymerization during growth cone outgrowth (Schmidt and Strittmatter, 2007). In the presence of ligand, this activity is blocked by the inactive form of CRMP which binds to alpha2-chimaerin and switches Rac1 to its GDP inactive state (Brown et al. 2004). The inactive CRMP induced sequestration of Rac proteins, blocks their regulation of cytoskeletal dynamics and thereby reduces Rac function in the growth cone (Schmidt and Strittmatter, 2007). Thus, the absence of CRMP protein might be expected to enhance Rac misexpression phenotypes by amplifying Rac-mediated actin polymerization. This prediction was
supported by enhancement of misexpression phenotypes produced by constitutively active
Rac1 or wild-type Rac2 in CRMP\(^{1}\) animals (Figures 2.6, 2.8, 2.9, and 2.10). These CRMP
effects were observed at all growth temperatures.

The misexpression data reported here support fly CRMP function upstream of Rac
activity. Other research data collected by Hall and colleagues suggests that CRMP functions
downstream of Rac1 in an alternate signaling pathway (Hall \textit{et al.} 2001). In neuroblastoma
N1E-115 cells, they observed that simultaneously expressing CRMP-2 with dominant active
Rac1 V12 inhibited Rac morphology, and in cells already expressing Rac1 V12 subsequent
transfection of CRMP-2 led to peripheral collapse of the Rac morphology, which involved
Rho activation (Hall \textit{et al.} 2001). Their data shows that the Rac2 misexpression phenotype is
suppressed by microinjection of wild-type CRMP-2, which is consistent with our Rac
misexpression data. The argument of whether CRMP functions upstream or downstream of
members of the Rho GTPase family could also depend on the signaling cascade in question.
In the mammalian Sema signaling pathway, CRMP functions upstream of Rac, and in the
mammalian lysophosphatidic acid (LPA) signaling pathway, CRMP functions downstream of
Rho (Figure 2.5).

The lack of a CRMP effect on Rho1 GTPase activity could imply that \textit{Drosophila}
CRMP does not regulate growth cone dynamics via the LPA signaling pathway during eye
development. However, it could just as easily be hypothesized that alternative negative
guidance cues function within the fly eye to carry out growth cone collapse in the absence of
native LPA signaling. No detection of a CRMP influence when placing constitutively active
Rho1 under control of \textit{sev-GAL4} in either a homozygous or heterozygous \textit{CRMP}\textsuperscript{supk1}
background makes these explanations more convincing (Figure 2.11). Another argument for
not observing suppression of the Rho1 overexpression phenotype produced by other
transactivators in CRMP\(^{1}\) animals could be due to the fact that the lethality is so severe and
occurs at such an early stage in development that it is inescapable. Since misexpressing
Rho1 resulted in such a persistent severe lethality phenotype at the embryonic stage, it is not
surprising that loss of CRMP function could not compensate for the severe errors that ensued.
It is impossible to distinguish between the severities of dead embryos and make an insightful
conclusion with regards to CRMP.
The inability to detect a \textit{CRMP}^{supK1} effect on the eye misexpression phenotypes produced by the various forms of Pi3K92E could be due to a variety of explanations. This result may perhaps be expected if PI3-kinase regulates cytoskeletal dynamics independent of CRMP via an alternate pathway during eye development. In addition, there are four PI3-kinase proteins in \textit{Drosophila} and the lack of a \textit{CRMP}^{supK1} effect could be due to the PI3-kinase chosen not interacting in the same pathway as CRMP in the cells targeted by the GAL4 drivers.

\textit{Future Direction}

Because only the CRMP form of the two encoded \textit{CRMP} gene products, CRMP and DHP, is expressed in the nervous system, the ability of \textit{CRMP}^{supK1} to suppress/enhance the misexpression phenotypes generated by neuron-specific GAL4 drivers indicates that these effects derive from CRMP, rather than DHP. Testing that assumption would be possible by genetically incorporating \textit{P\{UAS-CRMP\}} and \textit{P\{UAS-DHP\}} constructs in those tests. I predict that \textit{P\{UAS-CRMP\}} would block the suppression/enhancement effect, whereas \textit{P\{UAS-DHP\}} would not. For example, if the normal eye phenotype seen in \textit{GMR-GAL4>UAS-Ras5-1 CRMP} \textsuperscript{-} animals could be changed back to the rough eye phenotype observed in \textit{GMR-GAL4>UAS-Ras5-1 CRMP} \textsuperscript{+} animals, by simply crossing the latter flies to flies that have the \textit{\{UAS-CRMP\}} construct, then CRMPs involvement in these signaling cascades would be further confirmed. These crosses would help pinpoint the CRMP protein in causing the enhancement or suppression and not the DHP protein since the \textit{CRMP}^{supK1} deletion is in a region common to both isoforms.

Further investigation into the role \textit{Drosophila} CRMP plays in signaling pathways that involve these proteins could be conducted by utilizing UAS constructs that contain a modified version of the CRMP protein. It has been shown that the C-terminal region of the protein is necessary for interactions with proteins like Sra-1, Numb and tubulin. It is also residues found in the C-terminal region of the CRMP protein that undergo the various phosphorylation events by members of the semaphorin and LPA signaling pathways mentioned previously, which result in an inactive form of the protein (Arimura \textit{et al.} 2000; Brown \textit{et al.} 2004; Uchida \textit{et al.} 2005). \textit{In vitro} mutagenesis has been done on the CRMP protein to create large C-terminal deletion mutants in hopes of generating a functionally
modified form of *Drosophila* CRMP. Misexpression studies with *GMR-GAL4* imply that the C-terminal region of the *Drosophila* CRMP protein is important to the functionality of the protein. Flies that possess these constructs could also be used to look for additional phenotypic modification of the misexpression phenotypes produced by the various signal transduction mediators that show a \( CRMP^{supk1} \) effect.

Finally, the misexpression experiments could be repeated to see if the results collected using \( CRMP^{supk1} \) are reproducible using the other unambiguous \( CRMP^{supla1} \) mutant allele, if so; the data would provide additional verification that CRMP interacts with members of the signaling pathways tested.
Excision mutagenesis screen for \textit{CRMP} region mutations

\begin{align*}
G_0 & \quad \varnothing \ \text{w; } P[EP] \text{crmp}^{EP3238} \quad \times \quad \varnothing \ \text{w; } \text{TMS, Sb } P[\Delta2-3]/Dr \\
G_1 & \quad \varnothing \ \text{w; } P[EP] \text{crmp}^{EP3238} \quad / \quad \text{TMS, Sb } P[\Delta2-3] \quad \times \quad \varnothing \ \text{w; } \text{ri} \\
G_2 & \quad \text{single } \varnothing \ \text{w; } **/\text{ri} \quad \times \quad \varnothing \ \text{w; } \text{r}^{70b26}; \text{ri crmp}^{\text{supA4}}/\text{TMS, Sb ri } [\text{Cr}^+] \\
G_3 & \quad \text{examine } \varnothing \ \text{w; } \text{r}^{70b26}; \quad **/\text{ri crmp}^{\text{supA4}} \text{ for suppression of r wing phenotype}
\end{align*}

\textbf{Figure 2.1. P-element mobilization screen for \textit{CRMP} deletion mutants.} The parental line, \(P[EP] \text{CRMP}^{EP3238}\), has a P-element transposon inserted in the 5'UTR region of exon 1 of the \textit{CRMP} gene. This transposon also has the \(w^+\) marker resulting in flies with orange eyes. When crossed with a fly line that carries P-element transposase (\(\Delta2-3\)) (cross \(G_0\)), imprecise excision mutants can subsequently be generated and identified by white eye phenotype (\(G_2\) males). Single \(G_2\) males, each representing a unique excision event, were crossed to \(w/r^{70b26}; \text{ri crmp}^{\text{supA4}}/\text{TMS, Sb ri } [\text{Cr}^+]\) females and the wings of non-\(Sb\) and non-\(ri\) male progeny were examined for normalized wings. This \(G_2\) cross ensures that the excision resulted in a non-functional copy of the \textit{CRMP} gene due to the suppression of the rudimentary wing phenotype indicating a loss of DHP activity in these animals. This screen resulted in the \textit{CRMP}^{\text{supaal}} suppressor mutant that was further characterized.
Figure 2.2. CRMP loss-of-function mutations. All mutations were selected by their DHP-null phenotypes. In the above diagram, the double black line represents the CRMP gene region, blue blocks represent CRMP exons, blue dashed blocks represent the alternative E9 exons, pink bars underline the location of the CRMP mutations, and the green triangle shows the insertion site of the original P element used to generate the supΔIa1 mutant. supK1 is a 153 bp deletion that removes the E4/I4 junction, apparently resulting in a frameshift. The supΔIa1 mutation is a complex rearrangement that deletes most of the CRMP ORF.
Figure 2.3. Novel CRMP\textsuperscript{supK1} transcript. The primer combination used in RT-PCR to analyze the CRMP cDNA made from wild-type (2u) animals, homozygous supK1 animals, heterozygous supK1 animals or a control CRMP cDNA sample is show in the CRMP gene diagram. These primers span the CRMP\textsuperscript{supK1} deletion region (pink square). Primer one is complementary to sequence in exon 2 and the antisense primer 2 is complementary to sequence in exon 8. A wild-type CRMP cDNA sample should produce a 650bp band after PCR utilizing these primers. Lane 2 shows the expected 650 bp band from wild-type cDNA sample. Lane 3 shows the absence of the normal 650 bp band and a novel smaller RT-PCR product for the homozygous supK1 mutant sample (red circle). Lanes 4 and 5 contain PCR product from heterozygous supK1 animals, which result in a 650 bp band. Lane 6 contains control CRMP cDNA and produces the expected 650 bp band. Lane 7 contains the 1Kb Plus Ladder for comparison.
Figure 2.4. *CRMP\textsuperscript{supK1}* embryogenesis. *supK1* embryos were carefully staged and sequentially stained using antibodies against the CNS axons (BP102 from DSHB). The mutant embryos were compared to their wild-type siblings (A). Stage 13 and 16 homozygous mutant embryos show no obvious defects during neurogenesis (B & C). The mutants exhibit normal axon scaffold formation at the midline and no gaps in the longitudinal connectives.
Figure 2.5. A model for pathways involving CRMP. CRMPs exist in two states within the cell, an inactive or active state (A and B). During growth cone collapse triggered by LPA or Sema signaling, CRMP is found in a phosphorylated inactive state (A, purple stars). In this phosphorylated state CRMP undergoes a conformational change that perturbs its interaction with proteins like Numb, Sra, and Tubulin; thus preventing actin and microtubule polymerization leading to axon collapse. During growth cone extension and branching, the growing neurite encounters adhesion molecules and positive guidance cues that promote a CRMP active conformation, which allows for actin and microtubule stability through CRMPs interactions with Numb, Sra, and Tubulin (B).
Figure 2.6. Expression of constitutively active \textit{Rac1} under the control of a \textit{sevenless} GAL4 driver in a wild-type or \textit{CRMP} mutant background. Pictures of adult male eyes (A-F) are shown. The genotypes are as follows: (A) \textit{w; sev-GAL4/+; UAS-Rac1.V12 CRMP}\textsuperscript{supK1}/TM3 raised at 19°C, (B) \textit{w; sev-GAL4/+; UAS-Rac1.V12 CRMP}\textsuperscript{supK1}/CRMP\textsuperscript{supK1} raised at 19°C, (C) \textit{w; sev-GAL4/+; UAS-Rac1.V12 CRMP}\textsuperscript{supK1}/TM3 raised at 25°C, (D) \textit{w; sev-GAL4/+; UAS-Rac1.V12 CRMP}\textsuperscript{supK1}/CRMP\textsuperscript{supK1} raised at 25°C (E) \textit{w; sev-GAL4/+; UAS-Rac1.V12 CRMP}\textsuperscript{supK1}/TM3 raised at 29°C, (F) \textit{w; sev-GAL4/+; UAS-Rac1.V12 CRMP}\textsuperscript{supK1}/CRMP\textsuperscript{supK1} raised at 29°C. The misexpression phenotype in a wild-type genetic background (A, C, E) is enhanced in a \textit{CRMP}\textsuperscript{supK1} homozygous genetic background (B, D, F).
Figure 2.7. Misexpression of dominant negative Rac1 using the sevenless GAL4 driver in a wild-type or CRMP mutant background. Pictures of adult male eyes (A-F) are shown. The genotypes are as follows: (A) w; sev-GAL4/+; UAS-Rac1.N17 CRMPsupK1/TM3 raised at 19°C, (B) w; sev-GAL4/+; UAS-Rac1.N17 CRMPsupK1/CRMPsupK1 raised at 19°C, (C) w; sev-GAL4/+; UAS-Rac1.N17 CRMPsupK1/TM3 raised at 25°C, (D) w; sev-GAL4/+; UAS-Rac1.N17 CRMPsupK1/CRMPsupK1 raised at 25°C (E) w; sev-GAL4/+; UAS-Rac1.N17 CRMPsupK1/TM3 raised at 29°C, (F) w; sev-GAL4/+; UAS-Rac1.N17 CRMPsupK1/CRMPsupK1 raised at 29°C. The misexpression eye phenotype in a wild-type genetic background (A, C, E) is comparable to the misexpression eye phenotype in a CRMPsupK1 homozygous genetic background (B, D, F).
Figure 2.8. The eyeless GAL4 driver was used to drive the expression of wild-type Rac2 in a wild-type or CRMP mutant genetic background. Pictures of adult male eyes (A-F) are shown. The genotypes are as follows: (A) w; ey-GAL4/+; UAS-Rac2<sup>EP3118</sup> CRMP<sup>supK1</sup>/TM3 raised at 19°C, (B) w; ey-GAL4/+; UAS-Rac2<sup>EP3118</sup> CRMP<sup>supK1</sup>/CRMP<sup>supK1</sup> raised at 19°C, (C) w; ey-GAL4/+; UAS-Rac2<sup>EP3118</sup> CRMP<sup>supK1</sup>/TM3 raised at 25°C, (D) w; ey-GAL4/+; UAS-Rac2<sup>EP3118</sup> CRMP<sup>supK1</sup>/CRMP<sup>supK1</sup> raised at 25°C (E) w; ey-GAL4/+; UAS-Rac2<sup>EP3118</sup> CRMP<sup>supK1</sup>/TM3 raised at 29°C, (F) w; ey-GAL4/+; UAS-Rac2<sup>EP3118</sup> CRMP<sup>supK1</sup>/CRMP<sup>supK1</sup> raised at 29°C. The misexpression eye phenotype in a wild-type genetic background (A, C, E) is enhanced in a CRMP<sup>supK1</sup> homozygous genetic background (B, D, F).
Figure 2.9. *GMR-GAL4* was used to drive the expression of wild-type *Rac2* in a wild-type or *CRMP* mutant genetic background. Pictures of adult male eyes (A-E) are shown. The genotypes are as follows: (A) *w; GMR-GAL4/+; UAS-Rac2\textsuperscript{EP3118} CRMP\textsuperscript{supK1}/TM3* raised at 19°C, (B) *w; GMR-GAL4/+; UAS-Rac2\textsuperscript{EP3118} CRMP\textsuperscript{supK1}/CRMP\textsuperscript{supK1}* raised at 19°C, (C) *w; GMR-GAL4/+; UAS-Rac2\textsuperscript{EP3118} CRMP\textsuperscript{supK1}/TM3* raised at 25°C, (D) *w; GMR-GAL4/+; UAS-Rac2\textsuperscript{EP3118} CRMP\textsuperscript{supK1}/CRMP\textsuperscript{supK1}* raised at 25°C (E) *w; GMR-GAL4/+; UAS-Rac2\textsuperscript{EP3118} CRMP\textsuperscript{supK1}/TM3* raised at 29°C. The *w; GMR-GAL4/+; UAS-Rac2\textsuperscript{EP3118} CRMP\textsuperscript{supK1}/CRMP\textsuperscript{supK1}* animals when raised at 29°C resulted in lethality. The misexpression eye phenotype in a wild-type genetic background (A, C, E) is enhanced in a *CRMP\textsuperscript{supK1}* homozygous genetic background (B, D).
Figure 2.10. *elav-GAL4* was used to drive the expression of wild-type *Rac2* in a wild-type or *CRMP* mutant genetic background. Pictures of adult male eyes (A-F) are shown. The genotypes are as follows: (A) *w* *elav-GAL4*; +; *UAS-Rac2*\(^{EP3118}\) *CRMP\(^{supK1}/TM3* raised at 19°C, (B) *w* *elav-GAL4*; +; *UAS-Rac2*\(^{EP3118}\) *CRMP\(^{supK1}/CRMP\(^{supK1}\)* raised at 19°C, (C) *w* *elav-GAL4*; +; *UAS- Rac2*\(^{EP3118}\) *CRMP\(^{supK1}/TM3* raised at 25°C, (D) *w* *elav-GAL4*; +; *UAS-Rac2*\(^{EP3118}\) *CRMP\(^{supK1}/CRMP\(^{supK1}\)* raised at 25°C, (E) *w* *elav-GAL4*; +; *UAS-Rac2*\(^{EP3118}\) *CRMP\(^{supK1}/TM3* raised at 29°C, (F) *w* *elav-GAL4*; +; *UAS-Rac2*\(^{EP3118}\) *CRMP\(^{supK1}/CRMP\(^{supK1}\)* raised at 29°C. The misexpression eye phenotype in a wild-type genetic background (A, C, E) is enhanced in a *CRMP\(^{supK1}\)* homozygous genetic background (B, D, F).
Figure 2.11. *sev-GAL4* was used to drive the expression of constitutively active *Rho1* in a wild-type or *CRMP* mutant genetic background. Pictures of adult male eyes (A-B) are shown. The genotypes are as follows: (A) *w; sev-GAL4/++; UAS-Rho1.V14 CRMP*supK1/*TM3* raised at 25°C, (B) *w; sev-GAL4/++; UAS-Rho1.V14 CRMP*supK1/*CRMP*supK1* raised at 25°C. The misexpression eye phenotypes in a wild-type genetic background (A) are unchanged in a *CRMP*supK1* homozygous genetic background (B).
Figure 2.12. **GMR-GAL4** was used to drive the expression of wild-type *Ras1* in a wild-type or **CRMP** mutant genetic background. Pictures of adult male eyes (A-F) are shown. The genotypes are as follows: (A) *w; GMR-GAL4/UAS-Ras1; CRMP^supK1/TM3* raised at 19°C, (B) *w; GMR-GAL4/UAS-Ras1; CRMP^supK1/CRMP^supK1* raised at 19°C, (C) *w; GMR-GAL4/UAS-Ras1; CRMP^supK1/TM3* raised at 25°C, (D) *w; GMR-GAL4/ UAS-Ras1; CRMP^supK1/CRMP^supK1* raised at 25°C (E) *w; GMR-GAL4/UAS-Ras1; CRMP^supK1/TM3* raised at 29°C, (F) *w; GMR-GAL4/UAS-Ras1; CRMP^supK1/CRMP^supK1* raised at 29°C. The misexpression eye phenotype in a wild-type genetic background (A, C, E) is suppressed in a **CRMP^supK1** homozygous genetic background (B, D, F).
Figure 2.13. Expression of dominant negative Ras1 using elav-GAL4, ey-GAL4 or GMR-GAL4 in a wild-type or CRMP mutant genetic background. Pictures of adult female (A-B) and male (C-D) eyes are shown. The genotypes are as follows: (A) w UAS-Ras.N17/elav-GAL4; +; CRMPsupK1/TM3 raised at 25°C, (B) w UAS-Ras.N17/elav-GAL4; +; CRMPsupK1/CRMPsupK1 raised at 25°C, (C) w UAS-Ras.N17; ey-GAL4/+; CRMPsupK1/TM3 raised at 25°C, (D) w UAS-Ras.N17; ey-GAL4/+; CRMPsupK1/CRMPsupK1 raised at 25°C, (E) w UAS-Ras.N17; GMR-GAL4/+; CRMPsupK1/TM3 raised at 25°C, (F) w UAS-Ras.N17; GMR-GAL4/+; CRMPsupK1/CRMPsupK1 raised at 25°C. The misexpression eye phenotypes in a wild-type genetic background (A, C, E) are unchanged in a CRMPsupK1 homozygous genetic background (B, D, F).
Figure 2.14. Misexpression of constitutively active *Ras* using the *sevenless* GAL4 driver in a wild-type or *CRMP* mutant background. Pictures of adult male eyes (A-F) are shown. The genotypes are as follows: (A) *w; sev-GAL4/UAS-Ras64B.V14; CRMP\textsuperscript{supK1}/TM3* raised at 19°C; (B) *w; sev-GAL4/UAS-Ras64B.V14; CRMP\textsuperscript{supK1}/CRMP\textsuperscript{supK1}* raised at 19°C; (C) *w; sev-GAL4/UAS-Ras64B.V14; CRMP\textsuperscript{supK1}/TM3* raised at 25°C; (D) *w; sev-GAL4/UAS-Ras64B.V14; CRMP\textsuperscript{supK1}/CRMP\textsuperscript{supK1}* raised at 25°C; (E) *w; sev-GAL4/UAS-Ras64B.V14; CRMP\textsuperscript{supK1}/TM3* raised at 29°C; (F) *w; sev-GAL4/UAS-Ras64B.V14; CRMP\textsuperscript{supK1}/CRMP\textsuperscript{supK1}* raised at 29°C. A difference in the misexpression eye phenotype between a wild-type genetic background (A, C, E) and a *CRMP\textsuperscript{supK1}* homozygous genetic background (B, D, F) is not detectable.
Figure 2.15. Misexpression of constitutively active Ras2 using the eyeless GAL4 driver in a wild-type or CRMP mutant background. Pictures of adult male eyes (A-D) are shown. The genotypes are as follows: (A) w; ey-GAL4/UAS-Ras64B.V14; CRMP<sub>supK1</sub>/TM3 raised at 19°C, (B) w; ey-GAL4/UAS-Ras64B.V14; CRMP<sub>supK1</sub>/CRMP<sub>supK1</sub> raised at 19°C, (C) w; ey-GAL4/UAS-Ras64B.V14; CRMP<sub>supK1</sub>/TM3 raised at 25°C, (D) w; ey-GAL4/UAS-Ras64B.V14; CRMP<sub>supK1</sub>/CRMP<sub>supK1</sub> raised at 25°C. Expression of constitutively active Ras2 using ey-GAL4 results in lethality in both CRMP<sup>+</sup> and CRMP<sup>−</sup> animals at 29°C. A difference is not detectable in the misexpression eye phenotype between a wild-type genetic background (A, C) and a CRMP<sub>supK1</sub> homozygous genetic background (B, D).
Figure 2.16. Expression of wild-type PI3-kinase92E using *ey-GAL4* in a wild-type or CRMP mutant genetic background. Pictures of adult male eyes (A-F) are shown. The genotypes are as follows: (A) *w; UAS-Pi3K92E. Exel/ey-GAL4; CRMP<sup>supK1</sup>/TM3* raised at 19°C, (B) *w; UAS-Pi3K92E. Exel/ey-GAL4; CRMP<sup>supK1</sup>/CRMP<sup>supK1</sup>* raised at 19°C, (C) *w; UAS-Pi3K92E. Exel/ey-GAL4; CRMP<sup>supK1</sup>/TM3* raised at 25°C, (D) *w; UAS-Pi3K92E. Exel/ey-GAL4; CRMP<sup>supK1</sup>/CRMP<sup>supK1</sup>* raised at 25°C, (E) *w; UAS-Pi3K92E. Exel/ey-GAL4; CRMP<sup>supK1</sup>/TM3* raised at 29°C, (F) *w; UAS-Pi3K92E. Exel/ey-GAL4; CRMP<sup>supK1</sup>/CRMP<sup>supK1</sup>* raised at 29°C. The misexpression eye phenotypes in a wild-type genetic background (A, C, E) are unchanged in a *CRMP<sup>supK1</sup>* homozygous genetic background (B, D, F).
Figure 2.17. Expression of wild-type PI3-kinase92E using GMR-GAL4 in a wild-type or CRMP mutant genetic background. Pictures of adult male eyes (A-F) are shown. The genotypes are as follows: (A) w; UAS-Pi3K92E.Exel/GMR-GAL4; CRMPsupK1/TM3 raised at 19°C, (B) w; UAS-Pi3K92E.Exel/GMR-GAL4; CRMPsupK1/CRMPsupK1 raised at 19°C, (C) w; UAS-Pi3K92E.Exel/GMR-GAL4; CRMPsupK1/TM3 raised at 25°C, (D) w; UAS-Pi3K92E.Exel/GMR-GAL4; CRMPsupK1/CRMPsupK1 raised at 25°C, (E) w; UAS-Pi3K92E.Exel/GMR-GAL4; CRMPsupK1/TM3 raised at 29°C, (F) w; UAS-Pi3K92E.Exel/GMR-GAL4; CRMPsupK1/CRMPsupK1 raised at 29°C. The misexpression eye phenotypes in a wild-type genetic background (A, C, E) are unchanged in a CRMPsupK1 homozygous genetic background (B, D, F).
Figure 2.18. Expression of constitutively active PI3-Kinase92E using *ey-GAL4* or *GMR-GAL4* in a wild-type or *CRMP* mutant genetic background. Pictures of adult female eyes (A-D) are shown. The genotypes are as follows: (A) *w; UAS-Pi3K92E.CAAX/ey-GAL4; CRMPsupK1/TM3* raised at 25°C, (B) *w; UAS-Pi3K92E.CAAX/ey-GAL4; CRMPsupK1/CRMPsupK1* raised at 25°C, (C) *w; UAS-Pi3K92E.CAAX/GMR-GAL4; CRMPsupK1/TM3* raised at 25°C, (D) *w; UAS-Pi3K92E.CAAX/GMR-GAL4; CRMPsupK1/CRMPsupK1* raised at 25°C. The misexpression eye phenotypes in a wild-type genetic background (A, C) are unchanged in a *CRMPsupK1* homozygous genetic background (B, D).
Figure 2.19. Expression of dominant negative PI3-Kinase92E using *ey-GAL4* or *GMR-GAL4* in a wild-type or *CRMP* mutant genetic background. Pictures of adult male eyes (A-D) are shown. The genotypes are as follows: (A) *w; UAS-Pi3K92E.A2860C/ey-GAL4; CRMPsupK1/TM3* raised at 25°C, (B) *w; UAS-Pi3K92E.A2860C /ey-GAL4; CRMPsupK1/CRMPsupK1* raised at 25°C, (C) *w; UAS-Pi3K92E.A2860C /GMR-GAL4; CRMPsupK1/CRMPsupK1* raised at 25°C, (D) *w; UAS-Pi3K92E.A2860C /GMR-GAL4; CRMPsupK1/CRMPsupK1* raised at 25°C. The misexpression eye phenotypes in a wild-type genetic background (A, C) are unchanged in a *CRMPsupK1* homozygous genetic background (B, D).
elav-GAL4 was used to drive the expression of wild-type sgg in a wild-type or CRMP mutant genetic background. Pictures of adult male eyes (A-F) are shown. The genotypes are as follows: (A) w elav-GAL4; UAS-sggB/++; CRMP\textsuperscript{supK1}/TM3 raised at 19°C, (B) w elav-Gal4; UAS-sggB/++; CRMP\textsuperscript{supK1}/CRMP\textsuperscript{supK1} raised at 19°C, (C) w elav-GAL4; UAS-sggB/++; CRMP\textsuperscript{supK1}/TM3 raised at 25°C, (D) w elav-GAL4; UAS-sggB/++; CRMP\textsuperscript{supK1}/CRMP\textsuperscript{supK1} raised at 25°C (E) w elav-GAL4; UAS-sggB/++; CRMP\textsuperscript{supK1}/TM3 raised at 29°C, (F) w elav-GAL4; UAS-sggB/++; CRMP\textsuperscript{supK1}/CRMP\textsuperscript{supK1} raised at 29°C. A difference is not detectable in the misexpression eye phenotype between a wild-type genetic background (A, C, E) and a CRMP\textsuperscript{supK1} homozygous genetic background (B, D, F).
Figure 2.21. *ey-GAL4* was used to drive the expression of wild-type *sgg* in a wild-type or CRMP mutant genetic background. Picture of an adult male eye (B) is shown. The genotypes are as follows: (A) *w*; *ey-GAL4/UAS-sggB; CRMP<sup>supK1</sup>/TM3* raised at 25°C is lethal and animals die as pharate adults without detectable eye structures (as shown in A) or pupae, (B) *w*; *ey-GAL4/UAS-sggB; CRMP<sup>supK1</sup>/CRMP<sup>supK1</sup> raised at 25°C. Animals with the former genotype either eclose as adults (as shown in B) or die as pharate adults with small eyes. Expression of *sgg* using *ey-GAL4* results in lethality in both CRMP<sup>+</sup> and CRMP<sup>-</sup> animals at 19°C and 29°C. The misexpression eye phenotype in a wild-type (*CRMP<sup>supK1</sup>/CRMP<sup>+</sup>*) genetic background (A) is suppressed in a *CRMP<sup>supK1</sup>* homozygous genetic background (B).
Figure 2.22. *GMR-GAL4* was used to drive the expression of wild-type *sgg* in a wild-type or *CRMP* mutant genetic background. Pictures of adult male eyes (A-F) are shown. The genotypes are as follows: (A) *w; GMR-GAL4/UAS-sggB; CRMP^{supK1}/TM3* raised at 19°C, (B) *w; GMR-GAL4/UAS-sggB; CRMP^{supK1}/CRMP^{supK1}* raised at 19°C, (C) *w; GMR-GAL4/UAS-sgg; CRMP^{supK1}/TM3* raised at 25°C is lethal and animals die as pharate adults, (D) *w; GMR-GAL4/ UAS-sggB; CRMP^{supK1}/CRMP^{supK1}* raised at 25°C. The black arrow in (C) points to the proboscis, the only detectable head feature in the pharate adult. Expression of *sgg* using *GMR-GAL4* results in lethality in both CRMP⁺ and CRMP⁻ animals at 29°C. The misexpression eye phenotype in a wild-type genetic background (A, C) is suppressed in a *CRMP^{supK1}* homozygous genetic background (B, D).
Figure 2.23. Expression of constitutively active sgg using sev-GAL4 in a wild-type or CRMP mutant genetic background. Pictures of adult male eyes (A-B) are shown. The genotypes are as follows: (A) \( w; UAS\text{-}sgg.S9A/\text{sev-GAL4}; CRMP^{\text{supK1}}/\text{TM3} \) raised at 25°C, (B) \( w; UAS\text{-}sgg.S9A/\text{sev-GAL4}; CRMP^{\text{supK1}}/CRMP^{\text{supK1}} \) raised at 25°C. The misexpression eye phenotypes in a wild-type genetic background (A) are unchanged in a \( CRMP^{\text{supK1}} \) homozygous genetic background (B).
Figure 2.24. *In vitro* mutagenized CRMP carboxyl terminus. Protein alignment of the C-terminal domain residues for *Pseudomonas* DHP, human DHP, *Drosophila* DHP, human CRMP-1, CRMP-2, CRMP-4, and CRMP-5, and *Drosophila* CRMP. Transgenic animals were generated by deleting subsets of the ~80 amino acid C-terminal domain (pink, green and blue triangles). Deletions remove the potential phosphorylation sites of *Drosophila* CRMP. Mammalian CRMP phosphorylation sites are highlighted by the pink asterisks.
Figure 2.25. CRMP misexpression phenotype. Pictures of adult male eyes (A-J) are shown. All animals were raised at 25°C and the genotypes are as follows: (A) w; UAS-DHP/GMR-GAL4; CRMPsupK1/TM3, (B) w; UAS-DHP/GMR-GAL4; CRMPsupK1/CRMPsupK1, (C) w; UAS-CRMP/GMR-GAL4; CRMPsupK1/TM3, (D) w; UAS-CRMP/GMR-GAL4; CRMPsupK1/CRMPsupK1, (E) w; UAS-CRMPmutΔ1/GMR-GAL4; CRMPsupK1/TM3, (F) w; UAS-CRMP/GMR-GAL4; CRMPsupK1/CRMPsupK1, (G) w; UAS-CRMPmutΔ2/GMR-GAL4; CRMPsupK1/CRMPsupK1, (H) w; UAS-CRMPmutΔ2/GMR-GAL4; CRMPsupK1/CRMPsupK1, (I) w; UAS-CRMPmutΔ3/GMR-GAL4; CRMPsupK1/TM3, (J) w; UAS-CRMPmutΔ3/GMR-GAL4; CRMPsupK1/CRMPsupK1. Misexpression of DHP and CRMP cDNA resulted in a mild rough eye phenotype (A and C). The phenotype was slightly suppressed in a homozygous CRMP
genetic background (B and D). Misexpression of the C-terminal deletion forms of CRMP using \textit{[GMR-GAL4]} resulted in a fused facet rough eye phenotype (E, G, and I). This phenotype was rescued (F and H) or partially rescued (J) in a homozygous \textit{CRMP} mutant background.
Table 2.1. CRMP interaction results at 25°C.

<table>
<thead>
<tr>
<th>(UAS-RIK92E,Exel8) (wt)</th>
<th>(sev&gt;Gal4)</th>
<th>(crisv&gt;Gal4)</th>
<th>(crm&gt;Gal4)</th>
<th>(GMR&gt;Gal4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal → Normal</td>
<td>Normal → Normal</td>
<td>Reduced Rough eye → Reduced Rough eye</td>
<td>Rough overgrown eye → Rough overgrown eye</td>
<td></td>
</tr>
<tr>
<td>(UAS-RIK92E,Exel8) (dominant negative)</td>
<td>Normal → Normal</td>
<td>Normal → Normal</td>
<td>Reduced Rough eye → Reduced Rough eye</td>
<td>Rough eye → Rough eye</td>
</tr>
<tr>
<td>(UAS-RIK92E,GAAX) (constitutively active)</td>
<td>Normal → Normal</td>
<td>Normal → Normal</td>
<td>Rough eye → Rough eye</td>
<td>Rough eye → Rough eye</td>
</tr>
<tr>
<td>(EP)Rac2^{2011} (wt)</td>
<td>Normal → Normal</td>
<td>Normal eye → Slight rough eye</td>
<td>Slight rough eye (kidney shaped) → No eye or small, reduced eye</td>
<td>Reduced Rough eye → Reduced Rough eye, very narrow</td>
</tr>
<tr>
<td>(UAS-Rac1,NN17) (dominant negative)</td>
<td>Narrow Rough eye → Narrow, Rough eye</td>
<td>Lethal → Lethal</td>
<td>Lethal → Lethal</td>
<td>Lethal → Lethal</td>
</tr>
<tr>
<td>(UAS-Rac1,NN13) (constitutively active)</td>
<td>Narrow, Reduced eye → Narrow, More Reduced eye</td>
<td>Lethal → Lethal</td>
<td>Lethal → Lethal</td>
<td>Lethal → Lethal</td>
</tr>
<tr>
<td>(UAS-Ras)S-1 (wt)</td>
<td>Normal → Normal</td>
<td>Normal → Normal</td>
<td>Normal → Normal</td>
<td>Rough overgrown eye, Fused ommatidia → Normal</td>
</tr>
<tr>
<td>(UAS-Ras,N17) (dominant negative)</td>
<td>Normal → Normal</td>
<td>Rough eye → Rough eye</td>
<td>Rough eye → Rough eye</td>
<td>Rough eye → Rough eye</td>
</tr>
<tr>
<td>(UAS-Ras84B,V14) (constitutively active)</td>
<td>Rough eye → Rough eye</td>
<td>Lethal → Lethal</td>
<td>Rough, overgrown eye → Rough, overgrown eye</td>
<td>Lethal → Lethal</td>
</tr>
<tr>
<td>(UAS-Akk8)MB14 (wt)</td>
<td>Normal → Normal</td>
<td>Slight Rough eye → Slight Rough eye</td>
<td>Lethal → Rough, Reduced eye, Partially Lethal</td>
<td>Lethal → Rough, Reduced eye, Partially Lethal</td>
</tr>
<tr>
<td>(UAS-Akk8)MB14 (constitutively active)</td>
<td>Rough eye → Rough eye</td>
<td>Lethal → Lethal</td>
<td>Lethal → Lethal</td>
<td>Lethal → Lethal</td>
</tr>
<tr>
<td>(UAS-Rho1,V14)2.1 (constitutively active)</td>
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<td>Lethal → Lethal</td>
<td>Lethal → Lethal</td>
<td>Lethal → Lethal</td>
</tr>
<tr>
<td>(UAS-Rho1,N19) (dominant negative)</td>
<td>Rough eye → Rough eye</td>
<td>Lethal → Lethal</td>
<td>Lethal → Lethal</td>
<td>Lethal → Lethal</td>
</tr>
<tr>
<td>(UAS-Akt1,Exel8) (wt)</td>
<td>No data</td>
<td>Normal → Normal</td>
<td>Rough eye → Rough eye</td>
<td>Rough eye → Rough eye</td>
</tr>
</tbody>
</table>

In this table, the interaction results reported in chapter two of the dissertation are summarized. The UAS signal transduction responders are listed in the first column and the GAL4 drivers are listed in the first row. The results are listed in a CRMP⁺ → CRMP⁻ order with respect to the genetic background. The results highlighted in red show a CRMP^{Pepk1} effect in misexpression phenotype outcome. The results highlighted in blue show lethal effects. These data suggest that CRMP interacts in the same signaling pathways as Rac1, Rac2, Ras1, and Sgg.
Chapter Three
Role of CRMP in *Drosophila melanogaster* Adult Behavioral Processes

**Introduction**

*Drosophila* learning and memory:

In this section of the dissertation, the performance of the *Drosophila* CRMP mutant lines, described in chapter two, in learning and memory tasks will be addressed. As previously mentioned, behavioral analysis of CRMP-1 knockout mice revealed a deficiency in spatial learning and memory and reduction in long-term potentiation (Su *et al.* 2007). These data suggest that CRMP may play a role in the synaptic plasticity underlying memory in adult animals. Since *Drosophila* has a single CRMP gene that possibly represents the more ancestral version of this protein, a simpler neuronal circuitry, and a vast array of genetic tools, the role of CRMP in learning and memory can be much better assessed using this model system. Much time and effort has gone into the development of a Pavlovian olfactory conditioning assay for assessing fly learning and memory (Tully and Quin 1985). In this elemental assay, animals learn to associate a conditioned stimulus (odors) with an unconditioned stimulus (shock). This aversive method was utilized to investigate a potential for CRMP’s involvement in learning and memory and to provide further confidence for such an accusation.

There are five temporal phases underlying *Drosophila* observed olfactory memory (Dubnau and Tully 1998). These five temporally, mechanistically and anatomically distinct phases making up memory formation include learning (LRN), short-term memory (STM), middle-term memory (MTM), anesthesia-resistant memory (ARM) and long-term memory (LTM) (Dubnau and Tully 1998). The current model for memory processing, based upon experimental evidence, suggests that LRN, STM and MTM all occur in a sequential pathway and that consolidation of ARM and LTM is split and occurs in two genetically distinct parallel pathways (Dubnau *et al.* 2003) (Figure 3.1). In addition to revealing distinct memory stages, genetic experimentation has also dissected out the neuronal circuitry involved in *Drosophila* Pavlovian olfactory associative memory, mostly implemented by a structure
called the mushroom body (MB) (Heisenberg et al. 1985; de Belle et al. 1994; Davis 2005) and more recent evidence suggests a structure found in the central complex (CC) called the ellipsoid body (Wu et al. 2007). A diagram of the entire circuit was retrieved from Tully’s and Dubnau’s 2005 review and can be seen in Figure 3.2. The MB is thought to be the site where the conditioned stimulus and the unconditioned stimulus are associated and is also the structure where many olfactory memory genes are predominately expressed (Margulies et al. 2005). The MB consists of three major types of Kenyon cells, whose axonal projections occupy distinct structures called the $\alpha/\beta$ lobe neurons, $\alpha'/\beta'$ lobe neurons, and the $\gamma$ lobe neurons. The conditioned stimulus (CS) is sent from the antennal lobe, the olfactory processing center, to the MB and a not so well studied structure called the lateral horn via cholinergic neuronal projections (Margulies et al. 2005). The unconditioned stimulus (US) reaches the MB via dopaminergic inputs to the calyx (MB dendritic field) and lobes (axon terminals) (Schwaerzel et al. 2003).

Here, we investigate the conservation of CRMP’s role in learning and memory by addressing questions regarding the involvement of the Drosophila CRMP gene in such processes. First, are any memory phases disrupted by loss-of-function mutations of the CRMP gene? Second, if a certain memory phase is dependent on CRMP, then can the mutant phenotype be rescued by genetically providing a wild-type allele of CRMP? What regions of the adult brain require CRMP for normal learning and memory? Finally, is a mutant phenotype due to a chronic abnormality resulting from aberrant neurodevelopment or an acute CRMP biochemical requirement in the adult brain during the memory consolidation process? These questions were answered by conducting appropriate experiments at the renowned Cold Spring Harbor Laboratory in the lab of Dr. Josh Dubnau. Many thanks to Josh and the following Dubnau lab members for providing the equipment, facilities and training to complete the experiments successfully: Dr. Hongtao Qin, Dr. Claudia Jurgensen, Dr. Allison Blum, Hilary Cox, Wanhe Li, and Mike Cressy.

Drosophila circadian rhythms:

In addition to assessing CRMP’s role in learning and memory, experiments were carried out to assess roles of the CRMP gene in other well studied Drosophila adult behaviors.
Locomotor activity rhythms are well characterized circadian behaviors in *D. melanogaster* and permit straightforward analysis of our mutant strains (Hamblen et al. 1986; Rosato and Kyriacou 2006). A series of experiments were conducted to examine CRMP’s potential in regulating *D. melanogaster* circadian rhythmicity and the preliminary results will be discussed in this chapter as well. The experiments were conducted at the University of Tennessee in Dr. Jae Park’s laboratory. Many thanks to Dr. Park for providing the equipment and facility needed to carry out the locomotor activity assay and for analyzing the raw data.

In *D. melanogaster*, a group of small ventrolateral neurons are the main pacemaker in the adult brain and serve as oscillators for daily activity rhythms (Chang 2006). The circadian clock is only part of a neuronal circuitry that makes up the circadian system that is responsible for maintaining an accurate 24 hour cycle. The circadian clock is coupled to input pathways for relaying external cues for entrainment purposes and output pathways for generating rhythms in behavior (Blau *et al.* 2007). It is the endogenous clock that maintains this 24 hour periodicity in constant darkness with flies exhibiting their normal times of peak activity at the onset of dawn and dusk. The purpose of the experiments described here are to determine whether mutations in *Drosophila CRMP* lead to arrhythmicity in constant darkness during the locomotor activity assay (reviewed in Shaw 2003).
Results

The CRMP gene is necessary for normal Drosophila learning and memory

No obvious morphological defects were observed in the Drosophila CRMP mutant lines. To analyze the learning and memory capability of the CRMP loss-of-function flies, they were subjected to the Pavlovian olfactory conditioning assay of Tully and Quinn in which animals learn to associate a conditioned stimulus (odors) with an unconditioned stimulus (footshock) (Tully and Quin 1985). In this behavioral assay, learning (“immediate memory”) is tested following one training session, 3 hour memory (MTM) is tested three hours post one training session, and LTM is induced by spaced training and assessed 24 hours after training. Three separate fly lines, each containing a different mutant allele of the CRMP gene, were analyzed base upon their performance in this task for the three memory phases mentioned. The CRMP mutant lines tested include CRMP\textsuperscript{supK1}, CRMP\textsuperscript{supLa1}, and P\{EP\}CRMP\textsuperscript{EP3238} all of which were previously described in chapter two. Before the lines were assayed, each was outcrossed for at least six generations to an isogenic white control strain \{w\textsuperscript{1118}(isoCJ1)\} which shows normal levels of olfactory learning and memory (Yin et al. 1995). The outcrossing enhances equilibration of the genetic background by removing possible second-site mutations in the CRMP mutant lines. The performance of the mutant lines in the aversive olfactory learning and memory assay was compared to the performance of the wild-type control strain \{w\textsuperscript{1118}(isoCJ1)\} in all cases.

CRMP mutants perceive and respond to odors and shock

Before CRMP mutants were tested for learning and memory ability, the necessary task-relevant sensorimotor responses were checked in these animals. The reason for this assessment is that learning in this assay is not directly observed but is determined based upon an association of two stimuli (Dubnau and Tully 1998). In this assay, olfactory acuity and shock reactivity are pertinent sensorimotor responses required for normal performance. Therefore, the fly lines were tested for their ability to sense and respond to olfactory stimulation and electrical shock. The CRMP\textsuperscript{supK1}, CRMP\textsuperscript{supLa1}, and P\{EP\}CRMP\textsuperscript{EP3238} mutants all avoided the aversive odors of octanol (Oct) and methylcyclohexanol (MCH) to
the same degree as the wild-type control animals using the same concentration of the odors that are administered in the Pavlovian olfactory conditioning assay (Figure 3.3, A-D). This result indicates that the perception of these odors and the activation of the motor circuits are normal in the mutants. One unique result was the CRMPsupK1 line showed a stronger avoidance towards MCH in the first round of experiments (Figure 3.3, B), but after repeating the experiment for another N=8 the strong avoidance was not detected (Figure 3.3, D). The same mutants were also examined for the ability to respond to electrical shock. The CRMPsupK1, CRMPsupIa1, and P{EP}CRMPEP3238 mutants perceived and avoided electrified grids used for delivering the negative reinforcement for learning (Figure 3.4, A-B). Again, all three CRMP mutants performed comparable to the wild-type control animals at avoiding the applied voltage, which is the same voltage used in the Pavlovian olfactory conditioning assay. In summary, CRMPsupK1, CRMPsupIa1, and P{EP}CRMPEP3238 appear to have normal sensorimotor functions required for aversive olfactory learning and memory tasks.

**Loss-of-function CRMP mutants perform poorly in learning task**

The CRMP mutant animals were first examined for a defect in learning using the olfactory conditioning assay. The control animals and the three CRMP mutants were given olfactory classical conditioning using the odors Oct and MCH as the CS and electrical shock as the US. Two minutes after a single aversive Pavlovian training session their memory was tested. The three CRMP mutant lines exhibited a defective conditioned response compared to the wild-type control flies at 2 min. after entrainment (Figure 3.5, A). Loss of just one copy of the CRMP gene did not result in learning impairment, indicating that the mutant phenotype is recessive. Given that the learning defect was apparent at 2 min. after training in homozygous mutant animals, the data suggest that CRMP is required for molecular function underlying STM.

To provide further supporting evidence that loss of CRMP leads to a 2 min. memory deficit, P{PYD2+} transgenic rescue of the CRMPsupK1 LRN phenotype was attempted. The P{PYD2+} transgene introduces a wild-type genomic copy of CRMP and is similar to the P{PYD2GFP} transgenic flies mentioned in the introduction except minus the GFP-tag (Rawls 2006). A variety of the P{PYD2+} transgenic lines, differing in genomic sites of
insertion, were subjected to the above behavioral screen in parallel with the homozygous supK1 mutant line. Two different PYD2+ transgene insertion lines were tested, P{PYD2}1/+; CRMPsupK1 and P{PYD2}3/+; CRMPsupK1, as well as the P{PYD2GFP}/+; CRMPsupK1 transgenic line. Since the CRMP gene encodes for both CRMP and DHP proteins, two additional transgenic lines were tested for rescue to help distinguish between the two gene products role in LRN. The P{PYD2GFPfs9a} transgene contains a frame shift mutation in exon 9a required for DHP mRNA production, and the P{PYD2GFPfs9b} transgene contains a frame shift mutation in exon 9b required for CRMP mRNA production. The five rescue transgenic lines produced PI results that were indistinguishable from that of the homozygous supK1 mutant line, and significantly different from the wild-type and heterozygous supK1 controls (Figure 3.5, B). Thus, rescue of the mutant phenotype was not achieved by any of these transgene lines. A possible explanation for such results could be that CRMP expression is tightly regulated during normal learning and the genomic constructs used to rescue the learning defect lack an important CRMP gene regulatory region (for example a cis-acting regulatory sequence required for the binding of a negative transcriptional regulatory protein).

CRMP mutants exhibit severely reduced performance in MTM

Since the CRMP loss-of-function animals displayed poor learning skills, the CRMPsupK1, CRMPsupIa1, and P{EP}CRMPEP3238 lines were examined for memory retention three hours after a single aversive Pavlovian training session. This experiment tested the MTM capability of the three independently derived CRMP mutant alleles. As seen with the learning task, the mutants showed a reduction in 3 hour memory in comparison to the control animals (Figure 3.6, A and B). MTM of the heterozygous supK1 mutant line appeared identical to that of the control (Figure 3.6, A). This result is not the first time a single gene mutant has tested defective for both immediate memory and MTM. Two mutant alleles of the rutabaga gene each produce Drosophila learning and 3 hour memory deficiencies (Livingstone et al. 1984; Zars et al. 2000; Schwaerzel et al. 2002).

CRMP mutant alleles fail to complement each other in LRN and MTM tasks
Animals that were trans-heterozygous for both mutant alleles were also tested for 2 min memory and 3 hr memory after a single aversive Pavlovian training session (Figure 3.7, A and B). The \( CRMP^{supK1}/CRMP^{supIa1} \) as well as homozygous \( CRMP^{supK1} \) and \( CRMP^{supIa1} \) animals all exhibited performance indices significantly lower than wild-type controls for 2 min memory (Figure 3.7, A) and 3 hr memory (Figure 3.7, B). The \( CRMP^{supK1/+} \) and \( CRMP^{supIa1/+} \) animals performed similar to the wild-type controls in both tasks (Figure 3.7, A and B). These data are consistent with the data reported in the previous two experiments (Figure 3.5, A and Figure 3.6, B).

**CRMP is also required for Drosophila Long-Term Memory**

If \( CRMP^{supK1} \) mutants are mutant for LRN and MTM, then it is possible that these animals also have defects in LTM. Only one form of consolidated memory was examined in the \( CRMP^{supK1} \) mutant line. This experiment employed ten repetitive aversive Pavlovian training sessions, with a fifteen minute rest interval between each training session (spaced training). The spaced training induces LTM that is protein synthesis sensitive and dependent upon CREB function (Yin et al. 1994; Tully et al. 1994). This training and T-maze testing method is identical to the one utilized in identifying protein tyrosine phosphatase-10D as a key protein in establishing LTM in *Drosophila* (Qian et al. 2007). The \( CRMP^{supK1} \) mutants showed reduced performance 24 hours after ten spaced training sessions in comparison to the wild-type animals (Figure 3.8). Collectively, the results reported here provide confidence that the \( CRMP \) gene is required for both STM and LTM processes, and is consistent with results gathered for other *Drosophila* STM and LTM mutants (Blum et al. 2009).

*Mushroom body expression of CRMP does not restore LRN in CRMP\(^{supK1}\) flies*

To test which \( CRMP \) gene product, CRMP or DHP, is the limiting factor for memory loss, the effects of expressing wild-type CRMP or DHP in the MBs using \{UAS-CRMP\} or \{UAS-DHP\} transgenes was observed in a homozygous \( CRMP^{supK1} \) genetic background. The endogenous expression pattern of CRMP in the *Drosophila* adult brain is unknown, thus a variety of MB specific GAL4 transactivators were chosen to misexpress these transgene
constructs. The GAL4 driver lines utilized include the following: c739, 201Y, OK107, 247, c747 and c309 (see Figure 3.10 for detailed expression pattern of each line) (Aso et al. 2009). Misexpressing UAS-CRMP under control of c739, 201Y, 247, or OK107 resulted in lethality or partial lethality; whereas misexpressing UAS-DHP with the same driver lines produced viable adults. Since no adults or very few adults emerged using the UAS-CRMP lines in combination with these drivers, these animals could not be tested for rescue of learning or memory. Fortunately, two GAL4 lines when crossed to UAS-CRMP produced testable adults. Both c309 and c747 label all lobe systems in the MBs (Aso et al. 2009). For c309, the expression is reported to be strong in the α/β and γ lobes, very weak in the α'/β' lobes, and outside the MB in the antennal lobe, triocerebrum, subesophageal ganglion, and optic lobes (Aso et al. 2009). For c747, the expression is reported to be preferentially strong in the α/βp (p, posterior subdivision), α/βs (s, surface subdivision) and γ neurons, weaker in the α/βc (c, core subdivision) and α'/β' neurons, and outside the MB in local interneurons of the antennal lobe, antennal nerve, optic lobes, pars intercerebralis, and subesophageal ganglion (Aso et al. 2009).

Figure 3.9 shows that both the {UAS-CRMP}/c309; CRMPsupK1 and {UAS-DHP}/c309; CRMPsupK1 combinations fail to rescue immediate memory to wild-type levels. This initial set of experiments conducted to restore learning in the CRMP mutant flies using the UAS-GAL4 system resulted in mean PI values below 56, compared to an 82 PI value produced by wild-type controls. The {UAS-CRMP}/c309; CRMPsupK1 line performed similar to the CRMPsupK1 homozygous mutants (p = 0.59). The {UAS-DHP}/c309; CRMPsupK1 line also produce performance scores indistinguishable from the CRMPsupK1 animals (p = 0.34). CRMPsupK1 flies with the {UAS-CRMP}4a or c309 insertion alone also displayed no rescue of the LRN defect (Figure 3.9). The next step will be to assess learning in {UAS-CRMP}/c747; CRMPsupK1 and {UAS-DHP}/c747; CRMPsupK1 animals. It is possible that misexpression with c747 will rescue the learning and memory defect of CRMPsupK1 mutant animals since this GAL4 driver line does not have an overlapping expression profile with c309 (Aso et al. 2009). Since the MB specific misexpression of wild-type CRMP cDNA using c309 in a mutant background produced performance results statistically similar to mutant animals, it is still uncertain whether CRMP or DHP is responsible for the learning and memory defects observed in homozygous CRMP mutant animals.
It is interesting that the \textit{c309} GAL4 misexpression of CRMP in the adult brain did not rescue the LRN defect of mutant animals. Recent expression profiling of this GAL4 driver line indicates that it exhibits expression in the region of the adult brain responsible for learning and memory consolidation (Aso \textit{et al.} 2009). To test the CRMP expression pattern of this GAL4 driver and provide insight into why it was incapable of rescue, GFP tagged constructs were used. Misexpression of $P\{UAS-CRMPGFP\}$ and $P\{UAS-DHPGFP\}$ with \textit{c309} resulted in an overall weak GFP signal in the MB lobes (Figure 3.11, A-D). These images also show very weak signal in the MB gamma lobes (Figure 3.11, B & D arrowhead). These data indicate that the level of CRMP and exact anatomical location may be important in memory formation reliant on CRMP. However, it is important to point out that the expression pattern was only observed in adult brains and the misexpression experiments just described do not address whether the rescue requires appropriate expression of CRMP during neurodevelopmental as as opposed to fulfillment of an acute biochemical need for CRMP in the adult.

\textit{No CRMP expression is detected in the mushroom bodies}

Since the MB is the anatomical site implicated in learning and memory formation, it is important to determine if CRMP is expressed in this adult brain region. Unfortunately, there is currently no antibody against fly CRMP, so the endogenous CRMP protein expression pattern cannot be detected. Rather, use was made of the \{PYD2GFP\textit{fs9a}\} transgene that contains a genomic DNA fragment spanning the \textit{CRMP} gene and expresses only a GFP-tagged CRMP protein (DHP expression is blocked by a frameshift mutation in exon E9a). Brains were dissected out of 1-3 day old adults and stained with antibody against Fasciclin II (FasII). Confocal imaging of the brains revealed no GFP expression in the MB structures, which were highlighted red from the FasII immunoreactivity (Figure 3.12, A). Wild-type \textit{\{w\textit{1118 (isoCJ1)\}} control brains underwent the same treatment and no GFP signal was detected in these animals either (Figure 3.12, B). The lack of GFP signal in the \{PYD2GFP\textit{fs9a}\} brain does not rule out the possibility that CRMP is expressed in the MBs. The fixation of the tissue or the FasII antibody staining could have quenched the endogenous GFP fluorescent signal or the fluorescent signal could be too weak to detect using this method. Furthermore, \textit{CRMP} mutant animals that possess this transgene still performed poorly in both olfactory
learning and memory tasks suggesting that the transgene does not functionally replace a wild-type copy of CRMP.

**Drosophila CRMP involvement in Circadian Rhythms**

CRMP loss-of-function mutants were assessed for their effects on circadian locomotor rhythms. The \( CRMP^{supK1} \), \( CRMP^{supIa1} \), and \( P\{EP\}CRMP^{EP3238} \) lines were subjected to a locomotor activity assay in which the adult animals were tested for maintenance of normal activity patterns in the absence of external environmental cues (Rosato and Kyriacou 2006). In this behavioral assay, 1 to 3 day old adult males are entrained and monitored for three to four days in 12 hr light/dark (LD) cycles; then, free-running activity is monitored in constant darkness (DD) for an additional eight days in which flies rely on their endogenous clock for normal circadian behavior. All the lines that were subjected to the assay were outcrossed for at least six generations to the isogenic white control strain \( \{w^{1118}(isoCJ1)\} \) (Yin et al. 1995). The performance of each mutant line used in the locomotor activity assay was compared to the performance of the wild-type control strain \( \{w^{1118}(isoCJ1)\} \) in all cases. Partial data collected on animals that die during the experiment was discarded. Only data gathered from animals surviving the entire experimental period are included in the results presented below.

**Certain CRMP mutants lack normal circadian rhythmicity**

We compared the behavior of the CRMP mutant lines with that of three genotypes: +/- wild-type controls, \( supK1/+ \) or \( supIa1/+ \) heterozygous lines, and \( \{PYD2\}; supK1 \) rescue lines. The data are presented in three ways, as tabulated data in Table 3.1, as averaged power values (strength of rhythmicity) for individuals of the same genotype under constant conditions (Figure 3.13), and as average activity histograms for each group tested under cycled conditions and constant conditions (Figure 3.14, A-H).

All genotypes tested showed normal period length during constant conditions (Table 3.1). Thus, CRMP does not appear to affect the basic circadian clock of Drosophila as measured in these experiments.
During the 12 hr LD cycles, wild-type males produced two obvious peaks of activity, which is consistent with normal patterns seen by other researchers (Hamblen-Coyle et al. 1992; Renn et al. 1999; and Bahn et al. 2009). The two maximal peaks consist of one morning peak at the onset of lights on and one evening peak around the time of lights off, both peaks were preceded by anticipation of the transition between light and no light or no light and light (Figure 3.14, A). In all CRMP heterozygous and homozygous mutant lines exposed to cycling conditions, both the morning and evening peaks appeared similar to those of wild-type control animals, and all lines displayed normal anticipation marked by gradual increase in activity prior to lights on and lights off (Figure 3.14, B-F). These results are not surprising, because regardless of genotype, LD behaviors give rise to 24 hr rhythmicity due to LD input driving the flies’ periodic movement. Only free-running activities are indicative of the endogenous clock function.

Under constant darkness, the wild-type animals maintain daily rhythmicity over the entire 8 days (Figure 3.14, A). Based upon periodogram analysis, 98% of individuals were rhythmic and the group produced an average power value of 83.7±4.7 (Table 3.1). In addition, both the supK1/+ and supIa1/+ heterozygous lines showed normal locomotor activity patterns during the 8 days of DD (Figure 3.14, B and C). Periodogram analysis revealed that 90% of supK1/+ animals were rhythmic with only one animal displaying weak rhythmicity and two animals displaying arrhythmicity. The mean power (P) value for the supK1/+ heterozygotes was 74.2±6.8 and indistinguishable from wild-type (p = 0.8) (Figure 3.13). 100% of the supIa1/+ flies showed rhythmic free-running locomotor activity and had a mean P score of 67.9±5.8 (p = 0.4 in comparison to wild-type) (Figure 3.13).

In contrast, CRMPsupK1 homozygotes are significantly arrhythmic under constant conditions and show substantial differences in daily activity patterns from those of wild-type. Homozygous CRMPsupK1 mutants exhibited normal entrainment when exposed to LD conditions, but displayed an abnormal free-running phenotype that was detectable the first day of DD exposure (Figure 3.14, D). The locomotor behavior under constant conditions was arrhythmic during the entire DD period, with random atypical peaks of activity. During DD, only 60% of CRMPsupK1 animals were rhythmic; whereas, 13% were weakly rhythmic.
and 27% were arrhythmic with an average P value of 29.4±5, which is statistically different
from wild-type (*p = 9.7e-14) (Table 3.1 and Figure 3.13).

A substantial fraction (17%) of $P{\text{EP}}/CRMP^{\text{EP3238}}$ flies also showed arrhythmic free-
running locomotor activity and 13% were weakly arrhythmic (Table 3.1). The P value for
$P{\text{EP}}/CRMP^{\text{EP3238}}$ (40.2±8.2) was significantly lower than wild-type (*p = 0.00004).

Dissimilar to the other homozygous CRMP mutant alleles, the $CRMP^{\text{supIa1}}$ mutant line
produced free-running rhythms under DD conditions very similar to the wild-type and
heterozygous mutant lines. The endogenous clock of these animals appears to be fully
functional. Only 2% of the flies tested were arrhythmic, while 98% were rhythmic. The
average P value (77.0±5.5) was also indistinguishable from wild-type (p = 0.9). Therefore,
two of the three CRMP mutant alleles produced locomotor activity suggestive of
arrhythmicity under constant dark conditions.

*Rescue of the arrhythmic CRMP$^{\text{supK1}}$ mutant behavior*

To test for transgene rescue of the CRMP$^{\text{supK1}}$ mutant arrhythmic phenotype, use was
made of the same $\{\text{PYD2}\}^+$ rescue transgene lines used in the learning and memory
experiments. One line, $\{\text{PYD2}\}l$, failed to rescue the free-running locomotor activity defect
seen in CRMP$^{\text{supK1}}$ flies. A large percentage (25%) of $\{\text{PYD2}\}l/\text{supK1}$ animals were
arrhythmic (Table 3.1). Their mean P value was 43.9±7.3, which is significantly lower than
control flies (*p = 0.00005) and comparable to homozygous supK1 flies (p = 0.105) (Figure
3.13). The $\{\text{PYD2}\}l/\text{supK1}$ flies also lacked a distinct daily activity pattern. The activity
histogram shows sporadic activity throughout the 24 hr period with subtle morning and
evening peaks (Figure 3.14, G). These flies also have a substantial increase in events per bin
(27.9) in comparison to wild-type, indicating overall hyperactivity.

Transgene rescue was achieved with a second $\{\text{PYD2}\}^+$ insertion line, $\{\text{PYD2}\}3$. The
mean P value of $\{\text{PYD2}\}3/\text{supK1}$ animals was 65.7±5.8, which is comparable to wild-type (p
= 0.097) and significantly different from homozygous supK1 mutant line (p = 0.000004)
(Figure 3.13). Overall, 83% of the flies tested rhythmic, 7% tested weakly rhythmic, and
10% tested arrhythmic (Table 3.1). The activity histogram shows a slight reduction in mid-day activity compared to the *PYD2* transgenic line and identifiable morning and evening peaks, but hyperactivity is also detected in this line (29.0 events/bin). These data are evidence that the CRMP gene product(s) plays an essential role as a potential clock messenger in *D. melanogaster*.
Discussion

**CRMP is required for *Drosophila* learning and memory and circadian rhythms**

In this chapter, behavioral assay results suggest that CRMP is playing a role in regulating events that depend on synaptic plasticity in the adult fly nervous system. The data collected from the aversive Pavlovian olfactory conditioning experiments support the notion that the *CRMP* gene is required for normal LRN, MTM, and LTM. The ability of multiple, unambiguous mutant alleles of *CRMP* singly and in combination to produce similar results provide additional confidence in this conclusion. Unfortunately, an attempt to rescue the LRN and MTM defects by expressing CRMP in the MBs was unsuccessful. This prevents the functional distinction between CRMP and DHP isoforms, which are products from the same gene, in the role of olfactory learning and memory.

Results obtained through the locomotor activity assay indicate participation of *CRMP* in regulation of circadian rhythms in *Drosophila* adult animals, a behavior that also relies on synaptic plasticity. Only two of the three *CRMP* mutant alleles, *CRMPsupK1* and *P{EP}CRMPEP3238*, produce significant arrhythmicity in animals. The frequency of arrhythmia among these mutant animals is similar to that observed in *pdf* null mutants (Renn *et al.* 1999; Bahn *et al.* 2009). However, other free-running features distinguish these mutants from others that disrupt circadian behavior. For example, both *CRMPsupK1* and *P{EP}CRMPEP3238* mutants display a normal period length in comparison to wild-type; whereas *pdf* null-mutants had a period length approximately 1 hr shorter than the wild-type control (Renn *et al.* 1999). The *CRMPsupIa1* mutant line demonstrates rhythmic free-running locomotor activity under constant dark conditions, which is similar to activity patterns produced by animals that harbor a wild-type copy of the *CRMP* gene. One possible explanation for this result is that the residual protein open reading frame of *CRMP* (e.g., exons E9a through E12) is expressed in *CRMPsupIa1* animals and that circadian clock function is somehow restored by this protein fragment. Another explanation is that the *CRMPsupIa1* line, despite the extensive backcrossing to which these lines were subjected, contains a genetic suppressor of the arrhythmia phenotype. The arrhythmic behavior of homozygous *CRMPsupK1* animals is rescued by providing an endogenous copy of the wild-type *CRMP* gene.
in the form of a transgene. The same $\{PYD2\}$ construct failed to rescue the learning and memory deficiency in homozygous supK1 animals, perhaps reflecting genetic position effects leading to differential expression of the transgene in different neural structures responsible for these behaviors.

Collectively, these data imply that the expression of CRMP is regulated differently depending on the brain region and behavior in question. To provide further insight into the spatiotemporal expression of the CRMP isoform, negative and positive regulatory elements associated with the gene need to be identified and manipulated. It is possible that negative trans-acting factors might be unable to bind their respective CRMP regulatory regions or that the rescue transgene lacks these regions, thus allowing for varying expression of the protein. This behavioral data collected on a variety of CRMP mutant animals finally provide mutant phenotypes associated with the CRMP protein and help support the idea that the gene indeed encodes for a CRMP protein.

**Future Direction**

Further studies need to be conducted to pinpoint CRMP expression in the adult brain regions associated with the behaviors of olfactory learning and memory and circadian locomotor rhythmicity, the MBs and ventrolateral neurons respectively. Structurally, these brain regions are intact and display normal morphology and projection patterns in supK1 and supIa1 mutant animals (Disc Large (Dlg) and Pigment-dispersing factor (PDF) immunostaining data not shown). Currently, no antibody against the Drosophila CRMP protein is available. Transgenic larval animals that possess a GFP-labeled CRMP-specific genomic construct, $\{PYD2GFPfs9a\}$, express tagged-protein ubiquitously in neural tissue (Figure 1.5B). Endogenous GFP expression from the same construct failed to show immunofluorescence in the adult MB structures; however, tissue fixation and the staining procedure could have quenched the fluorescent signal (Figure 3.12, A). Double immunostaining with antibodies against FasII (highlights MBs) and GFP in these same animal brains would be insightful. However, failure to detect CRMP expression in the MB structures does not discount CRMP’s role in learning and memory. The amnesiac protein, which functions in learning and memory, is not expressed in the MB, but rather the dorsal
paired medial neurons that project onto the MB lobes (Waddell et al. 2000). Perhaps there is more to learn about the olfactory memory circuit.

Since the lateral neurons are important pacemakers that regulate daily locomotor rhythms in *Drosophila* (Ewer et al. 1992), double staining with anti-PDF and anti-GFP antibodies should allow for examination of CRMP expression in these neurons. The later staining procedure would be most telling, due to the \(\{PYD2\}^+\) transgene rescuing circadian rhythm defects and not learning and memory defects in the mutant lines. Data from studies in mice show that mammalian CRMP isoforms are expressed in the adult brain, but due to the multiplicity of the CRMP protein and lack of *in vivo* behavioral data it is hard to correlate expression pattern with function.

Studies to determine how CRMP is involved in both STM and LTM processes still need to be carried out as well. Do CRMP dependent signaling cascades regulate these forms of memory by occurring in identical anatomical sites with similar temporal constraints or is CRMP necessary in distinct and functionally unrelated spatiotemporal regions depending on the memory process in question? It would be interesting to see if CRMP is also playing a role in ARM. This phase is the only memory phase not tested as part of this work. For quantification of ARM levels, 3 hr memory retention would be measured in flies that were anesthetized by cold shock for 2 min, 2 hrs after training. It would be very unexpected if CRMP is both acutely required for biochemical signaling necessary for memory formation and capable of regulating both forms of consolidated memory. Experimental evidence gathered so far suggests that consolidation of ARM and LTM is split and occurs in two genetically distinct parallel pathways, so it would be unlikely that CRMP regulates both ARM and LTM. Furthermore, our preliminary data provide confidence that CRMP is playing a role in LTM.

As previously noted, certain CRMP mutants were significantly less rhythmic than the wild-type control lines. Although it is clear that CRMP does not directly regulate the central clock due to these mutant animals not having a change in the period length and a complete loss of rhythmicity, which is seen in central clock mutants *per* and *tim* (Konopka and Benzer 1971; Hamblen et al. 1998; Rothenfluh et al. 2000). The CRMP\textsuperscript{supKI} animals did exhibit sporadic daily locomotor activity, which is abnormal. Evidence confirms that multiple
transmitters are required to produce normal circadian locomotor rhythms (Taghert et al. 2001), and CRMP could play a part in transducing the signals initiated by these transmitters to rely circadian time of day to downstream neurons. On the other hand, CRMP could just be a generic maintenance factor for rhythmicity and not a circadian effector molecule. However, further experimentation is necessary to pinpoint CRMP in such a role. Currently, the lab is attempting to rescue the supK1 arrhythmic phenotype by misexpressing wild-type CRMP in the key neurons involved in circadian rhythms by using {elav-Gal4}, {pdf-Gal4} (Park et al. 2000) and {tim-Gal4}. Data retrieved from this experiment will provide insight into the spatial requirement of CRMP in regulating circadian behavior.
Figure 3.1. The five phases of memory formation in *Drosophila*. (A) The observed memory retention seems relatively seamless over time when assessing memory as a behavior. However, research has shown that there are distinct memory phases underlying this observed memory curve. These include short term memory (STM), middle term memory (MTM), anesthesia resistant memory (ARM), and long term memory (LTM). (B) The current model for memory processing, based upon experimental evidence, suggests that LRN, STM and MTM all occur in a sequential pathway and that consolidation of ARM and LTM is split and occurs in two genetically distinct parallel pathways. It has also been shown that single-gene mutations (red), pharmacologic interventions (blue), and behavioral manipulations (green) affect specific memory phases. (Figure from Dubnau et al. 2003).
Figure 3.2. Neural circuitry involved in olfactory associative memory. The mushroom body (MB) (green structures) is site where the conditioned stimulus (CS) and unconditioned stimulus (US) are associated. The olfactory processing center is the antennal lobe (blue) and relays the CS to the MB via cholinergic neuronal projections (blue arrows). The US reaches the MB via dopaminergic inputs to the calyx (dendritic field) and lobes (axon terminals) (red arrows). (Figure from Margulies et al. 2005).
Figure 3.3. CRMP mutant flies test normal for olfactory acuity. Avoidance score produced by wild-type animals towards octanol are comparable to those of CRMPsupK1, CRMPsuppla1, and P{EP}CRMPEP3238 mutant animals (A and C). All three mutants avoid MCH to the same degree as the wild-type control animals (B and D). The avoidance of the odors indicates that the mutant animals do sense and respond to them. N=8 for all groups in each chart. Means and standard errors are shown.
Figure 3.4. *CRMP* mutant flies sense and respond to electrical foot-shock. *CRMP*<sup>K1</sup>, *CRMP*<sup>I1</sup>, and *P{EP}CRM*<sup>EP3238</sup> mutant animals respond to shock and produce PI scores similar to animals that possess a wild-type copy of the *CRMP* gene (A and B). All three mutants exhibit no defects in the sensorimotor responses necessary to perform normal in the aversive Pavlovian olfactory conditioning assay. n=4 for all groups in each chart. Means and standard errors are shown.
Figure 3.5. The CRMP gene is required to support learning, but rescue of CRMP mutant phenotype is unsuccessful. Male and female flies that were wild-type (w^{1118 isoCJ1}), heterozygous for CRMP^{supK1} or CRMP^{suplal}, or homozygous for CRMP^{supK1}, CRMP^{suplal}, or P/EP)CRMP^{EP3238} were tested for immediate memory after a single training session (A). The CRMP^{supK1/+] and CRMP^{suplal/+] lines exhibit learning scores statistically similar to wild-type (both p ≥ 0.49). All three CRMP mutant alleles exhibited performance indices (PIs) significantly lower than wild-type controls (*p ≤ 0.0000006). (B) The 2 min. memory mutant phenotype could not be rescued by supplying an endogenous wild-type copy of the CRMP gene in the form of a transgene (a variety of transgenic lines were tested, see text for details). The supK1 mutant animals carrying a copy of the transgene produced PIs significantly lower than wild-type (*p ≤ 0.008). n=8 for all groups in each chart. The mean ± SEM is plotted for each genotype.
Figure 3.6. The CRMP gene is required to support middle term memory. Male and female flies that were wild-type (w^{1118} isoCJ1), heterozygous for CRMP^{supK1}, or homozygous for CRMP^{supK1}, CRMP^{supLa1}, or P{EP}CRMP^{EP3238} were tested for 3 hour memory after a single training session (A and B). (A) The CRMP^{supK1/+} line produced a PI score statistically higher than wild-type (p = 0.02), indicating a better MTM; whereas, the CRMP^{supK1} line produced a PI score statistically lower than wild-type (*p = 0.000006), indicating an impaired MTM. (B) The other two CRMP mutant alleles also exhibited PIs significantly lower than wild-type (*p ≤ 0.00037) for 3 hour memory. N=8 for all groups in each chart. The mean ± SEM is plotted for each genotype.
Figure 3.7. Flies transheterozygous for the CRMP mutant alleles have defective 2 min. memory and 3 hr. memory. Male and female flies that were wild-type (w 1118 isoCJ1), heterozygous for CRMPsupK1 or CRMPsupIa1, homozygous for CRMPsupK1 or CRMPsupIa1, and transheterozygous for both mutant alleles (CRMPsupK1/CRMPsupIa1) were tested for learning and 3 hour memory after a single training session (A and B). (A) The CRMPsupK1/CRMPsupIa1 line produced a PI score statistically lower than wild-type (*p = 0.000000064), CRMPsupK1/+ , and CRMPsupIa1/+ indicating impaired LRN. (B) The CRMPsupK1/CRMPsupIa1 line produced a PI score statistically lower than wild-type (*p = 0.000019), CRMPsupK1/+ , and CRMPsupIa1/+ indicating impaired MTM. The two homozygous CRMP mutant alleles also exhibited PIs significantly lower than wild-type controls and indifferent from the transheterozygous line for (A) LRN (CRMPsupK1 *p = 0.000000027, CRMPsupIa1 *p = 0.0000000049) and (B) MTM (CRMPsupK1 *p = 0.000057, CRMPsupIa1 *p = 0.0059). N=8 for all groups in each chart. The mean ± SEM is plotted for each genotype.
Figure 3.8. The CRMPsupK1 mutants display long term memory defect. Male and female flies that were wild-type (w^{1118} isoCJ1) or homozygous for CRMPsupK1 were tested for 24 hour memory after spaced training. The CRMPsupK1 line produced a PI score statistically lower than wild-type animals (*p = 0.0017), indicating an impaired LTM. N=8 for both groups. The mean ± SEM is plotted for each genotype.
Figure 3.9. Broad expression of CRMP in the MBs fails to rescue the learning deficits observed in CRMP mutant flies. Memory was tested 2 minutes after a single training session. Immediate memory is not rescued in male and female flies that are homozygous for CRMP<sup>supK1</sup> by expressing wild-type CRMP in the MBs using c309 GAL4. The CRMP<sup>supK1</sup>, {UAS-CRMP}<sup>4a/c309</sup>; CRMP<sup>supK1</sup>, and {UAS-DHP}<sup>1b/c309</sup>; CRMP<sup>supK1</sup> lines all exhibit learning scores statistically lower than wild-type (*p ≤ 0.0002), implying that expression of CRMP or DHP is unsuccessful at restoring LRN. UAS-CRMP insertion line paired with c309, {UAS-CRMP}<sup>4a/c309</sup>; CRMP<sup>supK1</sup>, performed significantly worse than wild-type and similar to CRMP<sup>supK1</sup> animals (*p = 0.00004 and p = 0.59, respectively). UAS-DHP insertion line paired with c309, {UAS-DHP}<sup>1b/c309</sup>; CRMP<sup>supK1</sup>, also performed significantly worse than wild-type and similar to CRMP<sup>supK1</sup> animals (*p = 0.00001 and p = 0.34, respectively). Other lines with only the UAS constructs or GAL4 driver in a homozygous supK1 genetic background produced PI values significantly different from wild-type (*p ≤ 0.000002). N=6 for all groups. The mean ± SEM is plotted for each genotype.
Figure 3.10. Expression pattern of 25 GAL4 lines. Summary of the expression levels of 25 MB-GAL4s in various brain areas. Gray scale indicates subjectively evaluated signal intensity ranging from white (low) to dark grey (high). Brain areas evaluated include: MB, mushroom body; c, core subdivision; s, surface subdivision; p, posterior subdivision; a, anterior subdivision; m, middle subdivision; p, posterior subdivision; d, dorsal subdivision; AL, antennal lobe; CC, central complex; fb, fan-shaped body; eb, ellipsoid body; no, 88odule; pb, protocerebral bridge; OL, optic lobe; me, medulla; lo, lobula; lop, lobula plate; spr, superior protocerebrum; ipr, inferior protocerebrum; LH, lateral horn; optu, optic tubercle; vlpr, ventrolateral protocerebrum; plpr, posteriorlateral protocerebrum; vmpr, ventromedial protocerebrum; psl, posterior slope; pars in, pars intercerebralis; AN, antennal nerve; DE, deutocerebrum; TR, tritocerebrum; SOG, subesophageal ganglion. The GAL4 lines used for potential rescue of the CRMP mutant phenotypes in the Pavlovian olfactory conditioning assay are circled in blue. The GAL4 lines that result in lethality/partial lethality when used to misexpress CRMP are denoted by a red asterisk. (Figure from Aso et al. 2009).
Figure 3.11. *c309* GAL4-Driven GFP expression in the MBs of adult fly brains.  
Projections of adult fly brains focused on the MB lobe regions are shown at two different magnifications.  (A) *c309* GAL4 driving expression of a GFP tagged UAS-CRMP construct at 10X magnification.  (B) *c309* GAL4 driving expression of a GFP tagged UAS-CRMP construct at 20X magnification.  This genotype exhibits moderate expression of CRMP in the α/β (arrows) and γ lobes (arrowhead).  (C) *c309* GAL4 driving expression of a GFP tagged UAS-DHP construct at 10X magnification.  (B) *c309* GAL4 driving expression of a GFP tagged UAS-DHP construct at 20X magnification.  This genotype exhibits low expression of DHP in the α/β (arrows) and γ lobes (arrowhead).
Figure 3.12. **No CRMP transgene expression detected in the MB structures.** Projections of adult fly brains focused on the MB lobe regions at 20X magnification. Red signal shows FasII expression and highlights the α/β lobes and ellipsoid body. (A) Flies expressing a GFP tagged genomic copy of CRMP that will only produce a functional CRMP isoform, \( \{PYD2GFPfs9a\} \), show no CRMP expression in the MB lobes. A small amount of GFP signal (green) is detected outside the MBs possibly in nerve fibers. (B) Wild-type fly brain stained with antibody against FasII (red) serves as a control and shows no GFP expression.
Figure 3.13. Average values of varying behavioral rhythm strengths in normal, CRMP mutant lines, and \{PYD2\}-transgene rescued CRMP mutant lines. The mean power (P) values for the final eight days of constant darkness of the free-running period (see Table 3.1). The CRMP\textsuperscript{supK1/+} and CRMP\textsuperscript{supIa1/+} lines both produced an average P value comparable to wild-type (p = 0.8 and p = 0.4, respectively), indicating normal rhythmicity. The CRMP\textsuperscript{supK1} line produced a P score statistically lower than wild-type (*p = 9.7e-14), indicating arrhythmicity. Surprisingly, the CRMP\textsuperscript{supIa1} mutant allele exhibited a mean P comparable to wild-type controls (p = 0.9) for rhythmic activity. The \{EP\}CRMP\textsuperscript{EP3238} mutant line exhibited a mean P significantly lower than the wild-type control (*p = 0.00004), indicating arrhythmicity. The \{PYD2\}; supK1 line produced an average P value that was significantly different from wild-type control (*p = 0.00005) and indistinguishable from the CRMP\textsuperscript{supK1} mutant line, indicating a lack of rescue for arrhythmicity. However, the \{PYD2\}3 rescue transgene in a homozygous CRMP mutant background resulted in a P value similar to wild-type controls (p = 0.097) and significantly different than homozygous supK1 mutant line (p = 0.000004), indicating potential rescue. The n and average P values for all groups can be found in Table 3.1. The mean ± SEM is plotted for each genotype.
Figure 3.14. Locomotor activity of normal, CRMP mutant, and {PYD2}-rescued CRMP mutant flies. The average activity histograms above report relative levels of locomotion over a 24 hr period which is duplicated for visual purposes. Bars indicate average activity events per 30 min bin per fly. Gray shaded areas indicate the dark phases. The first three days of data collection occurred under a 12:12 hr LD cycle. The remaining eight days of data collection occurred under a 24 hr constant darkness. The no. of flies tested for each genotype can be found in Table 3.1. (A) +/+ wild-type control; (B) CRMP^{supK1}/+; (C) CRMP^{supIa1}/+; (D) CRMP^{supK1}; (E) CRMP^{supIa1}; (F) P[EP]CRMPEP3238; (G) {PYD2}1;CRMP^{supK1}; (H) {PYD2}3;CRMP^{supK1}. (Histograms were generated by Dr. Jae Park, University of Tennessee).
Table 3.1. Free-running behavior of different genotypes during days 4-11 under constant dark conditions.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>R (%)</th>
<th>WR (%)</th>
<th>AR (%)</th>
<th>Period</th>
<th>Power</th>
<th>Events/bin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2U</td>
<td>59</td>
<td>58 (98)</td>
<td>1 (2)</td>
<td>0</td>
<td>23.7±0.1</td>
<td>83.7±4.7</td>
<td>16.9±1.1</td>
</tr>
<tr>
<td>supK1/+</td>
<td>30</td>
<td>27 (90)</td>
<td>1 (3)</td>
<td>2 (7)</td>
<td>23.5±0.2</td>
<td>74.2±6.8</td>
<td>14.6±1.5</td>
</tr>
<tr>
<td>supla1/+</td>
<td>23</td>
<td>23 (100)</td>
<td>0</td>
<td>0</td>
<td>23.6±0.2</td>
<td>67.9±7.5</td>
<td>16.6±1.7</td>
</tr>
<tr>
<td>supK1</td>
<td>67</td>
<td>40 (60)</td>
<td>9 (13)</td>
<td>18 (27)</td>
<td>23.9±0.1</td>
<td>29.4±5.1</td>
<td>19.5±1.0</td>
</tr>
<tr>
<td>supla1</td>
<td>43</td>
<td>42 (98)</td>
<td>0</td>
<td>1 (2)</td>
<td>23.6±0.1</td>
<td>77.0±5.5</td>
<td>14.5±1.2</td>
</tr>
<tr>
<td>{EP3238}</td>
<td>23</td>
<td>16 (70)</td>
<td>3 (13)</td>
<td>4 (17)</td>
<td>23.1±0.2</td>
<td>40.2±8.2</td>
<td>17.0±1.7</td>
</tr>
<tr>
<td>{PYD2}1;supK1</td>
<td>32</td>
<td>22 (69)</td>
<td>2 (9)</td>
<td>8 (25)</td>
<td>23.5±0.2</td>
<td>43.9±7.3</td>
<td>27.9±1.4</td>
</tr>
<tr>
<td>{PYD2}3;supK1</td>
<td>42</td>
<td>35 (83)</td>
<td>3 (7)</td>
<td>4 (10)</td>
<td>23.6±0.1</td>
<td>65.7±5.8</td>
<td>29.0±1.3</td>
</tr>
</tbody>
</table>

In this table, the average performance of each genotype tested in the preliminary experiments for circadian locomotor activity is summarized. n equals the number of individuals tested for a given genotype. R, rhythmic, is individual flies that produced a power $\geq 10$; WR, weak rhythmic, is individual flies that resulted in $0 < \text{power} < 10$; AR, arrhythmic. Period is the length of the behavioral rhythm in hours (mean ± SEM). Power was defined as the amplitude of the peak above the significant line ($\alpha = 0.025$) in the chi-square periodogram (Liu et al. 1991) (mean ± SEM). Power is the strength of rhythmicity where an individual P value under 10 indicates that no particular rhythmic patterns were recognized from the animal’s activity during the time of constant darkness. The events/bin value is an indicator of the flies’ activity levels and provides insight into hyperactivity or hypoactivity (mean ± SEM).
Chapter Four
Conclusions and Discussion

In this dissertation, the functional role of the single CRMP protein in *Drosophila melanogaster* signaling and behavior was investigated. As mediators of pathways that regulate growth cone dynamics, vertebrate CRMP isoforms have been proposed to interact with a variety of proteins. This effort has contributed towards investigating these interactions by conducting studies in *D. melanogaster*, which offers an *in vivo* approach and a simplified system. Data presented suggest that the *Drosophila* CRMP gene product mediates signaling through multiple Rac pathways and the Ras/PI3-kinase/Akt/Shaggy pathway during eye morphogenesis. The results indicate that loss of functional CRMP affect eye morphology in directions (either enhancement or suppression) that are consistent with the known roles of mammalian CRMP in homologous pathways. Thus, our data are in agreement with the current model for CRMP’s role in signaling (Schmidt & Strittmatter, 2007). Since this model suggests that CRMP is important in neurological signaling events and expression data in both mammals and *Drosophila* detect this protein in nervous system tissue, the hypothesis that CRMP might play a role in behavioral processes that rely on synaptic plasticity was investigated. The results show that *Drosophila* CRMP is needed for normal learning and memory and circadian behaviors. Effects of CRMP on both types of adult behaviors might reflect interactive relationships between *Drosophila* circadian rhythms and memory (reviewed in Gerstner et al. 2009). Gerstner et al. 2009 documents work in additional species, including vertebrates, that provides evidence, both molecular and behavioral, of a conservation of this relationship between circadian rhythms and learning and memory formation.

CRMP Mediated Signaling

During Sema3A-induced growth cone collapse in mammalian neurons, phosphorylation of CRMP by a variety of kinases converts CRMP into an inactive state. In this inactive state, the association of CRMP with tubulin heterodimers, Numb, and with Sra-1/WAVE complex is reduced (Fukata et al. 2002, Arimura et al. 2005, Nishimura et al. 2003; Kawano et al.
Ultimately, this leads to the loss of cytoskeletal induced extension at the growing tip of the axon and axonal collapse (Schmidt & Strittmatter, 2007). The observation that the Ras1 and Shaggy misexpression phenotypes are suppressed in a CRMP<sup>supK1</sup> genetic background are consistent with CRMP acting downstream of the Ras/PI3-Kinase/Shaggy signaling cascade (Figures 2.11, 2.20 and 2.21). Phosphorylated CRMP has been shown to interact with α2-chimaerin in mammalian neurons, thereby sequestering Rac and consequently diminishing actin polymerization (Brown <i>et al.</i> 2004). In Drosophila, misexpression of a constitutively active form of Rac1 or wild-type Rac2 leads to eye defects that are enhanced in animals homozygous for the CRMP<sup>supK1</sup> mutation (Figures 2.6, 2.8, 2.9 and 2.10). These results are fully consistent with CRMP signaling upstream of Rac in pathways responsible for eye development. The misexpression phenotype produced by a dominant-negative form of Rac1 is unaltered in CRMP<sup>supK1</sup> flies, but absence of a supK1 effect is consistent with Rac functioning downstream of CRMP (Figure 2.7).

One caveat of the misexpression data is that the mutant phenotypes described may not entirely result from erroneous neuronal signaling. The eye phenotypes are outcomes of interactions in and between neuronal and non-neuronal cells alike; therefore, the results might not exclusively demonstrate a role for CRMP in neuronal signal transduction. However, it is important to point out that CRMP is restricted to neurons and DHP is non-neuronal indicating that neurons are involved in CRMP loss-of-function effects. Additional work is required to determine the mechanistic role of fly CRMP in neurogenesis. For example, direct analysis of CRMP influence on Sema signaling in neurons of embryos or Drosophila neuronal cell culture might be necessary to elucidate this role.

Further experiments are also required to prove that the eye morphology modifications are a result of loss of CRMP protein and not DHP protein. Although several lines of evidence support the view that CRMP exon (E9b) containing protein is the insect ortholog of vertebrate CRMP proteins, the null CRMP mutations used in this study lack both the CRMP and DHP forms. Therefore, additional experiments are necessary to distinguish the involvement of each protein in Rac as well as Ras/GSK3β signaling in the developing eye. Since transgenic lines that contain either the {UAS-CRMP} or {UAS-DHP} constructs are at hand, crosses with the same GAL4 drivers and UAS responders that produced positive results could be done to include these transgenes. Analysis for suppression/enhancement of the eye
defects in the presence of the transgenes with a CRMP mutant genetic background might distinguish which of the two proteins is responsible for the modification.

It will be interesting to determine which regions of the CRMP protein are required for such interaction events. In vitro mutagenesis experiments that were conducted as part of this work produced transgenic lines that lack a subset of the CRMP C-terminal region. These UAS responder lines could be utilized in the genetic interaction studies to test for a role of the C-terminal region in the signaling events mediated by CRMP. The C-terminal region of the protein serves as a good candidate region to test, due to the amino acid sequence being highly conserved and the site of an array of phosphorylation events via the kinases previously examined (Arimura et al. 2000; Hall et al. 2001; Brown et al. 2004; Cole et al. 2004; Cole et al. 2006; Arimura et al. 2005; Uchida et al. 2005). Another candidate region to test is located in the N-terminal region of the protein. Experimental mutagenesis on surface-exposed residues of CRMP-1 has shown that alanine substitutions in one domain (S4 & S5 or S5 & S6 linker, residues 46-57) of CRMP-1 caused Sema3A-independent COS-7 cell contraction (Deo et al. 2004). These residues are also found in other mammalian CRMP protein isoforms (Stenmark et al. 2007). In vitro mutagenesis on homologous residues in Drosophila will provide additional transgenic lines to test in the interaction study.

New Insights into CRMP’s Involvement in Behavior

In this study, the characterization of CRMP in adult behavior was investigated. The current model for memory formation in Drosophila proposes immediate memory, short-term memory, and middle-term memory occurring as sequential steps in a linear pathway and long-lasting forms of memory (LTM and ARM) branching into two parallel pathways (Figure 3.1). A variety of genes, chemicals, and environmental factors have been shown to influence the distinct phases that underlie observed memory. Our work has contributed to this understanding by providing evidence that the CRMP gene is required for normal 2 min memory, 3 hr memory and long-term memory formation. Considering the model for memory formation, it is possible that CRMP is required during the initial stages of memory acquisition; thus, disrupting not only the immediate recall but blocking the downstream memory consolidation as well.
Animals homozygous for any one of three independent mutant alleles of CRMP are deficient in learning and memory. Animals trans-heterozygous for CRMP<sup>osgK1</sup> and CRMP<sup>osplal</sup> are also deficient in learning and memory indicating that the two mutant alleles fail to complement. The memory defect is not restored in mutant animals by expressing the CRMP-encoding cDNA in the mushroom body using c309-GAL4. Additional MB specific GAL4-drivers, including c747, should be tested for rescue. Recent expression data from preliminary anti-GFP antibody staining of {PYD2GFPfs9a} adult brains suggests a spatial distribution of CRMP in regions of the nervous system necessary for memory formation (MBs), but do not identify a temporal requirement for CRMP in <i>Drosophila</i> learning and memory (data not shown). The next step would be to provide a wild-type copy of CRMP just before aversive olfactory conditioning. The TARGET® system is a great method that will address the acute need of CRMP in memory formation (McGuire <i>et al.</i> 2004). This system utilizes a temperature sensitive GAL80 allele to temporally regulate the UAS-Gal4 misexpression of CRMP-encoding cDNA. In this experimental set-up the animals are raised at 18°C, a temperature that restricts Gal4 activity, thus inhibiting the expression of the {UAS-CRMP} responder. Three days prior to training the adults are place at the permissible temperature of 30°C for Gal4 activity, enabling CRMP production in the nervous system. These animals then are tested for restoration of learning and memory in the absence of an endogenous functional copy of CRMP.

In flies, many genes have been identified in pathways contributing to learning and memory (Dubnau 2003). CRMP is a new addition to this list of proteins that act in learning and memory. The ability of CRMP to interact with other proteins in memory processes should be investigated. Most interesting is Ras-dependent signaling in memory formation, which has been documented in many different systems (Orban <i>et al.</i> 1999). The data presented in chapter two provide evidence that CRMP and Ras interact during fly eye morphogenesis. Other research focusing on the hematopoietic system in flies has shown via microarray analysis that CRMP is upregulated in larval hemocytes expressing activated Ras (Asha <i>et al.</i> 2003). If this relationship is true in fly aversive olfactory learning and memory, CRMP may act through Ras in these complex behaviors.
A high percentage of CRMPsupK1 flies are arrhythmic in the locomotor activity assay. The arrhythmic behavior of CRMPsupK1 animals is rescued by providing an endogenous copy of the wild-type CRMP gene in the form of a transgene. This same transgene is not sufficient to rescue the learning and memory defects in homozygous CRMPsupK1 animals, perhaps due to differences in expression in the relevant areas of the brain. A high percentage of flies that possess the {EP3238} P-element, which disrupts the CRMP gene, also test arrhythmic. Interestingly, the CRMPsupIa1 allele does not disrupt circadian locomotor rhythms with most of the flies screened exhibiting normal rhythmicity. One possible explanation is that the CRMPsupIa1 gene produces a protein product (N-terminal fragment) that is capable of regulating circadian activity in the adult brain. Quantitative RT-PCR followed by sequencing analysis would help identify the amount, size, and sequence of transcripts that are made in homozygous CRMPsupIa1 animals. Another explanation is that the CRMPsupIa1 strain contains a linked modifier gene that suppresses the effects of the mutation on circadian activity rhythm.

The daily locomotor activity patterns of the CRMPsupK1 and P{EP}CRMPEP3238 mutant lines are abnormal. These flies exhibit sporadic activity throughout the entire day during constant dark conditions, even though the flies are negative for hyper- or hypo- activity and exhibit a normal period length. A plausible explanation for this observation is that CRMP plays a role in maintaining the normal oscillator function that triggers the anticipatory behavior displayed in wild-type flies at lights-on and lights-off. It will be interesting to see if expressing CRMP specific mRNA in circadian clock neurons can restore the usual anticipatory morning and evening peaks of activity during constant darkness in CRMP mutant flies.

This research project has revealed that animals lacking CRMP display defects in both learning and memory and circadian rhythmicity. However, how this one protein can modulate both behavioral processes is unclear. It is possible the two behaviors are interrelated by CRMP activity. Previous research by Lyons and Roman 2009 reports that the ability of flies to form STM is under influence of the circadian clock. Animals exhibit a peak performance in the olfactory conditioning assay during early night time (Lyons & Roman, 2009). It is possible that CRMP is functioning in an output pathway in response to the central clock to regulate this peak performance in the Pavlovian behavioral assay. Therefore,
loss of CRMP function could result in the circadian modulation of STM being altered and explain the reduction in learning and memory performance. CRMP could be a key molecular effector of the circadian clock, and thus regulate the behavioral process of learning and memory. It is unknown how the clock confers temporal information to modulate cellular function and CRMP might be a potential candidate.

In conclusion, this work has provided the first evidence that the fly CRMP gene encodes the putative CRMP homolog and answers questions regarding CRMP’s role in D. melanogaster. These studies have opened the door to many questions regarding CRMP to be addressed in future experiments.
Fly strains and transgenes

All flies mentioned in this work were raised on standard cornmeal food and at 25°C unless otherwise noted. Additional information on most genes, balancers and lines used can be found at FlyBase (Tweedie et al. 2009). Strains containing the following transgenes were obtained from the Bloomington Drosophila Stock Center: UAS lines P{UAS-Rac1.V12}1, P{UAS-Rac1.N17}1, P{UAS-Ras}5-1, P{UAS-Ras.N17}TL1, P{UAS-Ras64B.V14}1, P{UAS-sggB}MB14, P{UAS-sgg.S9A}MB14, P{UAS-Rho1.V14}2.1, P{UAS-Rho1.N19}1.3, P{UAS-Akt1.Exel}1, P{UAS-Pi3K92E.Exel}2, P{UAS-Pi3K92E.CAAX}1 and P{UAS-Pi3K92E.A2860C}1; and Gal4 driver lines P{GawB}elavc155, P{GAL4-ey.H}3-8, P{ninaE-GAL4.GMR}12, P{sevEP-GAL4.B}7, P{GawB-GAL4}389, P{Cha-GAL4.7.4}19B, P{Ddc-GAL4.L}4.36, P{Pan-R7-GAL4}2, P{RN2-GAL4}P, and P{GawB-GAL4}1407. The {Rac2}EP3118 strain was obtained from the Szeged Drosophila Stock Center and it contain the P{EPgy2} enhancer trap transposon inserted into the 5' end of the Rac2 gene. The wild-type flies utilized in behavior experiments were Canton-S w1118 (iso CJ1) and were a gift from the Dubnau lab. The Gal4 enhancer trap lines c309, c747, c739, 247, 201Y, and OK107 used for behavioral rescue experiments were also a gracious gift from the Dubnau lab.

The mutant CRMPsupK1 and CRMPsupIa strains used were created in this lab and specifically for this effort. The CRMPsupK1 line was generated via HMPA chemical mutagenesis (Nairz et al. 2004) and selected based upon the mutation blocking suppression of the black body phenotype by the semidominant rSu(b) mutation (Rawls 2006). In this screen, chemically treated w/Y; b;+ males were crossed to w; b; ri CRMPsupA4 p+ P{rSu(b).cSa} females. Among the progeny, the CRMPsupK1 mutation was isolated from a rare black body animal. The P{EP}CRMPEP3238 line used in the P-element mobilization screen that created the CRMPsupIa imprecise excision mutant (see chapter 2 for cross scheme) and in behavioral studies was obtained from Exelixis. The EP(3)3238 strain has a copy of the P{EP} transposon inserted near the 5' end of the CRMP gene (Liao et al. 2000). The extents of
mutant deletions were determined by PCR analysis of genomic DNA from mutant animals, then by sequencing gel-purified PCR fragments (Rawls 2006).

In this study, a variety of \( P\{PYD2\}^+ \) modified transgenic lines that possess a genomic DNA copy of \( CRMP \) were utilized and are described in more detail elsewhere (Rawls 2006; Rawls and Morris, in preparation). These lines include: \( P\{PYD2\}_1 \), \( P\{PYD2\}_3 \), \( P\{PYD2GFP\} \), \( P\{PYD2GFPfs9a\} \), \( P\{PYD2GFPfs9b\} \) and \( P\{PYD2GFPsupK1\} \). The \( P\{PYD2\}_1 \) and \( P\{PYD2\}_3 \) lines are independently isolated insertions, differing in the genomic site of the transgene insertions on the second chromosome. \( P\{PYD2GFP\} \) contains an in-frame eGFP cassette inserted within exon 12 of the \( CRMP \) gene immediately upstream from the stop codon of the protein ORF. Two derivatives of \( P\{PYD2GFP\} \) were created with frameshift mutations within either exons E9a or E9b. \( P\{PYD2GFPfs9a\} \) was derived by deletion of a 26 bp \( SacII \) fragment within exon E9a and presumably only encodes for a functional CRMP protein. \( P\{PYD2GFPfs9b\} \) contains a 4 bp deletion within exon E9b that was created by \( SacI \) cleavage, exonuclease trimming, and blunt-end ligation of that site and only produces a functional DHP protein. The \( P\{PYD2GFPsupK1\} \) transgene was made by removing the wild-type 2.7 kb \( XhoI \) - \( EcoRV \) fragment of the \( P\{PYD2GFP\} \) transgene and replacing it with the 2.55 kb fragment of \( CRMPsupK1 \) mutant DNA.

\( P\{UAS-CRMP\} \) and \( P\{UAS-DHP\} \) are insertions of full-length cDNAs into the pUAST vector (Brand and Perrimon 1994). \( P\{UAS-CRMP\} \) contains the CRMP-encoding GH07678 cDNA and \( P\{UAS-DHP\} \) contains the DHP-encoding LP11064 cDNA (Rawls and Morris, in preparation).

**RT-PCR**

Mini-prep of total RNA of ~50 flies for each genotype was performed. The flies were homogenized in solution D (10g guanidinium thiocyanate, 11.9 ml DEPC-treated H\( _2 \)O, 0.53 ml 1M sodium citrate pH 7.0, and 1.1 ml of 10% sarcosyl, and add 1 ml of mixture to 7.2 \( \mu \)l 2-mercaptoethanol) on ice, followed by phenol/chloroform extraction, isopropanol precipitation, and ethanol wash. Recovered RNA was vacuumed dried, dissolved in 25 \( \mu \)l of DEPC-treated H\( _2 \)O, and stored at -70°C. 10 \( \mu \)l of total RNA and the PYD2I primer were
used for cDNA synthesis. The hybridization reaction consisted of the following: 3 µl 10X PCR buffer (100 mM Tris-Cl pH 8.3, 500 mM KCl, 30 mM MgCl₂, 1 µg/µl gelatin, and 150 µl dNTPs), 2 µl PYD2I primer, 10 µl RNA, and 15 µl of DEPC-treated H₂O incubated at 42°C for 30 min. The extension reaction entailed the following: 5 µl 10X PCR buffer, 10 µl 200 mM MgCl₂, 5 U AMV reverse transcriptase XL (Life Sciences, Inc. cat. # LME 704, con. 35,000 U/ml), 10 µl 20 mM DTT, and 25 µl of DEPC-treated H₂O, and 10 µl of extension reaction mix was added to hybridization reaction tube and incubated at 42°C for 30 min. The extension reaction was stopped by boiling samples for 5 min. A total of 40 µl of cDNA product for each sample was obtained and then 10 µl of cDNA was used in PCR amplification reactions. PCR program was set-up as followed: Cycle 1 (1X): 2 min at 94°C; Cycle 2 (35X): 1 min at 94°C, 1 min at 50°C, 3 min at 68°C; Cycle 3 (1X): 7 min at 68°C.

Primers used in RT-PCR experiment:
PYD2I: GTATACGAACGGCGAGCACTGG
PYD2V (Primer 1 in Figure 2.3): CCGTTAAGAAGGTGCCATTCGATTGCAG
PYYD2Qrc (Primer 2 in Figure 2.3): TGTCTTGGCAATGATGTCGCCATTCTC

In vitro mutagenesis

To engineer the C-terminal deletion CRMP mutants, nested-PCR was carried out to generate each mutant sequence fragment. The following PCR primers were used to generate the genomic DNA sequences for the C-terminal deletions:

mutΔ1new: CCAGAATCCAGAAGACCTTTCCGCCCCGCTTGCCCATGCTGCGGTTCT
mutΔ2: CCAGAATCCAGAAGACCTTTCCGGCTATGGAGAAGAAGACTCCTGCAA
mutΔ3: CCAGAATCCAGAAGACCTTTCCGGCTATGGAGAAGACTCCTGCAA
UASTEcoRc: GCTAAACAATCTGCAGTAAAGTGCAAG
PYD2Cc: GCGGAAAGTCTTCTGGATTCTGG
UASTXba: GCTCCCATTCATCAGTTCCATAGGTGG

The obtained individual fragments were first subcloned into the pBSIIKS+ vector, and then subcloned into the pUAStCRMP vector. The pBSIIKS+ vector was cut with BamHI and XhoI and ligated with the individual deletion fragments (2.8 kb). The pUAStCRMP vector
was cut with *Not*I and *Xho*I and ligated with each previously subcloned deletion fragment (2.8 kb). Each ligation product was transformed into *E. coli* DH5α cells for propagation of the construct. Plasmid DNA constructs were purified, reconfirmed by restriction analysis, and microinjected into pre-blastula *Drosophila* embryos. Unique transgenic lines were selected for by genetic crosses in which constructs were mapped to their respective chromosomes. Four independent lines for *P{UAS-CRMPmutΔ1}* were recovered, four independent lines for *P{UAS-CRMPmutΔ2}* were recovered, and two independent lines for *P{UAS-CRMPmutΔ3}* were recovered. The established transgenic lines were crossed to neuronal GAL4 drivers.

**Behavior**

*Aversive Pavlovian Olfactory Conditioning Assay*

All behavioral experiments were conducted so that the experimenter was blind to the genotypes being tested. The genotypes were also balanced and rotated equally throughout the experimentation process. Data in each chart represent independent sets of experiments, even if the genotypes and training paradigms are the same in some figures. For all figures, the experiments within each chart were performed in parallel. Olfactory associative learning was assessed by training 2 to 3 day old flies in a T-maze apparatus with a Pavlovian conditioning paradigm and testing 2 minutes after training (Tully and Quinn 1985). Training involves exposing the flies sequentially to one odor (CS+) paired with footshock and then a second odor (CS-) with no footshock. Testing entails exposure of the flies simultaneously to the CS+ and CS- in a choice spot of a T-maze and the flies were given two minutes to make a decision between the two odors. The flies were then trapped in either T-maze arm and counted. Middle term memory was also assessed using the same single training paradigm, but testing occurred 3 hours post training (Dura *et al.* 1993). Odors used were 3-octanol (concentration of 180µl diluted in 10ml mineral oil) and 4-methylcyclohexanol (concentration of 270µl diluted in 10ml mineral oil). Each individual n consisted of two groups of ~100 flies, each of which was shocked in the presence of one of the two odors. Thus, a single n consisted of ~200 flies, with half being trained to one odor and the other half being trained to the other odor. A half performance index was calculated by subtracting the
number of flies that chose correctly (odor that was unpaired with shock) minus the number of flies that chose incorrectly (odor that was paired with shock) and dividing that number by the total number of flies used in the experiment. A full performance index was calculated by averaging both reciprocal half performance indexes for the two odors. The final performance index reported in each chart for each experiment was calculated by averaging the full performance indexes for all replicas or n’s that were performed for a given genotype.

For long term memory experiments, animals were subjected to ten such training sessions that were spaced apart with 15 minute rest intervals (Tully et al. 1994). For this repetitive training protocol, robotic trainers were used and the flies were manually tested by placing them in the choice point of the T-maze apparatus 24 hours after training. All genotypes were trained and tested in parallel and were rotated between the robotic trainers to ensure a balanced experiment.

Sensorimotor Control Experiments

Olfactory acuity was examined by taking about 100 untrained flies and exposing them to a 2 minute test trail in the T-maze (Boynton and Tully 1992). The flies were given a choice between either OCT or MCH versus untainted room air. Shock reactivity was examined by taking about 100 untrained flies and exposing them to a 2 minute test trail in the T-maze, but this time each arm of the T-maze contained an electric shock grid (Dura et al. 1993). The flies were given a choice between shock (60V) versus no shock. In both cases, PIs were calculated as described above.

Circadian Locomotor Activity Assay

Locomotor activity rhythms of 1 to 3 day old adult male flies were monitored at 25°C as described in Rosato and Kyriacou 2006. The behavior of the flies was monitored over 3 to 4 days in 12 hrs light/dark (12:12 LD) conditions, followed by monitoring of free-running activity in constant darkness (DD) conditions for an additional 7-8 days. The number of activity events, detected by infrared beam breakage, was recorded per half-hour bin, and average numbers of activity events per bin, per fly were calculated. This locomotor activity of individual flies was monitored using Drosophila activity monitors (Trikinetics). The
measurement of locomotor activity was determined by using 7-mm-diameter locomotor monitors equipped with dual detectors to allow free movement of the flies (Trikinetics; Rosato and Kyriacou, 2006). Data analysis was done with ClockLab software (Actimetrics).

Statistics

The behavioral data collected using the odor discrimination paradigm were normally distributed and therefore, could be analyzed by analysis of variance (ANOVA). JMP 5.1 statistical discovery software was utilized to perform One way Anova and Student’s t tests, with means comparison made between each genotype. Statistical significance in the figures represents a significant decrease in performance in comparison to the wild-type control levels and is signified with p-values \( \leq 0.05 \). Error bars in the data graphs represent the standard error of the mean. For the behavioral data collected using the locomotor activity assay, a Dunnett’s post hoc analysis was performed. The Dunnett’s post hoc analysis, comparing all average values to the wild-type control line, produced the following outcomes: \( p<0.01 \) stocks are significantly different, \( p>0.01 \) stocks are not significantly different. Error bars in the data graphs represent the standard error of the mean.

Immunohistochemistry

Embryo staining technique

Approximately 100 staged and dechorionated embryos (exposure to 50% bleach for 2 minutes) were fixed in heptane containing 37% ethanol free formaldehyde for 40 minutes at room temperature (RT). Fixed embryos were then hand devitellinated and stored at 4°C in TBTA. To sort embryos based upon genotype, they were placed in polyclonal rabbit anti-GFP primary antibody (1:500 dilution in TBTA) for 2 hr at RT (Torrey Pines Biolab, cat. # TP401). TBTA is 50ml 10X TBS, 250µl tritonX-100, 5g bovine serum albumin, 0.1g sodium azide adjusted to 500ml with dH2O. After 2 quick washes in TBTA followed by a 2 hr wash in TBTA, the embryos were placed in secondary antibody (1:500 dilution in 2° diluent (10% 10XTBS, 20% Tween20, 10% 3M NaCl in dH2O)), AlexaFluor 488 anti-rabbit,
for 2hr at RT (Molecular Probes, cat. # A21206). Subsequent to staining, the embryos underwent a 2 hr rinse in 2° diluent at RT. Embryos were sorted based upon GFP fluorescence vs. non-GFP fluorescence. To stain the CNS axons, embryos were placed in 1:15 dilution of BP 102 primary antibody in TBTA (Developmental Studies Hybridoma Bank at the University of Iowa) for 2 hrs at room temperature, followed by an overnight (ON) wash in TBTA. The embryos were rinsed 2 X in 2° diluent and placed in 1:1000 HRP coupled anti-mouse (Santa Cruz) for 2 hr at RT. The secondary antibody was removed by 2 quick washes and 1 overnight wash in 2° diluent. After anti-CNS staining, the embryos were placed in DAB for 10 minutes, rinsed in 1XPBS and mounted on slides using Vectashield® mounting medium (Vector Laboratories, cat. # H-1000).

Adult brain staining technique

Ten brains for each genotype of 2 to 3 day old adult flies were dissected in phosphate-buffered saline (PBS) and fixed in PBS containing 4% paraformaldehyde overnight at 4°C. Brains were placed in a vacuum for 20 minutes to remove air from tracheae and then were blocked in penetration/blocking buffer consisting of 1XPBS, 2% Triton and 10% normal goat serum (Jackson ImmunoResearch Laboratories, Cat. 005-000-121) for 2 hours at 4°C. Then the dissected fly brains were placed in primary antibody (1:20 dilution in Dilution Buffer containing 0.25% Triton and 1% normal goat serum in PBS) for overnight at 4°C. After rinsing brains with Washing Buffer (1% Triton, 3% NaCl in 1XPBS) for 4 X 10 minutes at room temperature, the brains were placed in secondary antibody (1:200 dilution in Dilution Buffer) for overnight at 4°C. The following antibodies were used: monoclonal anti-Fasciclin II-s antibody 1D4 (Developmental Studies Hybridoma Bank at the University of Iowa) as primary antibody for FasII staining, Cy3 conjugated AffiniPure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Cat. 115-165-003) as secondary antibody. Lastly, the brains were washed by washing buffer 4 X 10 minutes at room temperature, treated with FocusClear (CelExplorer Labs, Cat. FC-101) for 10 minutes and mounted onto slides using MountClear (CelExplorer Labs, Cat. MC-301).

Image Acquisition and Processing
The adult fly eye images were taken on a Nikon SMZ1500 dissecting scope with a SPOT camera and adjusted in Adobe Photoshop. Embryo images were taken on a Nikon E800 microscope with a SPOT camera. The confocal stacks of brains were acquired using the ZEISS LSM 510 confocal microscope. The following confocal settings were used: 20X lens or 40X water immersion lens, 1µm spacing in the z axis and 1024X1024 resolution in the x and y axes. The Cy3 signal is captured by HeNe 543nm laser and GFP signal is captured by Argon/2 488nm laser. All brains were scanned from the anterior to the posterior. The data were processed into projected images by LSM Image Browser Rel.4.2 (ZEISS) and later arranged into figures using Microsoft PowerPoint.


Bloomington Drosophila Stock Center at Indiana University.


genomic fragments that restore circadian and ultradian rhythmicity to per$^0$ and per$^-$ mutants. *J. Neurogenet.* 3: 249-291.


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