

**Investigation for novel
anti-apoptotic factors in the neurons of
*Drosophila melanogaster***

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Honors Thesis

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Abstract

Neurodegenerative disorders plague the global population in vast number with very few treatments and no cures. These disorders act through various different mechanisms ranging from overexpression of certain genes to premature neuronal death. With no cure yet to be discovered, there is a need to look at other possibilities of treatment besides medicine to treat side effects. In attempt to find a genetically controlled factor that will prevent induction of apoptosis, or programmed cell death (PCD), this study screened eighty transgenic RNAi lines of *Drosophila melanogaster* with the hope of finding an anti-apoptotic factor. CCAP neurons were targeted using a UAS-GAL4 system as these neurons play an important role in wing formation and remain intact through the dynamic reconstruction *D. melanogaster* undergoes transitioning from larva to adult. Although the study did not yield any anti-apoptotic factor, one gene stood out due to its effect on wing structure. When silenced through RNAi, pebbled inhibited wing expansion and appears to have ablated the neurons before *D. melanogaster* enters the pupal stage of its life. Therefore, this study's results suggest that pebbled affects CCAP neurons functionality.

Introduction

Neurological Disorders

Neurodegenerative disorders are pathological manifestations from disturbed neuron function, whether by immediate injury or chronic activity (Martin 2002). Underlying mechanisms for these disorders are being investigated, and some have discovered that select properties of certain neurons can lead to selective vulnerabilities for those particular neurons (Martin 2002). Neurodegenerative disorders have afflicted millions of people around the world costing billions for treatments and deaths (Martin 1999 and Jackson 2014). Some appear

hereditary like Huntington's while others are more spontaneous like Alzheimer's (Martin 1999). They can range from genetic mutations to posttranslational alterations ultimately leading to the death of the neuron via programmed cell death (Martin 1999). These disorders have no cure and limited therapies. The study of neurodegenerative disorders encounters many difficulties when applied to humans. The human brain is a complex system of connections and activity. The disorders themselves are difficult to understand, and their specific mechanisms are still relatively unknown. Also, there is an arduous process that must be followed before testing can even be considered on humans. Therefore, much of what is known about neurodegenerative disorders utilizes non-human species for experiments.

Drosophila as a Model Organism

A particularly noteworthy non-human species used in the study of neurodegeneration is the common fruit fly, *Drosophila melanogaster*. This species, generally referred as a pest outside a research facility, has been used as a genetic model for over a century pioneered by Thomas Hunt Morgan (Stephenson and Metcalfe 2013). It is an ideal test subject for experiments requiring large sample sizes as it has a short development time just about ten days from embryo to a sexually mature adult (Jennings 2011). Its contribution to understanding signaling pathways and other metabolic attributes has been an immense addition to the knowledge of human pathology alongside its own (McGurk et al. 2015). A number of their developmental genes correspond to similar genes among higher order organisms like humans (Jennings 2011). It has four distinct life stages that require dramatic changes in anatomy and physiology. From the start, these fruit flies begin as eggs. Once laid, an egg will typically hatch after about a day at 25°C as a larva. From there, the larva will eat and grow over a week or so before it decides it is ready to pupate. Once it has pupated, the larva that entered the stage will undergo a dynamic

metamorphosis and eclosion, or emerge from their pupal casings, to become an adult fly (Jennings 2011). Reconstruction creates an entirely new physical profile with different functions and capabilities. This study focused on genetic regulation of before and after reconstruction of the *Drosophila*'s central nervous system (CNS). The larval CNS consists of two lobes, making up the brain, and a ventral nerve cord (VNC) that transitions into an entirely new configuration with the brain taking residence in the head and the VNC in the thorax of the species (Fig. 1). *Drosophila melanogaster* has a complex neuronal network that is incredibly similar to vertebrates (McGurk et al. 2015). For this reason, studies in neurodegeneration can be modeled using this species.

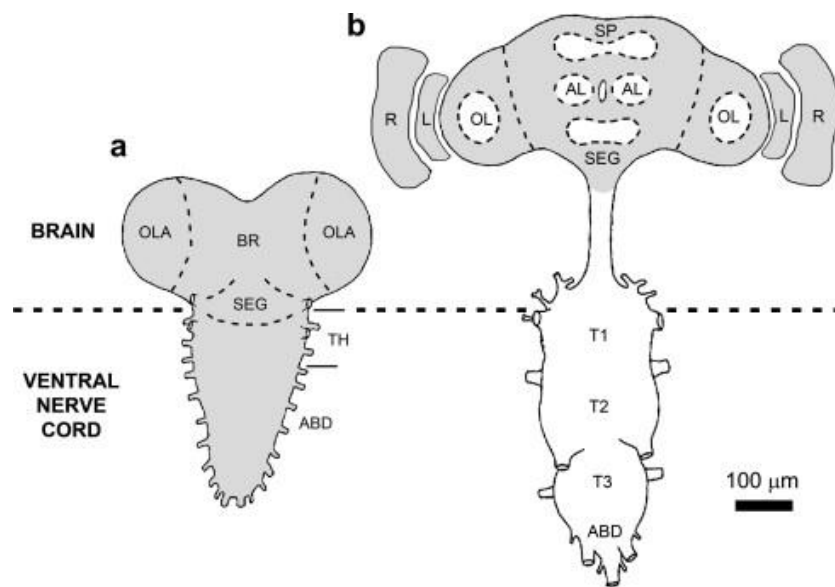


Figure 1. Schematic representation of *Drosophila melanogaster* third instar larva (a) and adult (b) CNS (from Strunov et al. 2013). OLA – optic lobe anlagen; BR – brain; SEG – subesophageal ganglion; SP – superior protocerebrum; AL – antennal lobe; OL – optic lobe; L – lamina; R – retina; ABD – abdominal neuromeres; TH – thoracic neuromeres; T1 – prothoracic neuromeres; T2 – mesothoracic neuromeres; T3 – metathoracic neuromeres.

CCAP Neurons

For the following experiment, focus turns to the Crustacean Cardioactive Peptide (CCAP) neurons found within the central nervous system (CNS) of *D. melanogaster*. These neurons are located along the VNC and into parts of the optic lobes (Fig. 1). CCAP neuron activity have been observed in larval, pupal, and adult stages of life which means it somehow survives the drastic changes that occur between each life stage for the fruit fly (Nichols et al. 1999). This is a key feature that was utilized within this experiment. First discovered in crustaceans in 1987, these neurons produce several different kinds of neuropeptides that act as regulators of some essential physiological activities and contain receptors for other important neuropeptides such as ecdysis-triggering hormone (ETH) and eclosion hormone (EH) (Dulcis et al. 2005, Baker et al. 1999). A majority of these expressed neuropeptides are peptidergic and highly conserved including bursicon and partners of bursicon (burs and pburs), CCAP, and myoinhibitory peptides (MIPs) (Kim et al. 2015). These particular neuropeptides hold key roles in adult ecdysis such as bursicon which is essential in wing expansion and cuticle tanning and hardening (Peabody et al. 2008). CCAP are involved in the circulatory system as well as visceral organ activity in *Drosophila* (Dulcis et al. 2005, Yip et al. 1997). Some studies have shown that restricted cardiac function could negatively affect the act of eclosion which is when the fully formed adult fly emerges from its pupal casing (Park et al. 2003). Effect on *Drosophila*'s ability to eclose was observed during the crosses made during this experiment and acted as a measure for determining how transgenic crosses performed.

Apoptosis

As mentioned before, the fruit fly undergoes dramatic changes in its morphology. Going from a wriggling larva to a flying adult takes a significant amount of time and energy for the species. Not only that, there are certain processes that are required for *Drosophila* to transition

smoothly between each stage of life. One process essential for this metamorphosis to occur is accomplished via a mechanism known as programmed cell death (PCD) and more specifically a type of PCD known as apoptosis. Apoptosis is not specific to *D. melanogaster*. It is seen in nature in a variety of species, including as humans, as a fundamental mechanism essential for development and homeostasis management (Fuchs and Steller 2011). Defects in its regulation has been shown to cause a range of pathologies in humans ranging from genetic disorders to diseases and even cancer (Fuchs and Steller 2011). *D. melanogaster* uses three known apoptotic genes to aid in its transition from larva to adult during the pupal stage. These genes, called *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*, activate signaling pathways that induce apoptosis and are considered proapoptotic genes (Fuchs and Steller 2011). Once apoptosis is induced it cannot be stopped within its target whether that be a cell or tissue. Regulation of this mechanism, therefore, is essential to ensure the “programmed” portion does not affect cells or tissues that do not require apoptosis. This regulation is suspected in protecting the CCAP neurons from death unlike their surroundings and the focus of this experiment. The exact factors that are required for their preservation from this deadly mechanism require further investigation.

Research Strategy

For this experiment, several genes were screened in the hope of finding anti-apoptotic factors that allow CCAP neurons to bypass apoptotic processes as *Drosophila* transition from larva to adult. Previous studies have shown that the genetic removal of CCAP neurons causes defects in their ecdysis behavior (Park et al. 2003). Genetic crosses were made using the bipartite UAS-GAL4 system in which genes within the CCAP neurons will be knocked down or silenced via RNA interference or RNAi. If the gene that has been knocked down is important for CCAP survival, then the *Drosophila* will not continue past the pupal stage as a result.

Materials and Methods

UAS-GAL4 system

Directed gene expression is an important technique required by scientists to study specific genes. However, there were limitations to previously most common gene expression manipulation methods. While the first method could induce expression, heat shock promotion required a heat shock promotor as well as heat shocking the transgenic animal while yielding low levels of expression (Brand and Perrimon 1993). The second method used transcriptional regulatory sequences from a defined promotor which permitted expression in specific cells but were limited in availability as well as being toxic for the organism (Brand and Perrimon 1993). Therefore, the UAS-GAL4 system was established. With this system, targeted gene expression was done in a temporal and spatial manner making it an incredible genetic tool (Duffy 2002).

Discovered in yeast, the 881 amino acid protein, GAL4, acts as a transcriptional regulator that is sequence-specific within DNA (Kakidani and Ptashne 1988, Duffy 2002). This protein produced by the *gal4* gene regulates transcription by binding to four sites known as Upstream Activating Sequences (UAS) which behaves similarly to enhancer elements in eukaryotes (Duffy 2002). Through this mechanism, transcription of specific genes in specific tissues can be activated (Kakidani and Ptashne 1988). This prokaryotic mechanism was replicated in the *Drosophila* genome by transformation, a type of horizontal gene transfer that involves uptake of foreign DNA sequences and integrating into recipient's own genome (Fischer et al. 1988). Further studies would also implement this machinery into other model organisms, like zebrafish.

The UAS-GAL4 system was made into a bipartite method where the parental lines, one expressing the GAL4 driver sequence and the other contains the UAS responder sequence, are

maintained separately, but their resulting progeny after mating contains the combined system where they will express both the driver sequence and responder sequence revealing the targeted gene products (Brand and Perrimon 1993, Duffy 2002) (Fig. 2). The bipartite aspect of this system is key to studying genes whose products could be detrimental to the organism if activated rendering loss of stock (Duffy 2002). Hence, this system has become an essential technique used by the majority of geneticists. In this experiment, the system was used in conjunction with RNA interference, RNAi, to knock down or silence suspected genes aiding in the protection of CCAP neurons through metamorphosis.

RNAi

The ability to knock down, or silence, expression of genes is another method of gene expression manipulation that was more recently characterized than the UAS-GAL4 system (Sen and Blau 2006). RNA interference, RNAi, utilizes transcription of double-stranded RNA (dsRNA) which breaks down via enzymatic activity into short interfering RNA segments (siRNA) which mark certain mRNA strands for destruction (Grishok et al. 2001). This destruction prohibits translation of the specific protein the target mRNA specifies (Fig. 2). Not only does it halt translation, but there are studies that show a posttranscriptional silencing of the corresponding gene (Grishok et al. 2001). RNAi's ability to silence genes has been incredibly useful for isolating gene function.

Therefore, in order to elucidate whether a gene was necessary or not for CCAP neuron protection, each gene that was investigated was replaced with this RNAi gene which would knock down the function of the gene. The phenotypic effects that resulted from this knock down could then be observed. This method is also not restricted to only *Drosophila* but has also shown to be effective in other species such as mammalian cells (Nielsen and Nielsen 2013). Therefore,

if a gene were to present itself as producing a protective factor against apoptosis within *Drosophila*, then the gene's homologue in human cells could also be observed and tested to have the same effect within neurons.

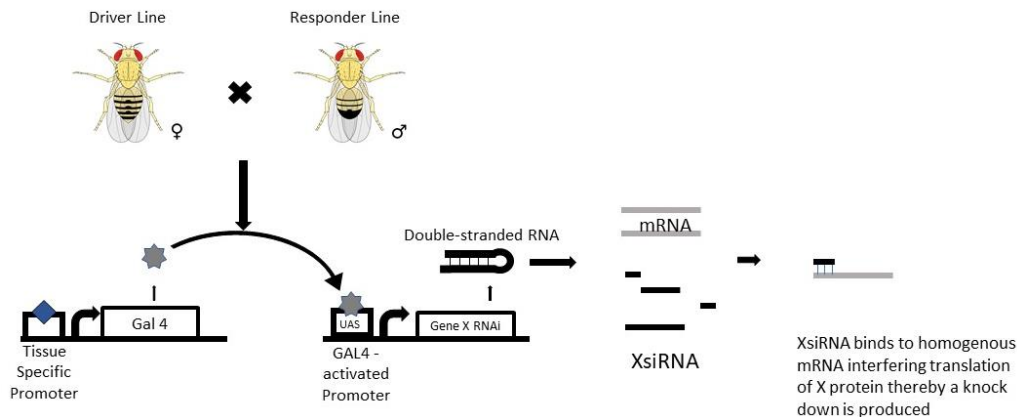


Figure 2. Visualization of UAS-GAL4 system. One parent contains the driving Gal4 sequence, referred to as the driver line. The other parent contains the corresponding UAS sequence, referred to as the responder line. The two sequences insert themselves in their designated position and begin expression within the targeted tissue. The *gal4* encodes for the GAL4 protein which binds to the GAL4-activated promoter, initiating transcription of the targeted gene that has RNAi added into it. Expression of the RNAi produces a dsDNA which is sliced into subsequent short-interfering RNA sequences that bind to the target gene's mRNA and halts translation of the targeted protein.

Crossing transgenic lines

Six males from test lines were crossed with five UAS-mCD8GFP; CCAP-gal4^{III} virgins for each crossing. The test lines were ordered from the Bloomington Drosophila Stock Center, specifically using their TRiP lines which is the Transgenic RNAi Project. These lines have been verified to be transgenic RNAi lines. The genes chosen for this screening were considered due their previously identified functions as being a transcription factor or part of a signaling pathway directly affecting gene expression such as the Jun-Kinase (JNK) pathway. By crossing these transgenic lines that contain the necessary UAS with virgins that expressed CCAP-GAL4 driver, they ensured these transgenic lines targeted the CCAP neuron tissues. Some of these lines were

heterozygous segregating which means that the lines required the utilization of balancer chromosomes.

Balancer chromosomes are used to suppress recombination with homologues that would otherwise lead to lethal or sterile mutants (Lattao et al. 2011). These balancers contain dominant markers that can be seen in all adults, but they also contain a subset of markers that will indicate homozygous mutant larvae from heterozygous (Lattao et al. 2011). The negative control for this experiment is normal development of the pupae (Fig. 3). This results in a subsequently successful eclosion by a fully developed adult. The positive control for the results were measured against was *UAS-grim*. Premature expression of *grim* leads to total pupal death (Fig. 4). If the resulting progeny from the test crosses produced the same effect, then the gene would be considered to be essential in preventing apoptotic expression within the CCAP neurons.



Figure 3. Normally developed pupa. This pupa has easily distinguishable features such as the eyes within the left portion of the pupa casing. There are also furled wings that have will expand once eclosion occurs denoted by the darkened portions seen in the midsection of the pupa casing.



Figure 4. Premature overexpression of *grim*. The head region, denoted by the black circle, has completely collapsed and condensed with no distinguishable features to indicate development. The dorsal region shows that the pupa has completely detached and shriveled away from the side leaving the casing completely hollow at the dorsal region.

Results

In an initial screening, eighty RNAi lines were crossed with UAS-mCD8GFP; CCAP-gal4III (U-mG; CCAP-gal4), which drives the expression of the RNAi genes in CCAP neurons as well as one for GFP (Fig. 5). The UAS-mCD8GFP produces a GFP signal in the CCAP neurons. GFP is a green fluorescent protein isolated from jellyfish and is used as a marker for tissue visualization in various organisms (Stretton et al. 1998). After screening the different genes ranging in functions from development to transcriptional controls, there were no knocked down genes that yielded any probable anti-apoptotic factors as all larvae grew into adulthood. However, some of these genes resulted in eclosed adults exhibiting unusual wing formations. One particular gene stood out. Pebbled (*peb*), which codes for Hindsight (Hnt), is a homologous to the human zinc-finger transcription factor RREB-1 that has been seen to be expressed during embryonic development as well as later during the adult phase acting as a regulator (Sun and Deng 2007, Baechler et al. 2016). This particular stock of knocked down pebbled, *peb*^{RNAi}/TM3*, Sb, Tb (*peb*^{RNAi}), was heterozygous segregating using a dominant larval markers, tubby (Tb), and a dominant adult marker, stubble (Sb), contained in different loci within the third chromosome called TM3*. Tubby results in larvae and pupae appearing squished compared to their wild type counterparts (Fig. 6B). Stubble refers to the stunted bristle length that adult *Drosophila* have lining their backs (Fig. 7B).

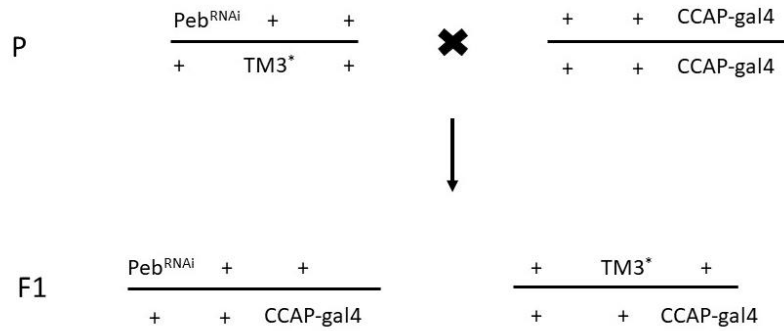


Figure 5. Genetic scheme of pebbled-RNAi cross with CCAP-gal4. Crossing the heterozygous *peb*^{RNAi} that contains Tb and Sb on the third chromosome with homozygous CCAP-gal4 results in two genotypes in the F1 generation. One expresses the *peb*^{RNAi} while the other does not. The one that does not express *peb*^{RNAi} will have the tubby and stubble phenotypes while the one that does express *peb*^{RNAi} will not have those phenotypes.

The *peb*^{RNAi} parent for this cross are homozygous lethal where the pupae did not eclose and died (Fig. 6A). The heterozygous form of this stock appeared tubby and progressed to eclosion (Fig. 6B). For the CCAP parent, pupae appeared normal as they developed towards the adult stage (Fig. 7C). After crossing the heterozygous *peb*^{RNAi} with homozygous CCAP-gal4, roughly half of the F1 generation express the *peb*^{RNAi} (96/200) while the other half will not (104/200) (Fig. 5). Those expressing *peb*^{RNAi} progress to eclosion with a pupal length similar to the CCAP parent (Fig. 6E). Those that did not express *peb*^{RNAi} served as the genetic control as they express the normal *pebbled* function in the CCAP neurons and identified as such by expressing the tubby phenotype during pupal stage (Fig. 6D).

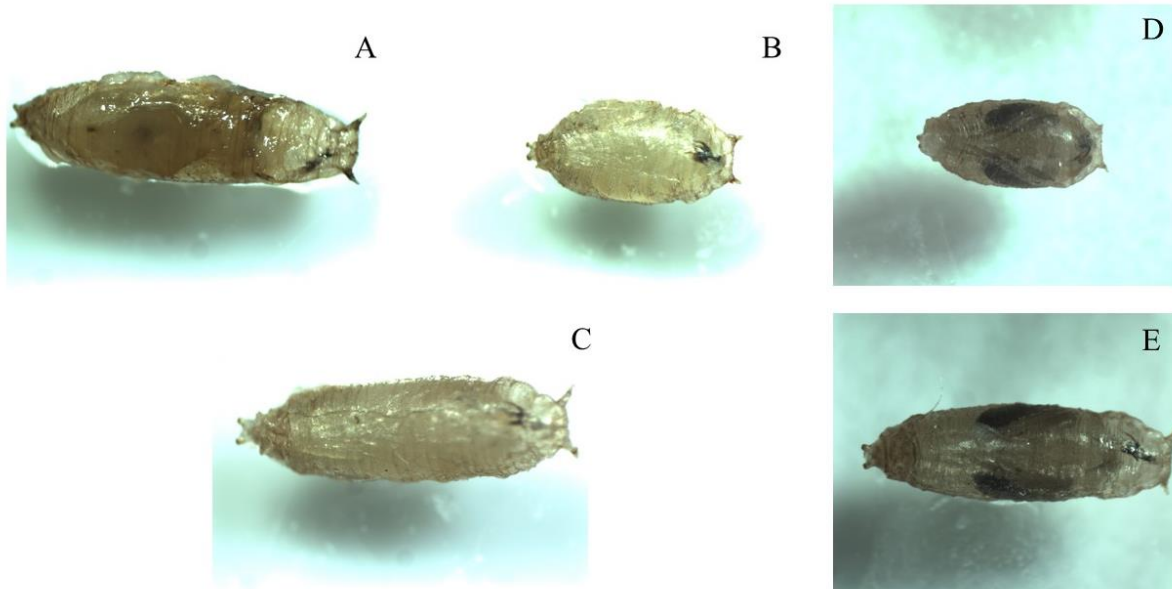


Figure 6. Composite picture of pupae structures. A) Homozygous lethal appearance of Pebbled 33943/ TM3^{*}, Sb, Tb parent. B) Heterozygous tubby (Tb) appearance of Pebbled 33943/ TM3^{*}, Sb, Tb parent. C) U-mG; CCAP-gal4^{III} parent D) Tubby appearance of Pebbled 33943/ TM3^{*}, Sb, Tb, crossed with U-mG; CCAP-gal4^{III} not expressing *peb*^{RNAi}. E) Homozygous appearance of Pebbled 33943/ TM3^{*}, Sb, Tb, crossed with U-mG; CCAP-gal4^{III} expressing *peb*^{RNAi}.

Once eclosed, the adult wing structures were most notable. For the CCAP parent, after eclosion the adults exhibit a crinkled wing formation with long, thin, bristles along the back and strong red eye color (Fig. 7A). The *peb*^{RNAi} parent, after eclosion, has straight wings with shortened, thick, bristles – typical of the stubble phenotype – along its back and a more orange eye color (Fig. 7B). Two crosses were made in this experiment. One crossed *peb*^{RNAi} males with CCAP virgins while the other crossed CCAP males and *peb*^{RNAi} virgins. All pupae eclosed reaching the adult stage. Adults that did not have the tubby phenotype during the pupal stage had severely shriveled wing structures that bent down close to their bodies and remained shriveled even after several days (Fig. 7D). Their eye color ranged from light orange in color to strong red and had long, thin, bristles like the CCAP parent (Fig. 7C). The pupae that did have

the tubby phenotype eclosed with straight, expanded, wings with short bristles similar to the *peb^{RNAi}* parent (Fig. 7D).

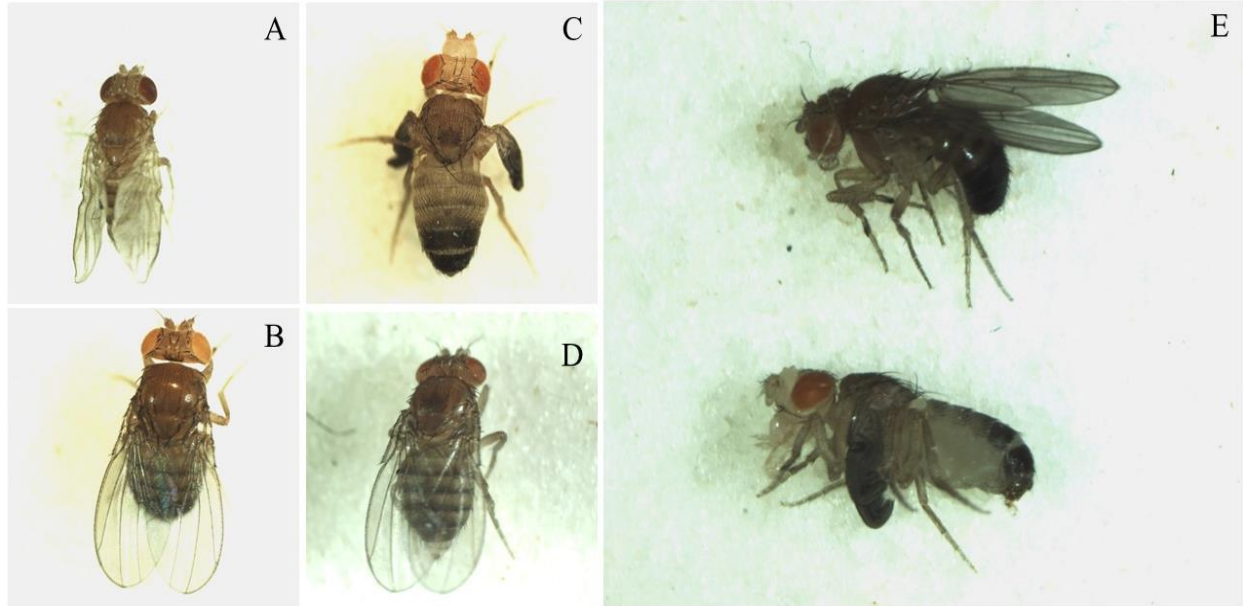


Figure 7. Composite image of eclosed adult wing structures. A) Presentation of homozygous U-mG; CCAP-gal4^{III} parent. B) Presentation of heterozygous Pebbled 33943/ TM3^{*}, Sb, Tb parent. C) Presentation of *peb^{RNAi}* expressing adult from Pebbled 33943/ TM3^{*}, Sb, Tb, crossed with U-mG; CCAP-gal4^{III} parent. D) Presentation of adult not expressing *peb^{RNAi}* from Pebbled 33943/ TM3^{*}, Sb, Tb, crossed with U-mG; CCAP-gal4^{III}. E) Comparison between the two adults resulting from Pebbled 33943/ TM3^{*}, Sb, Tb, crossed with U-mG; CCAP-gal4^{III}.

Dissection of the larval central nervous system (CNS) and subsequent GFP staining allowed for the visualization of the CCAP neurons in the CCAP parent as they produced GFP from the UAS-mCD8GFP combined with CCAP-gal4 (Fig. 8A). Roughly five to six CNS were dissected from each test cross and stained similarly for visualization. Larvae from the test crosses were indiscriminately dissected before undergoing fixation and staining, as in larvae with the tubby phenotype and those without were dissected together from each test cross. However, the test cross using *peb^{RNAi}* males did only contained CNS samples (n = 10) from larvae expressing

peb^{RNAi} while the test cross using *peb*^{RNAi} virgins had CNS samples (n = 12) from both those expressing *peb*^{RNAi} and those that were not (n_{peb} = 7, n_{Tb} = 5).

GFP staining of the test cross larval CNS revealed that when *peb*^{RNAi} males were crossed with CCAP-gal4 virgins, there appeared to be no presentation of CCAP neurons (Fig. 8B). Crossing *peb*^{RNAi} virgins with CCAP-gal4 males, GFP staining of the larval CNS had a portion of the dissected CNS not presenting CCAP neurons and the other portion did present CCAP neurons (Fig. 8C and Fig. 8D).

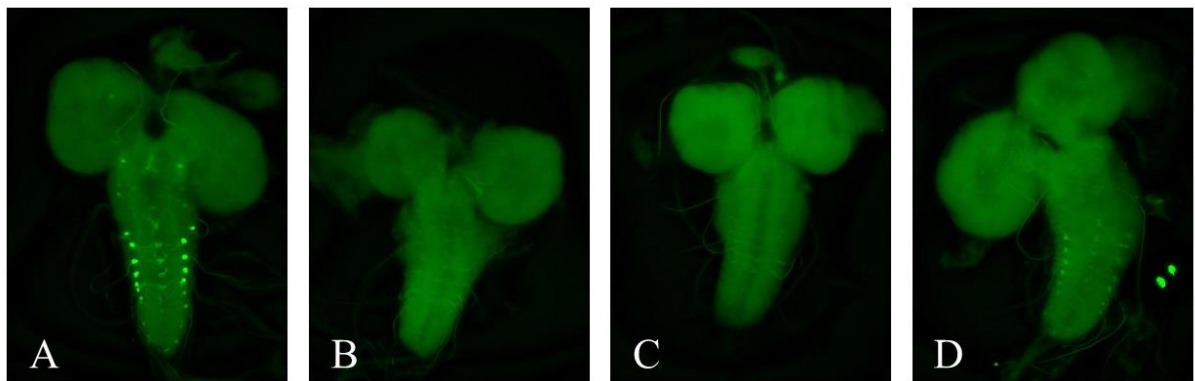


Figure 8. GFP stained larval CNS. A) U-mG; CCAP-gal4 parent showing the CCAP neurons. B) Test cross using *peb*^{RNAi} males and CCAP-gal4 virgins showing no neurons. C) Test cross using *peb*^{RNAi} virgins and CCAP-gal4 males showing no neurons. D) Test cross using *peb*^{RNAi} virgins and CCAP-gal4 males showing CCAP neurons.

Discussion

This study investigated eighty different genes to determine if any of them code for a factor that prevents apoptosis induction in CCAP neurons. These particular neurons survive through the metamorphosis as the *Drosophila* transition from larval stage to adult stage. Metamorphosis requires extensive reprogramming which involves the restructuring of the *Drosophila*'s neural circuit (Lee et al. 2013). Ultimately, this study hypothesized that if there is a protective factor preventing apoptosis from occurring during a dramatic change such as

metamorphosis then there could be a potential homolog within humans protecting against neurodegeneration. These protective factors could then be targeted for neurodegenerative disorder treatments. To determine their functions, the genes were silenced using RNAi. Though no anti-apoptotic factor revealed itself during this investigation, one particular gene appeared to indicate importance in the neuronal development.

Pebbled (*peb*) encodes for a highly conserved nuclear zinc-finger transcriptional protein expressed during different stages of the *Drosophila*'s life. Homolog expression has been identified in a variety of organisms, both invertebrates and vertebrates including the Ras-responsive element binding protein 1 (RREB-1) in humans (Ming et al. 2013). During embryonic development, *peb* expression occurs in the midgut and extraembryonic membrane and aids in germ-band retraction and positioning of forming body parts (Yip et al. 1997). *Pebbled* is required for eye development in adults as it assembles the five-cell preclusters, the timing of their neuronal determination, and subsequent rotation (Pickup et al. 2009). Studies have shown that *pebbled* is suggested to express in specific tissues and negatively autoregulates which many transcriptional factors have been shown to do to maintain homeostatic levels (Ming et al. 2013 and Rosenfeld et al. 2002).

From this study, knocking down *pebbled* in the CCAP neurons affected the *Drosophila*'s ability to expand its wings (Fig. 7C). These results suggest that *pebbled* somehow affects the CCAP neurons or parts of *Drosophila*'s neural circuitry to these neurons. One possible target of *pebbled* could be the neuropeptide production in CCAP neurons as these neurons produce neuropeptides such as bursicon and partners of bursicon which are responsible for cuticle tanning and wing expansion. CCAP neurons release bursicon before wing expansion hastening the hardening of the expanding cuticle suggesting that the hormone induces apoptosis of the wing

epidermis (Kimura et al. 2004). Analysis of the test cross larval CNS using GFP staining shows that expression of GFP has been hindered resulting in the CCAP neurons either not appearing, or they have been ablated. Two test crosses were made where one used *peb*^{RNAi} males crossed with CCAP-gal4 virgins and the other used the *peb*^{RNAi} virgins crossed with CCAP-gal4 males. Interestingly, the cross using *peb*^{RNAi} females resulted in a population that expressed GFP signal in the CCAP neurons while another population did not (Fig. 7C and D). Compared to the CCAP parent, signal was not as robust in the test cross population that did visualize the neurons, but this may have been due to the time between staining and visualizing. GFP signal degrades over time and faster when exposed to fluorescent light.

Exactly how pebbled affects these CCAP neurons was not determined during this study and will require further testing to elucidate its relationship between the two. The next step would be to repeat dissections on larval CNS but separate the genetic control from the test cross to confirm the presence of CCAP neurons when pebbled is knocked down. Also, dissections on adult ventral nerve cord (VNC) will also be required to determine the presence or lack of these neurons after the organism has undergone its metamorphosis. These neurons reposition into the thorax area on the VNC after neural reconstruction that occurs during the transition from larva to adult (Fig. 1). Further investigation will be needed to understand the pebbled gene more and see if its function translates to human homologs as the search for treatments and cures for neurodegenerative disorders continues.

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