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Investigating the absence of a dietary response at the neuromuscular junction in larval Drosophila melanogaster

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Investigating the Absence of a Dietary Response at the Neuromuscular Junction in Larval *Drosophila melanogaster*

by

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Abstract

Research in *Drosophila melanogaster* (*D. melanogaster*) has been growing in order to identify the fundamental processes of human disorders of the central nervous system such as Alzheimer's disease, diabetes, and other neurological disorders at a molecular level. Altered insulin signaling itself has been linked to widespread nervous system dysfunction including cognitive dysfunction, neuropathies, and susceptibility to neurodegenerative disease. However, knowledge of the cellular mechanisms underlying the effects of insulin on nervous system function is still incomplete. The focus behind investigating the insulin signaling pathway is derived from our observations in the adult *D. melanogaster* neuromuscular junction (NMJ) to changes in diet; these studies exhibit decreased excitatory post-synaptic potentials (EPSP) in the adult that show a decrease in neurotransmission response when insulin signaling is increased, whilst larva do not share the same response. These findings have led us to try to understand why adult *D. melanogaster* changes in neurotransmission are observed when diet is manipulated, but the larval responses are not changing in the same manner. This investigation will compare the adult D*rosophila* mechanism of insulin signaling within the NMJ to evaluate the larvae of its difference of components shown or not shown at the pre-synaptic bouton of the NMJ. This investigation evaluated the components of the insulin signaling pathway in the larval *D. melanogaster* in order to identify the absence of mechanical structures in the larva that do not allow for a similar response seen in the adult *Drosophila*.

Introduction

The use of *Drosophila melanogaster* has long been known as a truly powerful and simple model system for studying the pathogenesis of human neurodegenerative disorders as a proof-of-concept model before progressing the research into higher order mammal studies. The model

organism, *D. melanogaster i*s one of the most efficient models to use in comparing with human neurological disorders in *Drosophila*. In comparison to studying in mice, *D. melanogaster* is a simpler system that avoids the expensive costs that are associated with mice studies (**Eaton et al., 2017**). Another benefit of the *Drosophila* model system is that the genes from the *Drosophila* model are highly conserved with genes from the human genome (**Chan et al., 2000; Eaton et al., 2017**). The high conservation of genes between the human and *Drosophila* genomes allows for homology in the signaling pathways of both organisms (**Chan et al., 2000; Wagner et al., 2015; Eaton et al., 2017**). Their complex nervous system also provides a simple model system to research human neurodegenerative diseases on *Drosophila* as conceptual proof and then apply their understanding to the human nervous system (**Chan et al., 2000, Wagner et al., 2015; Eaton et al., 2017**). As studies investigated structural changes in adult *Drosophila*, they focused on the quantitative and qualitative measurements of their structural morphology in the synaptic boutons throughout their life cycle (**Beramendi, 2007**). Synaptic boutons, also referred to as synaptic terminals, are the end projections of the axon terminal where vesicles of neurotransmitters are stored and released to aid in neuronal communication. It is important to note that there has been a significant finding that the larval mechanisms were found to be structurally different in the synaptic boutons (**Beramendi et al., 2007**). This has led proposed studies to question the structure of protein components in the adult *Drosophila* pre-synaptic boutons in comparison to the larval *Drosophila* morphology.

An important structural aspect of the pre-synaptic bouton at the last stage of larval development, the third-instar larvae, is that they contain glutamatergic synapses. The human central nervous system (CNS) can be compared with *D. melanogaster* because they are also comprised of glutamatergic synapses in their CNS pre-synaptic boutons (**Smith et al., 2011; Niciu**

et al., 2011). Comparing the human CNS mechanism of neurotransmission has partly narrowed down the mechanisms of the insulin signaling pathway behind the responsiveness of the NMJ to diet. Researching the larval *Drosophila* melanogaster will investigate how the fundamental structures in the larval stages are organized and manipulated in order to further contribute to the understanding of the human neuromuscular junction (NMJ) at all stages of life in response to dietary changes.

Insulin Signaling Pathway

Neurotransmission is the process by which a chemical messenger, a neurotransmitter, is released from a neuron and transmitted across the synapse to another neuron or the target cell. In the NMJ, the insulin signaling pathway begins with the muscle cell reacting to the insulin receptor and creates the mechanism that is important for regulating glucose metabolism in the body and maintains normal blood sugar. The insulin signaling pathway begins with an insulin receptor (IR) which is activated by the insulin ligand. When activated, an insulin receptor substrate, like chico, creates a signaling cascade throughout the pathway. An initial post-synaptic target is the rapamycin (mTOR) complex which positively regulates insulin signaling and is directly involved in the synthesis of new synapses (**Penney et al., 2012; Stoica et al., 2011; Takei and Nawa, 2014; Weston et al., 2012; Mahoney et al., 2016)**. Another component in the pathway is the FOXO family transcription factors, which has been observed to be negatively affected by insulin via the phosphorylation of Akt in flies (**Puig et al., 2003; Teleman et al., 2005; Yamamoto and Tatar, 2011; Mahoney et al., 2016**). Studies have also found that FOXO is needed for the synaptic growth and vesicle recycling in larval *Drosophila* motor neurons (**Howlett et al., 2008; Nechipurenko and Broihier, 2012; Mahoney et al., 2016**). Mahoney et al., 2016 introduces evidence that insulin signaling negatively regulates presynaptic neurotransmission through the

FOXO-dependent regulation of the transitional inhibitor, eukaryotic initiation factor 4e binding protein (4eBP) (**Mahoney et al., 2016**). Another component that is known to regulate synaptic exocytosis in response to insulin is the Complexin (CPX) protein (**Mahoney et al., 2016**). These components can be regulated and manipulated in *Drosophila* to copy neuropathies, such as diabetes, and in turn be used to investigate the effects of dietary restriction on neurotransmission.

Dietary Restriction

Dietary restrictions have been evaluated throughout the years on mammals and invertebrates to investigate how age-dependent diseases respond to this manipulation of therapeutic effects that lengthen their lifespan with neurodegeneration. The insulin signaling pathway is examined at a molecular level to investigate how neurons are damaged by dietary habits that are often neglected and maltreated. Today's studies exhibit how insulin signaling plays a role in the neurotransmitter release in adult *D. melanogaster* at the NMJ (**Chen et al., 2008; Duan et al., 2003; Kerr et al., 2009; Steinkraus et al., 2008; Halagappa et al., 2007; Rawson et al., 2012; Mahoney et al., 2016**). At the molecular level of the NMJ, the insulin signaling pathway focuses on the direct and indirect relationships between the proteins c*hico*, the insulin receptor substrate, and Complexin (CPX), then eukaryotic initiation factor-4e (eif-4e) and its translational inhibitor eif-4e binding protein (4eBP). It is observed that chico was only beneficial when responding to a high protein diet, which reduces the amount of insulin signaling and improves neurotransmission (**Mahoney et al., 2016**). In contrast, it is found that chico is downregulated by downstream $4eBP^{RNAi}$ in the 2X diet reducing the excitatory post-synaptic potentials (EPSPs) responses in which decreases neurotransmission (**Mahoney et al., 2016**). This study continues to create a better understanding of how these proteins work together while manipulating diet: It is observed that in a high protein diet, 4eBP is reduced in transcription causing an increase in CPX

and ultimately a decrease in neurotransmission (**Mahoney et al., 2016**). From all of these discoveries of the adult *Drosophila*, it is found that none of these responses are observed in the larval *Drosophila* (**Mahoney et al., 2016**).

Changes in the insulin signaling pathway have been studied in the adult *Drosophila* in order to investigate the responsiveness of the NMJ to manipulations in diet as well as due to modifications in insulin signaling (**Mahoney et al., 2016**). This study has narrowed down on the control of neurotransmission in the adult *Drosophila* Ciberial Muscle 9 (CM9) NMJ, which is a motor neuron that is located on the fly's proboscis, nostril projection, by measuring synaptic output from the neurons at the CM9. This study used electrical recording that observed a high protein diet reduces the release of neurotransmitters (**Mahoney et al., 2016**). This study also found that there was a lack of dietary response at the larval NMJ; the adult *Drosophila* NMJs exhibited significant changes from the low-protein diet (1X), 50 g of active yeast, to the high-protein diet (2X), 100 g of active yeast, where the 2X diet decreased the EPSPs by over half of the 1X diet EPSP response (**Mahoney et al., 2016**). The contradictory elements that there is active neurotransmitter release with calorie diet restrictions in adult *Drosophila* whereas the larval *Drosophila* shows no change in neurotransmission from the control to the 1X 4eBP (**Mahoney et al., 2016**). The main question has concerned if larval *D. melanogaster* has similar responses in comparison to what is examined and expected in adult D. *melanogaster*. This study will investigate what proteins of the insulin pathway are affected or not affected when investigating dietary responses in the larval *Drosophila* and if they are present considering there have been a lack of responses in the larvae.

Methods

Diet food preparation: 300 mL of DiH_2O was boiled on a hot plate. After the water began to boil, 5 g of agar was sprinkled into the water and then 25 g of sucrose was added. In a separate container, for the 1X diet, 50 g of yeast was added to 25 g of corn meal with 200 mL of DiH_2O. For the 2X diet, 100 g of yeast and 300 mL of DiH_2O with the 25 g of corn meal was mixed. After the diet was mixed, the container of the diet food was added to the boiled water mixture for 15 minutes at 85^{o}C. While it cooled, gloves were used to set aside 1.5 mL of propionic acid under the fume hood into a small beaker and covered with aluminum foil until use. In an Erlenmeyer flask, 1.5 g of Tegosept was measured out on a scale and then dissolved in 15 mL of ethanol. When the diet mixture was cooled to 70^{o}C, the propionic acid and the Tegosept ethanol solution was added to the overall mixture. The mixture was stirred, and then 5 mL of the diet was pipetted into each vial. After pipetting, the vials were wrapped in cheese cloth overnight, and then placed in an airtight bag in the refrigerator until use.

Dissection of Larvae: Three third instar larvae were taken from one of the diet condition vials and placed on an agar plate. Phosphate buffered saline (PBS) was poured into the agar plate dish enough to cover the larvae. Using a pin, the head of the larvae was pierced and quickly after another pin was placed on the tail of the larvae to pin the body down. After pinning the larvae, a horizontal incision below the abdomen proximal to the tail was cut with scissors. Then a vertical incision was made all the way to the head of the larvae; this incision should be in between the two tracheae careful not to scrape or pull out any of the muscles. The specimen was washed with PBS to discard the intestines. Forceps were used to delicately remove the insides of the body cavity. It was crucial to not scrape or injure the muscles inside. Then, using four pins, the four corners of the larvae's body were pinned and stretched to expose the internal muscles of the larvae. One more wash with

PBS was completed to rid any leftover intestines. 1 mL of bouin solution was pipetted onto the dissected specimen. After five minutes in the bouin solution, the pins were removed, and the specimen was put into a microcentrifuge tube labeled with its diet condition and then stored. The solution was discarded in centrifuge tubes to be thrown away. This dissection process was completed with three larvae for each diet condition: control, 1X, and 2X. These dissections were then stored to complete the antibody staining with 4eBP and CPX.

Antibody Staining to observe 4eBP levels: After the dissections, one wash was completed by replacing the 1 mL of PBS in the microcentrifuge tube of each diet condition and placing it on the rocker for five minutes. After five minutes, the primary antibodies were micropipetted into each tube. Each diet condition tube received 1:100 uL concentration of 4eBP and 1:200 uL concentration of futsch. Then, the tubes were put into a cold room at on a rocker overnight. For the secondary staining, three washes for five minutes each were conducted on each tube using a pipette. Next, 1 mL PBS was discarded and replaced in each tube. Each diet condition tube received 1:200 uL concentration of goat anti-mouse 488 gfp and 1:200 uL concentration of goat anti-rabbit 555 rfp. The tubes were placed on a rocker with foil covering the top to avoid light exposure for 2 hours at room temperature. Next, the mounting procedures were followed.

Antibody staining to observe CPX levels: After the dissections, one wash was completed by replacing the 1 mL of PBS in the microcentrifuge tube of each diet condition and placing it on the rocker for five minutes. After five minutes, the primary antibodies were micropipetted into each tube. Each diet condition tube received 1:100 uL concentration of CPX and 1:200 uL concentration of futsch. Then, the tubes were put into a cold room at on a rocker overnight. For the secondary staining, three washes for five minutes each were conducted on each tube using a pipette. Next, 1 mL PBS was discarded and replaced in each tube. Each diet condition tube received 1:200 uL

concentration of goat anti-mouse 488 gfp and 1:200 uL concentration of goat anti-rabbit 555 rfp. The tubes were placed on a rocker with foil covering the top to avoid light exposure for 2 hours at room temperature. Next, the mounting procedures were followed.

Mounting the specimens: Three washes for each diet tube were performed for five minutes each wash on the rocker with 1 mL of PBS. After the last wash, the PBS was replaced to fill to 1 mL. Then, each diet condition specimen was mounted onto three separately labeled slides with their concentrations and their respective antibody staining with the muscles facing upwards. It was taken into consideration that the underneath light is off to limit exposure to light for the fluorescence antibodies. Then, 50 uL of Vectashield was micropipetted onto the slide in one droplet. The coverslip was then placed to spread the Vectashield throughout each sample. To dry the slides, the slides were covered in foil to rest in the refrigerator for five minutes. Then, the slide was taken out to trace a nail varnish around the sides of the coverslip. The slide was placed back into the refrigerator for five minutes. When the slides were dried, they were put into the storage container to be viewed in the future for the results under the fluorescence microscope to record the results.

Results

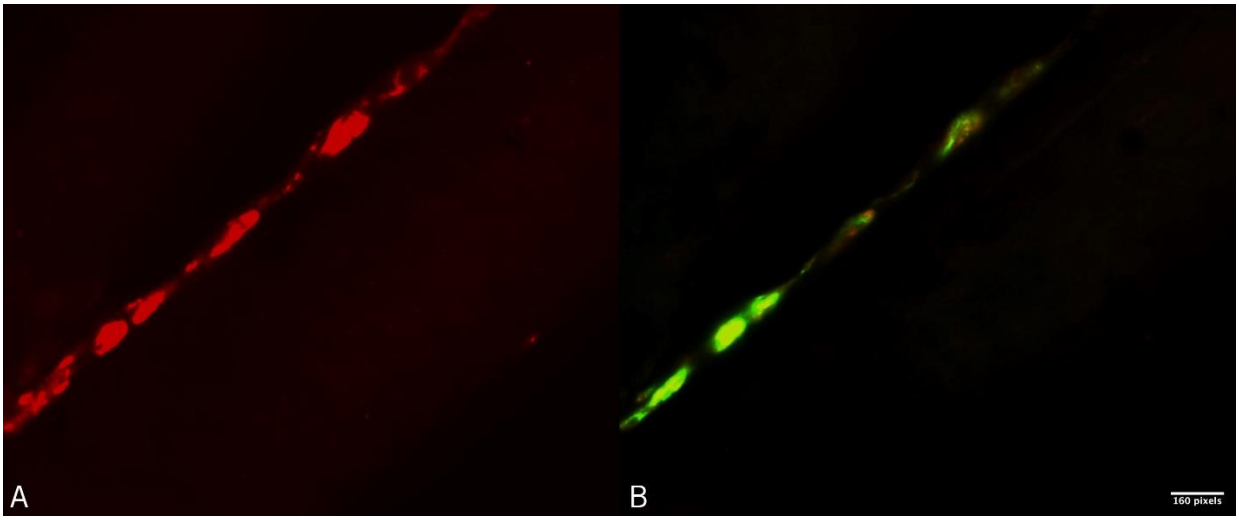

Figure 1: Larvae *Drosophila* Control W1118. The left image exhibits the red fluorescent protein (rfp) staining of vesicular glutamate (vGLUT) in the pre-synaptic bouton of the NMJ. The right image exhibits the green fluorescent protein (gfp) staining of a pre-synaptic marker, Futsch.

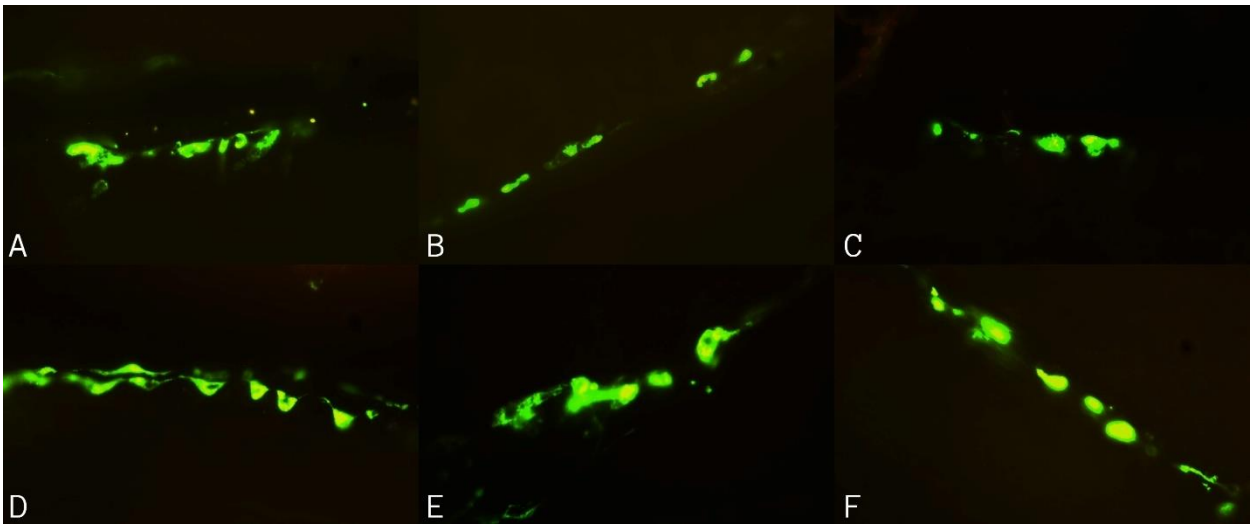

Figure 2: Larvae *Drosophila* Florescence Response to Condition. Immunofluorescent images of synapses from animals raised on a 1x or 2x diet, Futsch, green upper and lower panels. (A) 4eBP Control; (B) 4eBP 1X; (C) 4eBP 2X; (D) CPX Control; (E) CPX 1X; (F) CPX 2X

Conclusions and Future Work

The investigation of larval *D. melanogaster* at the NMJ exhibited that dietary changes, 1X and 2X diets, do not affect larval synaptic transmission due to the proposed absence of the exocytotic machinery proteins, 4eBP and CPX, in the pre-synaptic bouton. Figure 2 shows that there was no positive staining for the red-fluorescently tagged proteins tagged to 4eBP and CPX, which would be indicative in the presence of the protein in the presynaptic terminal. This is surprising given the clear co-localization of these proteins in the adult NMJ. This may be because these proteins are simply not turned on this early in development and are turned on later in the life of the fly. There is also the possibility that there may be differences in the manipulations of

Complexin. In addition, it is likely that the effects of Complexin that we observe require the co-translation of other exocytotic components. Further analysis of the animals via the use molecular methods such as western blot for protein analysis or PCR for gene specific analysis will help us understand the absence of these proteins in the larval neuromuscular junction.

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