Neuromodulation of aggression behavior by Neuropeptide-F in *Drosophila melanogaster*

Tyler Gopee

Wallkill, New York

A Thesis Presented to the Honors Program of the State University of New York, New Paltz in Candidacy for graduation with honors

Biology Major

8 April 2020

Dr. Aaron T. Haselton 26 January 2020

Acknowledgements

I would like to express my gratitude to SUNY New Paltz for letting me fulfill my dream of being a student here. I would also like to thank the Biology Department for turning me from a curious student to inquisitive researcher. To my fellow lab members Naveeda, Gladis, and Jahed, I am extremely grateful for you guys always having my back and pushing me to succeed. I would like to thank all my friends and family for your support. Most of all I would also like to thank my mentor Professor Haselton for always fostering creativity in an environment where the sky is the limit as long as you can build your way up to it.

Table of Contents

Abstract

Neuromessenger proteins such as Neuropeptide-F (NPF) have been linked to aggression in animals such as the fruit fly *Drosophila melanogaster*. To better understand NPF's influence on aggression, we investigated the direct effects of NPF release on fly fighting aggression behaviors. Utilizing targeted gene-expression techniques that can be confirmed via antibody-based immunohistochemical tissue analysis, we created a transgenic line of flies with light-inducible ion channels in NPF-secreting cells. Upon confirmation, utilizing optogenetic tools we performed fly fight bioassays in a ring with limited food to see the direct effects of NPF on aggression through light activation. The results of this study suggest the secretion of NPF impinges upon aggression circuits enough to depress aggression levels in flies.

Introduction and Background

Understanding the neurochemical basis of behavior is an important and rapidly developing topic within the field of animal behavioral neuroscience. Recent findings in the fruit fly, *Drosophila melanogaster*, have demonstrated that specific brain regions contain neural circuitry critical for the regulation of aggression behaviors (Hoopfer et al. 2015). The neural architecture of these circuits has been partially resolved and many neuromessenger molecules that modulate neuronal activity in these regions have been identified. Specifically, neurons that release the neuromessenger neuropeptide-F, a neuropeptide, onto identified aggression-regulating neuronal circuits have been shown to have an effect on aggression behaviors and whether it enhances or depresses aggression is still disputed (Dierick et al. 2007; Lee et al. 2004). To investigate the effects Neuropeptide-F signaling has on aggression we will take advantage of standard targeted gene-expression techniques and optogenetic tools to express light-activated ion channel proteins (channelrhodopsin 2 proteins) in the membranes of neuropeptide-F-secreting neuromodulatory neurons. Immunofluorescence imaging will be used to confirm the specificity of transgene expression within neuropeptide-F-secreting neurons. Upon confirmation we will conduct aggression behavior assays that have been developed for flies (Chen et al. 2002). Male fruit flies, in particular, exhibit aggressive behaviors toward one another when competing for access to food resources and mating opportunities. The stereotyped combat exhibited by male flies, which consist of sequences of lunges and hitting, are easily studied and individual aggression can be ranked based on fight analysis of recorded interactions. With this we desire to see the effect of NPF on aggression while fruit flies fight.

Materials and Methods:

Creating transgenic lines:

Using the GAL4/UAS system transgenic fly lines were created. GAL4 is a yeast-specific transcription factor that will bind to the enhancer element UAS (upstream activating sequence), effectively turning on expression of genes downstream of that UAS nucleotide sequence. The expression of GAL4 protein can be controlled by tissue-specific promoters, and thousands of different GAL4 "driver" lines are available from the Bloomington Drosophila Stock Center (BDSC).

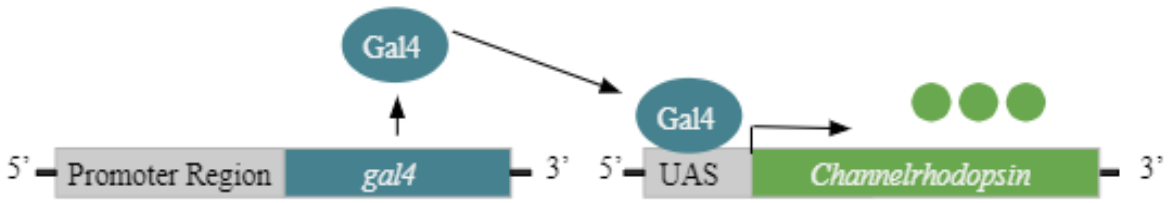


Figure 1. UAS/GAL4 expression system. Cartoon diagram of the region of DNA which was targeted for transgenic expression of channelrhodopsin in *D. melanogaster*. The native gene promotor, or driver gene, controls the GAL4, while the *UAS* promoter controls expression of a target gene, in this case *Channelrhodopsin*.

Two strains of flies were crossed by collecting virgin females of one strain within 8 hours of emerging from pupae and mating them with adult males of another strain. Flies were allowed to mate for 2 days before passing to further vials.

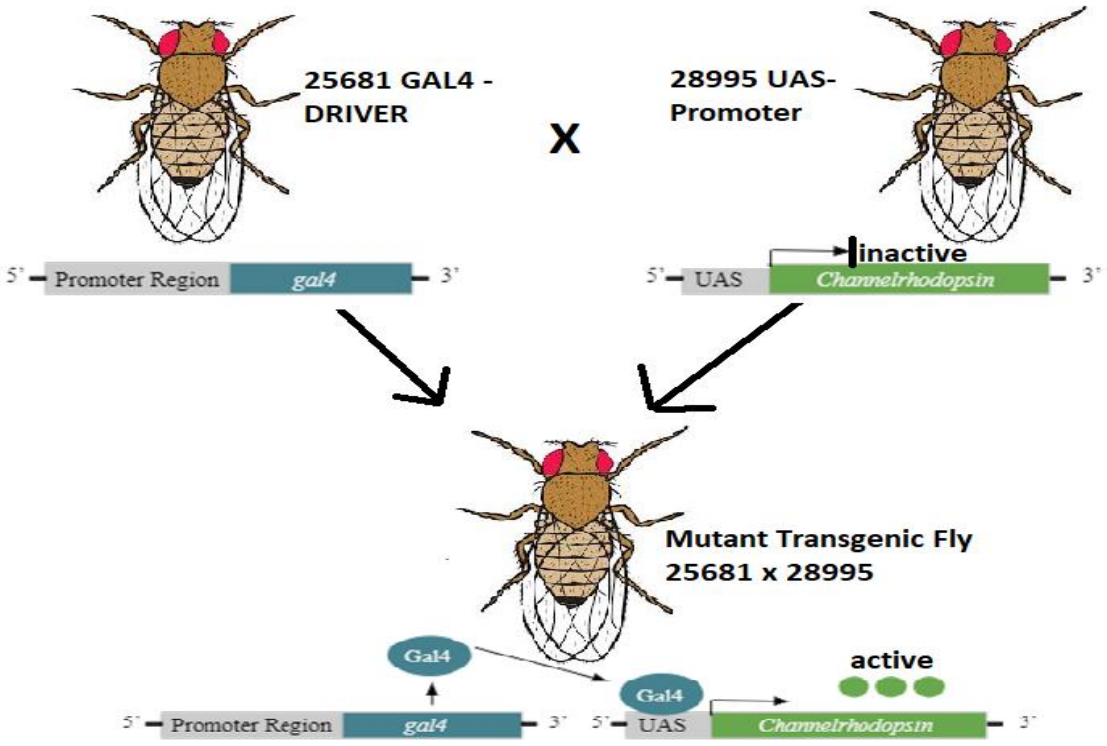


Figure 2. Cross design. Diagram showing transgenic fly cross with fly line 25681 that has tissue specific expression of GAL4 in the neuropeptide F-secreting neurons. GAL4 when paired with UAS promoter line such as 28995 drives transcription and eventual creation of Channelrhodopsin in desired regions of NPF secreting cells in mutant fly.

Rearing Transgenic Line:

The first cohort of 25681x28995 flies were passed on regular Drosophila fly diet every two days in the dark. The P-generation (Parents) mated on regular diet, therefore the offspring were reared on regular fly diet without retinal. Retinal also known as retinaldehyde, is a form of vitamin A (Ardevol 2018).

Figure 3. **Photoisomerization of retinal**. Light hits pi-bonds of trans retinal converting it to cis retinal. Retinal's rapid changes are important to rhodopsin function.

Adult flies 0-5 days old were isolated for 10 days in retinal diet mini fly vials in the dark to make them socially naïve, and more likely to be aggressive upon social contact. Retinal diet was made by heating up 5ml standard fly diet and adding 50µl of Retinal solution (10% Retinal in Ethyl Alcohol).

For Cohort 2 flies, P-generation mated on retinal diet, then were collected and isolated just as in in Cohort 1.

Immunohistochemical Preparation and Imaging:

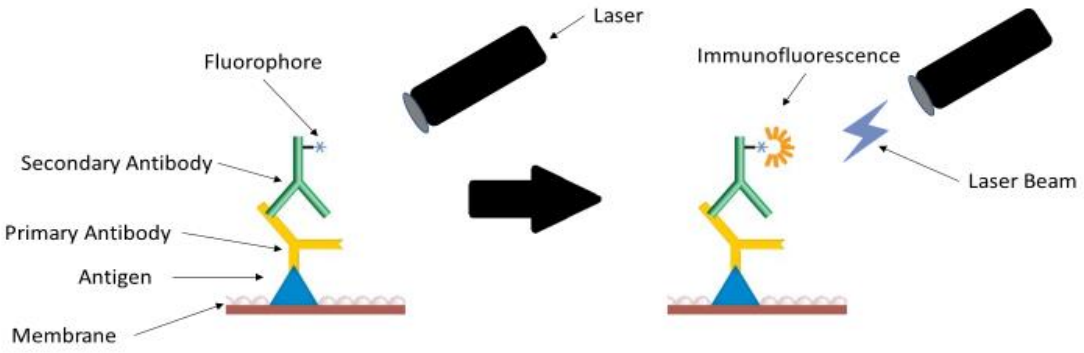


Figure 4. **Cartoon illustrating immunofluorescence at specific location on tissue**: Primary antibody binds to specific epitope of surface membrane protein antigen. After which, Secondary

antibody that has fluorophore binds to primary antibody. Upon light excitation by laser beam at specific wavelength, light is emitted at fluorophore. This light can be viewed on a fluorescence microscope.

To procure tissue for immunohistochemical preparation transgenic flies were taken from crosses and sacrificed by soaking in 70% ethanol. Flies were dissected in PBS (Phosphate Buffered Saline) and neural tissue was removed and fixed in 4% phosphate buffered paraformaldehyde overnight at 4°**C**. After fixing, tissues were washed 6 times for 1 hour in PBST (Phosphate buffered saline plus 0.5% Triton X-100) with agitation at room temperature. Tissues were left in the last solution change overnight in PBST at 4°**C**. After soaking, tissues were blocked in 10% NGS (Normal Goat Serum)/PBST for 1 hour with agitation at room temperature. Blocked tissues were then treated with primary antibody solution (1:200 dilution of designated primary antibody in 10% NGS/PBST) and left to soak for 3 days at 4°**C**. After primary antibody treatment, tissues were washed 5 times for 1 hour in PBST with agitation at room temperature. After washing, tissues were blocked again in 10%NGS/PBST for 1 hour with agitation at room temperature. After blocking, tissues were treated with secondary antibody solution (1:200 dilution of designated primary antibody in 10%NGS/PBST) and left to soak with agitation overnight in the dark. After secondary antibody treatment, tissues were washed 3 times for 30 minutes in PBST with agitation at room temperature. After washing, tissues were cleared in glycerin series in 40%,60%, and 80% glycerin washes for 30 minutes each with agitation. After clearing, tissues were mounted in 80% glycerin on slide. Mounted slides were left in the dark at 4°**C** to avoid decomposition of samples and fluorophores (Johnson 2012)

In reference to imaging, samples were analyzed using Leica Microsystems Laser Scanning Spectral Confocal Microscope. A laser at a specific wavelength was shot at the tissue sample to excite Cy3 flourophores to emit green light that is seen on the scope.

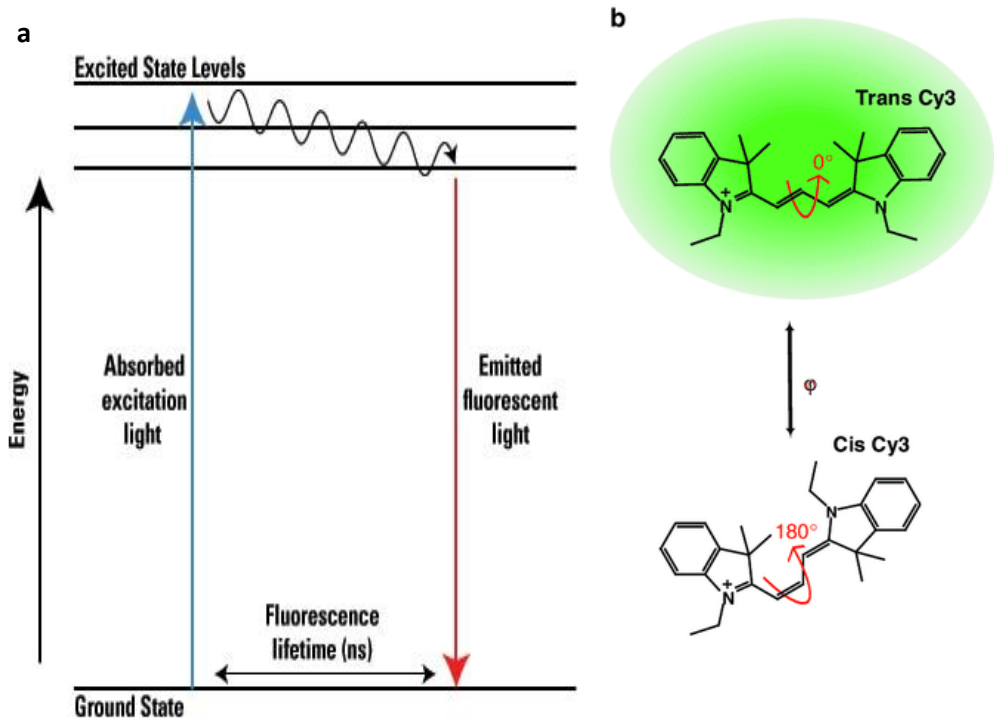


Figure 5a and 5b. **Excitation and emission of Light by Cyanine-3**: Cyanine-3 (Cy3) is a fluorescent molecule commonly utilized in biomedical imaging. Normally Cy3 is in the Trans lower energy state, and with use of laser at 532 nm is excited to the higher energy Cis state (Rashid et al. 2019). Due to the instability of the Cis state, the molecule shifts back to the Trans state and with this emits fluorescent light at 570 nm.

Images of tissue samples with fluorescent imaging were taken in series moving in z-axis through the tissue to produce an image that clearly shows us where proteins of interest were found in the sample. This series was overlaid to produce a Z-stack.

Fly Fighting:

Aggression bioassays were performed in a ring with blue light (470 nm) nearby to be turned on

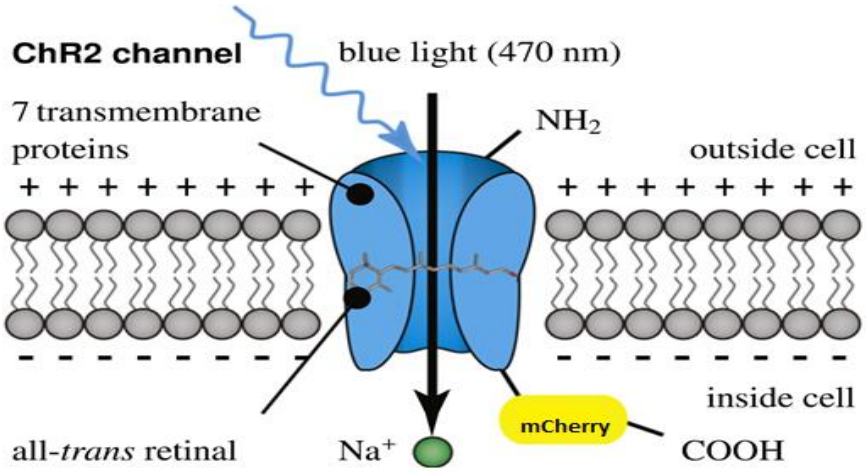


Figure. 6: **Optogenetic light excitation of Channelrhodopsin 2 ion channel**: Upon Photoisomerization of all-trans retinal as seen in Figure 3, ion channel opens letting Sodium ions rush inside cell. This change in electrochemical potential activates certain neural cells allowing for the flushing of Neural circuits with Neuropeptides such as NPF

Socially naïve flies retrieved from isolation were placed into arena one at a time.

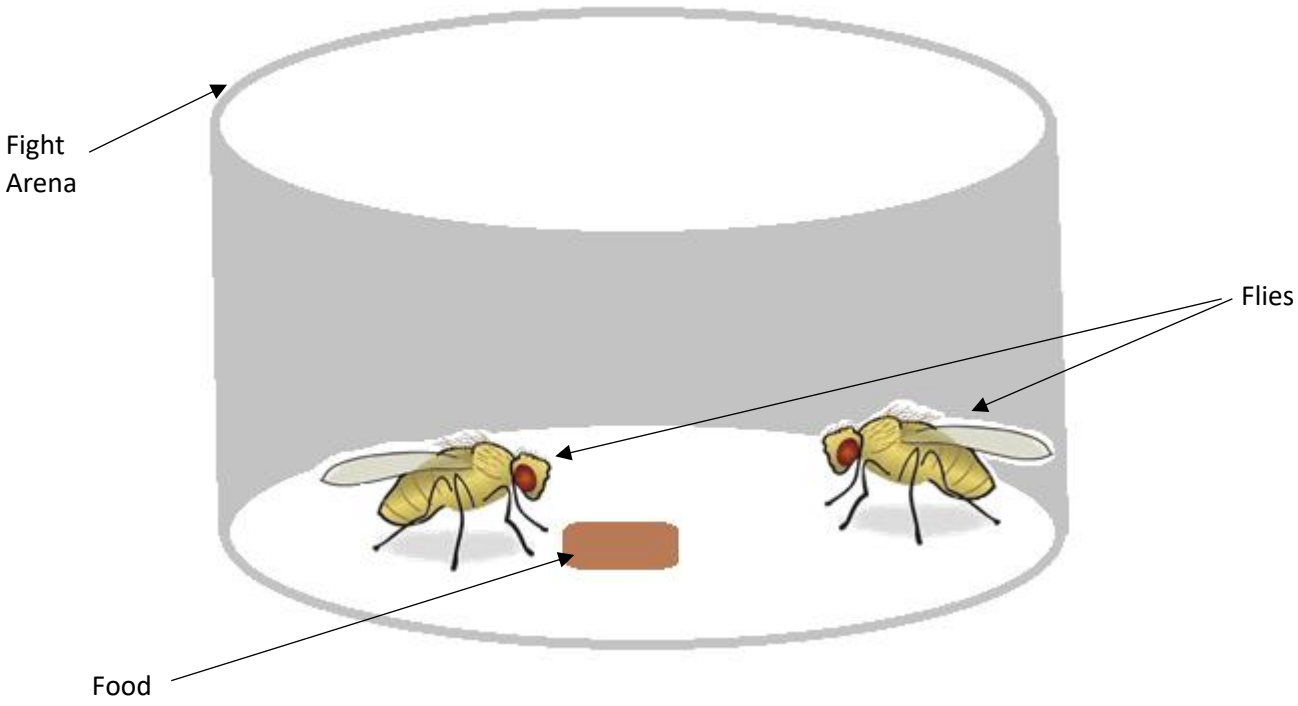


Figure. 7: **Aggression Bioassay Fight Arena Set-up**: Two fruit flies are place one by one into fight arena where the walls are constructed out of acrylic, the floor made out of 2% Agar gel, and plexiglass ceiling stopping the flies from flying out. Yeast paste food is placed in center of ring for the two flies to fight over.

The first fly called the ‘guard’ is placed first. The guard is given about 2 minutes to acclimate then the next fly called the ‘intruder’ is placed inside the same ring. Upon entry of the intruder the flies fight, and after recording for ten minutes flies are removed from the ring and sacrificed by placing in freezer. For control fights the intruder is just placed into the ring with no treatment, and for experimental fights intruder flies are activated by being placed under focused LED array light at around 470 nm for 20 seconds. LED array was controlled using Powerlab system and Labchart 8 to manipulate the light.

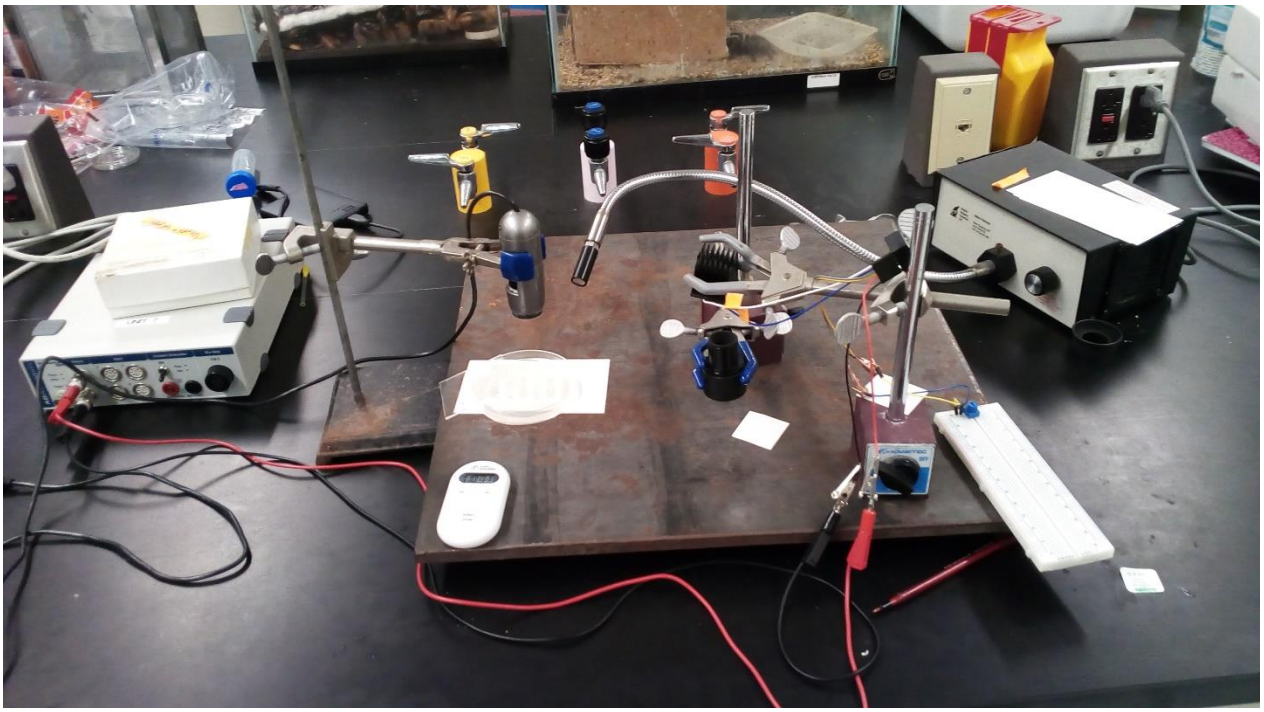


Figure. 8: **Bioassay full set-up**: AD Instruments Powerlab connected to laptop running lab chart 8 utilized to function LED array. Each fight was recorded on video via Dinocam microscope camera and saved on computer to be analyzed later. LED light array was focused under lens to shine high intensity Blue light onto treated flies. Gooseneck lamp was utilized to maintain just enough brightness for camera to pick up the video of each fight while room was kept dark.

Results:

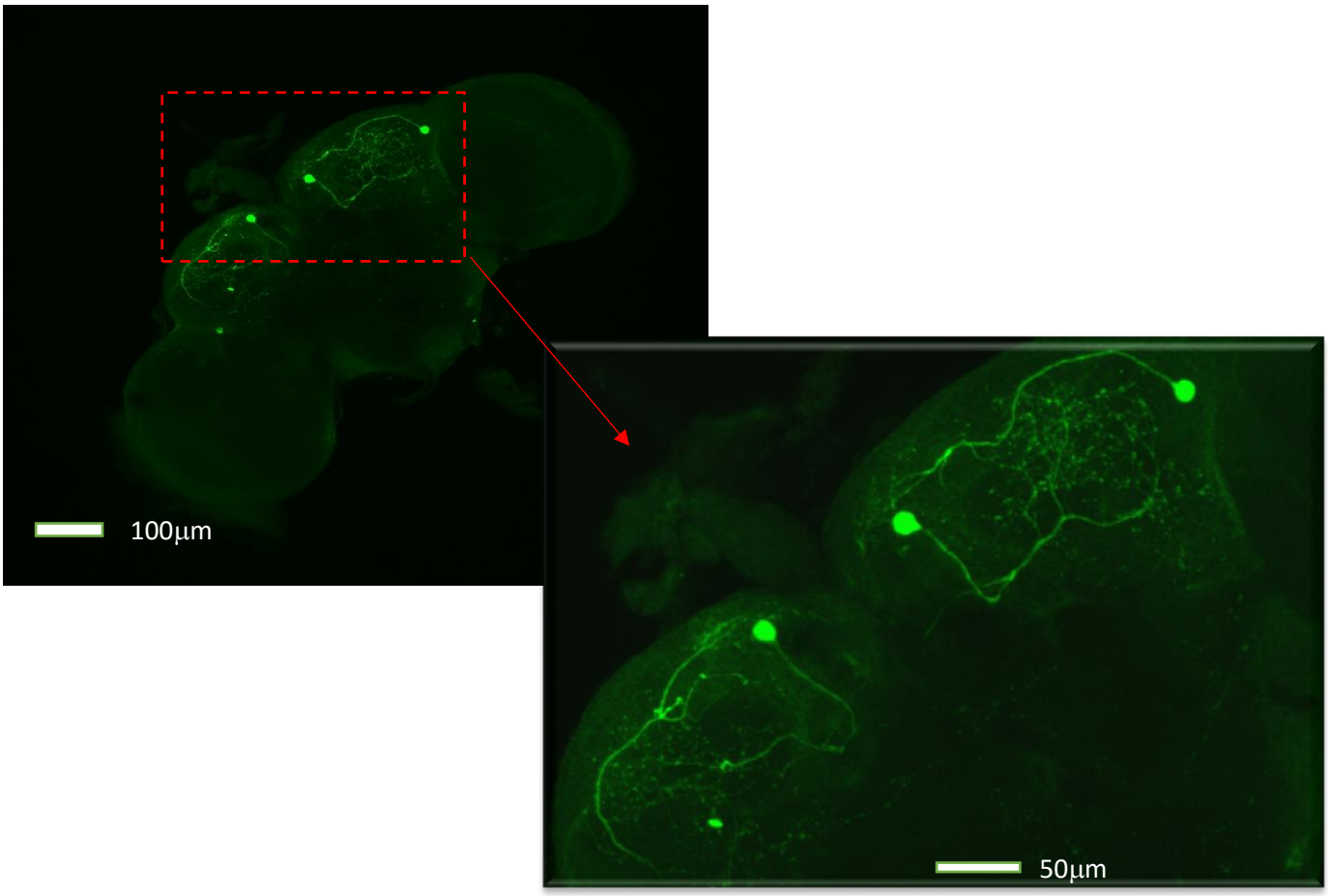


Figure. 9: **Confocal images of Adult Fruit fly brain**.: Adult Fruit fly confocal z-stack overlay of brain tissue prepared as explained in methods. Tissue was treated with anti-mCherry Primary antibody that binds to mCherry on Channelrhodopsin transgene complex visible in Figure 6. Secondary antibody had Cy3 fluorophore and was hit with laser on scope to fluoresce. Bars show relative size with image on left being Image of whole brain, and on the right a zoom in on L1 NPF cells expressing (Lee et al. 2020). Expression of ChR2 is visible in characterized NPF-secreting cells in adult flies.

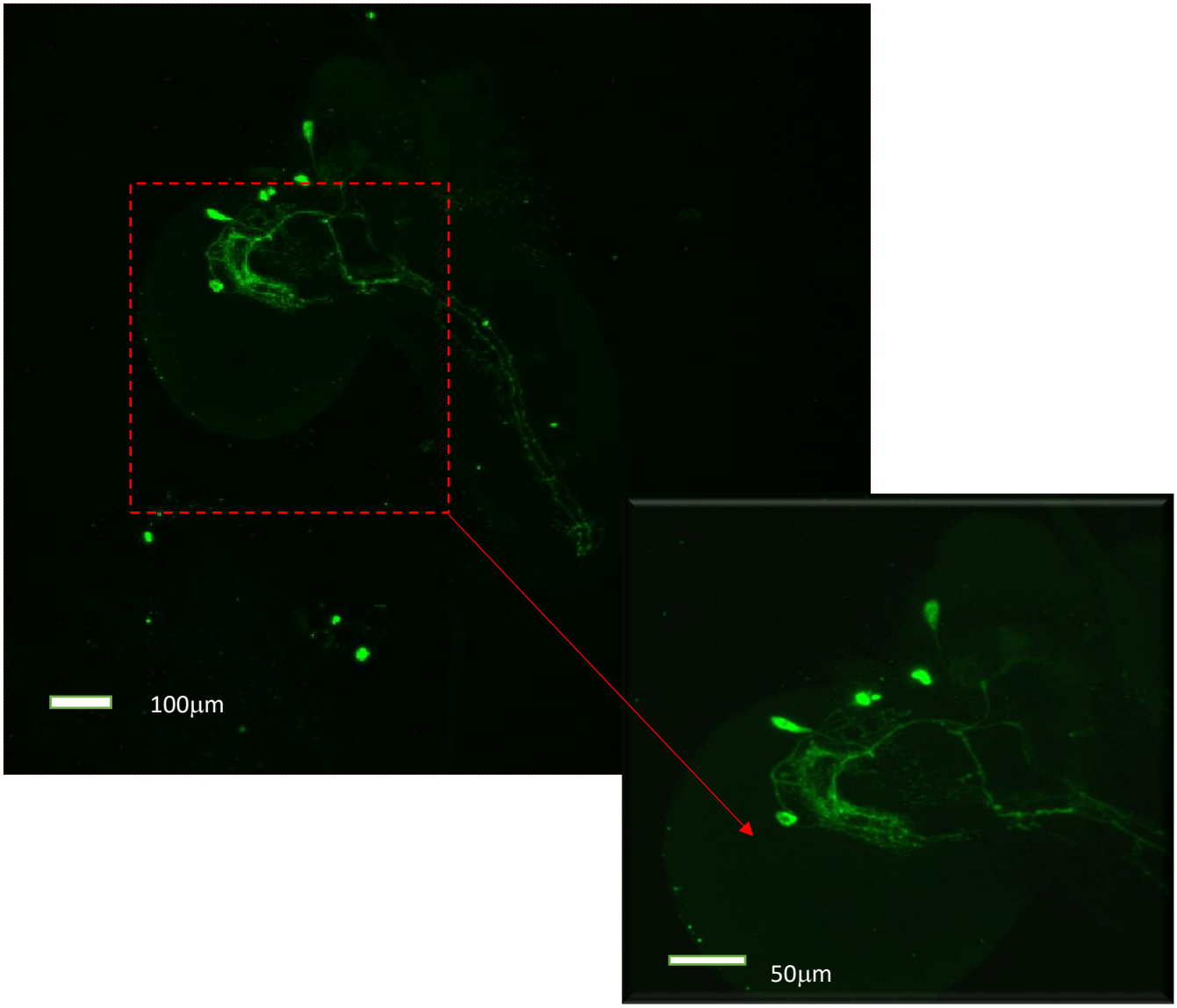


Figure. 10: **Confocal images of Larval Fruitfly Central Nervous System**.: Larval Fruitfly confocal z-stack overlay of neural tissue prepared the same as in Figure 9. Bars show relative size with image on left being Image of whole CNS, and on the right a zoom in on cells expressing NPF. Expression of ChR2 is visible in characterized NPF-secreting cells in larvae.

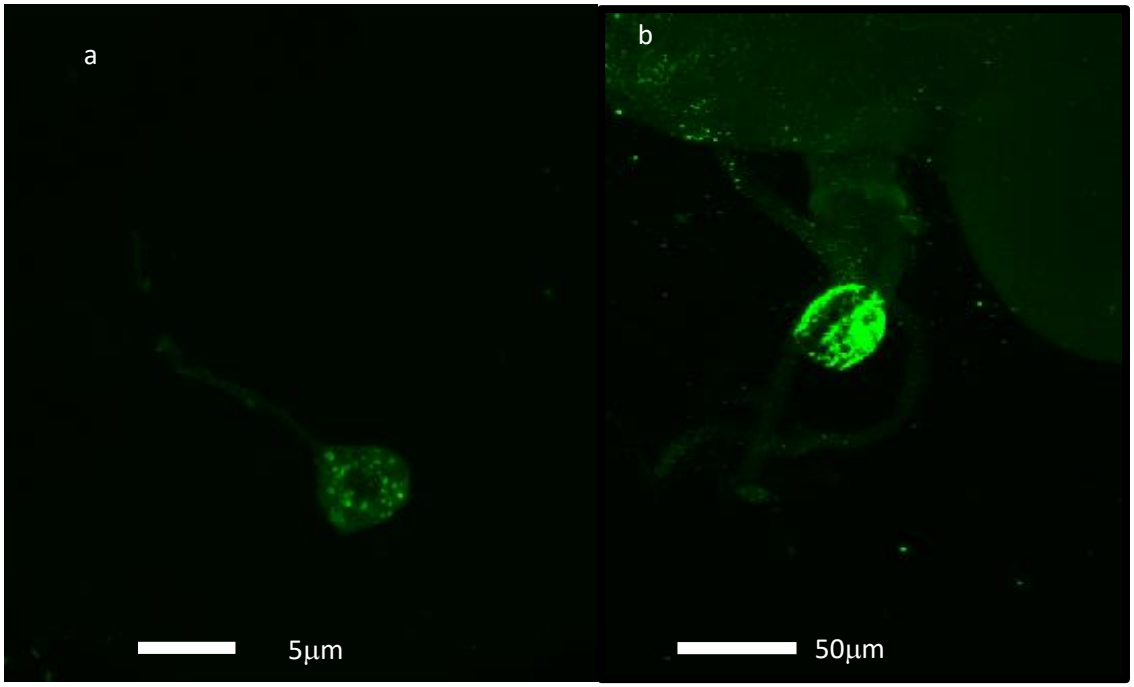


Figure. 11a and 11b: **Confocal images of Neuron and Section of Ventral Nerve Chord:** Adult fruitfly confocal z-stack overlay of neural tissue prepared the same as in Figure 9. Bars show relative size with Figure 9a showing individual Neuron. Figure 9b shows section of ventral nerve chord coming off the brain. There looks to be individual vesicles with visible expression of ChR2 in the individual neuron, with vesicle starveling down the axon. Figure 9b shows strong fluorescent signal at ventral nerve chord section possibly showing large amount of expression related to ChR2 where NPF may be expressed in large amounts to be sent out into the fly body to go systemic.

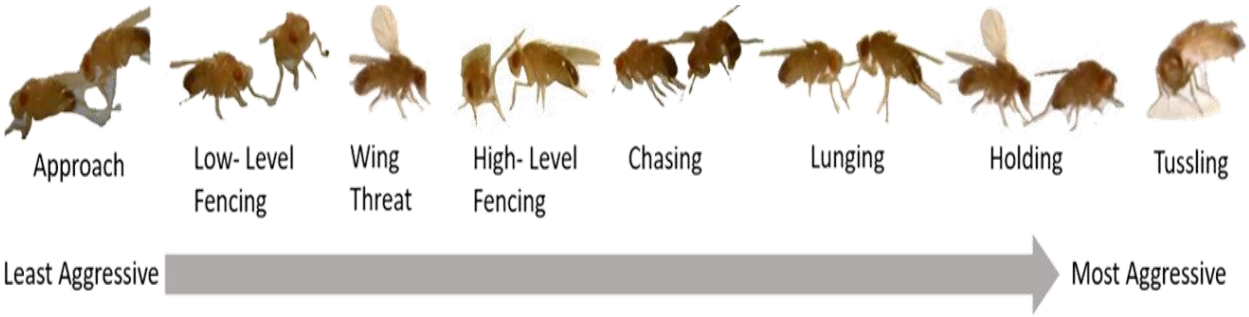
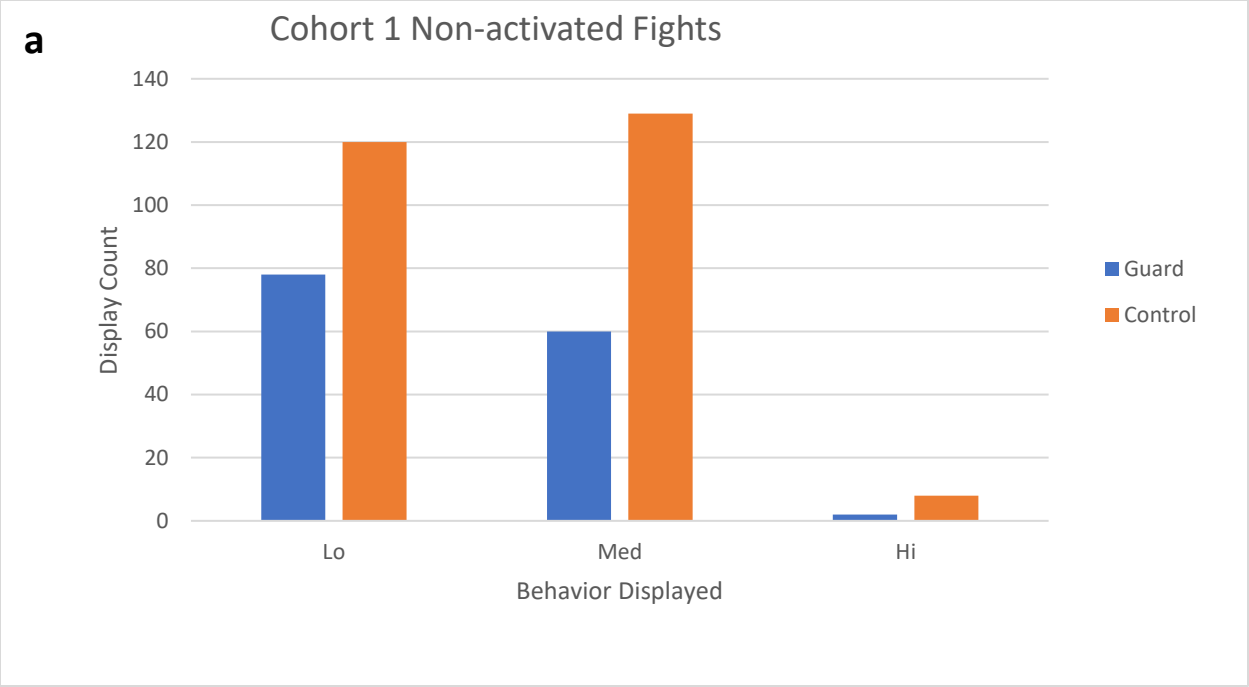
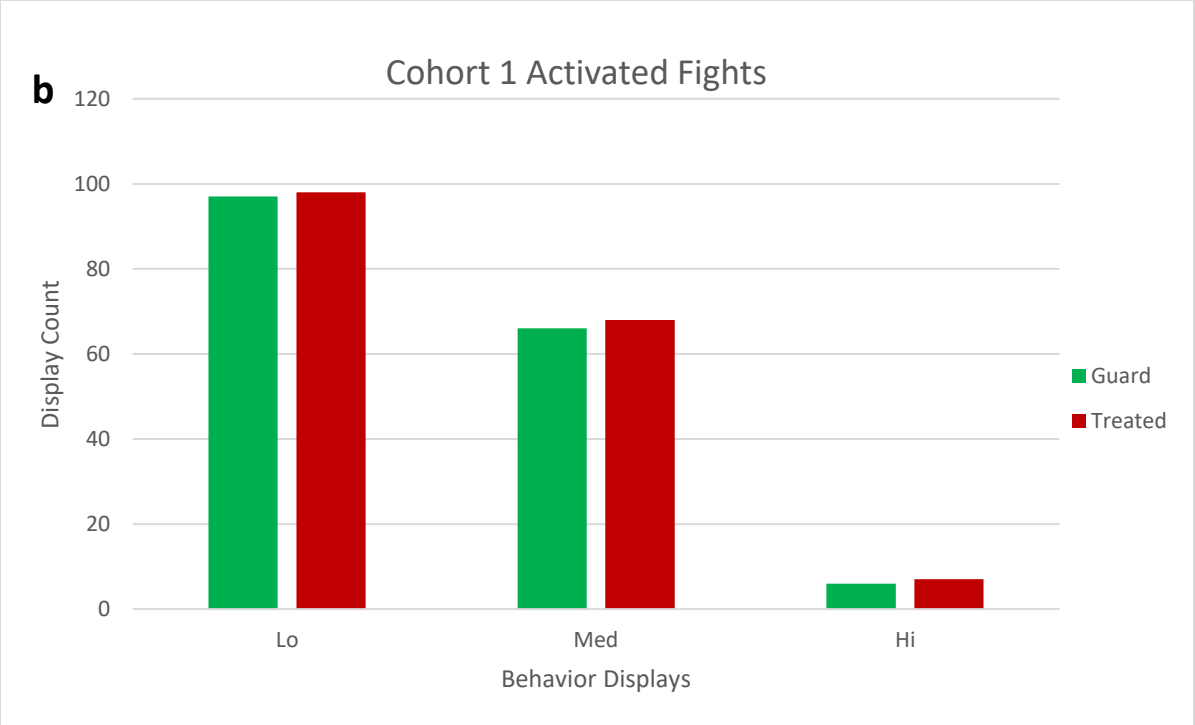
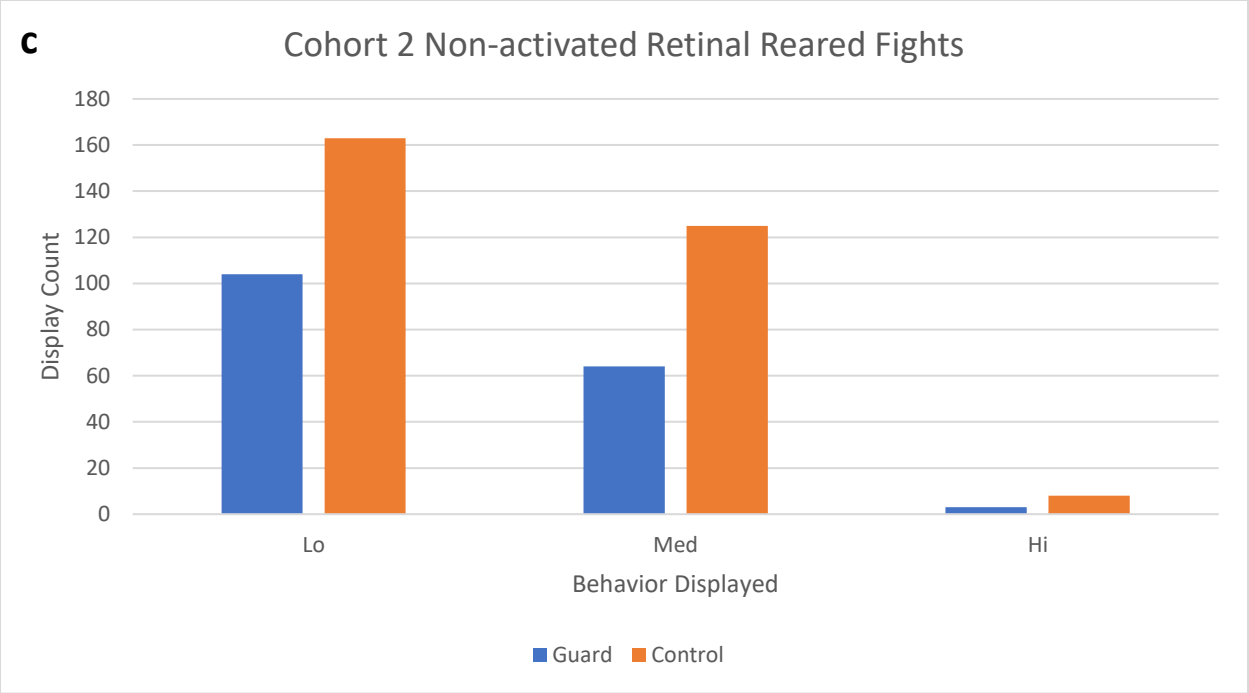


Figure 12. **Representative behavioral displays of *D. melanogaster***: Ethogram is based on Chen et al. 2002 Fruit flies- a model system for the study of aggression. Each behavioral display of a male fruit flies is ranked from least to most aggressive. Scores were tallied depending on the frequency of display. From least to most aggressive displays in order are: Approach, Low-Level Fencing, Wing Threat, High-Level Fencing, Chasing, Lunging, Holding, Boxing, and Tussling. Tallied scores from watching Fly fights were then organized by relative tiers of aggression. Lo-level aggression behaviors were Approach, Low-Level Fencing, and Wing Threat. Med-level aggression behaviors were High-Level Fencing, Chasing, and Lunging. Hi-level aggression behaviors were Holding, Boxing, and Tussling.


a
Cohort 1 Non-activated Fights
140
120
100
80
60
40
20
0
Display Count
Lo
Med
Hi
Behavior Displayed
Guard
Control


b
Cohort 1 Activated Fights
120
100
80
60
40
20
0
Display Count
Lo
Med
Hi
Behavior Displays
Guard
Treated


c
Cohort 2 Non-activated Retinal Reared Fights
180
160
140
120
100
80
60
40
20
0
Display Count
Lo
Med
Hi
Behavior Displayed
Guard
Control

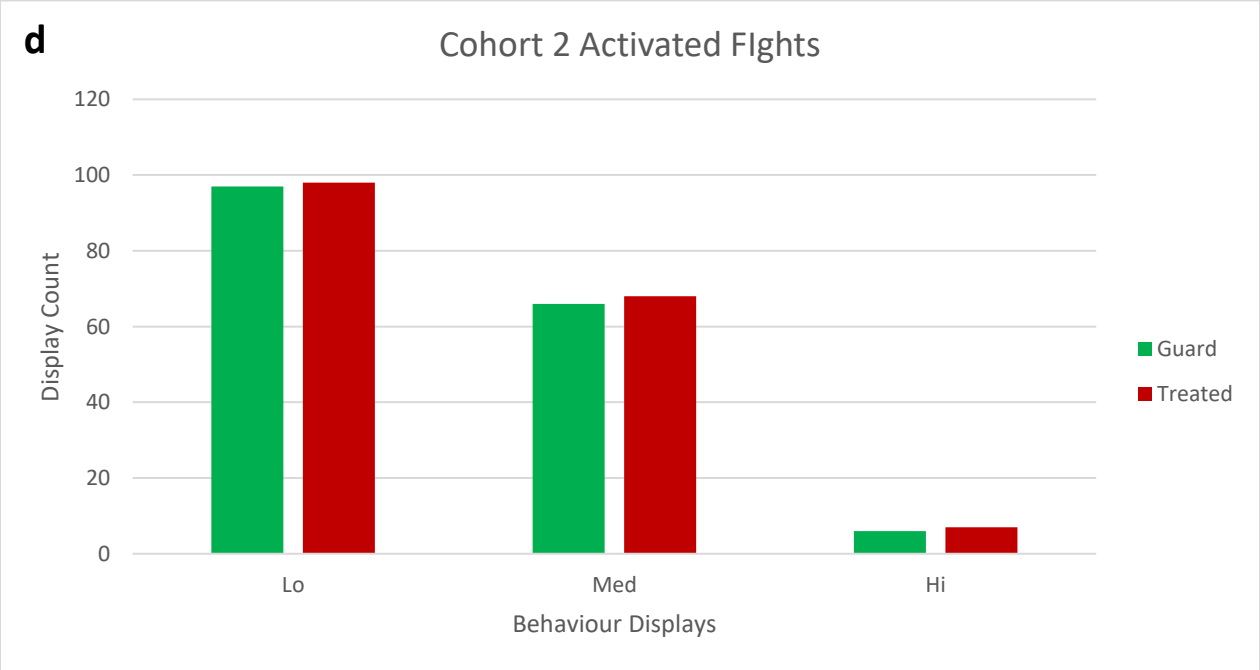

d
Cohort 2 Activated FIghts
120
100
80
60
40
20
0
Display Count
Lo
Med
Hi
Behaviour Displays
Guard
Treated

Figure 13a-d. **Aggression Bioassay Results**: Fight aggression behavior data from non-activated control fights (a&c), as well as activated fights (b&d) from Cohorts 1 and 2 are visible above. Cohort 1 flies were raised on regular diet then spiked with retinal, Cohort 2 was reared on retinal diet. Lo-level behaviors, Med-level behaviors include, and Hi-level are characterized as explained in Figure 12. Both initiated behavior and response behaviors are grouped in the same behavior display. Defensive actions are not included in the graph. Aggressive behaviors differed significantly between control and treated males in both cohorts (χ^2, $p<.01$) found by using Chi-square test.

Looking at our results, we found that we had definite expression of Channelrhodopsin 2 in areas related to aggression circuits seen in prior papers as seen in Figures 9 and 10. Under that assumption the UAS-GAL4 expression system was fully functional and operational. Overall, we saw a decrease in aggression for both cohort 1 and 2 when activated, as seen in Figure 13. Retinal reared flies in Cohort 2 showed a stronger decrease in aggression behavior when compared to cohort 1. In general, a statistically significant difference in patterns of aggressive behavior was recorded between treatment and control flies.

Discussion:

Our original goal was to see the effect of neuropeptide-F on aggression behaviors in *D. melanogaster.* We desired to see the effects of the direct release of the neuromessenger NPF onto identified aggression-regulating neuronal circuits to see if it enhances or depresses aggression (Dierick et al. 2007; Lee et al. 2004).

In our results, we were able to utilize optogenetic tools to turn on cells to release NPF into their proper neural tracts and view the effects *in vivo* via aggression bioassay. After watching and tallying all aggression behaviors visualized over the fights done, there was a statistically significant difference between control fly fights and experimental fly fights for both cohorts, as seen in Figure 11. In general, the aggression diminishing effects of NPF were more obvious in retinal reared fights seen in Cohort 2 data compared to Cohort 1.

Previous research has shown NPF to have different effects on *D.melanogaster* aggression. NPF itself is an orthologue of neuropeptide-Y (NPY) of vertebrate nervous systems, which has nearly the same sequence, but it ends in phenylalanine (F) rather that tyrosine (Y). Neuropeptide Y has been suggested to serve a modulatory role in aggression in mammalian systems (Karl 2004). Due to this similarity we can attempt to draw conclusions about the behavior of neural molecules in model organisms such as *Drosophila* and connect it to our internal aggression mechanisms within us. There are 26 NPF secreting neurons per adult vertically dissected hemibrain in male fruit flies found distributed in at least eight different clusters, but in females only 20 NPF-expressing neurons are found per hemibrain (Lee et al. 2006). Some of these cells are visible in our adult brain image visible in Figure 9, and some of the clusters can be seen in larval imaging in Figure 10. It was assumed previously that these clusters of NPF secreting cells were only found in the brain and there seemed to

be no expression in the ventral nerve chord or ventral ganglia; however, looking at images in Figure 10 and Figure 11b one may think otherwise (Lee et al. 2006). Looking at the images of the larval tissue, one could see axon tracts going down into the larval ventral ganglion. Figure 11b shows a strong signal at the section of ventral nerve chord seen in the image. It is very possible that there are cells expressing NPF down the ventral ganglia and ventral nerve chord and allowing for systemic dumping of neuromessenger molecules to effect behavior. To follow up, we could do full adult CNS dissections connecting adult brain to adult ganglion and see where NPF expressing cells are found along those possible neural tracts. Since NPF is related to food, it may also be a good idea to dissect out an entire Gastrointenstinal tract connected to the brain of the fly to see where expression may be seen there.

NPF has been implicated in multiple behaviors in *Drosophila* including foraging and feeding, which explains why food as a stimulus works as an easy way to stimulate aggression (Hermann 2012). Alcohol sensitivity, end of day locomotor activity patterns, and a longer mating duration of male flies housed with other male flies in mating arenas are also behaviors influenced by NPF (Kim et al. 2013; Nässel et al. 2010). NPF is suggested to hold aggression in check in male flies, and may account for the sexual dimorphic difference in number of NPF cells with 26 per hemibrain in males compared to 20 per hemibrain in females (Dierick et al. 2007). When it comes to aggression pathways there is a single pair of serotonergic proteolipid protein (PLP) neurons that enhances aggression in *Drosophila* (Alekseyenko et al. 2014). Those serotonin (5HT) neurons release onto serotonin receptor bearing neurons(5HT1A). 5HT1A receptors act by decreasing Cyclic adenosine monophosphate (cAMP) synthesis and inhibiting neurons expressing these receptors. Activation of neurons with 5HT1A receptors on their surfaces inhibits higher-level aggression behaviors seen in Fly fights, such as lunging and boxing. In these aggression circuits 5HT released by activation of

5HT-PLP neurons may release higher-level aggression by inhibiting a tonically active descending inhibitory pathway that is seen in both invertebrate and mammalian models (Kravitz 2015). The 5HT-PLP cell bodies are located on the posterior surface of the *Drosophila* brain, but their arbors form a dense neuropil throughout the ventrolateral protocerebrum and also branch out towards the central complex structures. The 5HT-PLP neurons do not co-express NPF; however, NPF neurons has been reported to innervate the fan-shaped body of the central complex and to partly surround the mushroom body neuropil regions. Both of these brain regions are nearby arborization fields of the 5HT-PLP neurons that show a link between NPF and serotonergic aggression circuits (Alekseyenko and Kravitz 2014). In general, more expression of serotonin in the brain of *Drosophila* feeds behaviors related to feeding, locomotion, and mating, NPF circuits impinge on those activities in a system of checks and balances. That decrease in overall aggression related to food, is seen in our results in Figure 11.

What is interesting is how the results of the retinal reared flies differed from the flies that were raised on regular fly diet. Looking at Figure 11a and 11b vs Figure11c and Figure 11d, Cohort 2 flies that were raised on retinal had a larger difference in inactivated compared to activated fights than Cohort 1. Cohort 2 flies hatched out of eggs and grew up on retinal diet their entire lives, while Cohort 1 only had retinal in the 10 days preceding fights. Trans-retinal is required for the Channelrhodopsin-2 complex as seen in Figure 6. It could be assumed that just spiking flies with retinal wasn't enough to activate all the Channelrhodopsin-2 light gated channels due to the actual Retinal molecule that undergoes a conformational change wasn't in large supply during development. Figure 3 shows that light hits Trans-retinal in the light reaction that allows for Channelrhodopsin to function. The Channelrhodopsin transmembrane helix domain covalently anchors retinal via a Schiff base linkage where light absorption triggers a *trans*-to-*cis* isomerization of the C13−C14 double bond

of the retinal chromophore. The strained 13-*cis* retinal induces conformational changes in the protein that open a channel through sodium ions can pass activating the neuron (Ardevol and Hummer 2018). Flies in Cohort 1 didn't have as strong a difference between control and experimental fights due to not consuming enough Trans-retinal while developing as a larva. Transretinal is absolutely necessary for Channelrhodopsin development and is absolutely necessary to even have a shot at proper activation of light activated ion channels.

In reference to why fruit flies behave aggressively toward each other in such a stereotyped pattern this has many origins according to past researchers. In the wild fly fights occur naturally among males in competition for mating opportunities or food. Females also fight for areas to lay eggs as well, but easily visible combat is more exaggerated in males. With the motivation of food it makes sense that male flies would fight for resources. If two males were placed in a ring with a female fly they would probably fight in a similar manner for mating opportunities. It may be an interesting follow up to this food-based aggression study to replicate it with a decapitated female in the fight arena and to redo the fight procedure testing NPF effects on aggression in relation to potential mating opportunities directly. Both food based and mating opportunity-based aggression is heavily related to smell and olfactory reception (Lee et al. 2020). It may also be an interesting experiment to modulate aggression with various smells and see the effects on adult flies. To further check our aggression bioassay results, it may be beneficial to redo aggression bioassays with flies having different light induced ion channel such as using CsChrimson that opens with excitation of red light.

In reference to the larval brain imaged in Figure 10, it is obvious that even at lower stages of development that *Drosophila* has NPF secreting cells active. In theory, the behaviors of larvae could also be monitored for changes when NPF is expressed. Larvae just like adult flies may have

changes in locomotion related to NPF or even changes in feeding behavior (Hermann 2012). Using a similar setup we could visualize transgenic larvae feeding and moving in regards to NPF expression and its effect on those behaviors.

According to the Bloomington Stock center there may possibly be NPF expression in the midgut as well as where it is found presently. We can theoretically check for the effect of NPF expression in midgut cells by shining light on flies while fully alive in open vivisection. It may be possible to see a physiological response to NPF expression in relation to muscle contraction. One could potentially take a suction electrode and monitor a small section of muscle cells to see for changes in muscle contraction when a live transgenic fly is hit by light to express NPF.

Also, in another general direction, understanding aggression at this level may allow us to better understand diseases related to aggression such as Alzheimers, Dementia, Bipolar Disorder, and Epilepsy (Lane 2011). Understanding of disease at the molecular/cellular level in coordination with fear and aggression is the basis of all Neuropsychiatry in this domain.

Conclusion:

Overall, we learned that neuropeptide-F impinges upon aggression circuits within the nervous system of *Drosophila melanogaster*. Neuromessenger proteins such as itself works in a system of checks and balances within the brain of the fruit fly to keep it in check and regulate some of its most primal of feelings. The need to mate, feed, fight, or flee are parts of our most basic drives as animals. In our bodies we don't have NPF, but we have NPY that functions similarly to invertebrates such as *Drosophila*.

We aren't that different from fruit flies when it comes down to it, and we both have similar mechanisms that drive our aggression through serotonin-based pathways in both our nervous systems. Flies just like humans have the capability to get angry enough to fight one another through acts of violence. Flies compete just like us in a system of limited resources to attempt to get what they need and want.

In this day and age of extraordinary tools that allow us to view nervous systems in action at the molecular level it is our prerogative to explore the unknown using *Drosophila* not to just understand their behavior better, but to also better understand how we behave as well.

References:

Alekseyenko, O. V., Chan, Y.-B., de la Paz Fernandez, M., Bülow, T., Pankratz, M. J., & Kravitz, E. A. (2014). Single serotonergic neurons that modulate aggression in *Drosophila*. *Current Biology : CB*, *24*(22), 2700–2707. https://doi.org/10.1016/j.cub.2014.09.051

Alekseyenko, O. V., & Kravitz, E. A. (2014). Serotonin and the search for the anatomical substrate of aggression. *Fly*, *8*(4), 200–205. https://doi.org/10.1080/19336934.2015.1045171

Ardevol, A., & Hummer, G. (2018). Retinal isomerization and water-pore formation in channelrhodopsin-2. *Proceedings of the National Academy of Sciences*, *115*(14), 3557–3562. https://doi.org/10.1073/pnas.1700091115

Baier, A., Wittek, B., & Brembs, B. (2002). *Drosophila* as a new model organism for the neurobiology of aggression? *The Journal of Experimental Biology*, *205*(Pt 9), 1233–1240.

Baxter, C. M., Barnett, R., & Dukas, R. (2015). Aggression, mate guarding and fitness in male fruit flies. *Animal Behaviour*, *109*, 235–241. https://doi.org/10.1016/j.anbehav.2015.08.023

Bloomington Drosophila stock center. (n.d.). Bloomington *Drosophila* Stock Center. Retrieved March 16, 2020, from https://bdsc.indiana.edu/Home/Search?presearch=25681

Chen, S., Lee, A. Y., Bowens, N. M., Huber, R., & Kravitz, E. A. (2002). Fighting fruit flies: A model system for the study of aggression. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(8), 5664–5668. https://doi.org/10.1073/pnas.082102599

Dierick, H. A., & Greenspan, R. J. (2007). Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nature Genetics*, *39*(5), 678–682. https://doi.org/10.1038/ng2029

Fruit fly aggression studies have relevance to humans, animals. (n.d.). ScienceDaily. Retrieved March 15, 2020, from https://www.sciencedaily.com/releases/2006/09/060920192543.htm

Gene knockdown affects aggression in the fruit fly. (n.d.). Baylor College of Medicine. Retrieved March 15, 2020, from https://www.bcm.edu/news/molecular-and-human-genetics/gene-knockdown-aggressive-fruit-fly

Hermann, C., Yoshii, T., Dusik, V., & Helfrich-Förster, C. (2012). Neuropeptide F immunoreactive clock neurons modify evening locomotor activity and free-running period in *Drosophila* melanogaster. *The Journal of Comparative Neurology*, *520*(5), 970–987. https://doi.org/10.1002/cne.22742

Hoopfer, E. D., Jung, Y., Inagaki, H. K., Rubin, G. M., & Anderson, D. J. (2015). P1 interneurons promote a persistent internal state that enhances inter-male aggression in *Drosophila*. *ELife*, *4*, e11346. https://doi.org/10.7554/eLife.11346

How interacting with females increases aggression in male fruit flies. (2019). https://www.caltech.edu/about/news/how-interacting-females-increases-aggression-male-fruit-flies

Johnson, M. (2012). Antibody shelf life/how to store antibodies. *Materials and Methods*, *2*. https://doi.org/10.13070/mm.en.2.120

Karl, T., Lin, S., Schwarzer, C., Sainsbury, A., Couzens, M., Wittmann, W., Boey, D., Hörsten, S. von, & Herzog, H. (2004). Y1 receptors regulate aggressive behavior by modulating serotonin pathways. *Proceedings of the National Academy of Sciences*, *101*(34), 12742–12747. https://doi.org/10.1073/pnas.0404085101

Kim, W. J., Jan, L. Y., & Jan, Y. N. (2013). A pdf/npf neuropeptide signaling circuitry of male *Drosophila* melanogaster controls rival-induced prolonged mating. *Neuron*, *80*(5), 1190–1205. https://doi.org/10.1016/j.neuron.2013.09.034

Kravitz, E. A., & Fernandez, M. de la P. (2015). Aggression in *Drosophila*. *Behavioral Neuroscience*, *129*(5), 549–563. https://doi.org/10.1037/bne0000089

Lacaille, F., Hiroi, M., Twele, R., Inoshita, T., Umemoto, D., Manière, G., Marion-Poll, F., Ozaki, M., Francke, W., Cobb, M., Everaerts, C., Tanimura, T., & Ferveur, J.-F. (2007). An inhibitory sex pheromone tastes bitter for *Drosophila* males. *PLOS ONE*, *2*(8), e661. https://doi.org/10.1371/journal.pone.0000661

Lane, S. D., Kjome, K. L., & Moeller, F. G. (2011). Neuropsychiatry of aggression. *Neurologic Clinics*, *29*(1), 49–vii. https://doi.org/10.1016/j.ncl.2010.10.006

Lee, G., Bahn, J. H., & Park, J. H. (2006). Sex- and clock-controlled expression of the neuropeptide F gene in *Drosophila*. *Proceedings of the National Academy of Sciences*, *103*(33), 12580–12585. https://doi.org/10.1073/pnas.0601171103

Lee, K.-S., You, K.-H., Choo, J.-K., Han, Y.-M., & Yu, K. (2004). *Drosophila* short neuropeptide F regulates food intake and body size. *The Journal of Biological Chemistry*, *279*(49), 50781–50789. https://doi.org/10.1074/jbc.M407842200

Lee, S., Jones, W. D., & Kim, D. (2020). A cyclic nucleotide-gated channel in the brain regulates olfactory modulation through neuropeptide F in fruit fly *Drosophila melanogaster*. *Archives of Insect Biochemistry and Physiology*, *103*(1). https://doi.org/10.1002/arch.21620

Lim, R. S., Eyjólfsdóttir, E., Shin, E., Perona, P., & Anderson, D. J. (2014). How food controls aggression in *Drosophila*. *PLOS ONE*, *9*(8), e105626. https://doi.org/10.1371/journal.pone.0105626

Marder, E., O'Leary, T., & Shruti, S. (2014). Neuromodulation of circuits with variable parameters: Single neurons and small circuits reveal principles of state-dependent and robust neuromodulation. *Annual Review of Neuroscience*, *37*(1), 329–346. https://doi.org/10.1146/annurev-neuro-071013-013958

Nässel, D. R., & Winther, Å. M. E. (2010). *Drosophila* neuropeptides in regulation of physiology and behavior. *Progress in Neurobiology*, *92*(1), 42–104. https://doi.org/10.1016/j.pneurobio.2010.04.010

Palavicino-Maggio, C. B., Trannoy, S., Holton, K. M., Song, X., Li, K., & Nevo, E. (2019). Aggression and courtship differences found in *Drosophila* melanogaster from two different microclimates at Evolution Canyon, Israel. *Scientific Reports*, *9*(1), 1–9. https://doi.org/10.1038/s41598-019-40701-8

Pu, Y., Zhang, Y., Zhang, Y., & Shen, P. (2018). Two *Drosophila* neuropeptide y-like neurons define a reward module for transforming appetitive odor representations to motivation. *Scientific Reports*, *8*(1), 11658. https://doi.org/10.1038/s41598-018-30113-5

Rashid, F., Raducanu, V.-S., Zaher, M. S., Tehseen, M., Habuchi, S., & Hamdan, S. M. (2019). Initial state of DNA-Dye complex sets the stage for protein induced fluorescence modulation. *Nature Communications*, *10*(1), 1–14. https://doi.org/10.1038/s41467-019-10137-9

Roote, J., & Prokop, A. (2013). How to design a genetic mating scheme: A basic training package for *Drosophila* genetics. *G3: Genes|Genomes|Genetics*, *3*(2), 353–358. https://doi.org/10.1534/g3.112.004820

Siwicki, K. K., & Kravitz, E. A. (2009). Fruitless, doublesex and the genetics of social behavior in *Drosophila* melanogaster. *Current Opinion in Neurobiology*, *19*(2), 200–206. https://doi.org/10.1016/j.conb.2009.04.001

Trannoy, S., & Kravitz, E. A. (2017). Strategy changes in subsequent fights as consequences of winning and losing in fruit fly fights. *Fly*, *11*(2), 129–138. https://doi.org/10.1080/19336934.2016.1259041

Zhou, C., Rao, Y., & Rao, Y. (2008). A subset of octopaminergic neurons are important for *Drosophila* aggression. *Nature Neuroscience*, *11*(9), 1059–1067. https://doi.org/10.1038/nn.2164