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Blocking Neuropilin-1 in *Drosophila melanogaster* as a Possible Treatment for Pain and SARS-CoV-2

Ania Kelegama[1] and Leya Joykutty[#]

[1]American Heritage School, Plantation, FL, USA
[#]Advisor

ABSTRACT

This study aims to validate a method of neuropilin-1 blocking in *Drosophila melanogaster* to aid the development of chronic pain treatment as well as increase scientific understanding of SARS-CoV-2 cell entry. If *Drosophila melanogaster* are exposed to monoclonal antibodies (mAbs) used for targeted chemotherapy against neuropilin-1, then this targeted protein blocking method may allow for the development of new pain treatment and possible SARS-CoV-2 treatment. mAbs were microinjected into mutant flies to block NRP-1 activity. Then, a thermal nociception and von frey assay were done to test *drosophila* nociception. Finally, an IHC assay was performed to quantify protein activity. Overall, the hypothesis was supported as both nociception assays showed significant data proving mutant flies had delayed or no reactions to stimuli. The Von Frey assay did show some varying results, but the data is still significant. However, the IHC did show that there was still some NRP-1 activity in the mutant flies. NRP-1 was able to be partially blocked by mAbs. The collected data may apply to the expansion of research in pain treatment as well as COVID-19 research. Chronic pain is a prevalent area of research that is still not completely solved today. The fight against the SARS-CoV-2 is an ongoing fight and further research is mandatory in finding treatments for this deadly virus.

Introduction

Purpose

The purpose of this experiment is to validate a method of neuropilin-1 protein blocking through monoclonal antibodies in wild-type and Neto-insert *Drosophila melanogaster* to expand the field of pain treatment as well as to develop possible cellular level treatments for SARS-CoV-2. By experimenting with neuropilin-1 (Neto), a new door in pain treatment could be opened, along with discoveries in SARS-CoV-2 and membrane entry.

Background Research

A September 2020 study of SARS-CoV-2 revealed a protein spike of neuropilin-1. This protein has recently been discovered to function with pain receptors in COVID-19 patients with arthritis and osteoporosis. Pain signaling processes are inhibited as spike proteins bind to neuropilin-1, causing the protein to become inactive. As recorded in the study, arthritic SARS-CoV-2 patients reported numbness and decreased pain. Preliminary reports showed that SARS-CoV-2 spike proteins had binded to neuropilin, supporting the conception that the virus may enter nerve cells through neuropilin-1, as well as the ACE2 protein which has already been reported (Khanna, 2020). Scientists hypothesize that blocking neuropilin-1 could not only block pain but also limit the entry of SARS-CoV-2 into cells, as spike proteins use this protein to enter nerve cells.

Neuropilins are class 1 transmembrane glycoprotein receptors for central nervous system class 3 semaphorins, which provide epulsive or attractive signals for neurons. Neuropilin-1 (NRP-1) was found to be involved in neural crest migration and axon growth during the development of the vertebrate nervous system (Graziani & Lacal, 2015). It was recently discovered that this protein also serves as a coreceptor for vascular endothelial growth factor (VEGF) and interacts with VEGF receptor tyrosine kinases VEGFR1 and VEGFR2 (Tata & Ruhrberg, 2015). Past studies involving neuropilin research have shown strong evidence that NRP-1 acts as a tumor-promoting factor. Through many cancer studies, increased NRP-1 levels are linked to tumor aggressiveness, advanced disease stage, and poor prognosis. It has been expressed in the following cancers: prostate, lung, pancreatic, or colon carcinoma, melanoma, astrocytoma, glioblastoma, and neuroblastoma (Graziani & Lacal, 2015).

After these cancer discoveries were made, several methods of counteracting the tumor-promoting effects of NRP-1 were explored utilizing recombinant sNRP-1, class 3 semaphorins, monoclonal antibodies (mAbs), peptides and peptidomimetics, small interfering RNAs (siRNAs) or microRNAs. A high-affinity monoclonal antibody was capable of targeting NRP-1, therefore inhibiting VEGF-A-induced migration of human endothelial cells and tumor formation (Graziani & Lacal, 2015). This data led researchers to speculate that certain tumor progression could be inhibited by using anti-NRP-1 and anti-VEGF factors such as monoclonal antibodies. This research might also apply to other studies linked to neuropilin-1.

Most organisms have developed complex conserved mechanisms to allow for the best survival in hostile environments. Nociception is the acute detection of noxious or harmful stimuli to avoid potential tissue damage. Pain, a subjective experience, is different from nociception, the objective neural processes of encoding and processing noxious stimuli. For this study, *Drosophila melanogaster* are being employed as animal subjects due to their genetic compatibility with humans; it is estimated that 75% or more of human disease genes have conserved homologs in *drosophila*. *Drosophila* nociceptors (pain receptors) are found in each stage of *drosophila* life, and there is evidence of morphological and functional resemblance to vertebrate nociceptors, such as characteristic naked nerve endings. In both vertebrates and this invertebrate model, dendrite endings cover the entire epidermis and allow the organism to sense and respond quickly to harmful stimuli. This enables the organism to avoid tissue damage and potential injury. Therefore, because of the many similarities in vertebrate and invertebrate nociception, *Drosophila melanogaster* was chosen as an experimental model.

Hypothesis

If Neto-insert *Drosophila melanogaster* are exposed to monoclonal antibodies used for targeted chemotherapy against neuropilin-1, then this targeted protein blocking method may allow for the development of new pain treatment and possible SARS-CoV-2 treatment.

Methods

Maintenance

Obtain wild-type red-eye *Drosophila melanogaster* from Carolina Biological Supply Company and Neto mutant *Drosophila melanogaster* (Bloomington Drosophila Stock Center, stock #18542). Feed both groups using Carolina Biological White Fly Medium. Allow flies to reproduce until enough larvae are present to experiment with.

Injection

Prepare 10 mutant larvae and 10 wild-type larvae for microinjection with WPI Nanoliter microinjector. Monoclonal antibody solution may be mixed with dye to allow for in vivo viewing of successful microinjection. Orient larvae in

agar plate to immobilize them. Insert microneedle at the aortic junction near larvae's head. 50 nL of solution or a buffer solution as a control (10 mM Tris–HCl, pH 7.5) are injected using the nanoinjector. After injection, let larvae recover in appropriate vials in horizontal position (to avoid flies getting stuck to the medium), and verify the appearance of melanization spots at the injection site. Allow injected flies to recover for 2-3 days and record data from the following nociception assays.

Thermal Nociception

Fifteen fly larvae from control and mutant were collected and placed in a 35-mm Petri dish and then touched with a soldering iron heated to 46°C. Record the reaction time of each group. Wild-type larvae respond to heat insult within a few seconds with a stereotypical rolling response.

Von Frey Filament Assay

Using a calibrated von frey fiber, touch larvae until they pause their normal feeding behavior; a force of 45 mN induces the stereotypic rolling reaction. Record whether the control and mutant group react to 45 mN force.

Tissue Embedding and Sectioning

Set apart 10 flies from control and mutant group for use in immunofluorescence microscopy. Place each group of flies in metal mold. Cover the entire tissue block with cryo-embedding media (e.g. OCT or Tissue Freezing Medium). Slowly place the base mold containing the tissue block into liquid nitrogen till the entire tissue block is submerged into liquid nitrogen to ensure tissue is frozen completely. Store the frozen tissue block at -80°C until ready for sectioning. Transfer the frozen tissue block to a cryotome cryostat (e.g. -20°C) before sectioning and allow the temperature of the frozen tissue block to equilibrate to the temperature of the cryotome cryostat. Section the frozen tissue block into a desired thickness (typically 5-10 µm) using the cryotome. Place the tissue sections onto glass slides suitable for immunohistochemistry (e.g. Superfrost). Fix the tissue sections with a suitable fixative. One of the commonly used fixation methods for frozen tissue sections is to immerse the slides in pre-cooled acetone (-20°C) for 10 mins. Wash slides in PBS for 5 mins. Repeat 3 times.

Blocking and Immunostaining

Incubate cells with 1% BSA, 22.52 mg/mL glycine in PBST (PBS+ 0.1% Tween 20) for 30 min to block unspecific binding of the antibodies. Incubate cells in the diluted primary antibody in 1% BSA in PBST in a humidified chamber for 1 h at room temperature or overnight at 4°C. Decant the solution and wash the cells three times in PBS, 5 min each wash. Incubate cells with the secondary antibody in 1% BSA for 1 h at room temperature in the dark. Decant the secondary antibody solution and wash three times with PBS for 5 min each in the dark. Analyze slides using a Nikon Eclipse Ti microscope and capture images using a compatible microscope camera. Analyze the intensity/pixel of the images by the Image Analyzer Pro software. Calculate average intensity/pixel using Microsoft Excel.

Results

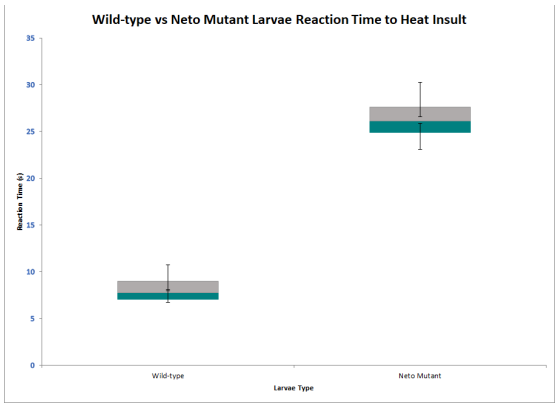

Figure 1. Reaction Times for Thermal Nociception Assay. Comparison between the reaction times of wild-type and mutant larvae groups after a heat probe was touched to the larvae. As can be seen, there is a significant difference between the reaction times of these larvae.

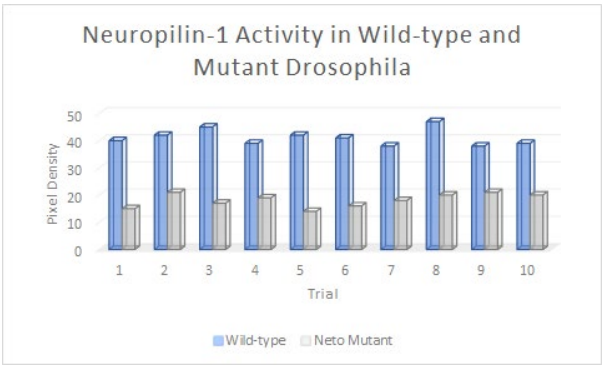

Figure 2. Neuropilin-1 Activity in the *Drosophila.* Difference in protein activity between wild-type and mutant drosophila after an immunofluorescence assay was performed. Protein activity was significantly higher in wild-type which had not received monoclonal antibodies. Some protein activity was seen in the mutant group, however.

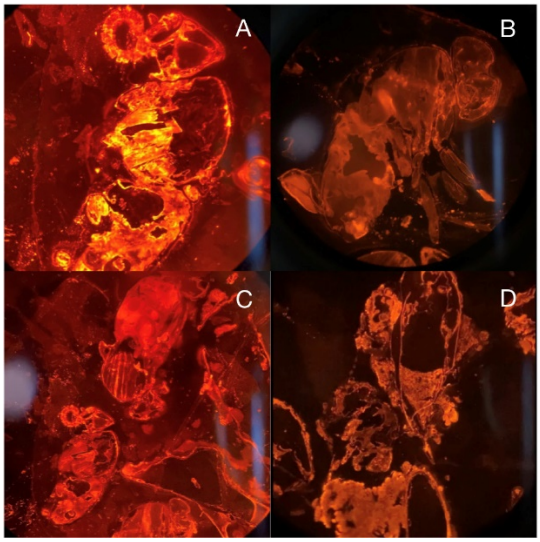

Figure 3. Immunofluorescence Assay detecting NRP-1 activity in *drosophila.* Images A and C depict the neuropilin-1 activity in wild type flies while the images B and D show the mutant NRP activity. It is clear to see that wild-type flies had higher protein activity.

Table 1. Data collected from the Von Frey filament assay.

Trial	Wild type	Neto Mutant
1	Yes	No
2	Yes	No
3	Yes	No
4	Yes	No
5	Yes	No
6	No	No
7	Yes	Yes
8	Yes	No
9	Yes	No
10	Yes	Yes

Data represents whether each larvae group reacted or not to a 45 mN force. Wild-type reacted 90% of the time, while mutant only reacted 20% of the time.

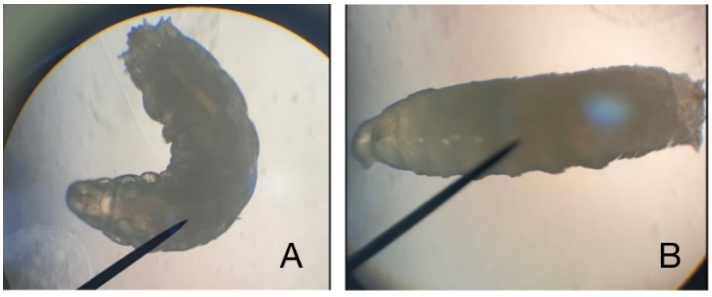

Figure 4. Comparison of larvae from Von Frey assay. Being exposed to pressure by a von frey filament, larvae (A) exhibits stereotypical rolling. Larvae (B) on the other hand does not show a response to the von frey filament.

Conclusion

The purpose of this project was to test a possible mechanism of neuropilin-1 blocking in *Drosophila melanogaster* to expand the fields of pain treatment research as well as COVID-19 research. To observe *Drosophila* nociception a thermal nociception assay and a Von Frey filament assay were performed on two groups of larvae to differentiate

between the control group and the neuropilin (Neto) mutant. After these nociception assays were done, an immunofluorescence assay was done to determine whether neuropilin-1 activity had decreased in mutant flies that had received monoclonal antibodies targeting NRP-1. Monoclonal antibodies were administered through microinjection into larvae. In conclusion, the monoclonal antibodies were successful in partially blocking NRP-1 in the mutant group which is supported by data from both the thermal nociception assay as well as the Von Frey assay. During the Von Frey assay, wild-type larvae reacted to stimulus 90% of the time, while mutant larvae only reacted 20% of the time. Both assays showed delayed or no reaction in mutant *drosophila* to noxious stimulus that was applied. The means of these nociception assays were significantly different, allowing the scientist to reject the null hypothesis. Data collected from immunofluorescence microscopy showed that wild-type flies had significantly increased NRP-1 activity compared to the mutant group who received monoclonal antibodies. However, the mutants still had some protein activity, supporting the idea the monoclonal antibodies were partially effective in this experiment. In summation, *Drosophila* proved a worthy model of nociception and were able to successfully uptake monoclonal antibodies. The hypothesis was partially supported in the end.

Limitations

Limitations in this experiment include organization of assays, drosophila as biological models, and complex equipment used. Nociception assays in the flies had varying results, and although the means of the collected data were significantly different, more trials should have been performed to validate the results of the assays. Each fly may have also had a different reaction to noxious stimuli; *drosophila* are biological organisms that are unique to themselves and, therefore, may have exhibited varying levels of nociception. The first step of the experiment, injection of monoclonal antibodies, may have also been a limiting factor. Since *drosophila* are incredibly small model subjects, proper microinjection of these organisms may not have been perfect. It is slightly unclear whether monoclonal antibodies were correctly injected into the flies. This project could have been improved by using a larger, more complex model organism, increasing the number of trials performed, and mastering the use of complex instruments used in this experiment.

Applications

The results of this experiment can be applied to the fields of COVID-19 research and pain research. The purpose of this project was to identify whether or not monoclonal antibodies could block neuropilin-1 activity in *Drosophila melanogaster.* The collected data which supports the hypothesis, to a point, is viable for researchers studying new treatments for chronic pain as neuropilins play a central role in nociception and pain signaling. Chronic pain is a prevalent issue in the medical world, and proper drug treatments have been developed; however, finding possible natural treatments would benefit patients experiencing chronic pain disorders such as osteoporosis or arthritis. Current narcotics can become highly addictive, therefore, finding non-drug pain treatments would benefit patients avoiding narcotics or those who are already addicted. The results of this data also apply to the COVID-19 pandemic currently ongoing around the world. With the SARS-CoV-2 virus rampaging the globe with its deadly symptoms, it is necessary to research and find explanations for the virus's cellular mechanisms of entry into human cells. Across the globe, nearly 90 million people have contracted the virus and almost 2 million people have died. Vaccines and hospital treatments are known today, however, having a better understanding of the virus's complete cellular function would aid in treatment development. Because of past discoveries linking the ACE-2 receptor to SARS-CoV-2 entry, researchers also discovered that neuropilin-1, a transmembrane glycoprotein acts as an open door to the SARS virus. *Drosophila* proved to be a cheap, reliable, and effective model of nociception. Therefore, further studies utilizing these invertebrates as test subjects in nociception assays for pain or SARS-CoV-2 research are validated.

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