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ARTICLE

Activation of a Receptor for Insulin-like Peptide Decreases Fictive Locomotion in Fruit Fly Larvae

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INTRODUCTION

The Human Nervous System

At a basic level, the human nervous system can be broken down into two categories: the central nervous system, composed of the brain and spinal cord, and the peripheral nervous system, made up of nerves that extend throughout the body. These systems work conjunctively to complete a multitude of actions performed by the body (Haven, P. et al.; 1991). The specialized cells that compose the nervous system are known as neurons. Neurons allow for the transmission of chemical and electrical messages between nerve cells. The space where this chemical transmission occurs is known as a synapse. Synapses occur between various cells within the body, including motor neurons and muscle cells, where the synapse is known as the neuromuscular junction (NMJ) (Haven, P. et al.; 1991).

Neuropeptides

Chemical messages transmitted between nerve cells are found in the brain in the form of neuropeptides. Neuropeptides are short chains of amino acids that can be found in the form of neurotransmitters, such as dopamine or serotonin, or as neurohormones, such as oxytocin or vasopressin (Haven, P. et al.; 1991). Neurotransmitters and neurohormones have a similar function in the brain, however, they differ in the way in which they transmit messages. Neurotransmitters are released from a presynaptic cell directly into the synapse, where they can bind to receptors in the postsynaptic cell (Figure 1A). Neurohormones differ in that they are released from a neuron directly into the bloodstream, where they are circulated and can bind to distant target cells (Figure 1B) (Haven, P. et al.; 1991). There are many human diseases that involve disturbances in neuropeptide signaling, either as a result of changes in peptide levels, or the effects on peptide receptors. Understanding how neuropeptides function in the nervous system should provide a better understanding of the role that neuropeptides play in disease and may eventually lead to treatments. Common diseases such as Alzheimer's disease, Huntington's disease, and narcolepsy rely on research involving peptide signaling to develop a clearer understanding of disease mechanisms (Lewis, D. A. et al., 1987).

Drosophila as a Model System

The fruit fly species, *Drosophila melanogaster*, is a popular model system used for neurological research. Peptide receptors found in *D. melanogaster* are very similar to peptide receptors found in humans. This has allowed *D. melanogaster* to be used in labs worldwide for research involving peptide signaling, genetics and behaviour. Many studies use *D. melanogaster* larvae found in the 3rd instar stage, which occurs directly prior to pupation in the fly life cycle, to study muscle movement during fictive locomotion (crawling-like behaviour) (Ormerod, K. et al., 2018).

Insulin & *Drosophila* Insulin-like Peptide (diLP)

This study has focused specifically on studying a role for insulin as a neuropeptide in the nervous system. The human neurohormone, insulin, can be compared to homologous peptides found in *D. melanogaster*, known as *Drosophila* Insulin-like Peptides (diLPs). Insulin in humans serves the purpose of regulating glucose levels in the bloodstream to prevent hyperglycemia or hypoglycemia and allowing cells to receive glucose as useable energy (Goodenough, J. et al., 2017). In fly, diLPs are primarily responsible for growth regulation during embryogenesis, especially development of the epidermis and nervous system. There are 8 known diLPs found in *D. melanogaster* larvae. These peptides have effects on metabolism, reproduction and many other key components of the fly life cycle. There is evidence to suggest that diLPs 1-7 act through the *Drosophila* Insulin Receptor (DInr), whereas diLP 8 acts through an alternate receptor known as Lgr3 (Brogiolo et al., 2001). Studies have shown that diLPs can act as neurotransmitters or neurohormones in the nervous system. diLPs 2, 3 and 5 can be found in neurons projecting to areas that release hormones into the hemolymph, which is a blood-like fluid found in fly. Alternatively, diLP 7 can be found in neurons in the abdominal nerve cord, which supplies muscle cells directly through neurotransmitters (Garelli et al., 2015; Nässel & Winther,

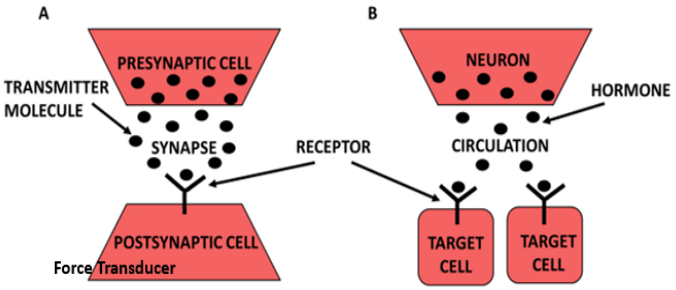

Figure 1. Neurotransmitter release from presynaptic cell into synapse (A) and neurohormone release into bloodstream (B).

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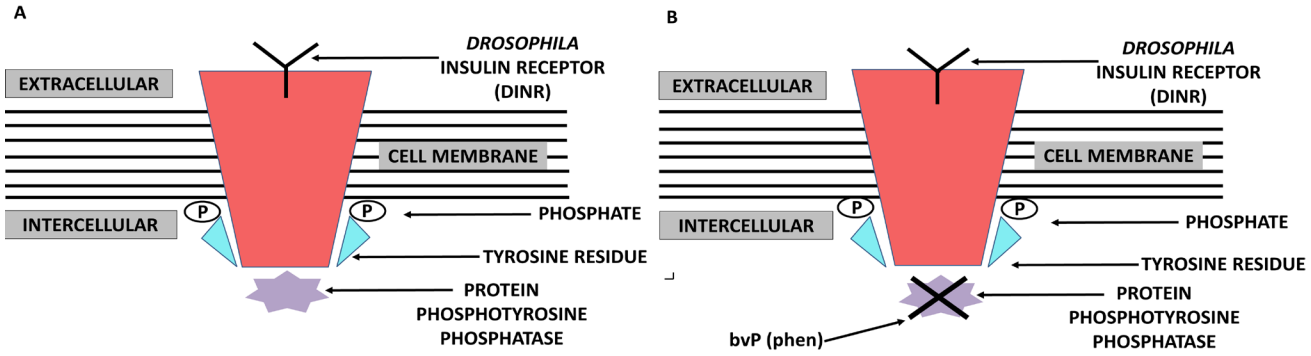

Figure 2. Receptor activity inhibition by phosphotyrosine phosphatase (A) and inhibition of phosphotyrosine phosphatase using the synthetic inhibitor bvP (B).

2010.).

Insulin Receptor (IR) & Drosophila Insulin Receptor (DInr)

Insulin in humans acts by binding insulin receptors as a neurohormone. The Insulin Receptor (IR) is a tyrosine kinase receptor (Trk) with intrinsic action. As insulin binds, tyrosine residues undergo autophosphorylation. This in turn allows for IR to activate the Insulin Receptor Substrate (IRS-1). This action works similarly in other receptors as well, such as Insulin Growth Factor 1 (IGF1) (White et al., 1994). IR is acted on by the enzyme protein phosphotyrosine phosphatase 1B (PTP1B) (Li et al., 2014). The action of this enzyme on IRS-1 can lead to different signaling pathways involving phosphoiniositol-3-kinase (PI3K) and mitogen-activated protein kinase cascades (MAPK). (Plum et al., 2005) There are many similarities between the IR and DInr regarding signaling pathways and mechanisms of action. The human receptors, IR and IGF-1, are homologs of DInr found in fly (Brogiolo et al., 2001). Similarly to IR, there is evidence to suggest that DInr is a Trk. At an intracellular level, the receptor contains tyrosine residues which phosphorylate one another to cause receptor activation. Under normal cellular conditions in the larvae, protein phosphotyrosine phosphatase (PTP1F) (an enzyme found in fly which is homologous to PTP1B found in humans) prevents receptor activation. This inhibition is caused by the enzyme removing the available phosphate groups, thus inhibiting receptor activity (Figure 2A) (Willoughby, L. et al., 2017). This study has used a synthetic phosphatase inhibitor known as bvP (phen) to attempt inhibition of the PTP1F enzyme, thus allowing for the tyrosine residues to phosphorylate one another. This phosphorylation would allow for the receptor to activate, providing a better understanding of the role of insulin in peptide signaling (Figure 2B). This study considers the effects of insulin receptor activation on the crawling behaviour of fruit fly larvae in order to develop a better understanding of the signalling pathway of insulin-like peptides.

METHODOLOGY

All larvae used in this study were in the 3rd instar stage, which is directly prior to pupation in the *Drosophila* life cycle. To prepare for dissection and trials, a physiological saline was created with a similar composition to that of hemolymph. The saline used for dissection and control trials was HL-6 1.5mM Ca^{2+} saline. In order to begin dissection, larvae were pinned to a tray in order to remain held down. With the dorsal side of the larvae facing upwards, pins were placed in the anterior and posterior ends on the fly (Figure 3C). All flies were dissected in a dish containing HL-6 1.5mM Ca^{2+} saline. Once the fly was pinned down, a cut was made in the dorsal epithelium, beginning posteriorly and moving towards the central nervous system. The visceral organs were then removed from the larvae and disposed of. The ventral nerve cord and nerve projections, composing the central nervous system, remained intact throughout the dissection. Excess saline and fly tissues were removed and replaced with fresh saline before beginning to record larval movements. In order to record, the anterior pin in the fly was removed and replaced with a force transducer (Figure 3A). This was used to measure larval contractions as a change in voltage over time. A catheter and pump were used to circulate fresh and excess saline through the recording dish (Figure 3B). In control trials, HL-6 1.5mM Ca^{2+} saline was applied to larval muscles for a period of 35 minutes at an approximate speed of 1mL/minute. bvP (phen) was diluted in HL-6 1.5mM Ca^{2+} saline into two concentrations of 10^{-4}M and 10^{-5}M. For each set of trials containing bvP (phen), HL-6 1.5mM Ca^{2+} without the drug was used for the initial 5 minutes of the trial, followed by 30 minutes of saline containing the drug (Figure 4). Larval contractions were recorded using DATA.Q software and analyzed by considering changes in contraction amplitude and frequency over time using a minute by minute analysis.

RESULTS

In control trials it was observed that there was a natural decrease in contraction amplitude and frequency over time (data not shown). This provided a baseline for comparison of the following two sets of trials using the concentrations of bvP (phen). When analyzed, it was observed that larval contraction amplitude decreased over time. The data was analyzed using a linear regression to compare the slope of the relation between contraction amplitude and time for each of the concentrations of bvP (phen) with the control group. The slopes were negative in all three groups, however,

when the control group was compared with each of the experimental groups, the results were not statistically significant (data not shown). In order for the results to be considered significant, the probability that the results would occur by chance must have been less than 5% ($P<0.05$). This indicates that receptor activation did not speed up or slow down the drop in larval contraction amplitude over time.

A linear regression was also used to examine the slope of the relation between contraction frequency and time in both control and experimental groups. In all three groups the data revealed negative slopes, indicating that the contractions became less frequent with time in the control and experimental trials. The slopes were then compared statistically in order to determine if the application of bvP (phen) altered the rate at which contraction frequency decreased over time. There was no significant decrease in contraction frequency determined between the control group and the 10^{-5}M concentration of bvP (phen). At the 10^{-4}M bvP (phen) concentration, however, the P value was 0.052, which is very near statistical significance. This suggests that when applied at a concentration of 10^{-4}M, bvP (phen) may accelerate the decline in contraction frequency over time (Figure 5A).

The possibility that the bvP (phen) takes time to act on the receptor and demonstrate full effect was next considered. In order to determine this effect, the data for the last 15 minutes of the control group and the experimental group for the 10^{-4}M concentration of bvP (phen) were compared. An analysis of variance (ANOVA) test was performed and revealed that there was a significant difference ($P<0.05$) between the 10^{-4}M bvP (phen) trials and control trials. This indicates that activating the DInr with a higher concentration of bvP (phen) significantly reduced contraction frequency compared to the control group, however, it required more than 15 minutes of exposure to the drug to obtain these results (Figure 5B).

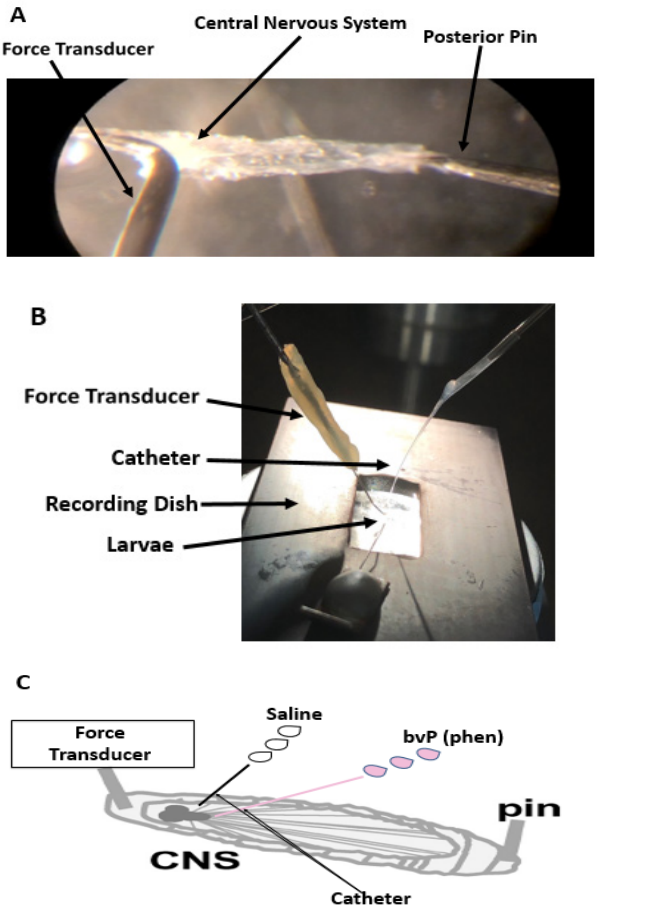

Figure 3. Placement of force transducer (A), catheter and pump apparatus (B), and positioning of larvae for pin placement (C).

DISCUSSION

Two plausible explanations that were considered for the recorded results are as follows; the movement of glucose in the fly can be considered as a result of activating DInr, and the possibility that diLPs act centrally as opposed to peripherally.

Under normal conditions muscle cells contain useable energy in the form of glucose, and store energy in the form of glycogen. It has been observed that when bvP (phen) is applied at the 10^{-4}M concentration, it causes a decrease in contraction frequency. It is possible that when this concentration of bvP (phen) is applied, it is able to activate the receptor as a result of a sufficient amount of the drug building up within the nervous system to meet the required IC_{50} (inhibitory concentration) value to elicit a physiological response. This could cause the useable energy to be moved into energy storage, reducing the amount of energy available for use by the fly, thus causing a decrease in the ability of the larvae to contract. This offers a potential explanation for the negative relationship between contraction frequency and time when bvP (phen) is applied at the higher concentration.

There was no significant change in contraction amplitude between control trials and experimental trials, thus there is no evidence to support the hypothesis that diLPs alter larval muscle performance by acting on the peripheral nervous system. The significant change in contraction frequency for the last 15 minutes of the trial when bvP (phen) was applied at the 10^{-4}M concentration suggests that the drug could be working centrally as opposed to peripherally. These larval movements are generated in response to bursts of impulses in motor neurons un-

Trials	Initial 5 Minutes	30 Minutes Remaining
Control	1.5 mm Ca^{2+} saline	1.5 mm Ca^{2+} saline
Experimental Group #1	1.5 mm Ca^{2+} saline	bvP (phen) 10^{-5}M
Experimental Group #2	1.5 mm Ca^{2+} saline	bvP (phen) 10^{-4}M

Figure 4. For each set of trials containing bvP (phen), HL-6 1.5mM Ca^{2+} without the drug was used for the initial 5 minutes of the trial, followed by 30 minutes of saline containing the drug.

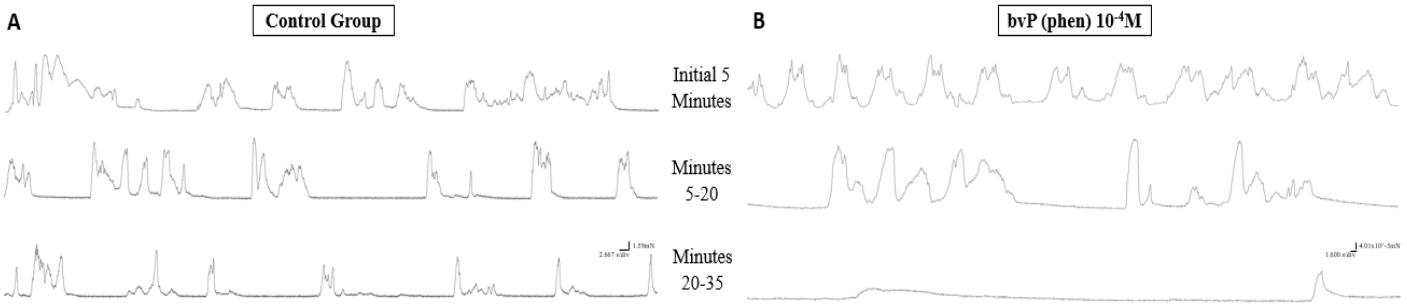

Figure 5.

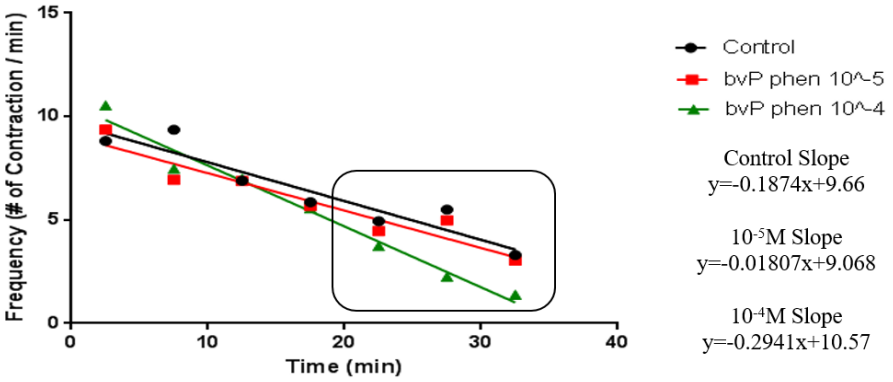

Figure 6.

der control of the central nervous syystem. Therefore, it can be determined that insulin receptor activation in the central nervous system could be responsible for the decrease in larval fictive locomotion when bvP (phen) is applied at the 10^{-4}M concentration. Determining this offers support to the hypothesis that bvP (phen) could affect the central nervous system as opposed to the peripheral nervous system. Therefore, one could expect that diLPs 1-7 act through the central nervous system.

Although successful in offering evidence to support the hypothesis that diLPs 1-7 in *D. melanogaster* may act through the central nervous system, there were also limitations to this study. The most difficult component to completing the project can be traced back to learning to dissect 3rd instar *D. melanogaster* larvae. Learning to dissect such a small organism by hand while working under a microscope and ensuring the quality of the dissections was highly challenging. The dissections performed were semi-intact, which involved the central nervous system remaining intact throughout the dissection. This specific dissection was difficult to learn in a short time period, and the quality of each dissection could have differed through the duration of the experiment. The time period for the recordings (30 minutes per recording) was also limited by the quality of the dissections. A higher quality of dissection would allow for flies to remain alive for a longer time period, allowing for testing to be performed regarding long term exposure to the drug.

FUTURE DIRECTIONS

This study has taken a fundamental approach to understanding the functionality and signalling mechanisms of diLPs 1-7 found in *D. melanogaster* larvae. Further experiments could be performed to develop a greater understanding of the role of insulin signalling in *D. melanogaster*. The trials could be performed once again while raising the concentration of bvP (phen) to 10^{-3}M, attempting to decrease the amount of time required to observe a significant decrease in larval contraction frequency. The trials could also be recorded for a longer time period (possibly 60 minutes), to allow for the physiological response of removing bvP (phen) from the catheter and replacing with saline to be observed. It is possible that larval contraction frequency would begin to increase following exposure to bvP (phen) if the drug was removed and replaced with saline. This test would allow for observations of the long-term effects of bvP (phen) on the central nervous system to be made. Another study that could be performed could involve applying bvP (phen) and observing the time until failure (death of the fly) in order to determine if the drug is slowing the frequency of larval contractions or possibly causing cell death within the fly. Upregulating the gene for insulin receptor expression in fly (using gene editing technology such as CRISPR/Cas9) could also be performed to allow for more insulin receptors to be activated, potentially causing a more rapid decrease in contraction frequency when bvP (phen) is applied. Alternatively, RNAi (RNA interference) could be used to reduce insulin receptor expression in neurons, attempting to prolong contractions when bvP (phen) is applied.

CONCLUSION

Neuropeptides play a key role in the functionality of the nervous system in both humans and *D. melanogaster*. There are a number of human diseases that involve disturbances in neuropeptide signalling. Through developing an understanding of how neuropeptides signal in a model system such as *D. melanogaster*,

it sets a basis for further research involving neuropeptides in the treatment of human neurological or neurodegenerative diseases. This study offers evidence to support the idea that diLPs 1-7 found in *D. melanogaster* most likely act through the central nervous system. This project provides a solid foundation for many future experiments to further solidify the findings of this study and to help better elucidate the functionality of neuropeptide signaling in *D. melanogaster* which could eventually be applied to human disease models.

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ZOE GAGNON

My name is Zoe Gagnon and I am an 18 year old student from Port Colborne, Ontario. I am currently pursuing an Honours Bachelor of Science degree at Brock University in the Neuroscience Co-op Program. Throughout high school I always had a great interest in science, specifically biology and different sectors of the healthcare field. I have been able to pursue these interests through volunteering at my local hospital and holding a position as a Pharmacy Assistant at a local pharmacy. Through the Science Mentorship Program offered at Brock University, I was able to pursue these interests through conducting research at the university level, which I am eager to continue throughout the years to come.