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The effectiveness of Argireline as a synthetic BoNT questioned, as examined in the neuromuscular junction of the *Procambarus clarkii*

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ABSTRACT

SNARE proteins play an essential role in neurotransmission. Impaired SNARE function inhibits neurotransmission by blocking exocytic membrane fusion. Using intracellular microelectrodes, we measured the amplitude of the EPSP in the superficial exterior muscles of a crayfish in saline solution in the presence of a 6-mer peptide Argireline, a drug which researchers claim prevents the formation of the SNARE complex and therefore inhibits neuroexocytic transmission, and in the presence of a control solution containing Hyauronic acid. The results from this experiment were inconclusive.

INTRODUCTION

SNARE proteins are known to play an essential role in neurotransmission by driving exocytic membrane fusion. Vesicle-associated protein 2 (v)-SNARE synaptobrevin and target membrane (t)-SNARE syntaxin 1a and synaptosome-associated protein of 25kDa (SNAP-25) bind together to form the four-helix SNARE complex. This complex facilitates the binding of neurotransmitter-filled vesicles to the presynaptic membrane, allowing the vesicles to release their contents into the synaptic cleft (Zucker et al., 2009).

Impaired SNARE function inhibits neurotrans mission by blocking exocytic membrane fusion (Jung et al., 2007). SNARE function can be impaired by cleaving proteins within the SNARE complex. SNARE proteins are the specific substrates of eight clostridial neurotoxins, tetanus and botulinum (BoNT)A-G. Botulinum attenuates neuronal function by cleaving the soluble N-ethylmaleimide sensitive-factor attachment receptor (SNARE) protein. In recent years, Botox, a neurotoxin derived from botulinum, has been used extensively for therapeutic and cosmetic purposes, particularly for diminishing the appearance of wrinkles when applied to the face and other areas (Blanes-Mira et al., 2002). However, its high neurotoxicity poses serious problems and necessitates a viable alternative.

Whether the blockage of neuronal exocytosis can be achieved by preventing the interaction of SNARE proteins has been recently tested by Jung et al. (2007). This is achieved through the addition of synthetic or natural proteins that mimic the sequences of the SNARE motifs, which then compete with the componential SNARE proteins (Jung et al., 2007; Jung et al., 2008), preventing SNARE assembly. The inhibitory effects of SNARE mimicking peptides, especially the 6-mer peptide (Ac-EEMQRR-NH2) patterned after the N-end of the N-terminal domain of SNAP-25, coined Argeline (Blanes-Mira et al., 2002), on neurotransmission are extremely promising in pharmaceuticals and cosmetics as alternatives to Botox. Argireline has been shown to inhibit neurotransmitter release almost to the same extent of BoNT, although as a biosafe alternative.

In our experiment, the effect of Argireline on synaptic transmission in the neuromuscular junction of a crayfish was tested. The crayfish was used as our specimen due to its availability and economy, and provided an uncomplicated apparatus on which to test the effects of Argireline. Our goal was to determine if Argireline worked consistently with the way it is marketed. If Argireline works as it claims to, it should prevent neurotransmission in any model organism. We hypothesized that Argireline would inhibit neuronal activity in crayfish by blocking the formation of SNARE proteins, thus causing EPSPs to be smaller in amplitude than those of a control. Findings of this experiment were inconclusive and statistically insignificant, although as preliminary research they show that Argireline does not inhibit post-synaptic response

MATERIALS AND METHODS

Model Organism

Procambarus clarkii, supplied by Carolina Biologicals in North Carolina, were the preparations used. The crayfish, which were stored in ice, were cut, separating the abdomen from the cephalothorax. The abdomen was then cut along the sides of the exoskeleton at which point the swimmerets, intestines and surrounding tissues were removed, leaving only the superficial extensor muscles and dorsal exoskeleton. The preparation was then secured

with pins to a dissection dish and submerged in Ringer's Solution.

Solutions and Drugs

The Ringer's solution was made 24 hours before the experiment was conducted and was adjusted to a pH of 7.4 just before use. The composition of the Ringer's solution used was as follows: 5.4 mM of KCl, 196 mM of NaCl, 2.6 mM of $MgCl_2 \cdot 6H_2O$, 10 mM of Sodium Hepes Buffer, and 13.5 mM of $CaCl_2 \cdot 2H_2O$.

The water-soluble "Baseline" gel was comprised of Hyaluronic Acid. Ingredients for the gel were listed as "Water" and "Sodium Hyaluronate." The water-soluble "Argireline" gel was comprised of Acetyl Hexapeptide-3 with Hyaluronic Acid. The ingredients were listed as "Argireline (Acetyl Hexapeptide-3)" and "Sodium Hyalurolate." Both were obtained from Cellbone Technologies and were available for purchase at cellbone.com. During experimentation, both gels were stored in a dark, cold (approximately 5° F) environment.

Drug Administration

After dissection, 100 μL of "Baseline" gel was measured, applied, and spread onto the superficial extensor muscles and surrounding areas of the preparation. The preparation was then incubated at room temperature (approximately 72° F) for 5 minutes before being submerged in Ringer's solution. Intracellular recordings were then taken as described below. After "Baseline" data was collected, the preparation was rinsed in Ringer's solution, and 100 μL of "Argireline" gel was measured, applied, and spread onto the superficial extensor muscles and surrounding areas of the preparation. The preparation was once again incubated at room temperature before being submerged in Ringer's solution for a second round of intracellular recording.

Microelectrodes

To measure the change in voltage within "post-synaptic" muscle cells, microelectrodes were made from glass micropipettes (1.2 mm in diameter) pulled to a thin tip using a Pull-1 electrode pulled made by WPI, and filled with 3M KCl. They were then attached to an electrode holder and subsequently attached to a micromanipulator. The amplifier calculates the difference in voltage by measuring the voltage of the electrode in comparison to the reference electrode.

A stimulating electrode was used to stimulate the nerve paired with its corresponding muscle cell. The nerve of the given preparation was sucked into the stimulating electrode (1.2 mm in diameter with a small piece of polyimide tubing attached to the end) and stimulated with an SD9 Stimulator made by Grass Technologies. The stimulator generated a current that traveled down the stimulating electrode to the ground, which was secured in the dissection dish. The voltage of each stimulus varied from measurement to measurement.

Recording Methods

The procedure for intracellular recording was as follows. The electrode was first submerged into the Ringer's solution, tested for resistance, and then zeroed. Resistance was tested to ensure that our microelectrode had not broken; significant resistance was considered anything higher than 2 MΩ. The electrode was then inserted into a superficial extensor muscle. No data was collected from muscle cells with resting potentials less negative that -40 mV. The nerve was then stimulated, and measurements were recorded using SCOPE 4.0.3. This procedure was repeated for both conditions. Amplitudes were measured using SCOPE, comparing the difference in voltage between the cell's resting potential, and the height of its post-synaptic response.

RESULTS

We hypothesized, according to previous study conducted by Blanes-Mira et al. (2002), that the drug Argireline would significantly inhibit post-synaptic responses, as measured in the neuromuscular junction of the *Procambarus clarkii*. The aforementioned hypothesis was neither supported nor refuted by the findings of this experiment. Given our method of application, a coating of 100 μL of either Argireline (Acetyl Hexapeptide-3 with Hyaluronic Acid) or Baseline (Hyaluronic Acid) gel for each preparation, it was found that the presence of Argireline increased the amplitudes of the post-synaptic responses, however this data is not significant as n=2 and variance was high (Figure 1). In unpublished research (Goldsmith, Granera & Wolfe, 2009), Argireline seemed to increase post-synaptic potentials, however further study would need to be conducted to test whether the difference in means between Argireline and control conditions observed in this study (16.94 mV) and the findings of Goldsmith et al. (2009) are significant.

In an alternate method of application (as mentioned in the Figure 1 legend guide), it was found that Argireline's effects may have been time sensitive (Figure 1). The difference in means (28.11 mV) of Preparation 3's conditions, while equally statistically insignificant to the findings from Preparations 1 and 2, supported the proposed hypothesis of this study.

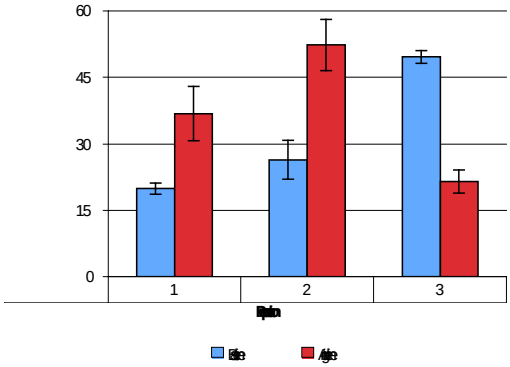

Figure 1. The effects of the drug Argireline on the neuromuscular junction of the *Procambarus clarkii*. Each bar represents an average of averaged EPSPs recorded at the same location. Data presented for Preparations 1 and 2 was collected from preparations using the methods previously described. For Preparation 3, 100 µL of Argireline was applied to the preparation and incubated at room temperature for 5 minutes before being submerged in high calcium (13.5 mM) Ringer's solution. After approximately 60 minutes, intracellular recording was performed as previously mentioned, represented above as "Baseline" for Preparation 3. Approximately 10 minutes later, 100 µL of Argireline was reapplied to the preparation and incubated and bathed in the same was as above. Intracellular recording was again performed as previously mentioned, represented above as "Argireline" for Preparation 3. Error bars represent 1 S.E.

DISCUSSION

We designed our experiment to determine if the drug Argireline would inhibit neurotransmission in a crayfish cell, consistent with how it is marketed. We hypothesized that the presence of Argireline would inhibit neuronal activity in crayfish by blocking the formation of SNARE proteins, thus causing EPSPs to be smaller in amplitude than those of a control. Our data did not support this hypothesis. The application of Argireline, as compared to Hyaluronic Acid on its own, increased amplitudes of EPSPs. While the data collected in this study is not extensive enough to be significant, it shows preliminary evidence that contradicts the marketing claims of Argireline.

This apparent contradiction, however, could be attributed to several things, not only limited to a flaw in the marketing of Argireline. Perhaps our method of drug administration was the cause of the contradictory findings, as it contributed heavily to the lack of data in this research. For example, early stages of this experiment included a 20 minute incubation time in a cool environment (5° F). Using this technique, intracellular recording was almost impossible. Perhaps then, the method of Argireline application was too strong, or at the very least unable to mimic the conditions of a human subject. However, this hypothesis too goes unsupported by Goldsmith et al. (2009), who found that Argireline increased post-synaptic potentials even when using a different method of drug administration.

The data in which Argireline inhibited post-synaptic potentials was conducted in a trial using two administrations of Argireline (Preparation 3). This may be the result of an increase in sensitivity of cells to allow the peptide to cross their membranes. Further research regarding Argireline's effectiveness with multiple treatments is needed, however, to support this hypothesis. If the results of this study were conclusive, and Argireline does facilitate synaptic transmission, they would be highly significant regarding both the marketing of Argireline and our understanding of how the hexapeptide works. The most readily drawn conclusion, then, would be that the Acetyl Hexapeptide-3 diminishes the appearance of wrinkles by a physiological mechanism other than inhibiting synaptic transmission.

Provided that our research supports the hypothesis that Argireline does not work as a synthetic BoNT, the question remains as to whether such a bio-safe peptide exists, the discovery of which would be highly lucrative.

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