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Increasing concentrations of acetyl hexapeptide-3 (Argireline) decreases EPSP amplitudes and slightly increases paired-pulse facilitation in the crayfish neuromuscular junction

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ABSTRACT

Many commonly used chemicals in cosmetic treatments are highly toxic. As a result, bio-safe synthetic alternatives to toxic cosmetic treatments, especially those concerning anti-wrinkle activity, are currently under investigation. It is thus crucial that the efficacy of these synthetic alternatives be ascertained. The synthetic hexapeptide Argireline (acetyl hexapeptide-3) is advertised to act as a safer alternative to commonly used cosmetic toxins and provide an effective mechanism for anti-wrinkle activity. This mechanism is the inhibition of Ca^{2+}-dependent exocytosis, specifically the inhibition of SNARE proteins (Blanes-Mira et al. 2002). Since Argireline is claimed to mimic commonly used neurotoxins, we wanted to investigate if increasing the concentration of this chemical produces an inhibitory effect on EPSP amplitudes and synaptic plasticity in the neuromuscular junction of crayfish. To test this, we used the method of intracellular recording to measure the EPSPs of the crayfish muscle fibers upon paired-pulse stimulation of a crayfish nerve. Our results show that the EPSP amplitudes decreased as the concentration of Argireline increased in crayfish saline. Contrary to our expected results for synaptic plasticity, our data show no form of synaptic depression. In fact, our results show a slight, yet mostly negligible, increase in facilitation as the concentration of Argireline increased. Our study ultimately attempts to establish the efficacy of Argireline as a synthetic hexapeptide in the crayfish neuromuscular junction and our results suggest that Argireline does in fact conclusively decrease EPSP amplitudes although also slightly increasing synaptic facilitation.

INTRODUCTION

Numerous techniques in cosmetic surgery utilize highly unsafe chemicals known as neurotoxins. One neurotoxin, known as the botulinum neurotoxin (BoNT), is specifically used in anti-wrinkle treatment. BoNTA, also known as BOTOX, is a highly toxic botulinum neurotoxin. In fact, BoNTA is the most potent toxin known to humankind (Blanes-Mira et al. 2002). These neurotoxins are used in anti-wrinkle treatments because they inhibit Ca^{2+}-dependent exocytosis by inhibiting the N-end of the N-terminal domain of SNAP- 25 located in the SNARE protein complex (Jung et al. 2008). These SNARE proteins are responsible for interacting with calcium, which ultimately fuses the neurotransmitter-filled vesicles to the membrane in the process of exocytosis. Since these SNARE proteins are inhibited, the synapse contains a smaller number of neurotransmitters. With less neurotransmitters in the synapse, there is a decreased amount of signals transmitted to allow certain muscles to contract. This inhibition decreases EPSP amplitudes (Jung et al. 2009). As a result, wrinkles will not form with this muscle paralysis.

In this study, we utilized a synthetic, non-toxic alternative chemical that carries out similar processes as the commonly used toxic chemicals. These chemicals also inhibit Ca^{2+}-dependent exocytosis by inhibiting SNARE proteins (Blanes-Mira et al. 2002). The alternative chemical that we use in our study is a synthetic hexapeptide called Argireline (acetyl hexapeptide-3). Since it functions through a similar mechanism, Argireline mimics the effects of neurotoxins. For the purpose of this study, we will use Argireline with the crayfish neuromuscular junction. We will do so because crayfish have a very simple nervous system. Also, crayfish show similar behaviors in synaptic transmission to that of the human nervous system. We sought to determine whether increasing the concentration of Argireline has the same effect as neurotoxins in that they decrease EPSP amplitudes in the neuromuscular junction of crayfish.

Past research has illustrated a relationship between the concentration of Argireline and EPSP amplitudes. Since Argireline is advertised as a bio-safe alternative due to its non-toxic nature (Jung et al. 2009), these relationships help illustrate the validity of these advertised claims. In a study by Blanes-Mira et al. (2002), synthetic peptides were used on rats as a

way to inhibit SNARE proteins from fusing synaptic vesicles to the membrane in exocytosis. This study measured the percentages of certain neurotransmitters in the process of neurotransmitter release. This was a way to determine the efficacy of these synthetic peptide inhibitors. Specifically, Argireline was used to inhibit the neurotransmitter catecholamine. This inhibition was measured during the process of neurotransmitter release. It was found that Argireline impaired this neurotransmitter release by approximately 30%, which caused a decrease in EPSP amplitudes. Comparatively, Jung et al. (2009) investigated the effects of neurotoxins of neurotransmitter release. These toxins impaired neurotransmitter release by almost 70%. Although neurotoxins show a larger percent inhibition, both neurotoxins and synthetic hexapeptide alternatives (such as Argireline) effectively perform inhibition on an organism's synaptic transmission and ultimately lowering EPSP amplitudes. Previous research from Jung et al. (2008) also indicates that synthetic peptides applied to specific regions of SNARE protein complexes in rats are excellent targets for the development of drugs to block SNARE-driven membrane fusion and neurotransmitter release. Specifically, synthetic peptides extracted from the N-terminal region of SNARE proteins showed significant inhibitory effects on neurotransmitter release, lowering the amplitudes of EPSPs. This is because these peptides blocked the N-terminal from forming SNARE complexes, which inhibits the SNARE-driven exocytosis process.

This research is important because it has the capacity to give the cosmetic industry insight on the efficacy of potential bio-safe alternatives for anti-wrinkle treatments. The research illustrates that Argireline and other synthetic peptides affect neurotransmitter release and EPSP amplitudes in the same way as commonly used toxic chemicals in cosmetics. With this new insight, patients will no longer have to be exposed to harmful cosmetic chemicals.

In our own research, we hypothesized that higher concentrations of Argireline applied in the neuromuscular junction of crayfish will cause a substantial decrease in EPSP amplitudes in the muscle fibers of crayfish. In addition to the decrease in the EPSP amplitude size, we hypothesize that higher concentrations of Argireline will create a large synaptic depression in the neuromuscular junction of crayfish. To test our hypothesis, we used intracellular recordings to measure the EPSPs of the crayfish muscle fibers. The EPSPs were generated from stimulating one of the crayfish's nerves. These measurements were recorded in environments with varying concentrations of Argireline in normal crayfish saline. The data we collected supported one of our hypotheses but not the other. Our data supported one of our hypotheses because the EPSP amplitudes decreased as the concentration of Argireline increased. The data did not support our other hypothesis because synaptic depression did not occur. In fact, the data showed an opposite effect. We observed a trend of a slight increase in facilitation as the concentration of Argireline increased.

MATERIALS AND METHODS

Crayfish preparation and dissection

In order to anesthetize the crayfish (*Procambarus clarkii*), we placed the crayfish in an ice bucket for about 30 minutes before the experiment; after the crayfish movement decreased, we dissected the crayfish tail using scissors. We cut the tail to the last segment, keeping the scissors as close as possible to the ventral surface. Then we removed the muscle mass so that only the exoskeleton of the dorsal surface and the four dorsal extensor muscles remained. Keeping the ventral surface up, we pinned the tail on a dissecting dish and covered it with the crayfish saline.

Preparation of experimental solutions

For each trial, we used 40 mL of low-calcium crayfish saline consisting of 5.4 mM KCl, 20.7 mM NaCl, 12.3 mM MgCl2*6H_2O, 6.5 mM $CaCl_2$*2H_2O, and 5 mM Sodium HEPES Buffer. The original 168.73μM Argireline solution (0.05% Argireline in 30 mL water) was added to 40 mL of crayfish saline. For our experiment, we used 10 concentrations of Argireline ranging from 0 μM to 10.298 μM.

Making microelectrodes

We used intracellular recording to collect data. To make microelectrodes, we used a PUL-1 microelectrode maker from World Precision Instruments to pull glass capillary tubes into microelectrodes. We then filled these microelectrodes with a 3 M KCl solution, removed air bubbles to reduce resistance, and attached the electrode to an electrode holder and a micromanipulator. Suction electrodes where also made using the same method (although not filled with KCl) but were then filed with sand paper to create an opening in the tip approximately 0.3 mm wide.

Method of stimulation

We used a paired-pulse stimulation of crayfish nerves, as opposed to tetanic stimulation, in

order to assess synaptic plasticity. Using paired-pulse stimulation, we can easily detect a change in facilitation in short-term plasticity since this method of stimulation quickly generates two EPSPs, side by side (Fioravante and Regehr 2011). Stimulation consistently occurred at a frequency of 0.5 Hz with a 60 ms delay between paired pulses and a 0.5 ms duration.

Acquiring EPSPs

We placed two reference electrodes in the solution and inserted the microelectrode that we prepared into the electrode holder connected to the micromanipulator. With the microscope and micromanipulator, we first entered the solution and zeroed the voltage. We inserted our electrode into the muscle cell, aiming for a lateral muscle, and recorded the voltage measured at the interior of the cell and stimulated the nerve of a crayfish using a suction electrode. We measured EPSPs of a postsynaptic muscle fiber of the crayfish neuromuscular junction for 5 minutes using a microelectrode filled with 3 M KCl. PowerLab 4/26 and Bridge Amp by ADInstruments transmitted the postsynaptic response to LabChart on MacBook Pro for analysis.

Experimental design

To test the effectiveness of Argireline concentrations on the synaptic plasticity of the neuromuscular junction, we recorded the percent change in EPSP amplitudes of crayfish muscle fibers under increasing concentrations of Argireline. For the first five-minute increment, we added 1 mL of a 168.73 μM Argireline solution to the 40 mL of crayfish saline. Throughout our 50-minute trial, we continually increased the concentration of Argireline. We recorded EPSP amplitudes for five minutes immediately following the application of an addition of 200μL of Argireline. Subsequent applications of Argireline were directly added to the accumulated solution in the dissecting dish, each given exactly 5 minutes for EPSP collection. By the end of the 50-minute run (approximately the amount of time allotted until the crayfish saline needed to be changed), we applied a total of 2.6mL to the original 40mL of crayfish saline. The final concentration of the solution was 10.298 μM.

This was done over one trial with one crayfish tail. To confirm that the results were not merely attributed to the passing of time, we conducted a control experiment. A different crayfish tail was submerged in 40 mL of the control crayfish saline and a nerve fiber was stimulated. EPSPs were collected using paired-pulse stimulation at 5-minute intervals over the course of 50 minutes.

Data analysis

For a statistical analysis, we calculated the mean of the EPSP amplitudes for both the first and second pulses and for the percent change in EPSPs. We also evaluated the percent change in the EPSP amplitudes:

$$\% \text{ Change} = \frac{[\text{EPSP} - \text{EPSP}_{\text{initial}}]}{[\text{EPSP}_{\text{initial}}]} \times 100\%$$

We made the calculations to find standard error of the mean for error bar analysis, p-values of t-tests for assessment of the significance of specific data segments, and correlation coefficients for application of correlation and linear regression analyses to evaluate relationships between variables.

RESULTS

Effects of Argireline on EPSP amplitudes

To determine if Argireline decreases EPSP amplitudes, we used paired-pulse stimulation to record EPSP amplitudes across the crayfish neuromuscular junction. A crayfish tail was submerged in 40 mL of crayfish saline and a nerve was stimulated for 5 minutes. For the next 5-minute increment, we added 1 mL of a 168.73μM Argireline solution to the initial 40 mL of crayfish saline and continually applied the paired-pulse stimulus. Then, for each subsequent 5-minute increment until 50 minutes, an addition of 200μL of Argireline was added to the solution as the crayfish was stimulated. The results from this dose-response curve indicate a decreasing trend of EPSP amplitudes in both the first and second pulse while concentrations of Argireline steadily increase (Figure 1). The second pulse, however, consistently remains at a higher mean EPSP amplitude throughout each increase in Argireline concentration indicating the persistence of synaptic facilitation through the trial.

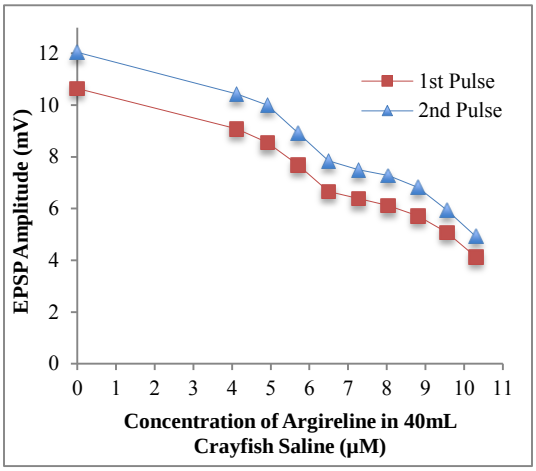

Figure 1. Decreased EPSP amplitudes (mV) through paired-pulse stimulation with increased concentrations (µM) of acetyl hexapeptide-3 (Argireline) in 40mL of the crayfish saline. Each point on the graph indicates the average EPSP amplitude over a sample size of 100 EPSP readings in one crayfish.

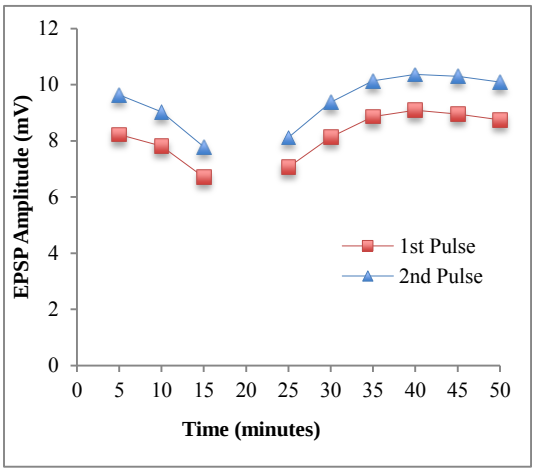

Figure 2. EPSP amplitude (mV) recorded at 5-minute intervals over 50 minutes in 40mL of the crayfish saline with no added Argireline. Each point on the graph indicates the average EPSP amplitude over a sample size of 100 EPSP readings in one crayfish (a different crayfish than in Figure 1). The measurement at 20 minutes is not included due to experimental inaccuracies at that point and loss of data. However, measurements were properly recovered after this point.

The decrease in EPSP amplitudes over time, however, cannot merely be attributed to the passing of time. We conducted a control experiment to interpret the effects of time on EPSP amplitude across the crayfish neuromuscular junction in the crayfish saline. As indicated by this study, a crayfish submerged in 40 mL of crayfish saline and being continually stimulated (paired-pulse) over the course of 50 minutes does not show an overall change in EPSP amplitudes over time (Figure 2). We observed an initial EPSP amplitude of 8.23mV and a final EPSP amplitude of 8.75mV. Thus, negligible change in EPSP amplitudes is attributed to the control experiment. Since the control EPSP values relatively stayed consistent (or only slightly increased), this suggests that the decrease in EPSP amplitudes in the trial with Argireline was due to Argireline and not the passing of time.

Effects of Argireline on percent change of facilitation in EPSP amplitudes

To determine if Argireline affects synaptic plasticity, we used paired-pulse stimulation to record the percent change of facilitation in EPSP amplitudes across the crayfish neuromuscular junction. Using the same procedure as before when determining the effect on EPSP amplitudes, we now utilized percent change in EPSP amplitudes to analyze our acquired paired-pulse EPSP amplitudes. The results from this type of dose-response analysis indicate a slight, though mostly negligible, increase in %Change of facilitation in EPSP amplitudes given a constant increase of Argireline concentration in crayfish saline (Figure 3).

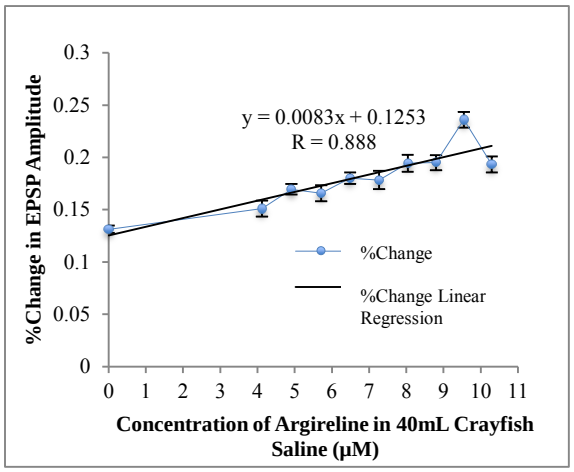

Figure 3. %Change in EPSP amplitudes (mV) through paired-pulse stimulation with increased concentrations (µM) of acetyl hexapeptide-3 (Argireline) in 40mL of the crayfish saline. Each point on the graph indicates the average %Change in EPSP amplitude over a sample size of 100 EPSP readings in one crayfish (the same crayfish as in Figure 1).

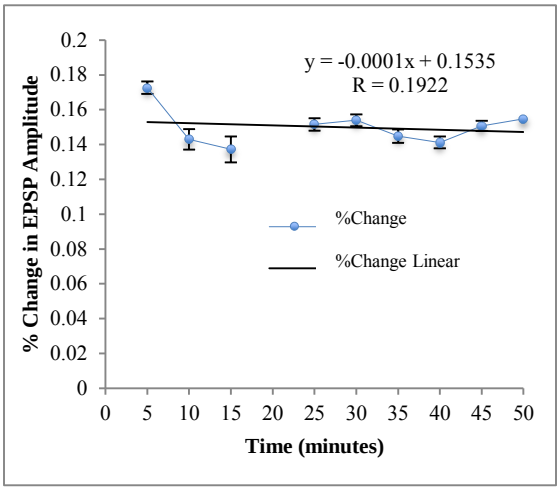

Figure 4. %Change in EPSP amplitude recorded at 5-minute intervals over 50 minutes in 40mL of crayfish saline with no added Argireline. Each point on the graph indicates the average %Change in EPSP amplitudes over a sample size of 100 EPSP readings in one crayfish (the same crayfish as in Figure 2). The measurement at 20 minutes is not included due to experimental inaccuracies at that point and loss of data. However, measurements were properly recovered after this point.

To analyze the statistical significance or insignificance of this trend, we used a correlation and linear regression analysis to determine if the increases in Argireline concentration presented any meaningful correlation to the increases in %Change in EPSP amplitudes. We calculated the following linear regression:

%Change = 0.0083[Argireline] + 0.1253

The correlation coefficient, or R-value, was found to be 0.888. This indicates that the %Change in EPSP amplitudes attain a relatively strong correlation to the calculated regression, which has a slope of 0.0083. Since the slope of this equation is near zero, at a very minimal increase, we came to the conclusion that increasing concentrations of Argireline has a negligible, or an only slight, effect on increasing %Change of facilitation in EPSP amplitudes in the crayfish neuromuscular junction.

Furthermore, when compared to control conditions of a different crayfish submerged in only 40 mL of crayfish saline, we can conclude that the lapse of time itself does not produce significant changes to %Change in EPSP amplitudes (Figure 4). This lack of %Change over time in the control condition can also be analyzed through a correlation and linear regression analysis. We calculated the following linear regression:

%Change = -0.0001[Argireline] + 0.1535

The correlation coefficient, or R-value, was found to be 0.1922. Although such a low correlation coefficient indicates a relatively low correlation of our data to our calculated linear regression, the slope of the regression is -0.0001, which is quite close to zero indicating a relative lack in change of %Change in EPSP amplitudes throughout this trial. From these calculations, we were able to conclude that time itself did not produce a significant increase or decrease in %Change.

DISCUSSION

Our results suggest that the synthetic hexapeptide Argireline decreases EPSP amplitudes in the crayfish neuromuscular junction. This was congruent with our hypothesis that upon increasing Argireline concentrations, there will be a decrease in EPSP amplitudes. However, we also found that Argireline produces a minimal, but mostly negligible, increase in %Change of facilitation in EPSP amplitudes. This contradicted our second hypothesis that upon increasing Argireline concentrations, there will be increased negative %Change in EPSP amplitudes and thus synaptic depression.

Previous studies also conclude that Argireline has an effect on EPSP amplitudes. Although contrary to our findings, Goldsmith et al. (2010) found that Argireline significantly increased the EPSPs of crayfish muscle fibers. Furstenau et al. (2010), though ultimately conveying inconclusive and statistically insignificant results, also implicated increases in EPSP amplitudes in crayfish muscle fibers as a result of Argireline application. The contradictory findings of these studies might be attributed to differing forms of Argireline used. Both Goldsmith et al. (2010) and Furstenau et al. (2010) used a solution of Argireline mixed with Hyaluronic Acid. Not only this, but both studies only allowed for tetanic stimulation of the crayfish thus only measuring the long-term and not the plasticity of the neuromuscular junction by means of paired pulse stimulation as we did in our study.

Although these two studies seem to provide antithetical findings to our own study, Blanes-Mira et al. (2002) found that Argireline specifically interferes with the assembly of the SNARE complex and inhibits Ca^{2+}-dependent exocytosis by almost 30%. These implications support the decrease in EPSP amplitudes we measured in our results. Decreased exocytosis by a synthetic hexapeptide, like Argireline, leads to lower neurotransmitter release in the presynaptic terminal and thus decreased neurotransmitter signaling in the postsynaptic terminal. This leads to decreased postsynaptic responses as we have shown in our study (Figure 1).

However, the slight, though mostly negligible, increase in %Change of facilitation in EPSP amplitudes is not supported by these previous studies. We propose that the slight increase in facilitation that we measured can be attributed to an insufficient bathing period in between additions of higher doses of Argireline. By only letting each new concentration bathe for five minutes and directly comparing these findings to an entire 50-minute control experiment, we cannot conclusively see the effects of each new Argireline concentration. Thus, in future research, by allowing each concentration to bathe for a 50-minute period, we can directly compare the results of such an experiment to that of the control experiment conducted in just crayfish saline. This will allow for more conclusive dose-response findings and a more clearly significant trend.

Furthermore, the implication of any study on Argireline is the assessment of the synthetic hexapeptide's efficacy as a BoNTA mimetic. Although our study cannot conclusively convey the efficacy of Argireline as a mimetic to highly unsafe cosmetic neurotoxins, we can attribute a portion of our results to implicate the potential for Argireline to be an effective bio-safe alternative. Our findings of a decrease in EPSP amplitudes by Argireline, as attributed by Blanes-Mira et al. (2002) to the inhibition of SNARE proteins and Ca^{2+}-dependent exocytosis, can be used to promote the use of Argireline as opposed to the highly toxic chemicals currently being used in the cosmetic industry.

Ultimately, despite finding relatively negligible effects on %Change of facilitation in EPSP amplitudes, Argireline did conclusively decrease EPSP amplitudes in the crayfish neuromuscular junction. Since the effect of Argireline on facilitation was very minimal, further studies should be conducted on the effects of Argireline on synaptic plasticity in the crayfish neuromuscular junction, specifically the effects on synaptic facilitation and/or depression using paired-pulse stimulation. In doing so, we would conduct a similar experiment to our study; but, as previously addressed, upon each addition of a higher Argireline concentration, we would allow the crayfish to be submerged and stimulated (through paired-pulse stimulation) for an entire 50-minute interval in only that specific concentration. This will allow for more consistent and comparable data across each concentration and the control experiment. This will more conclusively assess the short-term plasticity of the crayfish neuromuscular junction. Also, a different peptide may be used which also inhibits the N-terminal region of SNAP-25 in the SNARE complex in order to conclude if Argireline is only operating through the inhibition of these SNARE proteins. Higher concentrations of Argireline should be used if possible to complete these further studies.

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