Argireline decreases EPSP amplitude over time, and increases paired-pulse facilitation in a dose-dependent manner

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ABSTRACT

Wrinkles are an inevitable aspect of the aging process and an entire industry has developed to combat this sign of old age. The toxic nature of BOTOX has left researchers to develop bio-safe alternatives, such as Argireline. The effects of Argireline are produced through the inhibition of neurotransmitter release by preventing the formation of the SNARE complex in the presynaptic cell. Because Argireline functions by disrupting the formation of this mechanism, we tested its effect on synaptic transmission and short-term plasticity, as well as its effect over time. By measuring EPSP amplitude and paired-pulse facilitation under increasing concentrations and over time, we can better understand the SNARE protein’s role in these properties of cell communication. Our results show an observable decrease in EPSP amplitude and an observable increase in PPF, both in increasing concentrations for Argireline. In addition, we found a significant decrease in EPSP amplitudes over time, but no significant change in PPF over time.

INTRODUCTION

The formation of wrinkles is a natural, inevitable part of the aging process. As we age, our skin becomes drier, thinner, and deficient in its ability to protect itself from external damage. Wrinkles can form as a result of excessive stimulation of the muscle fibers in the face by pulling the skin inward (Blanes-Mira et al. 2002). Consequently, low doses of a popular botulinum neurotoxin (BoNT A), otherwise known as BOTOX, is widely used within the cosmetic industry in anti-wrinkle treatments (Jung et al. 2008). BoNT A neurotoxins alleviate the appearance of wrinkles by cleaving SNAP-25 in the SNARE formation, creating a neuromuscular paralysis that can last from weeks to months (Baskaran et al. 2013).

Under normal circumstances in synaptic transmission, calcium-sensor protein, synaptotagmin, activates the SNARE complex located in the presynaptic cell of the neuromuscular junction (Schiavo et al. 1997). The SNARE complex, made up of proteins called syntaxin, SNAP-25, and synaptobrevin (Jung et al. 2008), bind together like a zipper to execute exocytosis of the vesicles and release neurotransmitters into the synaptic cleft (Melia et al. 2002). Disabling any of the SNARE proteins results in a shutdown of the release of neurotransmitters, as the entire complex can no longer function (Chen and Scheller 2001). If less neurotransmitters are released, then the amount of signals transmitted to the postsynaptic cell will also be reduced, which can be observed by a decreased EPSP amplitude.

The SNARE complex and its involvement in synaptic transmission can be inhibited by various substances, such as acetyl hexapeptide-3, also known as Argireline (Blanes-Mira et al. 2002). This peptide is patterned after the N-terminal end of SNAP-25 and functions by inhibiting the formation of SNARE proteins, which in turn blocks the calcium-dependent exocytosis of neurotransmitters (Jung et al. 2008). Because it has been shown to reduce the intensity of wrinkles, Argireline is advertised to be a safer alternative to commercial BOTOX, which is under strict medical control due to its high toxicity (Blanes-Mira et al. 2002).

Past studies show contradicting and varied effects of Argireline on synaptic transmission and short-term plasticity. Goldsmith et al. (2010) found that the application of Argireline significantly increased EPSP amplitudes when compared to normal saline. In a similar experiment, Furstenau et al. (2010) observed an increase in EPSPs in the presence of Argireline; however, this is inconclusive as they found their data to be not statistically different. Therefore, both Goldsmith et al. (2010) and Furstenau et al. (2010) showed that the presence Argireline caused an observable pattern of increasing EPSP amplitude in the crayfish neuromuscular junction. On the other hand, Blanes-Mira et al. (2002) found that Argireline decreases the release of neurotransmitters in chromaffin cells, and thus, synaptic transmission; they found that 100 μM of Argireline inhibited up to 30% of the total catecholamine release, while almost 100% of the catecholamine was released without the addition of any drug. Jung et al. (2009) found that plant extracts paralyzed the muscles of rat phrenic nerve-hemidiaphragm preparations. An explanation of these results by Blanes-Mira et al. (2002) and Jung et al. (2009)
suggest that Argireline inhibits the release of neurotransmitters by inhibiting the formation of SNARE proteins.

Similarly, Dababneh et al. (2014) shows that increasing concentrations of Argireline decreases EPSP amplitudes but has only a negligible effect on the percent change of EPSP amplitudes during PPF. However, in their experiment, they soaked the crayfish tail in each concentration of Argireline for 5 minutes, which was not consistent with the time range for their control condition. They suggested that keeping the time range consistent between conditions would allow researchers to directly compare experimental results to the control results and observe a more clearly significant trend (Dababneh et al. 2014). Due to the varied research regarding the effect of Argireline on neuronal communication, we intend to determine whether increasing concentrations of Argireline causes a decrease in EPSP amplitude, a decrease in the ratio of the two EPSPs in PPF, and if Argireline’s effect is time-dependent.

Since Argireline functions by interfering with the formation of SNARE proteins, studying its effect on EPSP amplitude and paired-pulse facilitation (PPF), as well as its effect over time, could help us expand our knowledge on the distinct mechanism that is involved in these phenomena. PPF involved an increase in the release of neurotransmitter quanta (Zucker and Regehr 2002), so testing this could give us more insight on Argireline’s effect in this process of cell communication. Moreover, our findings on the role of SNARE proteins in synaptic transmission and plasticity can be applied to real-world, clinical applications such as anti-wrinkle treatments. Thus, the effectiveness and safety of these treatments can be better understood and developed. To test the effects of Argireline, we used the crayfish neuromuscular junction because their chemical synapses are comparable to those in humans (Dababneh et al. 2014).

Because the SNARE complex plays a key role in the release of neurotransmitters, we predicted that increasing concentrations of Argireline would cause a decrease in EPSP amplitude and EPSP ratio in PPF. We also predicted that Argireline would cause EPSP amplitudes and ratios to decrease over time. To test our hypothesis, we exposed the crayfish neuromuscular junction in increasing concentrations of Argireline. We then stimulated crayfish nerves and recorded EPSPs using intracellular electrodes. Our data showed that as Argireline concentration increased, there was an observable decreasing pattern of EPSP amplitude and an increasing pattern of PPF. In addition, we found that as Argireline had a mostly increasing effect over time on EPSP amplitudes but time did not cause a significant change in PPF.

MATERIALS AND METHODS

Crayfish tail preparation

Prior to the experiment, the crayfish Orconectes were anesthetized in an ice bath, thus, slowing down all metabolic processes. We removed the tail with a cut at the base. We cut along the ridges of the crayfish’s ventral surface on both sides of the abdomen, which was gently pulled at the ventral surface. A final cut was made at the base of the surface to completely remove it. After the exposed muscle mass and digestive track were detached, the dissected tail was pinned down to Sylgard in a Petri dish. We completely submerged the tail with the different concentrations of Argireline we were testing, and placed the Petri dish under the microscope lens (Leica Zoom 2000). The muscle we collected data from was the deep extensor muscle cells, located in the middle section of the dissected tail. We used a total of four crayfish, one for each condition (0, 10, 15, and 20 µM).

Preparation of experimental solutions

For each experimental trial, we used 40 mL of low calcium crayfish saline (in mM: 5.4 KCl, 20.7 mM NaCl, 12.3 MgCl2*6H2O, 6.5 CaCl2*2H2O, and 5 Sodium HEPES Buffer; pH: 7.4). The original Argireline solution (MakingCosmetics; 168.73 µM; 0.05% Argireline in 30 mL of water) was added to 40 mL of crayfish saline. We used 4 concentrations of Argireline: 0 µM (0 mL Argireline in 40 mL saline), 10 µM (2.37 mL Argireline in 40 mL saline), 15 µM (3.56 mL Argireline in 40 mL saline), and 20 µM (4.74 mL Argireline in 40 mL saline).

Microelectrode preparation

Glass intracellular microelectrodes were made using a PUL-1 electrode puller by World Precision Instruments. We filled one microelectrode with 3 M KCl using a fine syringe and attached it to the electrode holder, which was connected to the micromanipulator. We placed the tip of the electrode in the saline over the exposed crayfish muscles and measured the electrode resistance, which was amplified using a Model 3100 AC/DC Differential Amplifier by AD Instruments. Before we took resting membrane potential measurements inside the cell, we zeroed the amplified output. We used Lab Chart 8.0.5, which is coordinated with its hardware, Power Lab 26 T (both made by AD Instruments), to observe the voltage recordings from a MacBook Pro. If the resistance was between 10 to 20 MΩ, it indicated that the microelectrode tip was still intact. This intracellular electrode was used to measure EPSPs in the postsynaptic muscle cell of the crayfish.
Glass suction electrodes were also made using a PUL-1 electrode puller by World Precision Instruments. The fine tips of these electrodes were filed and dulled using sandpaper until the tip was approximately 0.3 mm wide. The suction electrode, which was connected to GRASS SD9 Stimulator by Grass Medical Instruments associated with Power Lab, stimulated the nerve.

Method of stimulation
To test the effect of Argireline on synaptic transmission and plasticity, we used paired-pulse stimulation. We specifically used this form of stimulation to test short term plasticity instead of other methods because it gives us EPSP 1s and EPSP 2s, which allows us to measure facilitation in ratios. This is significant because using a ratio to compare plasticity between increasing concentrations of Argireline eliminates the different EPSP readings that might occur across multiple crayfish and nerves. Because PPF causes two stimulations to occur close together, we can also utilize it to see apparent changes in facilitation. Each stimulation occurred with a 0.5 ms duration, a 60 ms delay between paired pulses, and a stimulation just above threshold.

Procedure
To test the effect of increasing concentrations of Argireline on synaptic transmission and plasticity, we measured the EPSP amplitude and EPSP ratio of crayfish muscle cells in 10, 15, and 20 µM. We submerged a dissected crayfish tail in 10 µM of Argireline saline solution, then proceeded to collect EPSPs in the muscle cells using four paired-pulse stimulations at each 10 minute interval for 60 minutes, including one measurement for 0 minute without the application of the drug. We proceeded with the experiment by using this same method with increased Argireline saline solution of 15 and 20 µM. For our control condition, we measured EPSP amplitudes in the crayfish muscle cells from 0 to 60 minutes. We soaked a dissected crayfish tail in 40 mL of low-calcium crayfish saline. At each 10-minute interval, we measured EPSPs inside a muscle cell using four paired-pulse stimulations. The control experiment was conducted to test if the crayfish muscle fibers would decay with increasing time, which would be indicated by decreasing EPSP measurements.

Data Analysis
To observe the effect of increasing concentrations of Argireline on synaptic transmission, we calculated percent change of EPSP amplitude. Percent change shows us how much the final amplitude (EPSP amplitude obtained at 0 minutes, before the addition of Argireline):

\[
\text{Percent change} = \left( \frac{\text{EPSP}_{\text{final}} - \text{EPSP}_{\text{initial}}}{\text{EPSP}_{\text{initial}}} \right) \times 100
\]

To observe the effect of increasing concentrations of Argireline on PPF, we calculated the ratio of the second EPSP to the first. EPSP ratio shows us the extent of facilitation:

\[
\text{EPSP ratio} = \frac{\text{EPSP}_2}{\text{EPSP}_1}
\]

Lastly, we expressed our data as mean ± S.E. in Figures 3 and 4. The statistical significance between the mean EPSP amplitudes of 10-30 minutes and 40-60 minutes were determined using two-tailed t-test to determine if Argireline had an effect over time on EPSP amplitude. P-values ≤ 0.05 were considered significant. We used this same method to determine if Argireline had a significant effect over time on PPF.

RESULTS

Effect of increasing concentrations of Argireline on EPSP amplitudes
To observe the effect of increasing concentrations of Argireline on EPSP amplitudes, we took EPSP measurements from time 0 and 40 for different concentrations of Argireline (0, 10, 15, 20 µM) to calculate the percent change of EPSP amplitudes. First, we measured EPSPs at 0 minutes, without the application of the drug, and continued to take measurements at 10 minute intervals until 60 minutes passed. We then computed the percent change under four concentrations of Argireline, 0, 10, 15, and 20 µM, which were 36.5%, 52.8%, 23.1%, and 92.7%, respectively.

Without Argireline, there was a positive percent change, but the presence of Argireline consistently resulted in negative percent changes, indicating that the Argireline decreased the EPSP amplitudes (Figure 1). Overall, EPSP amplitudes seem to decrease with an increase in Argireline concentration. Although there is an observable increase in percent change from 10 to 15 µM, it is unknown if this difference is significant because only one trial was conducted for each condition.
Figure 1. Percent change of EPSP amplitude with varying concentrations of Argireline (0, 10, 15, 20 μM). n=1. Each column in the graph is the percent change of EPSP amplitudes computed by using the EPSP amplitudes taken at 40 minutes (final EPSP) and the EPSP amplitude at 0 minutes (initial EPSP).

Effect of increasing concentrations of Argireline on paired-pulse facilitation
In order to determine Argireline’s effect on PPF, we stimulated the crayfish nerve using paired-pulse stimulation under varying concentrations of the drug. Using the same method as before, we obtained the EPSP ratios for each concentration at 40 minutes because at 20 μM concentration of Argireline, EPSPs were nonexistent from 50 minutes onward. The EPSP ratios at 0 μM, 10 μM, 15 μM and 20 μM were 1.32, 1.22, 1.44 and 1.54 respectively. Because EPSP ratio displays the difference between EPSP 1 and 2 in terms of PPF, a greater ratio indicates greater facilitation.

As shown in Figure 2, excluding 10 μM, the EPSP ratios seemed to have an overall increasing trend as the concentration of Argireline increased. Again, we were unable to determine if these differences were significant because only one trial was conducted.

Effect of Argireline on EPSP amplitude over time
We tested the effect of Argireline on EPSP amplitude over time by comparing the mean EPSP measurements obtained at 10-30 minutes to mean EPSP measurements obtained at 40-60 minutes for each concentration. Using the same method we used previously, we obtained EPSPs and calculated the mean EPSP amplitude of 10, 20 and 30 min. and the mean EPSP amplitude of 40, 50, and 60 min. Based on our data, excluding 15μM, EPSP amplitude significantly decreased as time increased, indicating that Argireline has a mostly increasing effect on EPSP amplitudes over time (Figure 3). In the control condition, the EPSP amplitude significantly increased, which simply indicates that the crayfish muscle tissue itself did not decay over time.

We conducted t-tests comparing the EPSP amplitudes in 10-30 minutes to the EPSP amplitudes in 40-60 minutes to observe the difference in amplitudes. At 0 μM, the mean EPSP amplitude in 10-30 minutes (x= 13.06 mV; SE= 1.89) was significantly smaller than the mean EPSP amplitude in 40-60 minutes (x= 19.86 mV; SE= 2.33; p ≤ 0.05). At 10 μM, the mean EPSP amplitude in 10-30 minutes (x= 9.95 mV; SE= 0.45) was significantly greater than the mean EPSP amplitude in 40-60 minutes (x= 6.03 mV; SE= 0.24; p ≤ 0.05). However, at 15 μM, the mean EPSP amplitude in 10-30 minutes (x= 3.39 mV; SE= 0.56) was not significantly different than the mean EPSP amplitude in 40-60 minutes (x= 2.44 mV; SE= 0.43; p > 0.05). At 20 μM, we found that the mean EPSP amplitude in 10-30 minutes (x= 2.33mV; SE=0.53) was marginally significantly greater than the mean EPSP amplitude in 40-60 minutes (x=0.41; SE=0.19; p=0.054). Therefore, we concluded that for most concentrations of Argireline that we tested, Argireline did decrease EPSP amplitude over time.

Figure 2. EPSP ratio from PPF at increasing concentrations (0, 10, 15, 20 μM) of Argireline. n=1. Each column on the graph represents the EPSP ratio (EPSP 2/EPSP 1) taken after 40 minutes following drug administration.

Figure 3. EPSP Amplitude measured in various concentration of Argireline (0, 10, 15, 20 μM) over time. n=3. For each concentration, the left column represents the mean EPSP amplitude of all EPSP measurements at 10, 20 and 30 minutes while the right column represents the mean EPSP amplitude of all EPSP measurements at 40, 50 and 60 minutes. The error bars represent the standard error for each condition and the height of the error bars represent the average of 3 measurements.
ARGIRELINE DECREASES EPSP AMPLITUDE AND INCREASES PPF

Effect of Argireline on paired-pulse facilitation over time
To test the effect of Argireline on PPF over time, we measured EPSP ratios from 10 to 60 minutes at 10 minute intervals using the same method as before to obtain EPSPs. We then compared the mean EPSP ratios from 10 to 30 minutes to those from 40 to 60 minutes. After conducting t-tests to compare the means of these two groups, we did not find a significant difference in EPSP ratios (Figure 4).

At 0 µM Argireline concentration, the mean EPSP ratio from 10-30 minutes (x̅ = 1.32; SE = 0.05) was not significantly different from mean EPSP ratio at 40-60 minutes (x̅ = 1.30; SE = 0.05; p > 0.05). In 10 µM Argireline concentration, the EPSP ratio at 10-30 minutes (x̅ = 1.19; SE = 0.004), was also not significantly different from mean EPSP ratio at 40-60 minutes (x̅ = 1.22; SE = 0.03; p > 0.05). Similarly, there was not a significant difference between the mean EPSP amplitude in 10-30 mins (x̅ = 1.45; SE = 0.03) and the mean EPSP amplitude in 40-60 min (x̅ = 1.73; SE = 0.04; p > 0.05) in 15 µM. Finally, at 20 µM Argireline concentration, the mean EPSP ratio at 10-30 minutes (x̅ = 1.49; SE = 0.003) also was not significantly different from mean EPSP ratio at 40-60 minutes (x̅ = 1.13; SE = 0.21; p > 0.05). Therefore, we concluded that Argireline had no effect on PPF over time.

Figure 4. EPSP ratio from PPF at increasing times of exposure to Argireline at various concentrations (0, 10, 15, 20 µM). n=3. For every concentration, the left column is the mean EPSP ratio of all the EPSPs taken at 10, 20 and 30 minutes while the right column is the mean EPSP ratio of all the EPSPs taken at 40, 50 and 60 minutes. The error bars represent the standard error for each condition and the height of the error bars represent the average of 3 measurements.

DISCUSSION

The effect of increasing concentrations of Argireline on synaptic transmission and PPF
To test Argireline’s effect on synaptic transmission, we compared the EPSP amplitudes under increasing concentrations of Argireline and found that there was a pattern of decreasing percent change in EPSP amplitude, excluding the 15µM Argireline condition. This matched our hypothesis that increasing concentrations of Argireline would decrease EPSP amplitudes. The results for 15µM may be attributed to the relatively low initial EPSP amplitude that we obtained (4.04 mV) when compared to the initial EPSP amplitudes we obtained from 0, 10, and 20 µM (11.95 mV, 12.93 mV, 10.5 mV, respectively). The unusually low initial EPSP amplitude might have prevented EPSP measurements that were recorded afterwards to experience a greater decrease in amplitude after 50 minutes.

Otherwise, this pattern aligns with Dababneh et al’s (2014) study that found that increasing concentrations of Argireline from 0 µM to 10.298 µM caused a significant decrease in EPSP amplitude. In another study, 100 µM of Argireline inhibited up to 30% of the total catecholamine release, compared to nearly 100% of catecholamine release without the application of any Argireline (Blanes-Mira 2002), further supporting our findings that Argireline has a decreasing effect on EPSP amplitudes. These results suggest that as Argireline concentration increases, the inhibition of SNARE proteins also increase, causing less vesicles to release neurotransmitters and thus, progressively decrease EPSP amplitudes.

To test Argireline’s effect on plasticity, we compared EPSP ratios for each concentration of Argireline and found that PPF increased in a dose-dependent manner, which contradicts our prediction that PPF would decrease with increasing concentrations of Argireline. Despite the slight observable decrease in EPSP ratio at 10 µM Argireline concentration, there is an overall upward trend from 0 to 20 µM Argireline.

Our results are similar to the findings of Dababneh et al. (2014), who found a slight observable increase in percent change between EPSP 1 and EPSP 2 in PPF from 0 to 10.298 µM. Young (2004) found that although BoNT A cleaves the SNAP-25 protein of the SNARE complex, submaximal doses of BoNT A-treated cultures still resulted in PPF. These studies, along with our results, may indicate that SNARE proteins do not play a critical role in the mechanisms that cause PPF. Fioravante and Regehr (2011) illustrates in her review article about the possibility that residual calcium increases the probability of release by binding to a sensor, separate from synaptotagmin, thus activating a site that is responsible for vesicular fusion. This unidentified sensor could explain the increase in facilitation that resulted from increasing concentrations of Argireline, even though the release of neurotransmitters was lowered as shown by the decreasing EPSP amplitudes in Figure 1. Still, further studies need to be conducted to determine how molecular release machinery, such as SNARE proteins, and calcium buffering in the terminal work together to yield short-term plasticity (Young 2004).
For both these tests on synaptic transmission and PPF, we used only one trial for each concentration, which prevented us from conducting statistical analysis. Although Figure 1 and 2 display an observable trend, it is unclear whether it was caused by Argireline or the differences in crayfish and their nerves. In addition, although our results showed that EPSP amplitude increased in 0 μM (Figure 1), it more importantly indicates that the crayfish muscle tissue did not decay with time. This shows us that time does not contribute to a decrease in EPSP amplitudes without the addition of Argireline.

The effect of Argireline on synaptic transmission and plasticity over time
To test Argireline’s effect on synaptic transmission over time, we compared the mean EPSP amplitude from 10-30 minutes to the mean EPSP amplitude of 40-60 minutes in different concentrations of Argireline, and found that our results mostly supported our hypothesis that Argireline would have an increasing effect on EPSP amplitude over time. For 10 and 20 μM of Argireline, the EPSP amplitudes between 10-30 minutes and 40-60 minutes were significantly different from each other, demonstrating that there was a significant decrease in EPSP amplitudes over time. However, the EPSP amplitudes at 15 μM Argireline were not significantly different with the increase in time. This could be attributed to the unusually low initial EPSP amplitude that we obtained under this condition.

Even though Argireline’s short peptide length (6-mer peptide) allows it to diffuse through the cell membrane (Blanes-Mira 2002), diffusion takes time to occur, meaning the cells cannot intake all of the peptide at once. As time increased, the amount of Argireline that was taken in by the crayfish muscle cell also increased. Since more SNARE complexes were inhibited from forming with the increase in time, the amount of neurotransmitters released into the synapse decreased.

To test Argireline’s effect on PPF over time, we compared the mean EPSP ratios at 10-30 minutes to the mean EPSP ratios at 40-60 minutes, and found that there was not a significant difference in EPSP ratios over time, which indicated that time had no effect on PPF. Our results did not support our hypothesis that as time increased, Argireline would have a decreasing effect on PPF. Presently, little is known to sufficiently explain these results. Future researchers should continue to test Argireline’s effect on PPF over time to further explore time’s effect on the role of the SNARE complex in PPF.

It is important to note that at 20 μM Argireline concentration, the EPSPs were virtually nonexistent after 50 minutes, causing a large decrease in the EPSP ratio in the 40-60 minute time period under this condition. This caused our standard error to be especially large relative to our other EPSP ratios. Furthermore, because we stimulated in 10 minute intervals and the 20 μM Argireline concentration caused EPSPs to disappear at 50 minutes, we assume that EPSPs must have diminished between 40 and 50 minutes.

Conclusion
In our experiment, we found that as time increased, Argireline significantly decreased EPSP amplitude and had no significant effect on PPF. However, we cannot conclusively determine if Argireline significantly decreased EPSP amplitude and significantly increased PPF in a dose-dependent manner. For that reason, we suggest that future researchers perform at least three trials for each concentration, with each trial stimulating a distinct nerve. This way, statistical analysis can be done to determine any significant differences between mean EPSP amplitudes and ratios for increasing concentrations of Argireline, which would give researchers a more accurate representation of Argireline’s effect on synaptic transmission and PPF. Conducting more trials would also account for any discrepancies in EPSPs that might occur across different nerves and crayfish. Further studies should also measure EPSPs in shorter time intervals between stimulations to more precisely identify when Argireline fully suppresses EPSPs, which would result in more accurate data. Finally, we suggest that future researchers start measuring EPSPs immediately after Argireline is applied (at 0 minutes), as opposed to starting at 10 minutes, to observe any immediate effects it may have on synaptic transmission or PPF. Because these findings can give more insight to present clinical applications, it is important that researchers continue studying the role of SNARE proteins in neuronal communication. Moreover, gaining a better understanding of this presynaptic mechanism can improve the effectiveness and safety of clinical treatments.

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