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The FMRFamide-Gated Sodium Channel's Effects on the EPSP Amplitude at the Neuromuscular Junction are Inconclusive

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ABSTRACT

Within the nervous system, neurons communicate at synapses via neurotransmitters transported between neurons until reaching the postsynaptic cell. They then bind to and activate specific proteins in the postsynaptic cell. Ion channels in the postsynaptic cell open and allow an influx of positive ions, depolarizing the cell, leading to an EPSP (Excitatory Postsynaptic Potential). FaNaC is a sodium-gated ion channel found in crayfish muscle cells that is activated by FMRFamide peptides. Since FaNaC allows positive ions into the postsynaptic cell, we sought to understand how it alters the strength of an EPSP by isolating the effects of FaNaC alone. We used NF1, a FMRFamide, to activate FaNaC. Additionally, we used a known FaNaC inhibitor, Amiloride, to see how EPSPs changed when FaNaC was not active. Protein kinases have also been implicated in the response to FMRFamide, so we utilized Staurosporine, a protein kinase inhibitor to isolate FaNaC and better understand its involvement in synaptic transmission. Our research potentially indicates that FaNaC decreases EPSP amplitude, but due to control discrepancies, it requires further research.

INTRODUCTION

To communicate within itself and alongside other systems of the body, the nervous system must use a combination of "all or none" electrical transmission and graded chemical transmission. Electrical signals occur within a single neuron in the form of action potentials, while chemical transmission occurs in the synaptic cleft, allowing communication from one neuron to another neuron or muscle fiber. Excitatory Postsynaptic Potentials (EPSPs) are a fundamental part of this communication process between two cells. EPSPs continue communication from one neuron to another by depolarizing the resting membrane potential of the postsynaptic cell enough to trigger the opening of voltage-gated sodium channels, starting a new action potential which will travel along the axon to a new cell (Pieroni, Byrne 1992). EPSPs occur in the neuromuscular junction, which increases the likelihood of an action potential in the muscle cell (Purves, Augustine, Fitzpatrick 2001). We experimented on crayfish extensor muscle cells because they do not have voltage-gated ion channels, and will not trigger an action potential, whereas other species would require toxins to stop action potentials. Also, the axons and extensor muscles are easily accessible, and crayfish are inexpensive.

Both presynaptic and postsynaptic cells in the neuromuscular junction require neurotransmitters to transport and transduce signals. Often, these come in the forms of neuropeptides. Neuropeptides are small chains of amino acids held together through peptide bonds. One group of neuropeptides are the FMRFamides. FMRFamide peptides are cardioexcitatory tetrapeptides and are essential to EPSP amplitude control (Duttlinger, 2003). NF1 is one of the FMRFamide-type neuropeptides and is the focus of our experiment. NF1 plays a role in the presynaptic cell in an EPSP, as it is the tetrapeptide that activates FaNaC, or FMRFamide-gated ion channels. These channels are gated to keep Ca^{2+} outside the presynaptic cell, which allows for a stronger EPSP when the cell is stimulated.

Previous research shows that the strength of an EPSP, measured by its amplitude, can be enhanced or suppressed using a variety of natural and synthetic chemicals (Pieroni, Byrne 1992). In particular, invertebrate FMRFamides significantly strengthen EPSPs (Friedrich, Molnar, Schiebe, Mercier 1998). The majority of research on NF1's effects on EPSPs has centered around its activation of kinases, which enhance EPSPs (Friedrich, Molnar, Schiebe, Mercier 1998; Papp 1996). However, NF1 also activates another more recently discovered protein, the positive ion channel FaNaC (Lingueglia 2006). Since both FaNaC and NF1 naturally occur in crayfish muscle cells, we ask whether FMRFamide's activation of FaNaC has any effect on the EPSP of crayfish muscle cells (Zhuang, Ahearn 1996). We want our research to connect the ideas presented in these various research articles. We also want these lab results to serve as a foundation for more in-depth experiments to advance the current knowledge of FaNaC's function in crayfish neuromuscular junctions.

We tested whether FaNaC affects the EPSP of crayfish extensor muscles. We hypothesized that FaNaC would increase the amplitude of the EPSP due to the

activation of FaNaC increasing the influx of positive ions into the cell, depolarizing the cell, activating voltage-gated calcium channels which would increase the amount of calcium entering the cell, therefore increasing the amount of neurotransmitter released and accepted by postsynaptic cell membrane protein channels.

We tested our hypothesis by submerging crayfish muscles in solutions, each containing amiloride or staurosporine with or without the presence of FMRFamide. We stimulated the extensor muscles in the crayfish tail and took EPSP recordings. Compiling this data, we were able to isolate FaNaC's effects on the EPSPs. Overall, our data suggests that there is an entity outside of kinases that, when activated by NF1, affects the EPSP amplitude; however, due to control data revealing unanticipated side effects of Amiloride, we cannot definitively conclude whether this effect is from FaNaC, only speculate that activated FaNaC decreases EPSP amplitude.

MATERIALS AND METHODS

Materials

We submerged our crayfish muscles in 100mL physiological saline containing 5.4 mM KCL, 196 mM NaCl, 2.6 mM $MqCl_2$-$6H_2O$, 10mM HEPES, 13.5 mM $CaCl_2$-$2H_2O$, and unique chemical inhibitors depending on the experimental trial. We used kinase inhibitor staurosporine at a concentration of 100 nM, FaNaC inhibitor amiloride at a concentration of 100 nM, and FMRFamide NF1 at a concentration of 10 and 50 nM in our experimental trials. We used a DC amplifier (A-M Systems 1600), a PowerLab (AD Instruments), an SD9 Square Pulse Stimulator (Grass Instruments), suction electrodes made of 1.2 mm heated and pulled borosilicate ground with sandpaper at the tip, and glass electrodes made of heated and pulled borosilicate capillary tubes with 1.2 mm diameter filled with 3M KCL (the highest possible concentration without precipitate used to lower resistance) to record data. We used a computer and the software LabChart, Datapad, and Microsoft Excel to store, process our data.

Dissection

We began by placing the crayfish in an ice bath. This action anesthetized the crayfish by drastically slowing its metabolism. After removing the crayfish from the ice bath, we used scissors to sever the tail from the rest of the body. We then used smaller scissors to cut along the border of the ventral and dorsal surfaces, separating the two at the base of the telson. Using our thumbs, we removed the bulk of the tail's muscle mass and intestines, leaving the dorsal extensor muscles and the connected axons. We then removed any remaining muscle mass, obscuring the extensor muscles using forceps. After completing our dissection, we used pins to secure the specimen to the dish, filling the dish with 100mL of physiological saline to simulate the extracellular fluid of the crayfish.

Maintaining axons and extensor muscles

Our primary method of maintaining our axons and muscles was to submerge the tail in physiological saline. The physiological saline is crucial because it simulates the extracellular fluids of a living crayfish and has the concentrations of ions necessary to allow for the continuation of normal physiological processes. However, the physiological saline must be changed consistently as the dissected tail continues to pollute the physiological saline throughout testing with metabolic waste products. To counteract this, we replaced the saline every 30 minutes with a new 100mL to maintain a consistent, accurate resting potential and EPSP. To replace the saline, we used the Simultaneous Liquid Uptake and Replacement Pump, removing the old solution with one side and replacing it with the other syringe.

Administering chemicals

After recording a successful EPSP, we administered chemicals by pipetting them into the physiological saline submerging the crayfish extensor muscles. We stirred the chemicals to disperse them into the physiological saline evenly, allowing them to permeate and affect the crayfish muscle. Recordings were taken immediately after introducing the chemical.

Recording data

We used two separate microelectrodes to produce and record EPSPs. We filled the first microelectrode with 3M KCl, leaving no air pockets (Dulhunty, 1977). We inserted the electrode into the micromanipulator, zeroed its voltage in the physiological saline. We checked the resistance of the electrode to see how much our current was disturbed. A high resistance could signal a broken electrode, which we would replace. We found between 5 and 15 megaohms an acceptable resistance. We looked through the microscope to observe where the tip punctured the muscle cell; a successful penetration of the muscle fiber showed a negative number on the DC amplifier after running through the PowerLab. We sanded the tip of our second microelectrode to create a wider-mouthed suction electrode. We attached the electrode to a syringe to suction the axons and to an SD9 Square Pulse Stimulator (Grass Instruments) to stimulate the axons and produce an EPSP in the muscle fiber. We used LabChart and DataPad software to record our results. EPSPs were recorded once every 30 seconds for 20 minutes.

Processing and presenting data

We chose to analyze each data point as a percentage of the first recording taken in each trial. This allowed us to compare trials despite differences in EPSP size. We presented data two ways to show the effects of FaNaC on overall EPSP strength and the prolonged effects of FaNaC on the EPSP. We used line graphs with time as the dependent variable in 30-second intervals and percent of the initial EPSP recording. This presentation showed the effects of FaNaC over time. We also presented our data in a bar graph, presenting the most significant percentage in each experiment to illustrate the effects of FaNaC on the EPSP overall.

In tests comprised of more than three trials, we used student's test, accepting p-values<.05 as statistically significant and p-values< .1 as marginally significant. We only used error bars represent standard error in graphs using three or more trials.

RESULTS

To determine whether FaNaC has any significant effect on EPSP amplitude, we ran a multitude of control and experimental tests using either Staurosporine, a kinase inhibitor, Amiloride, an FaNaC inhibitor, NF1, a neuropeptide that activates both kinases and FaNaC or a combination of the drugs. We compared the results from our trials to specific baselines to reach our conclusion.

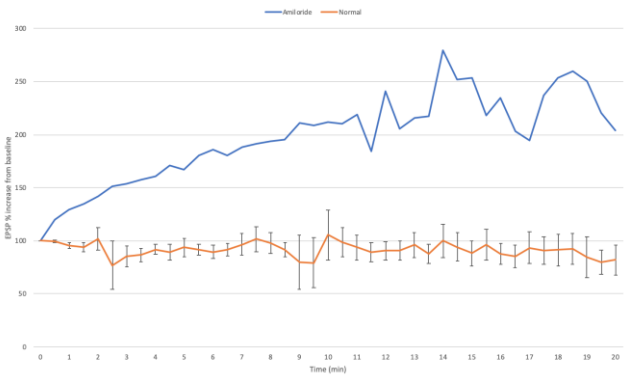

Fig. 1 shows the % change compared to the initial EPSP of crayfish extensor muscles submerged in saline solution containing Amiloride (blue) and unaltered physiological saline (orange). The Amiloride data represents the average of two trials and the unaltered data represent four trials. Bars represent the standard error of the average of EPSPs taken at the same time.

The Effects of Amiloride

As a control test to determine if Amiloride produced any unexpected effects on EPSP amplitude, we compared trials using just Amiloride to EPSPs from crayfish muscles in an unaltered solution. We submerged the crayfish extensor muscles in a saline solution with a 300 microMolar concentration of Amiloride and began recording EPSPs. We proposed that Amiloride alone would alter the EPSPs of the crayfish extensor muscles in a meaningful manner. The graph (fig. 1) indicates that Amiloride increases EPSP amplitude compared to EPSPs of crayfish muscles in unaltered physiological saline over time.

The Effects of Staurosporine

To determine whether Staurosporine exhibited any unexpected effect, we compared the EPSP amplitudes of trials using just Staurosporine to trials using crayfish muscle cells in unaltered physiological saline. In this test, we submerged the crayfish tail in the physiological saline and added 2.15μM Staurosporine. Once the Staurosporine was administered, we began recording EPSPs. Our null hypothesis stated that there would be no statistically significant difference between EPSP amplitudes in our unaltered trials and our Staurosporine trials. Figure 2 visualizes this data. From 0 minutes to 2 minutes, we observed a spike in the EPSP amplitude, which sank back down after minute 2. For the remaining time, the EPSP amplitudes were generally below the amplitude of the EPSP with no chemicals added. Consistent overlapping error bars and a p-test of .2 and above when comparing all data at a specific time represent no statistically significant data and supports the null hypothesis, indicating that Staurosporine has no unanticipated effects.

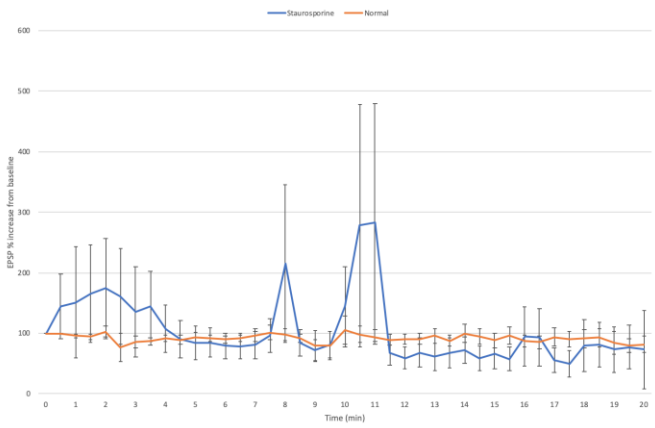

Fig. 2 shows the % change compared to the initial EPSP of crayfish extensor muscles submerged in saline solution containing Staurosporine (blue) and unaltered physiological saline (orange). The Staurosporine data represents the average of four trials and the unaltered data represent four trials. Bars represent the standard error of each EPSP taken.

The Effects of NF1

We conducted this control test to test for any unexpected effects of Staurosporine on the EPSP amplitudes by comparing it to a baseline of EPSP from crayfish muscles in an unaltered solution. We submerged our crayfish tail in a physiological saline solution with

either 10 or 50nM of NF1. After we added NF1, we began recording EPSPs. Our null hypothesis stated that there would be no statistically significant difference in EPSP amplitudes between trials using NF1 and trials using unaltered physiological saline. Figure 3 shows that crayfish muscle cells exposed to NF produced EPSP amplitudes that generally overlapped and remained close to one another.

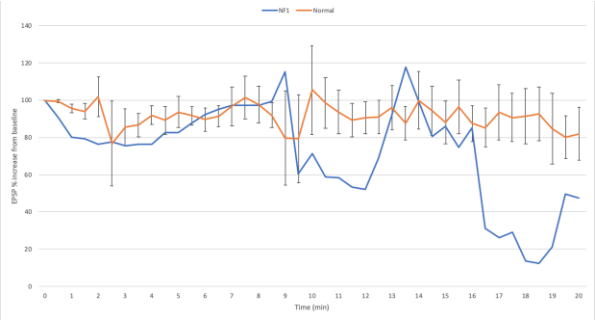

Fig. 3 shows the % change compared to the initial EPSP of crayfish extensor muscles submerged in saline solution containing NF1 (blue) and unaltered physiological saline (orange). The NF1 data represents 1 trial, and the unaltered data represents four trials. Bars represent the standard error of each EPSP taken.

The Effects of Staurosporine and NF1 over Time

In this experiment, we sought to determine whether anything other than kinases contributed to the amplification of EPSPs when NF1 is applied. To do this, we submerged crayfish extensor muscles in a 2.15 microMolar concentration saline solution and began to record EPSPs, which are displayed below (fig.4). Our null hypothesis stated that there would be no statistically significant difference between this data and our baseline data, EPSP amplitudes recorded from crayfish muscle cells submerged in unaltered physiological saline. The graph shows overlapping standard error bars from times t=0-13, from then on the EPSPs amplitudes of Staurosporine and NF1 increasing dramatically. Student's test gave p-values of around .09, one p-value taken using all data at one specific time. This semi-refutes our null hypothesis and gives us strong reason to believe that Staurosporine and NF1 EPSP amplitudes measured are different enough from EPSP recordings from muscle cells in unaltered physiological saline to indicate a potential additional NF1 activated entity amplifying EPSPs.

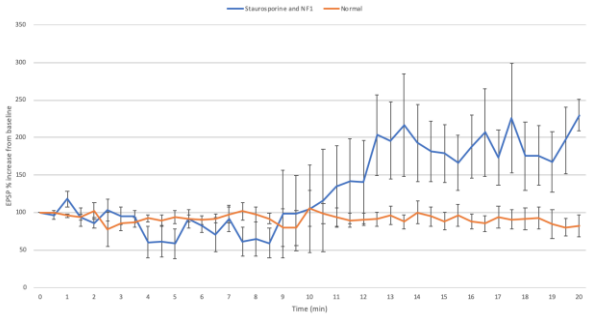

Fig.4 compares the effects of Staurosporine and NF1 on the EPSP of the crayfish extensor muscles (blue) to EPSPs of unaltered crayfish muscle cells (orange). EPSPs altered with Staurosporine and NF1 show a gradual increase over time as compared to the relatively unchanging, unaltered EPSPs. Bars represent standard error of the average of all the EPSPs taken at that specific time. We conducted four trials with staurosporine and NF1 and four trials using unaltered physiological saline.

The Effects of Amiloride and NF1 over Time

In this experiment, we looked at the effects of NF1 and Amiloride, a FaNaC blocker, on the EPSP amplitude of crayfish extensor muscles to look for any potential effects of FaNaC. We submerged the crayfish muscle cells in physiological saline and added NF1 and Amiloride until a 300 microMolar concentration of Amiloride and either a 10 or 50 microMolar concentration of NF1 was achieved. Looking at the graph (fig. 5), the two trials produce similar EPSP amplitudes for the first 9 minutes before the trials using Amiloride and NF1 quickly increased while trials using just NF1 remained steady. This indicates that the inhibition of FaNAC increase EPSP amplitude over time.

Fig. 5 compares the effects of Amiloride and NF1 on the EPSPs of crayfish extensor muscles (blue) to the effects of solely NF1 (orange). During times 18-19.5, we lost the EPSP and recorded from a different cell, accounting for the sudden drop in EPSP % change. The overall trend indicates that the addition of amiloride and NF1 increases the EPSP over time. Bars represent the standard error of the average EPSP at a given time. Amiloride and NF1 represents two trials while solely NF1 represents one trial.

The Effects of Amiloride, Staurosporine, and NF1

In this experimental test, we studied the effects to look for any effects recorded in previous data not contributed by kinases or FaNaC. To accomplish this, we added all chemicals at their prior concentrations to our

physiological saline and recorded EPSPs. The graph (fig. 6), when excluding points where EPSPs were unable to be recorded, shows the experimental trials are relatively close to the baseline of EPSPs from crayfish muscles in unaltered solution, only slightly higher over time. This indicates minimal effects outside of those caused by kinases and FaNaC: however, the lack of trials run for this experimental test makes the results less valid.

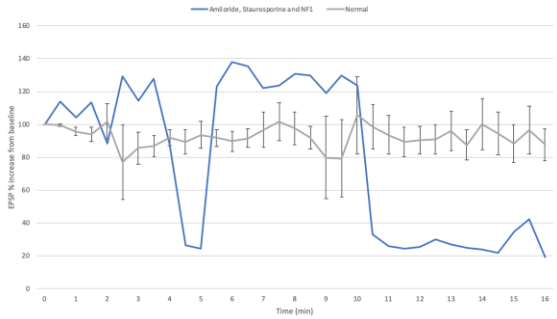

Fig. 6 compares the effects of all chemicals combined on the EPSP of the crayfish extensor muscles (blue) to EPSPs of unaltered crayfish muscle cells (grey). Bars represent standard error of the average of all the EPSPs taken at that specific time. We conducted one trial with the combination of inhibitors and NF1 and four trials using nothing.

Maximum Overall Changes in Control and Experimental Trials

Finally, we wanted to display the overall strengthening or weakening effects on EPSPs found within all of our experiments side by side. Figure 7 illustrates the compared average maximum increase from the initial EPSP of all tests. The trial "Normal" acts as a baseline for all other tests since it provided information on EPSP amplitudes without the influence of drugs. This graph serves as a summary of our individual data trials, showing them compared to one another to depict which inhibitor or combination of inhibitors caused the overall most substantial change in EPSP amplitude. This graph further displays the findings of our previous experiments in a more concise manner and allows all data to be compared.

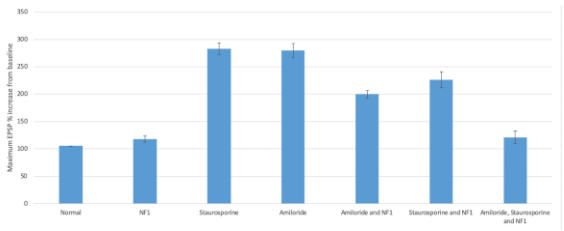

Fig. 7 showcases the effects of all of our experimental trials on the EPSP amplitude of our crayfish extensor muscles. Each bar represents the highest % change from initial EPSP amplitude from the averaged data of all trials. We arranged our trials in the following order. We ran four trials collecting data on the "Nothing" column, one trial for the NF1 column, four trials for the Staurosporine column, two trials for the Amiloride column, two trials for the Amiloride and NF1 column, four trials for the Staurosporine and NF1 column, and one trial for the Staurosporine, Amiloride and NF1 column.

DISCUSSION

Interpretation of Data

Our tests indicate that FaNaC may augment the EPSP amplitude, but the results remain inconclusive due to unanticipated side effects of the inhibitor Amiloride. Our test comparing EPSPs from crayfish muscles submerged in saline containing Staurosporine and NF1 versus muscles submerged in unaltered physiological saline indicate that something other than kinases contribute to the amplification of EPSPs. We drew this conclusion as the data reveals that to a significant degree, EPSP % increase from the initial reading is much higher over time in crayfish muscles submerged in the Staurosporine NF1 saline than the unaltered physiological saline. By using Staurosporine, an inhibitor that works by blocking the kinase function, halting their ability to change protein function via structural alterations, we isolate any remaining EPSP enhancement from unknown entities activated by FMRFamides. This initial research led us to believe that FaNaC potentially augmented the EPSP amplitude tested in our following experiment. Since our control comparing the effects of Staurosporine on crayfish muscle EPSPs and unaltered EPSPs showed no significant difference in % increase from the initial EPSP, we can assume with reasonable confidence that the results concluded from this experiment were caused by the blocking of kinases, not by any unintended effects of Staurosporine on the EPSP amplitude.

Our experiment comparing EPSPs from crayfish muscles submerged in saline containing Amiloride and NF1, versus muscles submerged in saline containing just NF1 initially showed with marginal significance that blocking FaNaC increased EPSP amplitude over time. This would potentially indicate that FaNaC channels, when activated, lower EPSP amplitudes. However, the control data comparing EPSPs from crayfish muscles submerged in saline and Amiloride compared to EPSPs from muscles submerged in unaltered physiological saline showed that with marginal significance, Amiloride increased the EPSP amplitude over time. This data conflicts with data collected from our control. As a result, we cannot confirm whether the EPSP amplitude augmentation in our experimental trial resulted from the inhibition of FaNaC, the unintended side effects of Amiloride, or a combination of both factors.

Figure 7 continues to support the evidence we found in figures 1-6. As shown, our highest EPSPs amplitudes recorded came from Amiloride and NF1, as well as Staurosporine and NF1. The patterns observed in our data

provide a potential answer that contradicts our hypothesis, in which we stated that activated FaNaC contributes to the amplification of EPSP amplitude in the presence of an FMRFamide. However, we would encourage more research using a different FaNaC inhibitor that has more limited excess effects on EPSPs' amplitude. Our data neither contradicts nor refutes any claims made by previous research as none exists in this exact niche.

Sources of Error

Throughout this experiment, we encountered multiple unforeseen difficulties, which likely affected our data in an unanticipated manner.

For some of our experiments and controls, we were unable to run a sufficient amount of trials, which makes our data less reliable as it represents a more limited number of repetitions. This issue stems from a lack of time and resources available to complete a desired number of runs and can be avoided in the future with additional funding and time.

During our recordings, due to muscle spasms and any additional movement of the lab table or pumping the electrode holder with the SLURP when replacing or removing the solution, sometimes electrodes slid out of cells, and EPSPs were "lost." This is reflected in our graphs by large, sudden drops. This primarily occurs due to the prolonged period and the fragility and general difficulty of working with organisms. We counteracted this by readjusting our electrodes and positioning them into new cells. However, this led to a different EPSP amplitude reading, which, when concerning the initial reading, sometimes created data discrepancies or data that was not accurate to what happened. In the future, we advise extreme caution when moving around the lab table and altering the setup of the experiment in any way to minimize these errors.

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