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Reversed State Sodium-Calcium Exchanger Contributes to Synaptic Facilitation in the Crayfish Neuromuscular Junction.

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ABSTRACT

The sodium-calcium exchanger (NCX) normally transports three Na^+ into the cell and one Ca^{2+} out of the cell. When the axon depolarizes during stimulation or an action potential, the action of the exchanger reverses (Araújo et al. 2007). We studied how the reversal of the sodium-calcium exchanger affected synaptic transmission and facilitation. The drug KB-R7943 prevents the reversal of the NCX with high specificity of action (Araújo et al. 2007). We acquired our data using intracellular recording of EPSPs at crayfish neuromuscular junctions while stimulating the associated nerves using paired pulses. We conducted our study by measuring EPSPs resulting from paired pulses in the superficial extensor muscle cells of crayfish using microelectrodes. We found that blocking the reversal of the NCX led to lower facilitation as compared with trials where reversal was not blocked. To support our observations regarding the effects of NCX reversal on facilitation, we repeated the experiment in a low-sodium environment and found the same trend that inhibition of NCX reversal lowered facilitation. This suggests that the calcium pumped into the cell by the NCX in its reversed state plays a role in facilitation. These findings help to understand the physiology of synaptic transmission and can serve as a base for further research aiming to rectify problems in synaptic transmission, such as those found in neurodegenerative diseases.

INTRODUCTION

Signal transmission between an organism's nervous and muscular systems is carried out by neurons sending signals through synapses by the means of neurotransmitters. Neurotransmitters are stored in vesicles in the axon terminal, the presynaptic component of the synapse. Depolarization resulting from an action potential opens voltage gated ion channels so that calcium, among other ions, can flow into the cell. Neurotransmitters are released from the vesicles into the synapse when calcium binds with synaptotagmin (Fernández-Chacón et al. 2001).

Various levels of intracellular calcium can strengthen or weaken synaptic transmission, which results in various behavioral and emotional changes responsible for shaping an organism's development (Fioravante et. al 2011). This is termed synaptic plasticity and implies the temporary weakening (depression) or strengthening (facilitation, augmentation) of synaptic transmission (Fioravante et. al 2011). Neurotransmitter release is a chance-based event because neurotransmitter vesicle fusion with the axonal membrane is caused by intracellular calcium binding to synaptotagmin (Fernández-Chacón et al. 2001). When an action potential causes an influx of calcium into the axonal terminal, the probability of neurotransmitter release is increased. In facilitation, subsequent stimulation evokes a larger release of neurotransmitters and, consequently, a larger EPSP. Katz and Miledi suggested that facilitation occurs because of residual calcium remaining form the initial influx of calcium during an action potential (1967). The subsequent influx of Ca^{2+} during the second stimulus adds to this residual calcium, resulting in an increased probability of calcium binding to regulator proteins, and subsequently neurotransmitter release (Katz and Miledi 1967). In our experimentation, we examined the role of the sodium-calcium exchanger in increasing the intracellular calcium in the axonal terminal and causing facilitation.

The sodium-calcium exchanger (NCX) is a transport protein that pumps three Na^+ ions into the cell and one Ca^{2+} ion out of the cell (Blaustein and Lederer 1999). Intracellular calcium activates the NCX (Araújo et al. 2007). However, during the depolarization phase of action potentials, the sodium-calcium exchanger reverses (Araújo et al. 2007). The reversed NCX pumps one Ca^{2+} into the cell and three Na^+ out of the cell, increasing the amount of residual calcium in the axon terminal (Lin et al. 2002). This increase in calcium may increase the amount of neurotransmitter released and the magnitude of the second EPSP (facilitation) in paired pulse stimulation. The NCX is far from the site of the neurotransmitter vesicles, and for this reason there is not an immediate effect of reversal of calcium flow (Lin et al. 2002). For this reason, we used paired-pulse stimulation, because by the time of the second pulse the calcium entering the cell will have had a chance to act on the vesicles.

KB-R7943 blocks the reversal of the sodium-calcium exchanger (Araújo et al. 2007). When the sodium-calcium exchanger is not reversed after depolarization, calcium continues being pumped out of the cell. This can reduce the size of the second EPSP in relation to the first because there will be less residual calcium in the synaptic terminal. We hypothesized that when NCX reversal is inhibited, the facilitation index will be smaller than that of the control.

To further implicate the sodium-calcium exchanger, we examined how the external ion concentrations affect the readiness of the NCX to reverse. We explored this query by examining the effect of altering external sodium concentration on the second EPSP during paired-pulse stimulation. A change in the extracellular concentration of sodium changes the gradients across the membrane which can alter the working of the NCX (Yu and Choi 2006). We hypothesized that if the external concentration of sodium was decreased to seventy-five percent of the normal concentration, the exchanger will reverse more readily because it will require less energy to move sodium out of the cell. This will cause an elevated level of intracellular calcium because the NCX will reverse sooner, and more calcium will be pumped into the cell. This change in intracellular calcium will lead to a higher facilitation index compared to that of the control.

We found that the facilitation index was smaller with KB-R7943 present than with the control. Also, we found that decreasing the sodium concentration led to a facilitation index that was higher than when KB-R7943 was applied but lower than the control. Additionally, when we used the treatment of low sodium and KB-7943 together, we got a facilitation index slightly higher than with just KB-R7943, but lower than with just seventy-five percent sodium.

MATERIALS AND METHODS

Crayfish storage

For our Experimentation we used *Procambarus Clarkii* due to their simplicity as organisms, segmentation, and multiple innervation which all contributed to a great variability of usable electrophysiological probing sites. The crayfish were stored in a glass aquarium with air bubblers to provide adequate oxygen. Their diet consisted of Special Kitty- brand cat food that was administered once per week.

Creating ringer's solution

The standard, full sodium Ringer's solution consisted of 196 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, and 2.6 mM $MgCl_2$.

We used approximately 100 mL of any solution in our dissecting dish, and replaced the solution after the same number of minutes as we had number of milliliters of the solution in the dissection dish.

To complete experimentation with low-sodium, we determined that 75% sodium of the standard ringer's solution is the optimal concentration to witness a greater readiness of the NCX to reverse. To make a 75% sodium solution, we mixed 100 mL of regular, full sodium ringer's solution with 100 mL of 8 mM NaCl, 98 mM LiCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, and 2.6 mM $MgCl_2$ solution. The second of the two mixed solutions had half the amount of sodium as the first, and it had 98 mM LiCl added to restore osmotic pressure. LiCl does not play a role in mimicking the crayfish's cellular environment, but the Li^+ has the same valence as Na^+ and serves to maintain the same osmotic pressure as the NaCl has in the standard Ringer's solution.

Since prior experimentation has shown that 5µM KB-R7943 is an effective drug concentration for halting NCX reversal (Fernández-Chacón et al. 2001), we used this concentration for our trials involving KB-R7943. We used DMSO as a vehicle to deliver KB-R7943 into the cells. We added the KB-R7943 in DMSO to the ringer's solution to administer the drug. We created a 5µM KB-R7943 solution by mixing 200 mL of standard or low-sodium ringer's solution with 20 µL of 50 mM KB-R7943 in DMSO.

To control for various interactions that the treatment solution may have had with the vehicle DMSO, we kept the amount of DMSO in solution steady, regardless of presence of KB-R7943. Thus, we added 20 µL DMSO to 200ml of our control trials and the 147 mM Na ringer's solution.

Dissection

We placed the *Procambarus Clarkii* in an ice bath for at least fifteen minutes until their locomotion was negligible or completely stopped. We then used dissection scissors to make a transverse cut across the junction of the tail and the crayfish body. Then, we made two ventrally superficial cuts along the junction of the ventral membrane of the crayfish tail and the dorsal shell from the initial opening at the superior end of the tail until the posterior end. These cuts fell in close proximity to the naturally-occurring ridges at the junction between ventral membrane and dorsal shell. We pulled off the cut ventral membrane by grasping the swimmerets and pulling away from the tail. Next, we removed the large phasic muscles in the crayfish by using a finger and squeezing out the muscles. We then secured the crayfish tail containing the superficial extensor muscles and the nerve segments to a

dissection dish and proceeded to experiment. (Crawdad 2005).

Creating Electrodes

We used both microelectrodes and suction electrodes in our experimentation. To make microelectrodes, we used a PUL-1 machine to pull glass tubes (diameter 1.2 mm) into microelectrodes. The tips of these microelectrodes were so fine that they could probe a single muscle fiber (Lindgren 2013). We then filled these microelectrodes with a 3 M KCl solution for conductance of electricity. The resistance of these microelectrodes was 5-10 MΩ.

To make suction electrodes, we pulled microelectrodes as described above and then we used sandpaper to file down the sharp ends to make blunt ends with a lumen large enough to suction a nerve. We filled these suction electrodes with our regular Ringer's solution during the process of suctioning a nerve.

Both types of electrodes were placed in electrode holders and ground wires were placed in the Ringer's solution.

Recording technique

We used superficial extensor muscles to record EPSPs in the *Procambarus Clarkii*. We first entered the solution with our microelectrodes and zeroed the voltage. Then, we inserted the electrode into the aforementioned muscles, aiming to have a lateral insertion. The lateral location was preferred because single innervation was more prominent as compared with central muscles, which tended to give action potentials (AP) instead of EPSPs because of multiple innervation (Lindgren 2013).

We used ADInstruments PowerLab 4/25 and MacLab BRIDGE Amp to amplify and transfer the electrical signals from the crayfish to the computer.

Nerve stimulation

We used a SD9 Stimulator to stimulate the suctioned nerves. Our stimulations were set at frequency 0.2 Hz, delay 20 ms, duration 0.055 ms, voltage 1 V, and paired-pulse stimulation.

Data acquisition and analysis

With the aforementioned preparations, we used the multiple recording function in the program Scope to record 60 pairs of EPSPs in a given muscle site. Then, we used another segment of the tail and another muscular site to conduct further trials.

Once we had our data, we used the average function of Scope to average the graphs of the 60 EPSPs to get rid of noise and to acquire the average EPSP magnitude of the 60 pairs. We measured from base to peak of both averaged EPSPs and then found the magnitude by finding the difference between base and peak of EPSP. We performed this averaging for all of our trials.

We used the facilitation index analysis method to determine the relative level of facilitation for each treatment. The formula we used was FI= $(EPSP_2-EPSP_1)/EPSP_1$.

RESULTS

To investigate the effects of the reversal of the NCX on facilitation, we used intracellular recording at crayfish neuromuscular junctions. We observed the effects of both the reversed and unreversed NCX on EPSPs in a full sodium ringer's solution and in a reduced sodium ringer's solution. To normalize results and to judge the changes in facilitation, we used the facilitation index analysis on our data, given by the formula FI = $(EPSP_2-EPSP_1)/EPSP_1$, where the numbers of EPSP correspond to the first and second EPSPs in the paired-pulse stimulations. We found that the facilitation index was lowered in the presence of KB-R7943. Furthermore, we found that when extracellular sodium was decreased, the facilitation index was lower than that with control normal concentration.

Facilitation Indices

	Control	KB-R7943	147 mM Na	KB-R7943 + 147mM Na
Trial 1	0.265625	0.86274	0.1199	0.069
Trial 2	0.10667	0.082251	0.1061	-0.0406
Trial 3		0.0491	0.0605	-0.0477
Average	0.1861475	0.0656755	0.0955	0.069

Table 1. The facilitation indices for each trial performed. We used the facilitation index method of analysis when analyzing the measured EPSPs. The analysis uses the formula FI= $(EPSP2_{-EPSP1)}$ $_{EPSP1}$to determine the increase of the second EPSP in a paired-pulse relative to the first. Figures in a light gray indicate unused facilitation indices. In these trials, the EPSP data was larger than the other data observed by a factor of approximately 3. The row of averages includes only the useable data, and the facilitation indices typed in light gray are omitted from calculations of averages.

Reversal of NCX in full sodium ringer's solution leads to facilitation.

We first established a facilitation baseline by measuring the facilitation occurring in the crayfish neuromuscular junction with no drug present and a standard crayfish ringer's solution. The average facilitation index was FI=0.1861475 (Fig. 1). The minimum and maximum facilitation indices were 0.10667 and 0.265625, respectively (Table 1). The control had the largest facilitation index, and this makes sense considering ringer's solution and the state of reversal of the NCX. The extracellular environment was closest to *in vivo* conditions in the crayfish body and the NCX was reversed and transporting calcium to the inside of the axonal terminal.

Prevention of NCX reversal with KB-R7943 decreases facilitation.

It has been shown that KB-R7943 selectively inhibits NCX reversal (Araújo et al. 2007). We used this selectivity to determine the effects of NCX reversal on facilitation by preventing reversal and observing changes in the facilitation index. We found the average facilitation index with KB-R7943 to be FI=0.06568, with minimum and maximum 0.0491 and 0.08225, respectively (Table 1). This facilitation index is smaller than that of the control and all of the other treatments (Fig. 1), indicating that the prevention of NCX reversal causes a decrease in facilitation. This is likely because the unreversed NCX pumps calcium out of the axonal terminal, thereby reducing the calcium available to bond with synaptotagmin and consequently to release neurotransmitters and evoke EPSPs.

Reduction of extracellular sodium leads to reduction of facilitation.

We conducted our experiments in a low-sodium ringer's solution to encourage the reversal of the NCX and to corroborate that the reversal encourages synaptic facilitation. We found that lowering extracellular sodium lowered overall facilitation indices, but the trend noticed in the control was still present (Fig. 1). .

First, we attempted to carry out the low-sodium trials with a 98 mM NaCl solution. However, no EPSPs were evoked in this solution. Thus, instead of reducing the sodium to half of the standard ringer's solution, we used a solution with 147 mM NaCl, which is 75% NaCl of the standard solution.

With the 147 mM NaCl solution, we found that the facilitation index was FI=0.0955, with minimum and maximum FI being 0.0605 and 0.1199, respectively (Table 1). The reduced sodium without KB-R7943 was lower than our control FI, but higher than both trials with KB-R7943 (Fig. 1). This suggests that while the NCX reversal aids facilitation, the reduction of extracellular sodium may impact unaccounted-for cellular processes.

Prevention of NCX reversal paired with low sodium leads to decreased facilitation.

We found the average facilitation index with KB-R7943 paired with seventy-five percent of the normal extracellular concentration to be FI=0.069 (Table 1). We were unable to get a range for the FI in this trial because the nature of our data suggested that the EPSPs we were observing were results of multiple innervation and were therefore unusable. This facilitation index is slightly higher than that of just KB-R7943, but lower than just seventy-five percent sodium (Fig. 1). This result agrees with previous results in that prevention of NCX reversal decreases facilitation (Fig. 1).

The two trials that were not used in our graph had EPSPs that likely came from multiple innervations of the same nerve. Consequently, the EPSP size was approximately triple the size of the EPSPs that came from a single innervation (Table 1). For this reason, we did not include two of the data sets from this trial. Because of the multiple innervation, they also had skewed facilitation indices (Table 1) which were not usable for our experimentation.

The result that we analyzed indicated that facilitation was lower with KB-R7943 added to solution than without, presumably because the reversal of the NCX was reduced. This reduction led to slowing of the flow of Ca^{2+} into the cell and less activation of synaptotagmin (Fernández-Chacón et. al 2001).

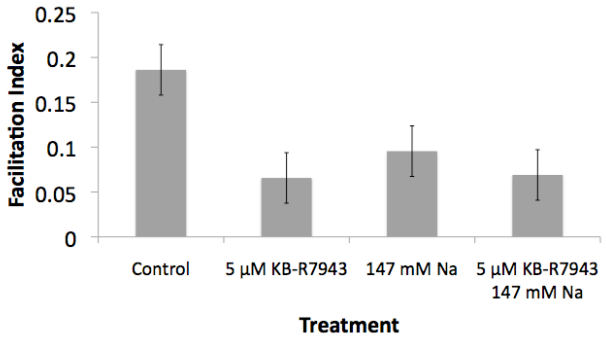

Figure 1. The average facilitation indices for paired-pulse stimulation for various treatments that affect NCX reversal**.** The range of facilitation indices for the control was 0.10667 to 0.265625 with an average of 0.1861475 (Table 1) (*n=2)*. The range for the KB-R7943 treatment was 0.0491 to 0.082251, with an average of 0.0656755 (Table 1) (*n=2*). The range for 147 mM Na was 0.0605 to 0.1199 with an average of 0.0955 (Table 1) (*n=3*). For the KB-R7943 + 147mM Na treatment, the only useable facilitation index was 0.069 (*n=1*). Error bars represent standard error of the mean of the facilitation indices. When the NCX reversal was prevented, then the facilitation indices were lower than those observed under conditions when the NCX reversed. This may be due to the NCX pumping calcium into the cell under reversed conditions, leading to facilitation. In the reduced sodium trials, it appears that there were other unaccounted-for cell processes that caused the facilitation index to decrease as compared with the control.

DISCUSSION

The reversed-state NCX contributes to facilitation.

Our first hypothesis is supported in that the facilitation indices of EPSPs under the influence of KB-R7943 were smaller than those without the presence of the drug. This suggests that the reversed-state NCX plays a role in synaptic facilitation, considering that it pumps in one Ca^{2+} for every three Na^{+} pumped out. This agrees with the mechanism for facilitation proposed by Matveev et. al (2004), which holds that the first wave of calcium influx during a potential is buffered by affinitive proteins, while

the second wave in a subsequent paired potential is able to bypass the saturated buffer proteins and instead activate synaptotagmin. Without the reversal of the NCX there is less facilitation; in this way KB-R7943 seems to hinder facilitation but does not completely stop it. These results are consistent with the findings of other studies such as those found by Araújo (2007).

Facilitation drops in a low-sodium environment.

Our second hypothesis is unanswerable because of the method of preparation used in this experiment. We expected to find a higher facilitation index in the preparations with low sodium as compared with the control, but we observed the opposite effect. However, there is insufficient data to conclude the reason for the drop in the facilitation indices of the low-sodium trials. It is possible that the NCX does more readily reverse, though there are likely other cell processes that are not accounted for. It is possible that there can be no potential forming presynaptically due to an insufficiently large gradient of sodium concentration across the membrane. However, since we did not measure action potentials in our experiments, we cannot conclude this. Another possibility is that the lowered sodium provides insufficient concentration gradient, but that this affects the postsynaptic cell and makes it unable to evoke a potential. .

However, even with the drop in overall facilitation indices, the trend of KB-R7943 hindering facilitation indices still held in a low-sodium environment. The average facilitation index for a low-sodium trial was lower with KB-R7943 added than a low-sodium trial without. This corroborates our hypothesis about the role of the NCX in facilitation, as the predicted result held true not only in an environment similar to that *in vivo* (standard ringer's solution), but also in an environment with low sodium.

Our results suggest that we should reject our second hypothesis. We found that when extracellular concentration of sodium is decreased to seventy-five percent of normal concentration, the facilitation index is actually smaller than that of the control, but still larger than the KB-R7943 treatment. However, it is quite possible that low sodium also affects other cell processes, not just the reversal of the sodium calcium exchanger, which could also affect facilitation.

It would be very beneficial in the future to perform the same experiments as we performed, only with the addition of monitoring action potentials in the presynaptic nerve. This would give information regarding the cell processes affected by the low-sodium environment and whether they are presynaptic or not. It may also be beneficial to lower extracellular calcium and observe the effects on the reversal of the NCX. According to the Nernst equation, lowering extracellular calcium may decrease the likelihood of reversal of the NCX because of the changes in the energy gradient for both Na^+ and Ca^{2+} would create an unfavorable energy balance for the reversal of the NCX. To improve our low-sodium trials, it would be helpful to control for all cell functions altered by the low sodium levels so that we could observe the effects of a lowered extracellular sodium concentration only on the NCX.

Furthermore, to understand the role of the calcium pumped into the cell by the reversed NCX, fluorescence techniques would be invaluable when used *in vivo* (Augustine et. al 1992). Down the line, we hope to transfect proteins that bind with calcium in the axonal terminal with green fluorescent protein in order to mark the location of calcium that is inflowing as a result of NCX reversal (Augustine et. al 1992). This, combined with staining for the location of the axonal terminal cell membrane and also tagging synaptotagmin and the NCX would show us a reliable path of calcium from the NCX to synaptotagmin. If we would be able to perform such staining and transfection *in vivo* or *in situ*, we would be able to determine exactly the impact of the NCX on intracellular calcium and on facilitation. .

Our current research, coupled with future research, will help to illuminate the mechanism of synaptic transmission in all organisms using the neurotransmitter glutamate. Since the crayfish neuromuscular junction uses glutamate, our findings about the NCX and facilitation extend to all organisms using glutamate as a neurotransmitter. By obtaining a full picture of the physiology of synaptic transmission, far-reaching innovations can be found that can help to rectify problems in synaptic transmission and the problems found in neurodegenerative diseases. .

Additionally, the crayfish neuromuscular junction uses glutamate to transmit signals. Glutamate is a universal and common neurotransmitter, and this research will have far-extending implications for other organisms that use glutamate. The study of the sodium-calcium exchanger can lead to further research in other organisms with respect to synaptic transmission and facilitation.

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