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# Effect of Lowered Extracellular Sodium Concentration on Sodium/Calcium Exchanger and EPSPs

KARAN DHINGRA
Department of Biology, Grinnell College, Grinnell, Iowa

## ABSTRACT

On a cellular level, simple learning processes are a result of facilitation of postsynaptic excitatory potentials (EPSPs). Facilitation occurs because of the presence of residual calcium in a presynaptic neuron, and various cell mechanisms regulate this amount, one of them being the sodium-calcium exchanger. In its forward state of action, the exchanger pumps calcium ions out, and in its reverse stage, it pumps calcium ions in. The working of the exchanger is dependent on the concentration and electrical gradients across the membrane; it flips from the forward state to the reverse stage when the cell membrane gets depolarized (during action potential). We decreased the extracellular sodium ion concentration to 75% of its original value in order to observe how that would affect facilitation. We failed to get any conclusive answers to the question because we did not have reliable control data to compare with, and thus were unable to make any judgments about whether facilitation increased or decreased in the experimental case with 75% Na. But we were able to verify the hypothesis that blocking the reversal of the exchanger leads to less facilitation, which has already been shown by previous studies.

## INTRODUCTION

Learning and adaptation are processes essential to the existence and survival of most multicellular organisms. Simple or involuntary learning, such as habituation and classical conditioning, occurs without the organism being aware of the change in behavior (Lin et al, 2002). On a cellular level, these learning processes are a result of facilitation of postsynaptic excitatory potentials (EPSPs) (Hawkins et al, 1982).

Facilitation, also known as neural facilitation or paired pulse facilitation, is a phenomenon that refers to the increase in the size of an EPSP evoked by an impulse when that impulse closely follows a prior impulse (Zucker & Regeher, 2002). It is attributed to the presence of residual calcium in the presynaptic neuron, after an action potential has passed through the cell and before the cell returns to its resting stage (Blaustein and Lederer, 1999).

As an action potential passes through a presynaptic neuron, the change in electrical potential across the membrane (depolarization) leads to the opening of voltage gated calcium ion gates, which further leads to an influx of calcium ions into the cell, which further causes vesicles containing neurotransmitters to bind to the presynaptic cell membrane and leads to neurotransmitter getting released into the synaptic cleft (Purves et al, 2012). A higher amount of residual calcium present in the presynaptic neuron leads to a larger EPSP.

Various cellular mechanisms influence the amount of calcium present in the presynaptic neuron – one of them is the sodium-calcium exchanger (Na/Ca exchanger). In the resting state of the cell, the exchanger works in what is called its forward direction, pumping 1 Ca ion out for every 3 Na ions coming in. It gets its energy from the incoming sodium, which, when coming into the cell, is moving in the positive direction of both its electrical and concentration gradient (Cao & Su, 2007).

The direction in which the Na/Ca exchanger works depends upon the chemical and concentration gradient across the membrane. During an action potential, the depolarization of the membrane causes the direction of action of the Na/Ca exchanger to flip. This is called the reverse mode of the exchanger, in which it pumps one Ca ion into the cell per 3 Na ions that go out (Lin et al, 2002).

The reversal of the Na/Ca exchanger happens during an action potential, and since the exchanger is present relatively far from the site of neurotransmitter release (Lin et al, 2002), the effect of the reversal is only seen on the second EPSP in a paired-pulse stimulation of the presynaptic neuron. Since the reversal leads to calcium being pumped in, it contributes positively to the amount of residual calcium present in the presynaptic neuron. Therefore, when the second action potential passes through the cell, a relatively higher amount of neurotransmitter gets released into the synapse, thus leading to the second EPSP being higher (facilitation).

Li et al, (2003) and Cao & Su, (2007) have done research in the past to determine more precisely the degree to which the Na/Ca exchanger affects facilitation; they did it by blocking the reversal of the exchanger with

the drug KB-R794 and then measuring EPSPs. Li et al, (2003) found that, in paired-pulse stimulation, the size of the second EPSP relative to the first EPSP decreased in the case in which the drug KB-R794 was applied, indicating that the exchanger directly influences facilitation. They did not notice any statistically significant differences in the size of the first EPSP between normal treatment and drug treatment.

Since the working of the Na/Ca exchanger depends on the electrical and chemical gradient across the membrane, changing the gradient would lead to a change in the likelihood of the reversal of the exchanger during an action potential, which would lead to a change in synaptic facilitation.

To experimentally determine this, we used a crayfish neuromuscular junction, lowered the extracellular concentration of sodium in presence of and in absence of KB-R794 (a drug that blocks the reversal of the exchanger), did paired-pulse stimulation and measured EPSPs. We hypothesized that lowering the extracellular Na concentration would lead to an increase in the likelihood of the reversal of the exchanger, because: the exchanger, in its forward state, pumps Ca ions out using the energy generated from the movement of the Na ions into the cell; if the concentration of Na is less outside, the concentration gradient and the electrical gradient across the membrane for Na decrease, making movement across the membrane for sodium relatively more difficult, and thus less energetic. And if the exchanger gets less energy to pump calcium outside, it is easier for it to flip and pump calcium inside. An increase in the likelihood of the reversal of the exchanger should lead to more facilitation, which we expected to see as an increase in the facilitation index in case of the lowered Na concentration as compared to control. The facilitation index is determined by the formula:

(Amplitude of 2$^{nd}$ EPSP – Amplitude of 1$^{st}$ EPSP)/(Amplitude of 1$^{st}$ EPSP)

One caveat to our approach is that changing extracellular concentration can change other aspects of the junction, making it impossible to determine whether a change in the exchanger actually lead to a change in the measured EPSPs. To control for any other random changes, we used KB-R794. We expected to measure a significant difference in the facilitation indices for lowered concentration with KB-R794 and without KB-R794, because, according to our hypothesis, the effect of the lowered concentration on the likelihood of the reversal of the exchanger causes change in facilitation, and not some other factor. If the facilitation indices had turned out the same for both, it would imply that changing the likelihood of the reversal of the exchanger does not affect facilitation.

We discovered that the facilitation index of one of the trials of control was much higher than the other one. The facilitation index of Trial 1 of control was almost twice than the facilitation index of Trial 2 of control, indicating that there was some significant error in measurement and experimentation techniques. The facilitation indices of both Trial 1 and Trial 2 for the treatment in which extracellular Na concentration is 75% of its value in the Ringer's solution were both higher than the facilitation index of Trial 2 of control, but there is only a slight difference. It is not adequate to provide any sort of conclusive evidence towards the correctness of our hypothesis, but it definitely points towards it. We also found that the facilitation indices were higher in the case of just 75% Na than 75% Na + KB-R794, which is more evidence for the claim that the exchanger causes facilitation.

## MATERIALS AND METHODS

*Solutions*

We used 100ml of the standard crayfish Ringer's solution for our control experiment. It was composed of 196 mM NaCl, 5.4 mM KCl, 3.5 mM $CaCl_2$, and 2.6mM $MgCl_2$. The pH of the solution was 7.4. For the lowered Na solution, the concentration of NaCl in the solution was changed to 147 mM and everything else was kept the same. In the lowered Na solution with KB-R794, the stock solution of KB-R794 with DMSO was added to the solution in an amount necessary to make the final concentration of KB-R974 in the solution 5μM.

*Dissection*

A crayfish was put into ice before dissecting it. The tail was cut off from the body and all extraneous tissue material was removed from the ventral side of the tail, revealing its superficial extensor muscles.

*Microelectrode Preparation*

We used two different kinds of microelectrodes: 1. Suction electrodes filled with the Ringer's solution sucked in from the dissection dish, used to stimulate the nerves, and 2. Sharp electrodes backfilled with 3M KCl, used to record EPSPs in the muscle cells. The electrodes were obtained from 1.2 mm x .68 mm x 4" capillary tubes. Before each set of measurements, we checked the resistance of the microelectrode setup and it feel in the range of 5-10 MΩ.

*Experiments*

The crayfish was pinned to a glass dish and 100ml of standard crayfish solution of 196 mM NaCl, 5.4 mM KCl, 3.5 mM $CaCl_2$, and 2.6mM $MgCl_2$ was added.

One particular section of the crayfish was cut open and the suction electrode was inserted close to the nerve, after which the nerve was sucked up. It was hooked up to a pulse generator, which was used to artificially stimulate the nerve bundles to generate action potentials. The sharp microelectrode was inserted into a muscle cell in the same segment, which was used to measure EPSPs.

All of the above steps were done similarly with the other two kinds of solutions: lowered Na with KB-R794 and lowered Na without KB-R794. Two trials of measurements were done for each kind of solution, and each trial involved 60 paired-pulse stimulations.

*Analysis*

The values for the EPSPs being measured by the sharp microelectrodes were obtained on a computer program called Scope on a Macbook Pro. We kept the frequency, duration of the pulses and the delay between pulses constant, but kept changing the voltage of the pulses to reach the threshold level in the nerve bundle and cause action potentials. The data from the 60 stimulations in each trial for each kind of solution was averaged out using a feature in Scope. We observed graphically the shapes and amplitudes of the two EPSPs in our program and obtained exact values for the latter.

## RESULTS

We obtained the average magnitudes of the first and the second EPSPs from our experiments for three different kinds of solutions: Case 1, Normal crayfish solution; Case 2, 75% Na solution; and Case 3, 75% Na with KB-R794. To ascertain whether lowering the Na ion concentration (which increases the likelihood of the reversal of the Na/Ca exchanger) actually increases facilitation, we compare the facilitation indices experimentally measured for Case 2 and Case 1. To make sure that it is the exchanger that is affecting facilitation in the case of 75% Na and not some other factor, we add KB-R794, which blocks the reversal of the Na/Ca exchanger, to the 75% solution. We compare facilitation indices for Case 3 with Case 2 for this purpose. The data for facilitation indices for Trial 1 and Trial 2 for each type of treatment are presented in Figure 1.

Facilitation Index = (Amplitude of 2$^{nd}$ EPSP – Amplitude of 1$^{st}$ EPSP)/(Amplitude of 1$^{st}$ EPSP)

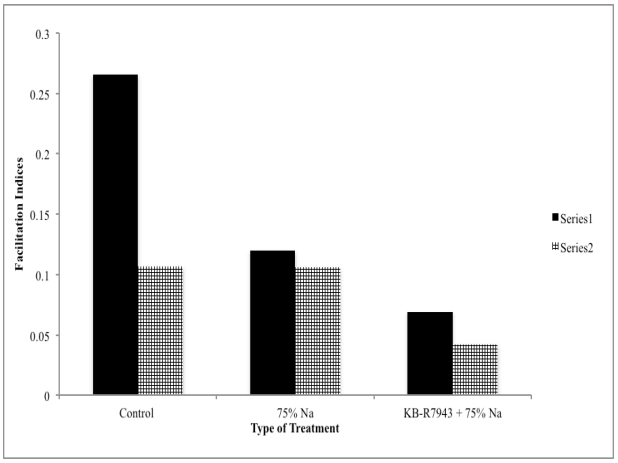


Fig. 1 Facilitation Indices v. Type of Treatment (Series 1 – Trial 1, Series 2 – Trial 2, n = 60)

As can be seen in the figure, the facilitation indices for Trial 1 and Trial 2 of the control experiment are significantly different. The Trial 1 value is more than twice the Trial 2 value. This indicates that there was some error with the experiment setup or something of that sort, because there is no other reason to have much higher facilitation if frequency, delay of the pulse is controlled for. Both Trial 1 and Trial 2 values of the 75% Na treatment are slightly higher than the Trial 2 value of our control experiment, which is what we hypothesized. But it is not very convincing because there is no way to tell which facilitation index value (Trial 1 or 2) is more correct for our control experiment.

The facilitation index values of both trials in Case 3 (KB-R794 + 75% Na) are significantly lower than the values in Case 2. This indicates that there definitely is a change in facilitation brought about by a quicker reversal of the Na/Ca exchanger that does not occur when the reversal is completely blocked with KB-R794.

## DISCUSSION

Our hypothesis was that lowering the extracellular concentration of Na ions would lead to more facilitation because the Na/Ca exchanger would reverse more readily. To measure the degree of facilitation, we used the metric of facilitation index. In our results, we observed that the facilitation indices for Trial 1 and Trial 2 of the control experiment were extremely different, indicating that we might have made some errors in our experiments, since there is no reason for two trials of similar nature to show such variation in facilitation when factors like frequency of pulse, delay between pulses were controlled for.

Trial 2 of the control was smaller than both the trials of the 75% Na experiment, which seems to support our hypothesis, but Trial 1 makes that really hard to say.

Some further control experiments might be able to solve this problem.

As already asserted by Lin et al, (2002) in their research, blocking the reversal of the sodium-calcium exchanger leads to less facilitation as the exchanger becomes unable to contribute to the residual calcium. Our data supports this view. The facilitation indices for KB-R794 + 75% Na are much lower than the facilitation indices for just 75% Na.

If further experiments are done to measure EPSPs more precisely, more interesting arguments could be made about how various changes in the mechanism of the sodium-calcium exchanger affect facilitation. Previous research on this topic has been focused on exploring only two states of the exchanger, reversed and unable to reverse. Tweaking the extracellular environment more could provide us with ways to manipulate the exchanger more subtly than that and see smaller effects in EPSPs, which are also probably very essential to various learning processes. It could provide us with more knowledge of how learning and behavior works on a cellular level since our current understanding of those is very limited.

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