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Inhibiting reverse-mode operation of the Na^+/Ca^{2+} exchanger does not affect long-term facilitation in crayfish neuromuscular junction

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ABSTRACT

High frequency stimulation of a crayfish neuron results in a long-term facilitation (LTF) of synaptic transmission of neurotransmitters across the neuromuscular junction. After LTF is induced, the Na^+/Ca^{2+} exchangers begin working in a reverse mode, expelling three Na^+ ions and admitting one Ca^{2+} ion into the cell. We hypothesized that inhibiting the reverse mode of the Na^+/Ca^{2+} exchanger with the chemical SN-6 would result in a decrease in postsynaptic EPSPs after low frequency (10 Hz) stimulation for a minute following high frequency stimulation (20 Hz) for 10 minutes. We then recorded EPSPs before and after both the long and short term LTF-inducing tetani. The difference in means of our EPSPs showed had no statistical significance ($p>0.05$).

INTRODUCTION

Perhaps one of the most fascinating qualities of neurons is their ability to track and respond to stimulation, altering their response as a result of past impulses (Fioravante and Regehr 2011). This trait, synaptic plasticity, provides the cells with a short-term "memory" in order to change its synaptic strength, resulting in an inhibitory or facilitated response (Fioravante and Regehr 2011). One form of synaptic plasticity is long-term facilitation (LTF), which occurs when a neuron is stimulated above 5 Hz for at least ten minutes, resulting in an excitatory response that lasts for hours (Sherman et. al. 1971, Dixon and Atwood 1989.) LTF results in an increased influx of Ca^{2+} ions into the synapse, which act as a signaling mechanism to augment the release of neurotransmitters, thus resulting in a facilitated postsynaptic response (Atwood and Wojtoweicz 1985).

The Na^+/Ca^{2+} exchanger (NCX), plays a crucial role in regulating Ca^{2+} influx. When functioning normally, it extrudes one Ca^{2+} in exchange for the admittance of three Na^+ ions. However, under the conditions of LTF, Zhong et al. (2001) propose that the exchanger operates in reverse-mode, admitting one Ca^{2+} ion and extruding three Na^+, thus contributing to LTF. The reverse-mode operation of NCX is activated when a cell is at its peak depolarization of an action potential, and also under ischemia, a condition of inadequate blood supply to cells (Iwamoto et al. 2004). According to Barrientos et al. (2009), the activation of the reverse-mode operation of NCX is significantly linked to Ca^{2+} overload during anoxia as well.

In an effort to investigate the link between LTF and the reverse-mode operation of NCX, Minami et al. (2007) used a drug, KB-R7943, to inhibit the reverse-mode operation of NCX in crayfish tonic limb neuromuscular junctions. Minami et al. (2007) measured the internal concentration of Ca^{2+} during LTF and hypothesized that the inhibition of the reverse-mode operation would antagonize Ca^{2+} accumulation, thus diminishing LTF. However, they found only a weak correlation between the inhibition of NCX with KB-R7943 and neurotransmitter release. They attributed this to the drug's inability to selectively inhibit the reverse-mode operation of NCX, as it inhibited the forward exchange as well. We used a more recently developed drug, SN-6, which was found to selectively inhibit the reverse mode operation of NCX, and also reported to effectively protect against ischemia and reperfusion injuries (Iwamoto et al. 2004). With SN-6, we hoped to successfully inhibit only the reverse-mode operation of NCX after inducing LTF. We tested these effects on the neuromuscular junctions (NMJs) of crayfish, because they are easily accessible, simple organisms with large, durable neurons.

We hypothesized that in the presence of SN-6, LTF would be effectively diminished as a result of the inhibition of the reverse-mode operation of NCX and the regulation of Ca^{2+} influx. If this was accomplished, we predicted that in the presence of SN-6, the antagonism of Ca^{2+} influx would be evident in a decrease of the amplitude of EPSPs after LTF was induced. We found that there was no significant decrease in the amplitudes of EPSPs after the induction of LTF in the presence of SN-6.

MATERIALS AND METHODS

Crayfish Preparation

We obtained one crayfish (Orconectes) for each trial of LTF induction. They were anesthetized in ice

water for approximately 30 minutes and the tails were removed by cutting just below the thorax. We then made two lateral cuts along the tail of the crayfish and one cut above the telson to remove the ventral portion of the carapace. We removed the flexor muscle and digestive tract from the remaining portion of the crayfish tail, revealing the deep extensor muscles. We placed the dissected crayfish tail into a petri dish and secured the tail to Sylgard gel with pins. The petri dish was then placed under the Leica Zoom 200 microscope for closer examination of the muscle. We then poured the desired crayfish saline solution into the dish, covering the crayfish tail. The saline solution was replaced every 30-45 minutes to keep the cells in prime experimental condition. We used 3 separate crayfish for our 3 control trials, and used 2 separate crayfish for our 3 SN-6 trials, conducting two trials on two nerves in one crayfish.

Solutions

The control group was tested in ~50mL low Ca^{2+} crayfish saline solution (5.4 mM KCl, 196 mM NaCl, 2.6 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM HEPES, 7.4 pH). The test solution was the same as the low calcium crayfish saline solution, but was also 20µM SN-6. The DMSO control solution was the low calcium crayfish saline solution with 100µM DMSO.

Electrode Preparation and Equipment

To prepare our microelectrodes, we used a World Precision Instruments electrode puller with the delay set at 3 and the heat set at 7, and a borosilicate capillary tube (resistance 10-20 MΩ). The resultant microelectrodes were then filled with 3M KCl using a syringe. Suction electrodes were prepared in the same manner except the tip was filed down to allow for the passage of a nerve. The microelectrode and suction electrode were then fixed into their respective micromanipulators. The reference electrodes were then inserted into the solution and resting potential was set to zero using A-M Systems, Inc. Model 3100 Intracellular Electrometer and was recorded by ADInstruments PowerLab 4/25.

Data Collection

We used a suction electrode to stimulate a nerve along the lateral portion of the crayfish tail. The suction electrode was positioned above the desired nerve which was then captured using the attached syringe. We then used a borosilicate microelectrode to penetrate a muscle cell in the region where the nerve was located. MacBook Pro Lab Chart 8.0.5 was used to measure the resting membrane potential once the microelectrode entered the cell.

We began by stimulating the nerve at different voltages to find the minimum voltage needed to create an EPSP that also would not cause the muscle to twitch excessively. We then stimulated the nerve just above that threshold voltage and measured the amplitudes of single EPSPs. We then induced a long-term tetanus, by stimulating the nerve at 20 Hz for 10 minutes and recorded the EPSPs immediately following the stimulation. Following the long-term tetanus, we induced a brief tetanus with 10 Hz stimulation for one minute. EPSPs were measured in the 7 second period of facilitation after tetanus, to investigate a change in the EPSP amplitudes after the LTF-inducing tetani (Minami et. al. 2007). The same procedure was done in both the control saline solution, as well as the SN-6 solution.

Data Analysis

The baseline EPSPs were calculated by finding the mean of EPSPs before stimulation for a given trial. In analyzing EPSPs before and after both sets of tetani, we accounted for differences in crayfish cells by normalizing the EPSP amplitudes to the baseline EPSP, and calculated the percent change of the EPSPs using the equation: $\%_{change} = ((EPSP_x - EPSP_{baseline})/EPSP_{baseline})*100$. Means were compared before and after long and short-term tetani, and were analyzed for significance using a two-tailed t-test, in which $p<0.05$ rendered a comparison statistically significant. Analysis for figures one and two consisted of a comparison of the mean EPSP amplitudes after the control solution was replaced with experimental solutions.

RESULTS

Our experiment used SN-6 to investigate the role of the reverse-mode operation of NCX on LTF. We first attempted to induce LTF by stimulating the nerve at 20 Hz for 10 minutes, followed by a brief tetanus of 10 Hz for 1 minute. We measured EPSP amplitudes before and after the long-term tetanus, and again after the brief tetanus. After obtaining three control trials, we tested the effects of the presence of SN-6 in saline solution after an LTF-inducing tetanus. In our SN-6 trials, we again stimulated the crayfish nerves with a long-term tetanus followed by a brief tetanus and measured its effects on the EPSP amplitudes.

SN-6 does not affect EPSP amplitudes after long-term tetanus

To determine if SN-6 affected the reverse-mode operation of NCX, we first attempted to induce LTF in order to activate the reverse-mode operation. The long-term tetanus comprised of a continuous 20 Hz stimulation for 10 minutes. However, in two of the three control

trials, we did not accomplish the induction of LTF, and rather observed a depression in the EPSP amplitudes. In each SN-6 trial, there was a decrease in the mean percentage change of EPSP amplitudes after the long-term.

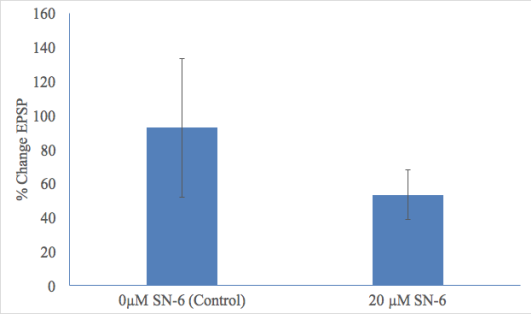

Figure 1. The percent change in EPSP amplitude of 0mM SN-6 solution and 20 mM SN-6 solution after long-term tetanus. The crayfish nerve was stimulated at 20 Hz for 10 minutes in an attempt to induce LTF. LTF was only expressed one of the three control trials, as the other two trials resulted in depression. n=3 control, n=3 SN-6 trial. There was no significant difference in the mean % change of the EPSPs before and after long-term tetanus with exposure to SN-6 ($p>0.05$). Error bars represent standard error of the mean.

LTF was not accomplished in two of the control trials; the mean percentage of EPSPs after long-term tetanus was 92.7% (S.E. 40.8%) of the baseline control EPSPs (Figure 1). There was also a decrease in percentage change in the SN-6 trials; the mean percentage of EPSPs after the long-term tetanus was 53.2% (S.E. 14.7%) than that of the baseline experimental EPSPs (Figure 1).

Because we reached depression rather than facilitation in some of the control trials, and the overall change in EPSPs after the long-term tetanus was negative, we cannot assume that the reverse-mode operation of NCX was activated, and therefore we cannot assume that SN-6 inhibited this mechanism. There was no significant decay in post-tetanic EPSPs, ($p>0.05$) and therefore we concluded that SN-6 does not affect synaptic transmission after a long-term tetanus.

SN-6 does not affect post-brief tetanus EPSP amplitudes following long-term tetanus

After applying a long-term tetanus to the cell, a brief tetanus was applied at 10 Hz for 1 minute. This second stimulation was applied in order to augment the facilitation gained from the long-term tetanus, which had not yet decayed (Minami et al. 2007). Approximately 5-10 seconds after the end of the long-term tetanus, the brief tetanus was applied to the same nerve as the long-term tetanus. Figure 4 shows that there was not a significant ($p>0.05$) decrease in EPSP amplitudes between stimulation at basal conditions and both long and short-term tetani in presence of SN-6.

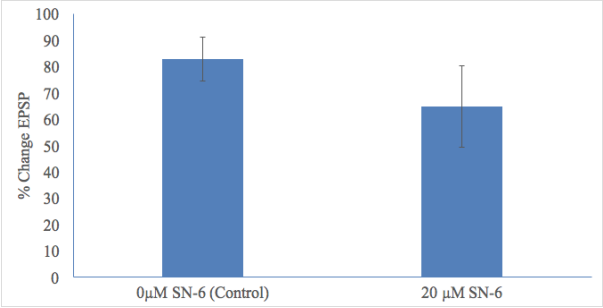

Figure 2. The percent change in EPSP amplitude of 0mM SN-6 solution and 20 mM SN-6 solution after both long and short-term. The crayfish nerve was stimulated at 10 Hz for 1 minute directly after the EPSPs taken during LTF. n=3 control, n=3 SN-6. There was no significant difference in the mean % change of the EPSPs ($P>0.05$). Error bars represent standard error of the mean.

LTF was not accomplished in all control trials; the mean percentage of EPSPs after brief tetanus compared to the baseline control EPSPs was 82.8% (S.E. 8.5%) and the mean percentage of EPSPs after brief tetanus in the SN-6 trials was 64.6% (S.E. 15%) compared to baseline EPSPs (Figure 2). Because there is lack of evidence of the reverse mode operation of NCX, we cannot assume that SN-6 successfully inhibited the reverse-mode operator. Because there was no significant percentage decrease in the EPSP amplitudes after both long and short-term tetani in presence of SN-6, we concluded that SN-6 did not have an effect on neurotransmitter release.

SN-6 does not affect EPSP amplitudes

Before measuring the effect of SN-6 on LTF, we wanted to ensure that SN-6 was not affecting mechanisms other than the reverse-mode operation of the NCX. To do this, we measured the amplitudes of EPSPs in a control DMSO solution, and compared them to the EPSP amplitudes in an SN-6 solution, consisting of 20 μM of SN-6 in DMSO solution.

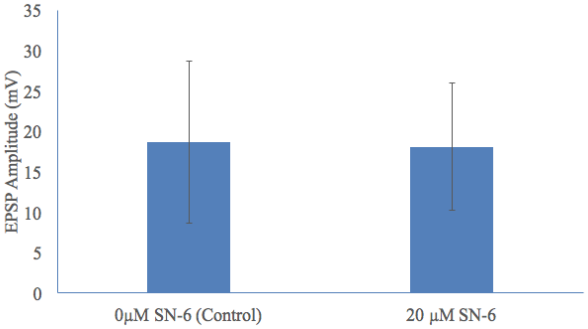

Figure 3. EPSP amplitudes of 0μM SN-6 solution and 20 μM SN-6 solution. n=3 control, n=3 SN-6. There was no significant difference in the amplitudes of EPSPs between the two solutions ($p>0.05$). Error bars represent standard error of the mean.

There was no significant difference in the amplitudes of the EPSPs between the two conditions ($p>0.05$); the mean of the EPSP amplitudes in the control condition, 18.6 (S.E. 10.0), was not significantly deviant from the mean of the EPSPs of the SN-6 solution, 18.1 (S.E. 7.9) (Figure 2). From these results, we concluded that SN-6 did not have an effect on EPSP amplitudes prior to the LTF-inducing tetani, and therefore no effect on neurotransmitter release.

DMSO does not affect EPSP amplitudes

Because we conducted our SN-6 trials with SN-6 dissolved in DMSO, we sought to ensure DMSO had no effect on the amplitudes of EPSPs. We created a DMSO solution, adding 50 µl DMSO to 50 mL of crayfish saline. We first measured amplitudes of EPSPs in the control saline solution, and then replaced the solution with the DMSO-saline solution, measuring the EPSPs once again.

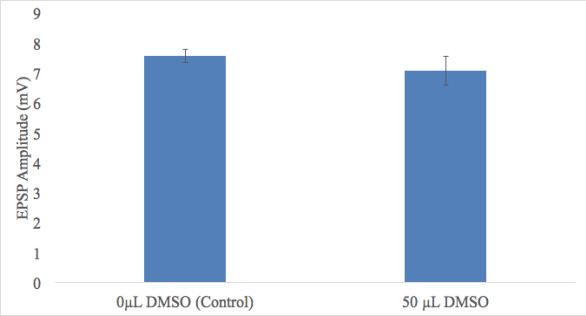

Figure 4. EPSP amplitudes before and after presence of DMSO in saline solution. n=3 control, n=3 SN-6. There was no significant change in amplitudes of EPSPs after saline was replaced with DMSO solution ($p>0.05$). Error bars represent standard error of the mean.

According to Figure 1, there was no significant change in the amplitude of EPSPs after the control solution was replaced with the DMSO-saline solution ($p>0.05$); the mean of the EPSPs in the control solution, 7.6, were not significantly deviant from the DMSO-saline solution, 7.1. From these results, we concluded that the presence of DMSO did not affect EPSP amplitudes, and thus did not have an effect on neurotransmitter release.

DISCUSSION

While investigating the role of the reverse-mode operation of NCX in LTF, we sought to inhibit the mechanism through application of SN-6 via an extracellular saline solution. We found that stimulating at 20 Hz for 10 minutes in conjunction with 10 Hz for 1-minute brief tetani was not wholly successful for the induction of LTF. Since we were unable to measure the internal concentration of Ca^{2+}, we could not be sure that the reverse-mode operation of NCX was activated. Furthermore, statistical significance was not found in the relationship between control condition EPSPs and SN-6 condition EPSPs taken after both long-term and brief tetani. These factors caused us to be unable to support our hypothesis that SN-6 would diminish LTF. This could be attributed to our inability to induce LTF in two of the three control trials.

The observed depression after the long and short-term tetani can possibly be explained by Dudek and Bear (1992), who observed the synaptic depression of hippocampal cells after excitatory input activity--stimulation from 10-50 Hz. They attributed this depression to a temporary modification of synaptic effectiveness, and observed that the depression was short-term, followed by the expected long term facilitation. It is possible that because we observed the synaptic response immediately after applying the tetanus, the cell was undergoing a depression as a result of temporary failure to activate NMDA receptors in the postsynaptic cell, and would show long-term potentiation in some time (Dudek and Bear 1992).

The lack of effect of SN-6 could be attributed to multiple other Ca^{2+} regulatory processes, such as the plasmalemma Ca^{2+} ATPase, as well as Ca^{2+} channels in other regions of the cell, including the endoplasmic reticulum (Beaumont et al. 2001). Both processes influence the internal concentration of Ca^{2+}, and could have contributed to the change in neurotransmitter release in the presynaptic cell. Further research should monitor these processes to determine their effect on internal Ca^{2+} concentration.

Our findings were similar to that of Beaumont et al. (2001), who also found a weak correlation between LTF and internal concentration of Ca^{2+}. They concluded that both LTF and internal concentration of Ca^{2+} are both variables extremely sensitive to past activity, rendering them resistant to strong stimulation. Kimura et al. (1999) also found that KB-R7943 inhibited the bidirectional NCX exchange in guinea-pig cardiac myocytes. They found that the NCX plays an important role in regulating intracellular Ca^{2+}. Kimura et. al (1999) discovered that the NCX, under certain physiological or pathological conditions, reverses its direction, in a similar manner to invertebrates. The research of Kimura et al. (1999) provides a link between NCX in crayfish NMJs and mammalian NCX.

More research should be done to investigate NCX in mammals to determine if there are human applications. There should also be more research using SN-6, a relatively new drug, while monitoring the internal concentration of Ca^{2+} and all of its regulatory processes. According to Barrientos et al. (2009), inhibitors of the

reverse-mode operation of the NCX may help alleviate ischemia-reperfusion injuries, along with skeletal fatigue and anoxia. Further research can determine whether SN-6 would be a safe drug for the inhibition of the reverse-mode operation of NCX in mammals, and eventually humans.

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REFERENCES

Atwood, H.L., and J.M. Wojtowicz. 1985. Correlation of presynaptic and postsynaptic events during establishment of long-term facilitation at crayfish neuromuscular junction. *Journal of Neurophysiology* 54:220-230.

Atwood, H.L., and D. Dixon. 1989. Adenylate Cyclase System Is Essential for Long-Term Facilitation at the Crayfish Neuromuscular Junction. *Journal of Neuroscience* 9:4246-4252

Barrientos, G., D.D. Bose, W. Feng, I. Padilla, and I.N. Pessah. 2009. The Na^+/Ca^{2+} Exchange Inhibitor 2-(2-(4-(4-Nitrobezyloxy)phenyl)ethyl)isothiourea Methanesulfonate (KB-R7943) Also Blocks Ryanodine Receptors Type 1 (RyR1) and Type 2 (RyR2) Channels. *Molecular Pharmacology* 76:560-568.

Beaumont, V., N. Zhong, and R.S. Zucker. 2001. Roles for mitochondrial and reverse mode Na^+/Ca^{2+} exchange and the plasmalemma Ca^{2+} ATPase in post-tetanic potentiation at crayfish neuromuscular junctions. *The Journal of Neuroscience*, 21:9598-9607.

Dudek, S.M., and M.F. Bear. 1992. Monosynaptic long-term depression in area CA1 of hippocampus and effects of N-Methyl-D-aspartate receptor blockade. *Neurobiology.* 89:4363-4367.

Fioravante, D. and W.G. Regehr. 2011. Short-term forms of presynaptic plasticity. *Current Opinion in Neurobiology* 21:269-274.

Hwang, H., T. Omura, and B. Clarke. 2014. Nitric oxide synthase inhibitor L-name decreases long-term depression at the crayfish neuromuscular junction. *Pioneering Neuroscience* 14:5-10.

Iwamoto T., Y. Inoue, K. Ito, T. Sakaue, S. Kita, et al. 2004. The Exchanger Inhibitory Peptide Region-Dependent Inhibition of Na^+/Ca^{2+} Exchange by SN-6 [2-[4-(4-Nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic Acid Ethyl Ester], a Novel Benzyloxyphenyl Derivative. *Molecular Pharmacology* 66:45-55.

Kimura, J., T. Watano, M. Kawahara, E. Sakai, J. Yatabe. 1999. Direction-independent block of bi-directional Na+/Ca2+ exchange current by KB-R7943 in guinea-pig cardiac myocytes. *J Pharmacol* 128(5): 969-974.

Minami, A., Y.F. Xia, and R.S. Zucker. 2007. Increased Ca^{2+} influx through Na^+/Ca^{2+} exchanger during long-term facilitation at crayfish neuromuscular junctions. *J Physiol* 585.2:413-427

Zhong N., V. Beaumont, and R.S. Zucker. 2001. Roles for mitochondrial and reverse mode Na^+/Ca^{2+} exchange and the plasmalemma Ca^{2+} ATPase in post-tetanic potentiation at crayfish neuromuscular junctions. *The Journal of Neuroscience* 21:9598-9607.