The Effects of Levetiracetam on Glutamatergic Synaptic Transmission

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Epilepsy is a neurological disorder characterized by recurring, unpredictable seizures. Its disease burden is high, ranking fourth in the world's neurological disorders behind tension-type headaches, migraines, and Alzheimer's disease. The commonly used antiepileptic drug levetiracetam (Keppra) reduces epileptic seizures; however, the exact mechanism is not known. Some studies suggest that sodium and/or potassium ionic channels are directly altered, reducing membrane excitability. Yet others suggest it interacts with synaptic vesicle protein SV2 to alter synaptotagmin's (a calcium sensor vesicle protein) action in the presynaptic nerve terminal to reduce excitability. The aim in this study was to examine whether synaptic transmission would be reduced in a model glutamatergic synaptic preparation. In this study, the glutamatergic synapses at crayfish neuromuscular junctions (NMJs) were used to assess the drug's action. The evoked excitatory junction potentials of the crayfish NMJ were unexpectedly enhanced within 20 min of stimulation with pulse trains following static incubation of 10 min exposure to 1 mM doses. Repetitive stimulation for 2 min and incubation for 10 min without stimulation did not show an effect. This project was an authentic course-based undergraduate research experience (ACURE) in a neurophysiology teaching laboratory with 16 students. It appears levetiracetam acts differently in different animal models and varied experimental conditions are required to note the effects.

Abbreviations: ACURE – authentic course-based undergraduate research experience; EJP – excitatory junction potential; NMJ – neuromuscular junctions; STF – Short-term facilitation

Keywords: invertebrate; crustacean; neuromuscular, glutamate

Introduction

Epilepsy is a neurological disorder of recurring, unpredictable seizures and ranks fourth in the world's neurological disorders behind tension-type headaches, migraines, and Alzheimer's disease (Beghi, 2016). Epilepsy is a complex disease and in some cases is linked genetically, but no one cellular defect encompasses the cause of all forms of the disease (Thijs et al. 2019). There are various types of prescribed antiepileptic drugs, and choice of prescription is based on the classification of a patient's seizure type and epileptic syndrome, as well as what is safest for their particular background (Abou-Khalil, 2008). One particular antiepileptic drug (AED) is levetiracetam (trade name Keppra), which is commonly used because of its effectiveness in reducing epileptic seizures (Boido et al., 2010). However, the exact mechanism of how it works is not known. Some reports state that it does not block voltage-dependent sodium channels and does not interfere with receptors associated with GABAergic or glutamatergic synaptic

transmission (Deshpande and Delorenzo, 2014). Other studies suggest that the compound blocks calcium channels in the presynaptic terminal of neurons which would reduce synaptic transmission (Vogl et al., 2012). Yet other investigations suggest that the compound is taken up in the presynaptic terminals when synaptic vesicles fuse to the presynaptic terminal and binds to a synaptic vesicle associated protein (SV2A) to reduce vesicle fusion events (Lynch et al., 2004). This lack of clarity regarding levetiracetam's mechanism of action requires further research.

Previous work looking at the mechanism of the ketogenic diet in reducing epileptic seizures showed that acetoacetate may interfere with glutamate's transport. Decreasing the vesicle package reduces nerve excitability (Juge et al., 2010; Stanback, 2019). Though some previous work focused primarily on the benefit of the ketogenic diet, there are other approaches, such as levetiracetam, that reduce synaptic transmission (through still unknown mechanisms). A recent study (Alshuaib et al., 2020) showed reduced action potential amplitudes when crayfish motor nerve axon preparations were treated with levetiracetam. This would suggest that sodium and/or potassium ionic channels are directly altered, reducing membrane excitability. The effects were noted in regions of the motor axon away from the distal synaptic terminal region of the axon.

This study, along with previous studies, utilizes the crayfish model because of the simplified structure of the glutamatergic crayfish neuromuscular junction (NMJ) as well as the easy accessibility of the axon for intracellular recordings (Alshuaib et al., 2020; Titlow and Cooper, 2018). The evoked synaptic potentials in the opener muscle fibers are graded and non-spiking. Repetitive stimulation results in an increased graded potential due to synaptic facilitation. In this preparation, short term synaptic facilitation is due to residual calcium in the presynaptic terminal during repetitive stimulation (Cooper and Cooper, 2009; Katz and Miledi, 1968).

SV2A is a synaptic vesicle protein highly expressed throughout the central nervous system (CNS) in mammalian preparations, specifically rodent models (Ciruelas et al., 2019). The SV2s bind synaptotagmin (a calcium sensor protein), but levetiracetam was found to reduce SV2A binding to synaptotagmin in particular cases (Ciruelas et al., 2019). The reduced binding could cause a decrease in the probability of transmission. The binding of levetiracetam to SV2 may have predictable responses when modulating neural activity, but we wished to characterize and confirm its effects in the crayfish synaptic NMJ model. It has not been established yet if the SV2 protein is present in crayfish motor neurons.

The NMJs of opener muscle in the walking legs of crayfish is a preparation which allows one to use an identified neuron, as only one excitatory motor neuron innervates the entire muscle. The rich history of this model in addressing synaptic physiology allows one to build on what is already known from the innervation of muscle types, mechanisms of synaptic facilitation, the quantal nature of synaptic transmission and modulation of synaptic release, as well as the cellular cascades involved in synaptic vesicle trafficking (reviewed in Cooper and Cooper, 2009). Synaptotagmin is present at crayfish NMJs, although SV2 has yet to be identified at the crayfish NMJ (Cooper et al., 1995). Potentially, understanding the cellular mechanisms behind how levetiracetam alters transmission in this crayfish model will aid in a better understanding not only of this preparation but of neurons in general. The hypothesis to be tested is that levetiracetam will reduce synaptic transmission in this model glutamatergic synaptic preparation.

Materials and Methods

Red swamp crayfish (*Procambarus clarkii*) were obtained from a distribution center in Atlanta, GA, and delivered to and bought from a local supermarket in Lexington, KY, USA. The crayfish (6-10 cm in body length and 12.5-25 g in body weight) were housed in individual standardized plastic containers with weekly exchanged dry fish food and aerated

water (20-21°C). Legs from different crayfish were used for each experiment. Twelve crayfish were used for control experiments (saline to saline exposure), six for exposure to levetiracetam at 100 µM and another six for exposure to 1 mM for the stimulation trains. Six more were used for the constant stimulation paradigm. This resulted in 30 crayfish being used to obtain the data presented. Another 16 crayfish were used for learning the dissection and the experimental conditions within the student classroom.

The crayfish walking leg opener neuromuscular preparation

The dissection and recording procedures were completed as described in Cooper and Cooper (2009). To summarize, the ventral cuticle of the propodite and the closer muscle were removed in order to expose the ventral surface of the opener muscle in the propodite cavity. In the meropodite segment, the cuticle covering the flexor muscle was removed. The apodeme (tendon) at the meropodite-carpopodite joint was cut from the flexor, thereby exposing the extensor muscle and the leg nerve. With the main leg nerve and extensor muscle exposed, the most dorsal branch of the main leg nerve in the proximal end of the meropodite segments contains the excitatory motor neuron to the opener muscle. This nerve branch is pulled into a suction electrode for stimulation.

The nerve branch was then selectively stimulated by a Grass stimulator in order to evoke action potentials in the excitatory axon. The distal muscle bundles (Figure 1) were impaled with a sharp intracellular electrode (20 to 30 megaohm resistance) filled with 3 M KCl, and the excitatory junction potentials (EJPs) were then recorded from the muscle fiber of interest.

Short-term facilitation (STF) in the EJPs was obtained by stimulating at 40 Hz for 25 stimuli within a train and repeating every 10 s. After adding levetiracetam, the preparation was incubated for 10 min before again stimulating at 40 Hz for 25 stimuli within a train every 10 s (Figure 2A). This paradigm is illustrated in Figure 2B. The amplitude of the 25th EJP in each train was obtained for further analysis.

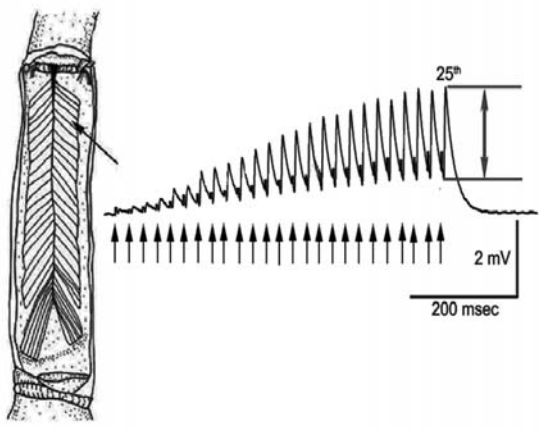

Figure 1: The opener muscle of a crayfish walking leg and electrophysiological response at NMJ. Representative trace of the excitatory junction potentials (EJPs) recorded with an intracellular electrode from the distal muscle fibers in opener muscle of a crayfish walking leg. The responses show a marked facilitation that occurs throughout the stimulation train delivered at 40Hz for 25 stimuli. The amplitude of the 25th EJP amplitudes (mV) is used for indexing the effect of levetiracetam.

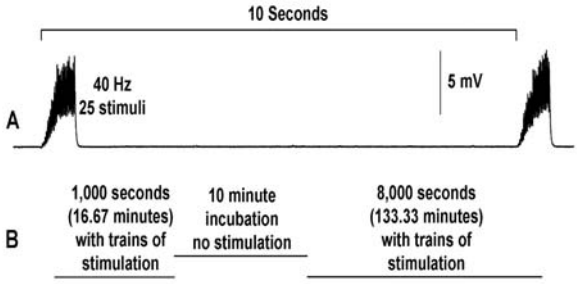

Figure 2: A stimulation train paradigm. One paradigm was to stimulate the motor neuron with trains every 10 seconds for 1,000 s followed by incubation of levetiracetam for 10 min without stimulation. Afterwards the stimulation of trains was repeated. The trains consisted of 25 stimuli delivered at 40 Hz. (A) Illustrates the trains of pulses and responses for the 10 s intervals. (B) A schematic of the entire stimulation paradigm over time.

A second paradigm involved stimulating the opener nerve at 20 Hz or higher continuously for 2 min, followed by switching the media to one containing levetiracetam and allowing an incubation of 10 min. After the incubation, stimulation at the same frequency was provided for another 2 min (Figure 3). The mean amplitude of the last 5 EJPs in the initial 2

min and the last 2 min of stimulation were used to assess the effects of levetiracetam for this paradigm.

For both stimulation paradigms, saline control experiments were performed with the same stimulation conditions, using only saline during the 10 min incubation time.

Data analysis

The rank sum pairwise test or a sign test was used to compare the differences in responses before and after exchanging solutions. When the assumption of normality held, a Student's T-test or an ANOVA was conducted to analyze the data. The analysis was performed with SigmaStat software. A p-value < 0.05 was considered statistically significant. To examine the consistency and reproducibility in analysis of the data, groups of class participants were blinded to the specific conditions of the experiments and were unaware of whether they were analyzing data from the saline trial or the compound of interest.

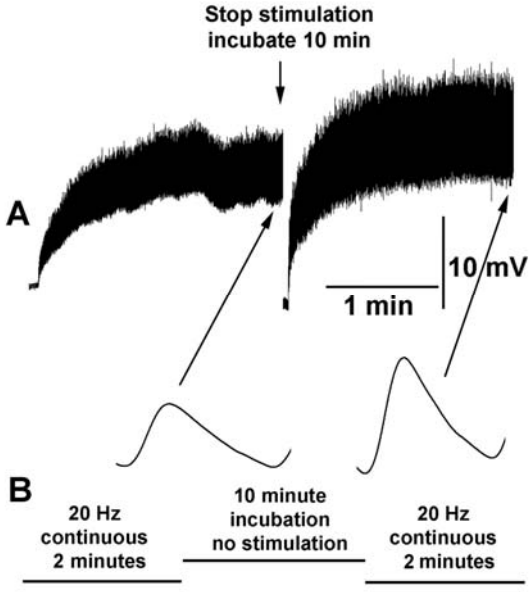

Figure 3: A constant stimulation paradigm. A second stimulation paradigm was to stimulate the motor neuron continuously for 2 min followed by incubation of levetiracetam for 10 min without stimulation. Afterwards the constant stimulation was repeated for 2 more minutes. (A) Illustrates the facilitated responses for each of the 2 min of stimulation with a 10 min break in between. (B) Representative EJPs at the end of each 2 min stimulation periods.

Results

The amplitudes of the EJPs tend to reach a plateau by the 25th stimuli when the nerve is stimulated at 40 Hz (Crider and Cooper, 2000; Desai-Shah et al., 2008; Cooper and Cooper, 2009). The evoked EJP responses on the opener muscle rapidly facilitate with repetitive stimulation as illustrated by a representative preparation (Figure 1). Thus, the amplitude of the 25th EJP within the stimulus train is used for assessment of the effect of levetiracetam on synaptic responses. Within 10 min of incubation in saline containing levetiracetam at 1 mM, the responses showed a gradual increase in amplitude after repetitive stimulation with pulsed trains (Figure 4). This paradigm was noted over a prolonged time frame of over 140 min after the static incubation without stimulation. With a prolonged stimulation time, there is a possibility of background facilitation which could be taking place despite the 10 s rest periods between each train. Thus, saline controls were performed over the same stimulation conditions. The saline controls and the lower concentration of levetiracetam (100 µM) did not show any significant change in the amplitude of the 25th EJP in the paradigm using trains of stimuli (Figure 5). However, the higher concentration of levetiracetam (1 mM) showed a significant increase in the amplitude of the 25th EJP compared to saline and the lower concentration of levetiracetam (Figure 5; $N=6$, $p<0.05$, ANOVA).

The second stimulation paradigm did not reveal any significant change in the average amplitude of EJPs as a before and after effect of exposure to levetiracetam compared to saline to saline control preparations ($N=6$, $p>0.5$, Student's T-test). As with the train stimulation paradigm, it was important to compare to saline only controls over the same experimental conditions (Figure 6).

Discussion

This study demonstrates that an incubation of levetiracetam at 1 mM for a

prolonged time followed by repetitive stimulation of pulse trains significantly enhanced synaptic responses. A lower concentration (100 µM) did not produce any significant changes in the amplitude of the EJPs from saline but was different to 1 mM in this paradigm. A second stimulation paradigm of continuous stimulation for 2 min, followed with a 10 min incubation and again continuous stimulation of 2 min, did not produce a significant effect in altering the amplitude of the

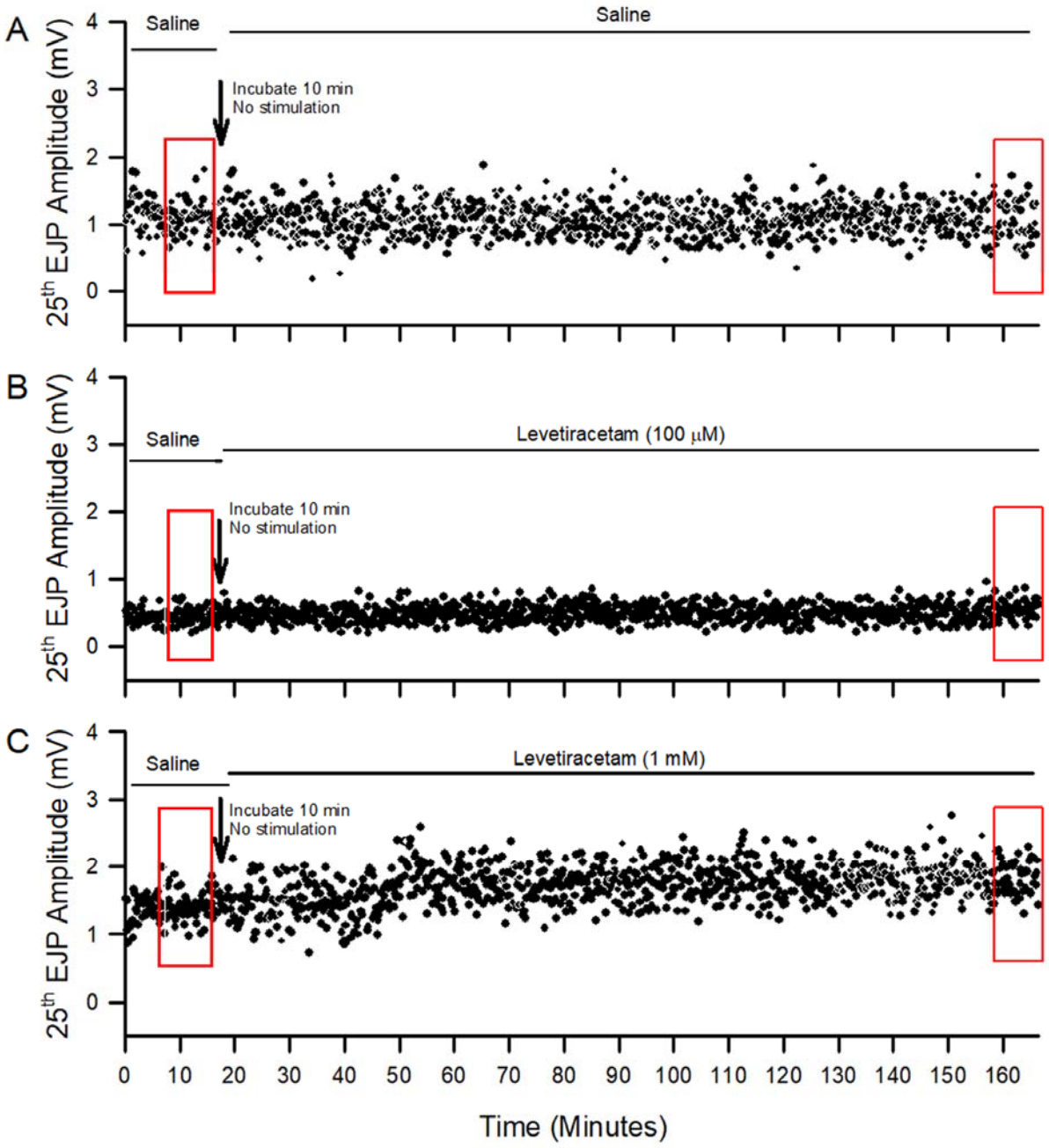

Figure 4: The effect of levetiracetam with a stimulus paradigm of pulse trains over a prolonged time. (A) Saline control experiments of the same stimulus conditions for examining the effects of levetiracetam at 100 µM (B) and 1 mM (C). The responses of the 25th amplitude of the EJP with the pulse trains is graphed over time. The average amplitude in the last 10 min of the initial saline exposure is obtained to compare to the last 10 min of response data after the 10 min in static incubation without stimulation followed by 130 min of return to the pulse stimulation paradigm as shown with the boxes over the data.

EJPs. This illustrates the effect of varying the stimulation conditions while testing the effects of compounds on synaptic transmission. This study did not support the hypothesis that levetiracetam would depress synaptic transmission in the crayfish NMJ. Thus, the actions of levetiracetam may work on other aspects of synaptic transmission than interfering with vesicle fusion, but perhaps promote synaptic transmission through yet a still unknown mechanism of action.

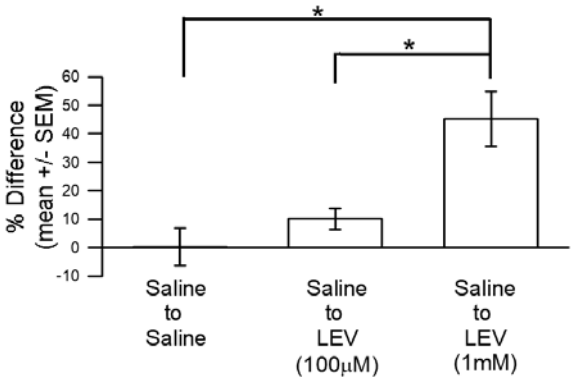

Figure 5: The percent differences in the EJP amplitudes for control saline and exposure to levetiracetam. The average percentage differences of the 6 preparations for each condition revealed that 1 mM exposure to levetiracetam resulted in a significant increase in the amplitude of the 25th EJP within the paradigm of pulse trains for the 100µM to 1 mM and from saline to 1 mM (N=6, $p<0.05$, ANOVA).

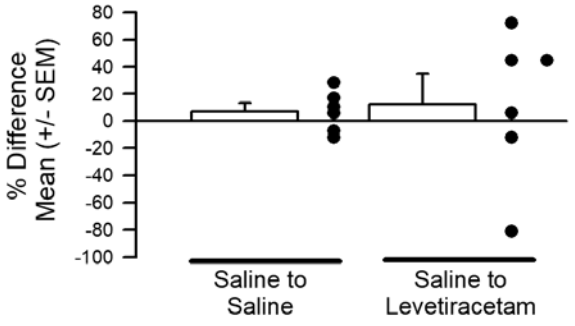

Figure 6: The effect of levetiracetam with the paradigm of continuous stimulation after 10 min of incubation. Saline to saline control experiments did not reveal any differences from saline to levetiracetam (1 mM) exposure. The mean (+/-SEM) percent differences in the average responses for each condition is shown along with the individual differences for each preparation as dots.

Considering that the mechanism of action for levetiracetam is not fully established but suggested to block presynaptic calcium channels (Vogl et al., 2012), or possibly be taken up into the presynaptic terminals to interfere with the evoked vesicle fusion process (Lynch et al., 2004), we expected a reduced synaptic transmission at the crayfish NMJ. The recent report of application of levetiracetam on the shape of action potentials in neurons of crayfish (Alshuaib et al., 2020) spurred this study to examine the effects on synaptic transmission at NMJs. The study of the effects of levetiracetam on the shape of action potentials of a motor neuron revealed that the effect was away from the nerve terminal, the location where it has been suggested to be taken up into the axon by synaptic vesicles during the fusion and recycling process (Meehan et al., 2011). There was a decrease in the amplitude of the action potentials in the inhibitory axon with an exposure time of 30 to 55 min with 100 µM. In addition, the study by Alshuaib et al., (2020) reported a decrease in postsynaptic inhibitory synaptic potentials. Thus, it would appear that the postsynaptic excitatory synaptic potentials would also show a decrease in amplitude. We predicted that there would also be a reduced amplitude in the evoked EJPs for the excitatory NMJ of the crayfish. However, Alshuaib et al., (2020) examined three preparations of the excitatory nerve and two of the three did not alter the amplitude of the EJPs or the shape of the action potentials in the excitor motor neurons. One can assume, the same period of exposure was used for the recordings of the EJPs and excitor motor neurons. In this study it took about 140 min to note a significant change in increasing the EJP amplitude with the paradigm of the pulse trains. It is likely the data set recorded with the second stimulation paradigm did not provide enough time for the effect of levetiracetam.

Future experiments could be conducted with more prolonged incubation conditions. However, it is important to use controls of saline only incubation over the same time frame of the investigation of the compounds being tested. The NMJs not only show short-term facilitation, which is due to residual calcium in the presynaptic terminal, but they also show long-

term facilitation (LTF) (Sherman and Atwood, 1971; Cooper and Cooper, 2009). Furthermore, there is a phenomenon which has been shown to occur for the high output NMJs which depresses synaptic responses, termed low-frequency depression, due to phosphorylation activity (Silverman-Gavrila et al., 2005). In speculation, it is possible that levetiracetam could block the inhibition of phosphatases 1A/2 inhibition and inhibit the depression of synaptic transmission. How levetiracetam is enhancing synaptic transmission remains to be discovered. Potentially the compound is enhancing LTF. Thus, if the mechanisms responsible for LTF (Lnenicka and Atwood, 1985) were blocked they may also alter the effect by levetiracetam. This remains to be examined in potentially future experimentation.

An additional future experiment could be to examine whether there is an increase in Ca^{2+} loading of the nerve terminal with calcium sensitive indicators when a high concentration of levetiracetam is used to accentuate the potential effects of the drug, even though this concentration may be higher than a therapeutic dose.

This study demonstrated that a prolonged incubation of levetiracetam at 1 mM results in promoting synaptic responses. A lower concentration did not have the same effect. A constant nerve stimulation, even at 1 mM exposure, was not adequate to enhance the synaptic response.

This project was conducted in a neurophysiology course to address novel experiments as educational course projects. This is referred to as ACURE (authentic course-based undergraduate research experiences; Malloy et al., 2017; Stanback et al., 2019; Wycoff et al., 2018), which is an approach that builds on the CURE (course-based undergraduate research experiences) philosophy (Bakshi et al., 2016; Linn et al., 2015). However, ACURE aids students in undergoing a more complete research experience. In addition, utilizing participants within a course setting to analyze data sets blind to the experimental conditions provides an additional level for interpretation of the findings.

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