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14. ABSTRACT We developed electrophysiological, two-photon laser scanning microscopic imaging and pharmacological tools to investigate the effects of levetiracetam, topiramate and carbamazepine on excitatory (glutamatergic) synaptic transmission and vesicular transmitter release at multiple synapses in <i>in vitro</i> brain slices from control and pilocarpine-treated epileptic rats and mice. We discovered that levetiracetam was more effective in reducing the frequency of excitatory synaptic transmission onto dentate granule cells in slices from chronically epileptic rats, while no significant effect was detected in the amplitude of mEPSCs, indicating a presynaptic site of action without postsynaptic effects on AMPA glutamate receptors. These data correlated well with findings in imaging experiments that LEV was more effective in suppressing the enhanced vesicular release of glutamate from mossy fiber terminals in field CA3 of epileptic mice, compared to non-epileptic control animals. These data indicate that presynaptically acting drugs such as levetiracetam may become a key piece in the arsenal of antiepileptic drugs in mesial temporal lobe epilepsy, and highlight the need for preventing the downregulation of sensitivity to levetiracetam observed with chronic administration in some patients. Thus, screening for a presynaptic site of action and assessment of chronic tachyphylaxis of presynaptic actions will be important to the discovery of novel and effective antiepileptic drugs.					
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2013-14 PROGRESS REPORT: DoD Grant - PR100534P1

Title: *New Treatments for Drug-Resistant Epilepsy that Target Presynaptic Transmitter Release*

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INTRODUCTION

Posttraumatic epilepsy is a major long-term complication of traumatic brain injury (TBI), that usually develops within 5 years of head injury (Schauwecker, 2012), and is often expressed as medically intractable hippocampal epilepsy (Turski et al., 1984; Muller et al., 2009). Posttraumatic epilepsy can develop after penetrating or severe non-penetrating brain injury. Although there are a variety of causes of traumatic epilepsy, the resulting chronic neurological condition is characterized by common features, including recurrent spontaneous seizures, neuronal damage, and, in ~30% of mesial temporal lobe epileptic (MTLE) patients, resistance to all available anticonvulsant drugs (Li et al., 2005; Burrone et al., 2006). ***Therefore, it is of critical importance to develop novel models to study post-traumatic epilepsy, to facilitate discovery of new treatments.*** During epileptogenesis, seizure-related functional and structural reorganization of neuronal circuits leads to both hyperexcitability of glutamatergic neurons and defective inhibition (Mello et al., 1993; Upreti et al., 2012). While many postsynaptic alterations have been demonstrated, there is surprisingly little known concerning dysfunction of presynaptic transmitter release machinery in epilepsy. The recent successful introduction of the antiepileptic drug levetiracetam (LEV; Boulland et al., 2007), which acts on presynaptic molecular targets, suggests that controlling dysregulation of presynaptic function could be a promising new therapeutic target for treatment of unresponsive epilepsies. While LEV binds to both the synaptic vesicle protein SV2a and N-type Ca^{2+} channels (Epsztein et al., 2005; Esclapez et al., 1999; Pacheco Otalora et al., 2006), its precise mechanisms of action are not understood.

BODY**HYPOTHESIS AND OBJECTIVES:**

During periods of intense neuronal activity such as seizures, a larger pool of vesicles could result in more glutamate being released and long-lasting aberrant excitation. We propose to explore the effects of seizures on transmitter release and the presynaptic action of AEDs on these changes. We will use electrophysiology and multiphoton confocal microscopy. Preliminary data indicate that SE induces long-lasting potentiation of synaptic vesicle release in epileptic rats. We hypothesize that successful AED treatment might prevent or reverse these seizure-induced molecular deficiencies (reduction of N-type VGCC, mGluR II and SV2a expression), and be antiepileptogenic as well. Our **central hypothesis** is that pharmacological regulation of glutamate transmitter release at presynaptic sites will be an effective, novel therapeutic strategy to ameliorate epileptogenesis and excessive synaptic excitation in epilepsy. The **long-term objectives** of this collaborative proposal are to: (1) identify the most effective AEDs which modulate presynaptic glutamate release, and (2) determine the presynaptic mechanism of action of the new AED LEV to modulate vesicular release properties. *Our central hypothesis is that pharmacological regulation of glutamate transmitter release at presynaptic sites will be an effective, novel therapeutic strategy to treat many cases of drug-resistant epilepsy, especially epileptogenesis following traumatic brain injury.* The **long-term goals** of this collaborative project are to: (1) identify the most effective antiepileptic drugs amongst compounds that modulate presynaptic glutamate release and (2) determine the presynaptic mechanism of action of the new antiepileptic drug **levetiracetam (LEV)**. ***In Year 3 of this proposal, we made meaningful progress on experiments with specific anticonvulsant drugs in the remainder of specific Aims 2 and 3 tasks that form our portion of year 03 of the collaborative project, as outlined below. We will fully complete the remaining experiments in the no-cost extension year.***

YEAR 03 Stanton Lab

Specific Aim 2: *Assess whether antiepileptic drugs acting on presynaptic sites can reduce or prevent seizure-induced long-term enhancement of vesicular release from mossy fiber boutons in MTLE.*

Working hypothesis: Epileptic rats exhibit enhanced pool size and release probability from the rapidly-recycling vesicle pool, and SV2a down-regulation contributes to this enhanced release. Chronic treatment with LEV or other presynaptic antiepileptic drugs during epileptogenesis will protect presynaptic function and normal glutamate release, reducing or preventing seizures. (October 1, 2012–September 30, 2013 = 12 months).

This aim will consist of: (a) chronic treatment with antiepileptic drugs after pilocarpine injection (b) two-photon confocal imaging of FM1-43 release from readily-releasable and total vesicle pools, and (c) two-photon imaging of vesicle release, recycling, and exchange rates of vesicle pools in pilocarpine-treated epileptic mice expressing synaptopHluorin (SpH), a genetically-encoded fusion protein of the vesicle protein Vesicular Associated Membrane Protein (VAMP2) and pH-sensitive Enhanced Green Fluorescent Protein (EGFP).

Research Goals:

Task 1. Assess the effect of chronic treatment with antiepileptic drugs acting on presynaptic glutamate release (LEV and other drugs characterized in Specific Aims 1) investigated by two-photon imaging of vesicular release of FM1-43 from individual mossy fiber terminals in hippocampal slices from non-epileptic and epileptic rats.

- **Subtask 1c.** Prepare hippocampal slices from control and epileptic rats chronically treated with antiepileptic drugs versus vehicle (Dr. Stanton and Dr. Zhang; months, 17-24).
- **Subtask 1d.** Image presynaptic release of FM1-43 from individual mossy fiber to test whether chronic treatment protects the kinetics of transmitter release in control versus epileptic rats (Dr. Stanton and Dr. Zhang; months 17-24).
- **Subtask 1e.** Statistical analysis of the experimental data (Dr. Stanton and Dr. Zhang, Months 17-24)

Task 2. Assess the effects of chronic treatment with antiepileptic drugs (identified in Aim 1) acting on presynaptic glutamate release on presynaptic transmitter release investigated by two-photon imaging of vesicular release, recycling, and exchange rates between vesicle pools in control and epileptic SpH mice treated with antiepileptic drugs.

- **Subtask 2d.** Image presynaptic release from individual mossy fiber presynaptic terminals to test whether chronic treatment protects normal kinetics of transmitter release in epileptic SpH mice (Dr. Stanton and Dr. Zhang; months 19-24).
- **Subtask 2e.** Statistical analysis of electrophysiology data (Dr. Stanton and Dr. Zhang, months 19-24).

Aim 2, Subtasks 1.c-e. Test whether chronic treatment with antiepileptic drugs prevents presynaptic dysfunction in chronic epilepsy

Experiments are nearing completion on the effects of acute administration of levetiracetam (LEV) on presynaptic vesicular transmitter release from excitatory Schaffer collateral terminals in hippocampal field CA1 using multiphoton laser scanning confocal imaging analysis of presynaptic release in both control and pilocarpine-induced epileptic transgenic SpH-expressing mice. In addition, the pilocarpine model of mesial temporal lobe epilepsy (MTLE) has been optimized in SV2A/SV2B knockout mice in the laboratory of Dr. Garrido.

LEV reduces enhanced vesicular release from mossy fiber boutons of chronically epileptic SpH-expressing transgenic mice expressing SpH at excitatory glutamatergic terminals

Levetiracetam (Keppra®, LEV) is a new class of antiepileptic drug exhibiting selective seizure protection in chronic animal models of epilepsy. LEV binds to the synaptic vesicle protein SV2A and can modulate excitatory transmission by a mechanism depending on the inhibition of presynaptic Ca^{2+} channels. It is also known that LEV targets SV2A expression of which is reduced by epileptogenesis in animal models and epileptic patients suffering mesial temporal lobe epilepsy (MTLE). By using 2-photon laser scanning confocal microscopy, we tested whether LEV was effective in reducing enhanced vesicle release in mossy fibers from control and epileptic transgenic SpH21 mice expressing synaptophysin (SpH) in mossy fiber boutons. Expression changes in SV2A, SV2B and SV2C were also analyzed in the laboratory of Dr. Garrido using immunofluorescence, western blotting and real-time quantitative PCR (qPCR).

To address the hypothesis of specific aim 2, we investigated whether chronic treatment with LEV (Kepra) can reduce the abnormally enhanced presynaptic release of vesicles in the mossy fiber glutamatergic pathway. Presynaptic transmitter (vesicle) release was investigated in 4 groups of SPH transgenic mice as follows: a) control mice injected with saline vehicle for 4 weeks (control no treatment=C-NT, n=5), b) pilocarpine-treated SpH mice that developed *status epilepticus* injected with saline (*status epilepticus* no treatment=SE-NT, n=5), c) control SpH mice injected with levetiracetam (see below) (control treated=C-T, n=4), and d) pilocarpine-treated SpH mice that suffered *status epilepticus* and were treated subsequently with levetiracetam intraperitoneally (see below) (*status epilepticus* treated= SE-T, n=4). The treatment schedule for levetiracetam and saline administration consisted of repetitive injections (100 µg/kg) in alternate days during 30 days. After the end of the treatment period, animals were sacrificed for a preparation of brain slices to measure changes in presynaptic transmission and to collect mRNA samples. Similar groups of animals were sacrificed for Dr. Garrido to measure changes in expression of SV2A, SV2B, and SV2C protein. Blood samples were collected (at endpoint) to measure plasmatic levels of levetiracetam using high-pressure liquid chromatography in treated versus non-treated groups.

Results: In experiments performed in collaboration with Dr. Garrido's laboratory, four different groups of animals were analyzed to determine whether chronic treatment with levetiracetam *in vivo* may reduce presynaptic vesicular transmitter release in the mossy fiber pathway from granule cells in hippocampus of pilocarpine-treated epileptic SPH transgenic mice. Changes of SpH fluorescence were induced by a train of 600 action potentials delivered to the mossy fiber pathway using a bipolar stimulating electrode. Images of stimuli-induced SpH fluorescence changes were detected using laser scanning confocal microscopy (see above). We first compared normalized peak fluorescence changes after stimuli in control versus pilocarpine-treated (suffering status epilepticus) group of animals that were injected with saline instead of levetiracetam for 1 month (**Figure 1A**). We detected a significant 4.4% increase in normalized peak fluorescence in status epilepticus (SE) group ($F_{\text{peak}} = 119.25 \pm 2.13\%$, $n = 4$, 106 boutons, 7 slices) when compared to saline-injected control group ($F_{\text{peak}} =$

114.18 ± 1.19 %, n = 5, 112 boutons, 10 slices) (**Figure A, b3**). These data is consistent with our previous findings that status epilepticus induce an abnormal increase in presynaptic vesicular release as measured by SpH fluorescence changes in transgenic mice (**Upreti et al., 2012**).

To determine if chronic treatment with levetiracetam can ameliorate or prevent such increase in presynaptic vesicular release, we treated pilocarpine-injected animals with levetiracetam (i.p, dose: 100 µg/kg) during one month immediately following *status epilepticus* and compared this group to control animals treated with a similar drug administration protocol. Analysis demonstrated no significant differences in of normalized peak fluorescence changes after stimulation of mossy fibers between these groups, indicating that chronic treatment with levetiracetam corrected presynaptic function abnormalities previously detected after *status epilepticus* (**Figure 1B**). (Plasma concentration of levetiracetam after treatment was assessed by high-pressure chromatography (HPLC) assays. No significant changes were detected between controls (57.1 ± µg/ml) versus and *status epilepticus* groups (47.9 ± 1.5 µg/ml, Student t-test, p > 0.05)).

Conclusions: Chronic treatment with LEV after *status epilepticus* can reverse abnormally enhanced presynaptic vesicular glutamate release at mossy fibers that may be responsible for epileptogenesis and hyperexcitability in mesial temporal lobe epilepsy. Accordingly, reduction in abnormally enhanced presynaptic vesicular release is possibly the main mechanisms of action of levetiracetam. Data in experiments from Dr. Garrido's lab indicate this effect may be mediated by LEV-induced up-regulation of SV2A that protects against *status epilepticus*. Further experiments will be performed in the no-cost extension period to elucidate how LEV during status protects vesicular release, by altering release probability and/or vesicle recycling.

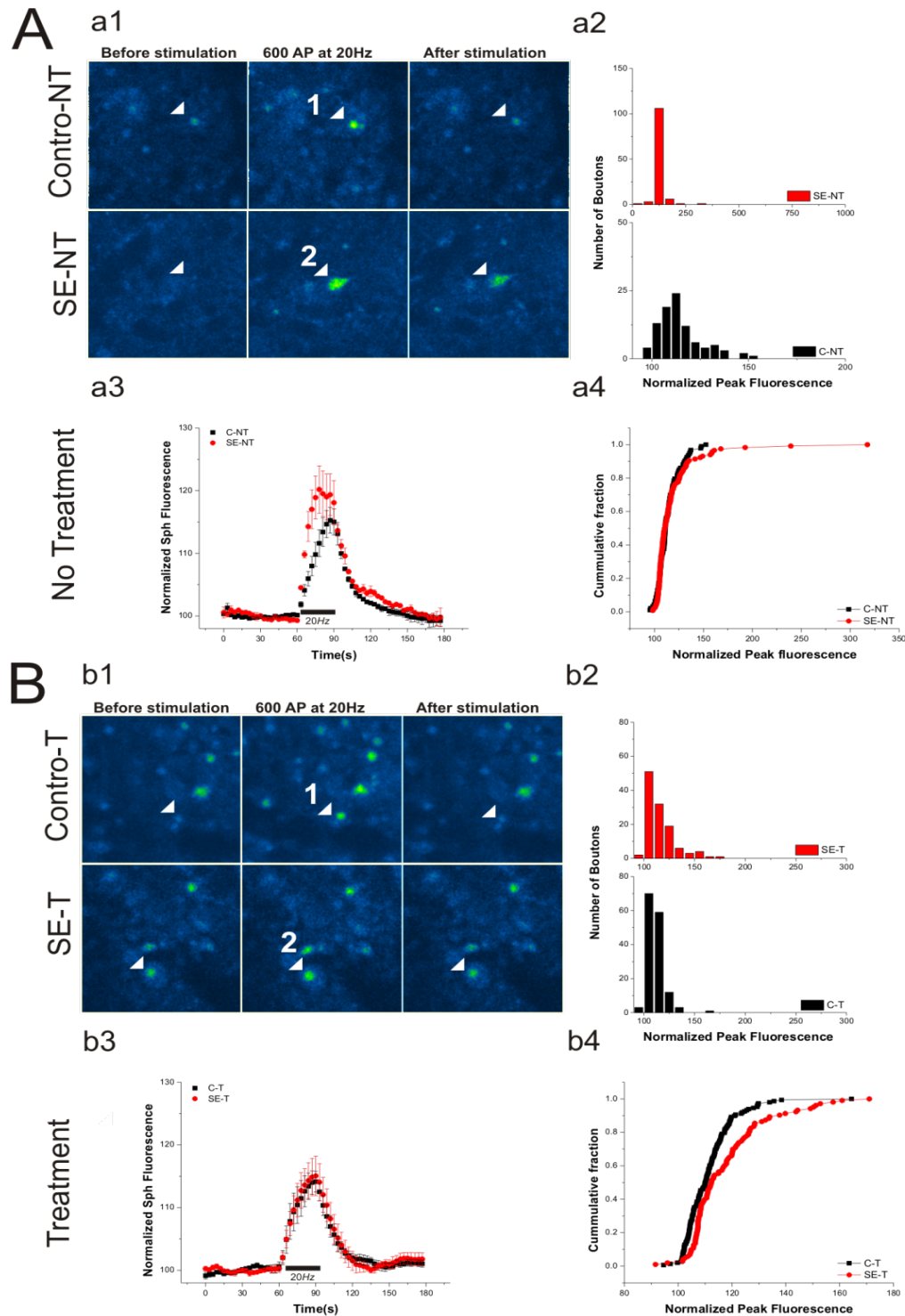


Figure 1. Effect of levetiracetam on the pilocarpine model to induced *status epilepticus* (SE) in SpH transgenic mice. **A.** *Stimuli-induced changes in presynaptic vesicular release at mossy fiber boutons in control and pilocarpine-treated SpH transgenic mice (no-treatment).* **a1.** Representative time-lapsed confocal images from control and post-*status epilepticus* SpH-expressing mossy fiber boutons MFBs in the proximal apical dendritic region of field CA3 in hippocampal slices of saline-treated control versus mouse one month after suffering SE treated with saline vehicle (lower row). First column: baseline imaging, second column: imaging during 600 action potential train stimulation, last column: recover of fluorescence changes 10 sec after end of stimulation. Notice larger increase in fluorescence changes after SE (compared arrowhead 1 to 2). **a2.** Frequency distribution histogram of normalized peak SpH fluorescence for all mossy fiber boutons after stimulation in control (black) compared to *status epilepticus* (SE) group (red). Notice a change in the distribution pattern,

specifically a large group of mossy fiber boutons that release more than 200% increase of baseline after *status epilepticus* while peak fluorescence changes in mossy fiber boutons from control saline-treated animals follow a normal distribution. **a3.** Normalized, evoked SpH fluorescence increases in response to a 600 pulse/20 Hz mossy fibre stimulus train, in MFBs from control (filled black circles, $F_{\text{peak}} = 114.18 \pm 1.19\%$, $n = 5$, boutons=112, 10 slices) versus post-status epilepticus (open red circles, $F_{\text{peak}} = 119.25 \pm 2.13\%$, $n = 4$, boutons=106, 7 slices). F_{peak} was significantly increased in post-status epilepticus slices ($P < 0.05$, Student's *t*-test; all values mean $\pm$ SEM). **a4.** Cumulative histogram distribution of normalized peak fluorescence changes in control (black) versus SE group (red) showing a left shift and a significant difference towards larger fluorescence peak changes (more release) in slices from post-status epilepticus animals ($D = 0.23$, $Z = 0.0305$, $p < 0.00197$, statistical comparisons using Kolmogorov-Smirnov test). **B. Chronic treatment with Levetiracetam (30 days period) after pilocarpine-induced status epilepticus normalized abnormally enhanced vesicular release at mossy fiber boutons.** **b1.** Time-lapsed images from representative experiments in slices from levetiracetam-treated control and epileptic SpH transgenic mice. Solid arrows indicate puncta corresponding to SpH-positive mossy fiber bouton that showed activity-dependent fluorescence changes during a 600 pulse/20 Hz stimulus train. **a2.** Frequency distribution histogram of normalized peak fluorescence for pooled mossy fiber boutons in control (black) versus pilocarpine-treated mice (red) chronically treated with levetiracetam after *status epilepticus*. **b3.** Representative time course of normalized, evoked SpH fluorescence increases in response to a 600 pulse/20 Hz mossy fibre stimulus train, in MFBs from control (filled black circles, $F_{\text{peak}} = 115.09 \pm 0.67\%$, $n = 3$, boutons=148, 9 slices) versus post-status epilepticus (open red circles, $F_{\text{peak}} = 116.25 \pm 1.27\%$, $n = 3$, boutons=106, 6 slices). F_{peak} was not significantly changed in levetiracetam-treated post-status epilepticus animals ($P > 0.05$, Student's *t*-test; all values mean $\pm$ SEM). **b4.** Cumulative frequency histogram of normalized peak SpH fluorescence between the levetiracetam-treated control and SE group (red). Kolmogorov-Smirnov two-sample test indicated a significant difference between both groups ($t = 2.7$, $DF = 18$, $p = 0.014$).

Research Goals:

The experiments in the remaining tasks below are currently underway to assess and compare the effects of acute versus chronic administration of LEV on excitatory synaptic transmission, presynaptic vesicular glutamate release and vesicular recycling rates in pilocarpine-induced epileptic rats and mice, and are expected to be completed in this no-cost extension year. This extension is critical because, in the past year, the two-photon laser scanning microscope in the NYMC core facility had to be disassembled and upgraded, which cost 4 months of down time for two-photon imaging. The TPLSM is now operational and, in fact, exhibits a 40% increase in sensitivity. Rats and SpH (Sp21 strain) expressing mice are in process of being chronically treated with therapeutic doses of LEV to examine release properties after 3-6 months of administration, and will soon be available for experiments. We will compare the ability of LEV versus carbamezipine administered during the silent epileptogenic period following pilocarpine-induced seizures, with the acute effects on glutamate release of LEV and carbamezipine when bath applied *in vitro* to slices taken from epileptic versus control mice. During this no-cost period, we plan to prepare and submit two full length publications to journals of high impact similar to our first publication from this work (Upreti et al., 2012), which was published in the internationally renowned journal *Brain* (5-Year Impact Factor 10.87). In addition, we have now successfully established a colony of the Sp64 strain of SpH-expressing mice, which express the vesicular release indicator synaptophluorin selectively in *GABAergic* inhibitory interneurons, which will allow us to compare the effects of LEV on excitatory and inhibitory presynaptic terminal release of glutamate versus GABA in control and epileptic mice.

Task 2. Assess the effect of acute versus chronic treatment with different antiepileptic drugs (LEV versus carbamezipine) acting on presynaptic glutamate release on presynaptic transmitter release investigated by two-photon imaging of vesicular release, recycling, and exchange rates between vesicle pools in control and pilocarpine-treated epileptic SpH mice treated with antiepileptic drugs.

- **Subtask 1b.** Systemic administration of antiepileptic drugs to different mice groups, versus vehicle controls, following pilocarpine administration. We will test chronic treatment with LEV versus carbamezipine (Dr. Stanton and Dr. Zhang; months 24-36).
- **Subtask 1c.** Prepare hippocampal slices from control and epileptic SpH mice chronically treated with antiepileptic drugs (Dr. Stanton and Dr. Zhang; months, 24-36).
- **Subtask 1d.** Image presynaptic release of FM1-43 from individual mossy fiber presynaptic terminals to test whether chronic treatment protects normal kinetics of transmitter release in epileptic SpH mice (Dr. Stanton and Dr. Zhang; months 24-36).
- **Subtask 1e.** Statistical analysis of the experimental data (Dr. Stanton and Dr. Zhang, Months 30-36)

KEY RESEARCH ACCOMPLISHMENTS:

- Discovery that chronic treatment of animals with levetiracetam (LEV) is effective in inhibiting the development of abnormally enhanced presynaptic vesicular release of glutamate from mossy fiber terminals of dentate granule neurons if applied during the silent post-seizure period after pilocarpine-induced *status epilepticus*.
- This finding correlates well with the discovery by Dr. Garrido's laboratory that chronic treatment with LEV, if applied during the silent post-seizure time window, also increased protein expression of its molecular SNARE protein target SV2A in mossy fiber terminals, and reduced seizure-induced increases in SV2C (gene expression of sv2c by qrtPCR) in hippocampus after pilocarpine-induced *status epilepticus*.
- The combined data from the Garrido and Stanton laboratories indicate that LEV can be effective in counteracting pro-epileptogenic changes in transmitter release and presynaptic molecules if administered shortly after a seizure insult (status epilepticus) that would otherwise lead to chronic epilepsy.
- Commenced studies using 2-photon laser scanning microscopy will evaluate the effects of acute versus chronic repeated administration of LEV and carbamezipine on vesicle release probability compared to rates of vesicular recycling in the readily-releasable vesicle pool.

REPORTABLE OUTCOMES:

National Meetings

Data from electrophysiological and pharmacological experiments were presented in 1 scientific meeting in 2013 (1-2) and new data has been submitted for presentation at another two meetings in 2014 (3-4).

1) *Inhibitory action of levetiracetam on CA1 population spikes and dentate gyrus excitatory transmission in pilocarpine-treated chronic epileptic rats.* **E. G. Sanabria**, L. Pacheco, J. Zavaleta, F. Shriver, L. M. Rambo, C. Upreti, **P. K. Stanton**. Society for Neuroscience Meeting in San Diego, CA, Nov 9-13, 2013

2) *Synaptic Vesicle Protein Expression in Sprague-Dawley Rats Treated with the Pilocarpine Model for Mesial Temporal Lobe Epilepsy.* Mayra Velazquez, L.F. Pacheco, **E. R. Garrido-Sanabria**. 25th Anniversary Conference, HENAAC Conference, October 3 - 5, 2013, New Orleans.

3) *Chronic treatment with levetiracetam upregulate SV2A and reduce abnormally augmented presynaptic vesicular release after pilocarpine-induced status epilepticus.* Luis F. Pacheco, Vinicius Funck, Nuri Ruvalcaba, Jose M. Rodriguez, Daniela Taylor, Rubi Garcia, Jason Zavaleta, Chirag Upreti, **Patric K. Stanton**, **Emilio R. Garrido-Sanabria**, the Military Health System Research Symposium, to be held on August 18-21, 2014.

4) *Treatment with levetiracetam ameliorates abnormal presynaptic vesicular release and altered presynaptic protein expression in a glutamatergic pathway after pilocarpine-induced status epilepticus.* **Emilio R. Garrido-Sanabria**, Luis F. Pacheco, Vinicius Funck, Nuri Ruvalcaba, Jose M. Rodriguez, Daniela Taylor, Rubi Garcia, Jason Zavaleta, Chirag Upreti, **Patric K. Stanton**. This abstract will be submitted to the 68th Annual Meeting of the American Epilepsy Society, Dec 5-9, 2014, Seattle, Washington.

CONCLUSIONS

In year 03, we completed the majority of our studies in Aim 1. We found that severe pilocarpine-induced seizures that result in long-term appearance of spontaneous epileptic seizures and enhanced presynaptic transmitter release upregulate the sensitivity of transmitter release to the anticonvulsant agent levetiracetam (LEV). Using two different fluorescent indicators to image, by 2-photon laser scanning microscopy, individual mossy fiber release sites in the hippocampal CA3 field, we found that vesicular glutamate release that is markedly enhanced in epileptic animals is significantly suppressed by acute administration of LEV. We have made significant progress in studies in Aim 2, showing that chronic administration of LEV during the silent post-seizure period when epileptogenesis occurs can prevent alterations in transmitter release, and these studies will be completed in this no-cost extension year, to test whether chronic administration of LEV or carbamezipine) during the post-pilocarpine development of the epileptic brain, can retard or prevent epileptogenesis, and if LEV is acutely anticonvulsant once an epileptic state has been established.

Recommended change: Now that we have begun implementing the pilocarpine seizure model in transgenic mice where the vesicle protein SV2A has been knocked out, we plan to determine whether a loss of SV2A and dynamin interaction might be responsible for seizure-induced enhancement in glutamate release, by examining the ability of the dynamin-inhibiting peptide dynasore to alter recycling in SV2A knockout versus wild-type mice. If we observe tachyphylaxis (desensitization) of anticonvulsant activity of LEV with prolonged administration, we propose to add experiment groups where multiple anticonvulsants are administered in an alternating paradigm, to determine whether LEV potency can be maintained and is correlated with prevention of the down-regulation of expression of SV2A.

Significance: While all but one antiepileptic drug acts by modifying postsynaptic neuronal excitability, our work indicates that extensive changes in presynaptic function are also associated with epilepsy. Increases in glutamate release, while certainly able to contribute to hyperexcitation in seizures, also have the potential to damage and even kill their postsynaptic target neurons. If we can find agents that can control presynaptic release, and do so even after development of epilepsy when release is enhanced, we would have a whole new class of treatments to help the 40% of epileptic patients who do not respond to any current therapeutics. Understanding the mechanisms by which seizures change presynaptic function is the essential first step towards this goal.

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