On the Development of a Technique to Isolate Newly Recycled Synaptic Vesicles for the Purpose of Studying the Mechanism of Action of the Anticonvulsant Drug Levetiracetam

Jacob T. Hyer

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

University of Washington

2015

Program Authorized to Offer Degree:

Department of Pharmacology

TABLE OF CONTENTS

Page

List of Figures

Number Page

List of Abbreviations

AED----------Anti-epileptic drug

Lev----------Levetiracetam

EEG----------Electroencephalogram

GABA----------γ-Aminobutyric acid

TCA----------Trichloroacetic acid

BSA----------Bovine serum albumin

DMEM----------Dulbecco's modified Eagle's medium

Dyn----------Dynamin

Mfn2----------Mitofusin 2

P38----------Synaptophysin

P65----------Synaptotagmin

SV2----------Synaptic vesicle glycoprotein 2

MS----------Mass spectrometry

Acknowledgements

I thank all those who have helped me on my graduate school adventure: my family for their love, encouragement, and valiant efforts to understand my research; my Seattle friends for the their incalculable benefit of allowing me to come up for air from the depths of academia; my classmates for the camaraderie and peer pressure; my labmates for their company, insight and entertainment; and the Department of Pharmacology for providing the venue and facilitating the details of this endeavor. Lastly, I would be remiss if I did not express my deepest gratitude towards my advisor, Sandra Bajjalieh, for her support, her advice, her anecdotes and for setting a vibrant example of passion for science.

Chapter 1: Introduction

Epilepsy

Epilepsy is a neurological disorder characterized by increased risk for spontaneous seizure activity in the brain, when excitatory neurotransmission becomes abnormally synchronous. The prevalence of epilepsy is about 1% in the global population. About 60% of cases have an unknown etiology. Epilepsy is highly heritable, but it is such a diverse disorder that there are many different ways that it can be inherited. There is evidence for autosomal dominant and recessive inheritance as well as X-linked and finally, polygenic inheritance[1]. In addition, epilepsy can be caused by injury or illness. About 30% of people with epilepsy are unable to manage their seizures with current drug therapies[2]. Since the mid-nineteenth century when the first antiepileptic drugs (AEDs) were discovered, pharmaceuticals have represented the standard treatment for epilepsy, but the prevalence of intractable presentations of the disease results in the need for patients to pursue alternative management strategies, including highly restrictive diets, invasive brain surgeries, therapeutic brain stimulation[3, 4] and even Mozart therapy[5].

Epilepsy was recognized as a disease as far back as ancient Mesopotamian civilizations. The Babylonian Code of Hammurabbi, dating back to the eighteenth century BC, grants a refund for a slave that is found to exhibit *bennu* with in a month of purchase[6]. This disease was related to *antasubbu* or “the hand of Sin,” which a 3,000 year old Accadian text describes as an attack with convulsions of the neck, limbs and face, foaming at the mouth, wide open eyes and loss of consciousness[6]. The Hippocratic Corpus contains a book written around 400 AD called *On the Sacred Disease*, which is mainly concerned with arguing against popular superstition peddled by magicians, wizards and charlatans. The author, who likely was not Hippocrates himself, recognized that epilepsy was hereditary, and believed it to be disease

with a physical cause like any other, with its etiology arising from a malfunctioning brain[6]. Granted, his explanations for this phenomenon were based on humorism, citing an excess of phlegm from the brain that rushed into the body and caused the attacks, but this does not lessen the impact of a first surviving recorded attempt to explain a partially psychological disease in terms of physical causes as opposed to the supernatural influences of gods, spirits or demons.

The recognition of epilepsy as a disease of the brain, or at least of the body, was important, as this emphasized attempts at medical treatments over the superstitions and incantations that were offered by magicians. Despite a rejection of superstition, the Greek physicians were still religious, and believed that the god Asclepius, whose snake entwined staff adorns many an ambulance today, could be invoked to help cure epileptic patients. They also borrowed from popular and magical remedies, providing rational explanations for their mechanism of effect and being unable to refute the apparent effectiveness of such remedies through observation alone. In this way, the physicians were pragmatic, accepting remedies and treatments that appeared to work, while rejecting supernatural explanations for their effects.

The Greek physicians of antiquity made several important observations regarding the treatment of epilepsy. One major focus of their interventions was a strict dietetic regimen. These dietetic strategies are the root of a modern day treatment strategy called the ketogenic diet. Another interesting observation made by these physicians was the preventive, ameliorative or even curative effect of quartan fever on the severity of epileptic disease. Finally, the Hippocratic physicians were aware of the importance of early intervention into epileptic disease, recognizing that early stages could progress to a more severe and intractable presentation of the disease. Epilepsies in children, they observed, might abate at the onset of puberty, but if they had not been cured or otherwise ended by puberty, seizures would likely persist and increase in frequency for the rest of the patient’s life.

The first modern pharmaceutical treatment for epilepsy was presented by Sir Charles Locock in 1857. His observation was that women with 'hysterical epilepsy' (now known as catamenial epilepsy) responded to treatment with potassium bromide. This treatment often resulted in bromism, a condition caused by bromine toxicity and characterized by rashes, gastrointestinal effects like anorexia and constipation, and psychiatric effects ranging from irritability to psychosis to coma[7]. Despite the side effects, bromides were effective and it was theorized that their benefit was the result of sedative effects on the heart's action[8]. Bromides were the standard of care, often in combination with agents such as digitalis, belladonna, zinc and iron[9]. Subsequently, the anticonvulsant properties of Phenobarbital were discovered by Albert Hauptmann in 1921 when he administered the barbiturate drug to his patients as a tranquilizer[7]. Phenobarbital was found to be more effective than bromides, and with a better side effect profile, it became the standard of care, although it was sometimes used in combination with bromides[10].

The next major AED to be discovered was phenytoin, which came out of one of the first screens using an animal model of seizures. Drs. Houston Merritt and Tracy Putnam found phenytoin to be effective in reducing electrically induced seizures in cats[11]. This marked the beginning of the use of animal seizure models in the development of anticonvulsant drugs. Phenytoin was effective at dosages that did not produce the sedative effects, while bromide and Phenobarbital needed to be administered at doses that prevented the cat from walking in order to raise their convulsive threshold[11]. Phenytoin also proved effective at treating more forms of epilepsy than these earlier compounds[12]. Despite these benefits, Phenytoin treatment also had drawbacks, including gross anatomical changes such as gingival hyperplasia and coarsening of facial features[13], as well as non-ideal pharmacokinetic properties including complicated drug interactions and a non-linear dose-serum concentration relationship due to saturable rate of metabolism[14]. Delayed effects such as carcinogenicity and teratogenicity are also a major concern for this drug[15-17].

Strategies developed for the treatment of epilepsy are generally geared towards dampening synaptic excitability in order to prevent the run-away neuronal activity that results in seizures. This means that most anti-convulsants have a sedative effect, due to global suppression of synaptic activity. Sedative side effects are a major reason that patients do not follow though with treatments, and therefore drugs with a more tolerable side effect profile would likely be more effective.

Therapies that treat neurological disorders suffer from a dearth of information on exactly how brains function. At its most basic unit, the brain is a collection of neurons that are communicating in concert. This communication is known as neurotransmission, or the process by which one neuron communicates with another (or many others). Chemical signals received by the dendritic arbor of a neuron serve to increase or decrease the probability that a neuron will fire an action potential that releases neurotransmitter into the synapses of its downstream connections. Excitatory neurotransmitters depolarize the neuron, pushing the transmembrane potential closer to the threshold required to initiate the chain reaction called an action potential that travels down an axon and results in release of neurotransmitter. Inhibitory neurotransmitters do the opposite, hyperpolarizing a neuron in order to decrease the liklihood that an action potential will be triggered. These conflicting signals are integrated at the cell body.

A greater understanding of the processes occurring at the synapse could contribute a great deal to our understanding of this disease. In addition, lev has shown some promise as a therapeutic for behavior disorders, and therefore the utility of lev-related compounds need not be limited to the treatment of epilepsy[18]. This research has the potential to uncover new drug targets in the synapse, help direct refinement of lev-related compounds and deepen our understanding of the brain.

Levetiracetam

Levertiracetam is an approved first-in-class AED from UCB, marketed as Keppra. It was approved by the FDA in 1999, and currently represents the most prescribed AED on the market, making up nearly a quarter of the market share for AEDs. Sales peaked in 2008 at $1.4 billion and in 2014, net sales were $726 million[19]. Keppra is not likely to lose much market share to erosion from the generic market, as neurologists in general are wary of switching patients to generic formulations. A 2011 study of 4 patients who switched to generic lev from Keppra showed that all for patients experienced an increase in seizure activity that was abolished upon switching them back to the branded drug[20]. Personally, I would be interested in a study comparing the effect of a true switch to a sham switch. Most studies comparing generic drugs to branded drugs are retrospective. In epilepsy, in particular, any single seizure carries a certain risk of lethality, and therefore studies have a responsibility to patients not to expose them to undue risks.

Levetiracetam was originally synthesized as an ethyl derivative of piracetam. Piracetam is an analog of cyclized γ-aminobutyric acid (GABA), and was one of the first compounds described as nootropic, meaning that it has neuroprotective, anticonvulsant and cognitive enhancement effects without the properties of a stimulant or sedative. Lev was originally tested in models of cognitive impairment in hopes that it would have better nootropic properties than piracetam, but this proved not to be the case[21]. A screen for anticonvulsant effects in audiogenic seizure-prone mice led to the discovery that lev had potent anticonvulsant activity, which held true over many animal models of epilepsy including genetic models, electric and chemical seizure induction and chemical kindling models[22].The therapeutic index for lev is highly favorable, with doses 50-100 times the therapeutic dose causing only mild sedation in mice[22]. Lev has a unique mode of action that inhibits burst firing of neurons while sparing normal neuronal excitability[23]. Clinical trials found that lev is effective as an adjuvant therapy

and is well tolerated, with few drug interactions[24]. This led to approval by the FDA and EMEA as an adjuvant therapy for a broad range of seizure types. While the EMEA has approved lev as a monotherapy based on non-inferiority trials comparing lev to approved treatments, the FDA does not recognize the validity of these types of trials and requires superiority trials that are placebo controlled.

The reason that lev is so unique and well tolerated has to do with its non-traditional mechanism of action. Lev was shown not to facilitate GABA, not to block sodium currents and to have no effect on low voltage activated calcium currents[25, 26]. Early trials characterizing the effectiveness of lev were also carried out with its R enantiomer, L060[22]. The apparent stereoselectivity of lev's effects suggested a specific binding site. Specific, reversible binding to rat central nervous system (CNS) membranes indicated a possible site of action that wasn't shared by any other drugs or endogenous compounds[27]. Subsequently, the protein that proved to be necessary and specific for lev binding to these membranes was the synaptic vesicle protein SV2A, the most common SV2 isoform[28]. Because of this, we expect lev's mechanism of action to be at the level of the synaptic vesicle.

Neuropsychiatric Considerations

The link between epilepsy and other neurological disorders has a long and muddy history. Going back to the Hippocratic texts, there is an association between epilepsy and madness. Neuropsychiatric comorbidities of epilepsy include depression, anxiety, suicide, psychosis and behavioral changes[29]. Depression is a major comorbidity of epilepsy where it is difficult to parse out the neurobiological etiologies of the depression from psychosocial and iatrogenic factors[30].

There is a complicated and curious interplay between epileptic activity and neuropsychiatric symptoms. Schizophrenia-like psychosis can emerge in a patient during nonconvulsive status epilepticus, although this might better be characterized as delirium[31]. There are also many documented cases of postictal or interictal psychotic symptoms. This phenomenon can be associated with forced normalization, where a patient experiences normal or at least improved EEG readings while experiencing psychoses[31, 32]. Other neuropsychiatric comorbidities also have a range of different presentations relative to seizure activity. Some people might experience symptoms that herald an oncoming seizure, while others experience it as a post-attack consequence.

Interactions between seizure activity and neuropsychiatric symptoms complicate analysis of the behavioral effects of lev treatment. Case studies illustrate the areas where this can be confusing. In one patient who was experiencing multiple, weekly seizures under treatment with valproic acid and lamotrigine, onset of lev treatment was coincident with a 2.5 month absence of seizure activity[33]. Subsequently, patient was admitted to the hospital with severe, unprecedented symptoms of depression including anhedonia and suicidal ideation[33]. Another patient whose seizure activity was strongly curtailed after beginning lev treatment was admitted to a hospital with symptoms of psychosis including auditory hallucinations and paranoid delusions[33]. Although these cases represent merely anecdotal evidence of an association between lev treatment and neuropsychiatric disturbance, they serve to illustrate the fact that individual responses to AED treatment will vary widely. A study of 288 patients undergoing lev treatment found that 37% of people experienced negative behavioral effects, with the most prevalent being increased aggression[33]. They also found that negative effects were associated with poorer seizure control[33].

Chapter 2: Isolation and Analysis of Newly Recycled Synaptic Vesicles

Introduction

Current state of the art for studying synaptic vesicles involves harvesting them in bulk from brain homogenate[34] or studying them in the context of synaptosomes[35]. In depth analysis of a specific sub-population of these vesicles, namely those newly recycled after exocytosis, requires techniques that have not yet been published in. Lev's hypothetical effect on protein trafficking in vesicle recycling should be most apparent at the level of isolated newly recycled vesicles.

The technique under development in the Bajjalieh lab depends on Dynole 34-2, a dynamin inhibitor that prevents fission of newly formed vesicles, trapping them at the plasma membrane[36]. Synaptosomes, stimulated to exocytose neurotransmitters in the presence of this inhibitor, were to be lysed to yield plasma membrane fragments with trapped recycling vesicle material or 'recycling intermediates.' This material should be separable from free vesicles from the readily releasable pool and the reserve pool via density gradient centrifugation. Incubation of recycling intermediates with recombinant dynamin should allow putative newly recycled vesicles to be separated from plasma membrane elements. Jia Yao had a working protocol, which relied on western blotting for synaptophysin as for marker of vesicles and Na^+/K^+ ATPase as a marker for plasma membrane, and she was able to show dynamin stimulated synaptophysin release.

Methods

Preparation of GST-Amph2-SH3 Beads for Dynamin Pulldown: BL 21 DE3 *E. coli* were transformed with 1μl of plasmid containing GST-Amph2-SH3 and plated onto Ampicillin (AMP).

A single colony was used to inoculate 50 mL LB + AMP and incubated overnight at 37°C in a shaking incubator. This overnight culture was split in two and spiked into two 500 ml SB + AMP cultures and further incubated for 3 hours. At 2 hours, 1mM IPTG was added to induce protein expression and the culture was allowed to express for 4 hours. After expression, the culture was centrifuged at 4000 rpm and 4°C for 20 min. Supernatant was discarded and the cell pellet was stored at -80°C. The pellet was later thawed on ice and resuspended in 50 mL lysis buffer (20 mM Tris - HCl (pH 7.4), 150mM NaCl, freshly added 0.1 M PMSF and 1 × Roche protease inhibitor cocktail). The cell slurry was lysed using a microfluidizer. Lysate was cleared by spinning at 15,000xg and 4°C for 20 min. Supernatant was collected, divided into aliquots and stored at -80°C. The GST-Amph2-SH3 supernatant was subsequently thawed on ice and incubated overnight at 4°C with Pierce glutathione agarose beads that had been equilibrated in 10 column volumes (CV) lysis buffer. The beads were washed the following day for use in Dynamin purification, undergoing 4 x 5CV wash steps with wash buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) and one 5CV equilibration step with the same buffer system as the Dynamin source(brain homogenate buffer or cell lysis buffer).

Dynamin Purification from Mouse Brain: Six mice were sacrificed by cervical dislocation and brains were immediately harvested and homogenized in 18ml homogenization buffer (150 mM NaCl, 20 mM HEPES pH 7.4, freshly added 4 mM DTT, 1 × Roche EDTA free protease inhibitor cocktail) in 30ml glass-Teflon homogenizer for ten strokes. Triton X-100 was added to a final concentration of 0.1%, and homogenate was incubated in the deli with agitation for 30 min to complete lysis. Homogenate was then centrifuged at 23,700 rpm (100,000 ×g) for 20 min in an SW40 Ti rotor to pellet debris. Supernatant was collected and saved as brain extract. Brain extract was incubated with 1.5 ml GST-Amph2-SH3 beads equilibrated in homogenization buffer while rotating for 1 hour at 4°C. Beads were washed 4 times with 7ml of wash buffer (200 mM NaCl, 20 mM HEPES pH 7.4, freshly added 1mM DTT). Dynamin was eluted in dynamin elution

buffer (1.2 M NaCl, 20 mM PIPES pH 6.5) with 1mM freshly added DTT. Five 3 ml elutions were performed, the first two being incubated for 20 min each and the remaining three being incubated for 10 min each at 4°C under agitation. The elutions were pooled and added to a 15 ml 10k MWCO centrifugal concentrator. The pooled eluate was concentrated to 500 ml, diluted to 10 ml with dynamin dilution buffer (200 mM NaCl, 20 mM HEPES pH 7.4), concentrated to 500 ml, diluted again with dynamin dilution buffer and finally concentrated to 500 ml, divided into aliquots and stored at -80°C.

Recombinant Dynamin Expression and Purification: Lentix 293T HEK cells were transfected with HA tagged mouse Dynamin 1 using a calcium phosphate transfection protocol. Cells were passaged into 15cm culture dishes with DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin G and 0.1mg/mL streptomycin sulfate and incubated at 37°C and 5% CO_2 until they reached 40-60% confluence. At 3 hours pre-transfection, the media was changed. Calcium phosphate crystals were prepared by mixing 30 μg of HA-mDyn1 plasmid with H_2O and then adding 93 μl of 2M CaCl2, then adding the Calcium/DNA solution dropwise to 750 μM of 2x HBS while vortexing gently. The crystals were allowed to form at room temperature for 20 min before being added dropwise to the HEK cells. The plate was swirled to evenly distribute the crytals over the culture and returned to the incubator. After 8-16 hrs, the media was changed. At 48 hours post transfection, the media was aspirated, the cells were washed with warm PBS buffer and then 2.5 ml of 0.25% trypsin were added to the plate. The cells were incubated briefly before 7.5 ml of DMEM was added to the plate. The cells were brought up and triturated, and then transferred into a 15 ml conical tube. A further 2 ml of DMEM was used to rinse left behind cells from the plate, which were added to the rest of the cells before they were pelleted at 2000 x g for 2.5 minutes. The media was aspirated and the cells were resusupended in 12ml warm PBS and then pelleted again under the same conditions. The PBS was aspirated, and the cells were resuspended in 1.5ml lysis buffer (250 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-

100, 20 mM HEPES, pH7.4) containing Roche cOmplete EDTA-free protease inhibitor cocktail. They were then sonicated for 25 pulses at 40% duty cycle and power level 5. The sonicate was transferred to a 2ml eppendorf tube and centrifuged at 4°C and 15,000 x g for 20 min. The sonicate supernatant was collected and added to 500 ml of Pierce Glutathione Agarose beads loaded with recombinant GST-Amph 2-SH3, washed and equilibrated in lysis buffer. The bead/supernatant slurry was rotated for 1 hour at 4°C. After incubation, the beads were washed 4 times with 2.5 ml of dynamin wash buffer (250 mM NaCl, 20 mM HEPES pH 7.4) containing 1mM freshly added DTT. Dynamin was eluted in dynamin elution buffer (1.2 M NaCl, 20 mM PIPES pH 6.5) with 1mM freshly added DTT. Five 1 ml elutions were performed, the first two being incubated for 20 min each and the remaining three being incubated for 10 min each at 4°C under agitation. Eluate was adjusted to pH 7 with concentrated Tris base upon removal from the beads. The elutions were pooled and added to a 15 ml 10k MWCO centrifugal concentrator. The pooled eluate was concentrated to 500 ml, diluted to 10 ml with dynamin dilution buffer (200 mM NaCl, 20 mM HEPES pH 7.4), concentrated to 500 ml, diluted again with dynamin dilution buffer and finally concentrated to 500 ml. The concentrated dynamin was divided into aliquots, flash frozen in a dry ice/ethanol slurry and placed in the -80°C freezer for later use.

Formation of PS Liposomes for Malachite Green Assay: Forty μl of 10 mg/ml L-α-phosphatidyl-L-serine in chloroform:methanol 95:5 solution were divided into aliquots into a silanized glass ampule and evaporated under N_2 gas. The dry lipid was resuspended in 1 ml 30 mM Tris/HCl pH 7.4 and sonicated on ice in a bath sonicator for 1 min. Two additional freeze/thaw cycles with vigorous vortexing while thawing were performed before the liposomes were ready for use in the malachite assay.

Malachite Green Assay for GTPase Activity: Purified Dynamin was tested for GTPase activity using a protocol adapted from one published by the Robinson lab in Methods in

Enzymology[37]. Stock solutions were assembled and stored as follows: Malachite reagent: Malachite green oxalate 1.5%, ammonium molybdate 15% in 6N HCl. 10x GTPase Assay Buffer: 100mM Tris/HCl, 100mM NaCl, 20mM $MgCl_2$, 0.5% Tween 20, pH 7.4, stored at 4°C. Guanosine 5' Triphosphate (GTP): 100x (30mM) stock prepared by dissolving 17 mg GTP in 1 ml 100mM Tris/HCl, pH 9.0 and adjusting to pH 7 using pH paper and μl amounts of 100mM HCl. Stocks were divided into aliquots and stored at -20°C. These GTP stocks are thawed and diluted to 10x (3mM) with 20mM Tris/HCl pH 7.4 for immediate use in the assay. 10x Leupeptin: 10 μg/ml in H_2O, stored at -20°C. 10x PMSF: 1mM PMSF stored at -20°C. Assays were preformed in 200 μl reactions. A master mix containing 10x GTPase Assay Buffer, 10x Leupeptin, 10x PMSF, 10x GTP, PS liposomes and H_2O was assembled in a 1:1:1:1:1:3 ratio on ice and divided into 160 μl aliquots. Dynamin samples were prepared at different concentrations in dynamin dilution buffer for total volumes of 40 ul. Dynamin samples or dilution buffer were added to the reactions and thoroughly mixed. The reactions were incubated at 30°C in a water bath. Activity was monitored over the course of the reaction by removing 30 μL aliquots from the reactions and adding them directly to 950 μl of malachite reagent plus 20 μl H_2O. After at least 20 min incubation at room temperature, OD_{620} was measured using a spectrophotometer.

Recycling Intermediate Preparation: Recycling intermediates are isolated from mouse brains using a protocol developed in the Bajjalieh lab and relying on a Percoll-based protocol for the isolation of rat synaptosomes from the lab of Dr. Walter Volknandt and adapted by Kristine Ciruelas and Jia Yao. Wild-type mice bred from SV2ABf12 KO heterozygous mice were raised and genotyped by Claire Schraeder. Three mice were sacrificed using cervical dislocation and brains were immediately harvested and kept on ice for the remainder of the protocol unless otherwise specified. Brains were homogenized in a Teflon/Glass homogenizer in Homogenization Buffer (HEPES Buffered Saline (Salt, Hepes), 1x Roche Complete Protease Inhibitor, 1mM EGTA) for 8 strokes. The brain homogenate was cleared by spinning in the

Beckman Avanti centrifuge by spinning at 1000 x g for 10 minutes in a swinging bucket rotor at 4°C. The supernatant from this step was loaded onto a discontinuous Percoll gradient consisting of four 2ml steps of increasing Percoll density: 10%, 24%, 27% and 31% Percoll. This gradient step was centrifuged for 7 minutes at 15,700 rpm and 4°C, with the deceleration rate set at 4 in a Beckman Ultracentrifuge using an SW40Ti rotor. Synaptosomes, verified by a positive western blot for synaptophysin and Na^+/K^+ ATPase, were found in a visible band that formed at the interface between the 24 and 27% Percoll steps and collected by eye for washing with HBS + 1mM EGTA. Collected synaptosomes were diluted into 12 ml HBS + EGTA and spun in the Avanti at 17,000 x g for 15 minutes at 4°C. The pellet was resuspended in 1 ml of HBS + EGTA by trituration with a p1000 micropipet and diluted to 12 ml again. The centrifugation was repeated, resulting in a pellet that was washed of residual Percoll. This pellet was resuspended in 1ml HBS + 1mM Ca^{++} by trituration with a p1000 micropipet and allowed to recover for five minutes at 30°C. Synaptosomes were stimulated with 1ml of High K+ Buffer(10 mM HEPES, 28 mM NaCl, 117.6 KCl, 1mM $MgCl_2$, 5mM d-glu pH 7.4) with 100mM Dynole 34-2 and incubated for 2 minutes at 30°C. Excitation was halted and hypotonic lysis initiated by addition of 18 ml of ice cold H2O and Dynole 34-2 was added to maintain the concentrations at 100 uM. 1 M HEPES pH 7.4 was added to bring the concentration to 5mM. Synaptosomes were homogenized for 3 strokes in a Teflon/glass homogenizer and incubated 30 min at 4°C. Synaptosomal lysis solution was further homogenized for ten strokes. Synaptosomal lysis solution was split into two aliquots of about 9 ml, and recycling intermediates were isolated by ultracentrifugation over two discontinuous sucrose gradients each consisting of two 10ml steps containing 0.8 and 1.2M sucrose (5mM HEPES pH 7.4). The gradients were centrifuged for 1 hour at 23,700 rpm and 4°C in the Beckman L8-80M Ultracentrifuge using the SW28 rotor. Recycling intermediates were collected from the interfaces between 0.8 and 1.2M sucrose steps.

Vesicle Release Protocol: Vesicles were released from Recycling Intermediates using recombinant dynamin and GTP based on a protocol developed by Jia Yao. Reactions of 240 μl were assembled on ice by combining 100 μL of recycling intermediates with dynamin, dynamin dilution buffer and GTP with $MgCl_2$. Final concentrations for GTP and MgCl were 1mM. These reactions were incubated at 37°C for 1 hour, and then plasma membrane material was pelleted via centrifugation for 30 min at 14,000xg and 4°C. Supernatants and pellets were collected separately and analyzed via Western blot.

Results

Dynamin Purification from Mouse Brain: It was found that dynamin purified from mouse brain contained synaptophysin, which was being used to detect the successful release of vesicles from recycling intermediates. Figure 1 shows the culmination and ultimate failure of efforts to remove this contamination from purified dynamin via more stringent wash procedures. This strategy for the acquisition of exogenous dynamin for use in release assays was thus abandoned.

Dynamin Expression and Purification in HEK cells: Human Embryonic Kidney cells do not express synaptophysin. This makes them an ideal method for generating dynamin free of confounding contamination. Figure 2 shows the results of the purification protocol, highly pure and concentrated dynamin with the major contaminant being the GST-Amph2-SH3 protein used to pull down the dynamin. Figure 6 (bottom, FF DYN) shows that this dynamin is lacking synaptophysin contamination.

Dynamin Activity Assessment and Handling: Poor activity from thawed dynamin stored in the -80°C freezer prompted concerns about the accuracy of the malachite green assay and the

preservative nature of the storage strategy being used. Alternative strategies for storing dynamin were tested (figure 3). It was determined that dynamin retained the most activity upon thawing when it was flash frozen in an ethanol/dry ice slurry (figure 3). Placing aliquots directly

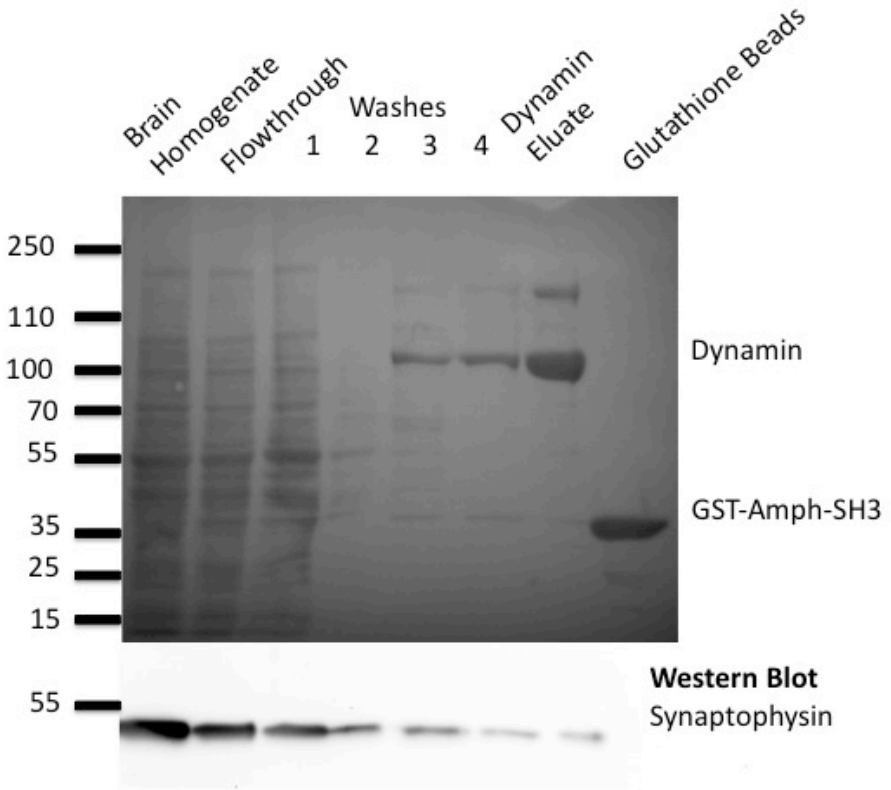

Figure 1: Coomassie and Western Blot Analysis of Dynamin Purification from Mouse Brain. Material was collected during the dynamin purification procedure to assay for yield and purity. Coomassie analysis of the material (top) shows that the material eluted from the GST-Amph-SH3 beads was greater than 90% pure, with the primary peak corresponding to the predicted molecular weight for Dynamin. Western blot analysis (bottom) using an anti-Synaptophysin antibody reveals that the pure dynamin is contaminated.

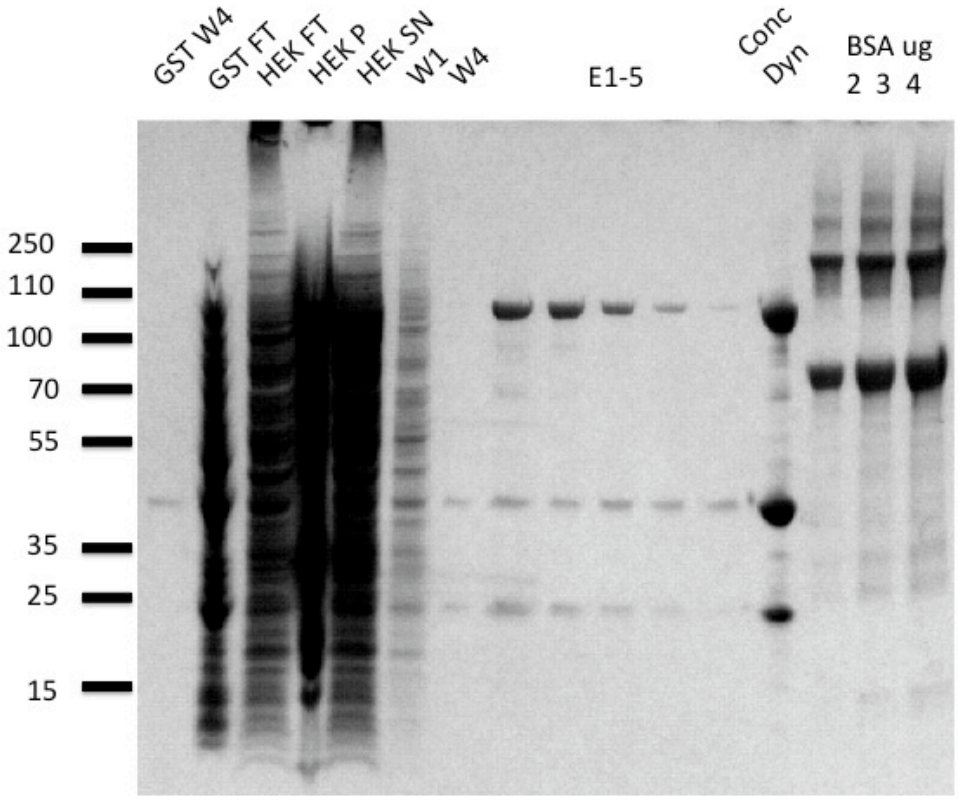

Figure 2: Coomassie Analysis of Dynamin from HEK Cells. Highly pure dynamin can be seen in the lane labeled conc dyn. The major contaminant, which runs between 35 and 55 kD, is consistent with the molecular weight of the GST-Amph2-SH3 affinity fragment used to pull down the dynamin from cell lysate.

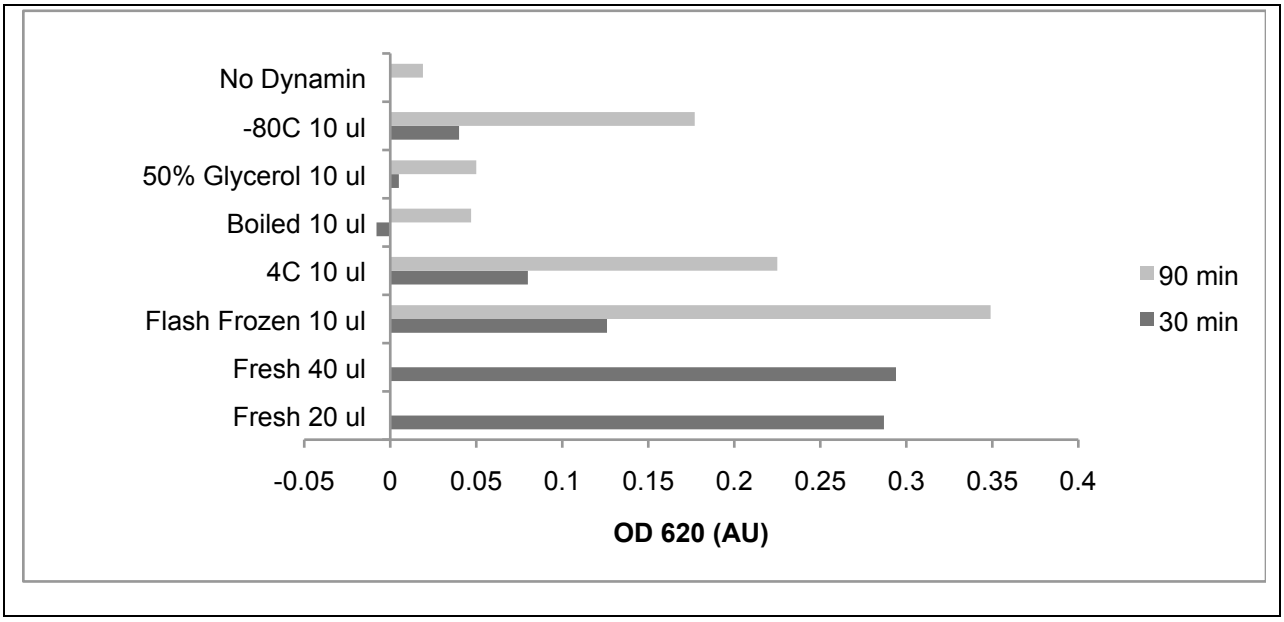

Figure 3: Malachite Green GTPase Activity Assay of Alternatively Handled Dynamin.

Dynamin prepared from HEK cells was divided into aliquots and either immediately assayed for activity (Fresh) or stored overnight under different conditions. The amount of concentrated dynamin added to each reaction is listed as well as storage conditions. Conditions -80C, and boiled were placed directly in the -80°C freezer after they were divided into aliquots, while the 50% glycerol condition was first diluted 1:2 with glycerol and then placed into the same freezer. Condition 4C was stored in the 4°C lab fridge. The flash frozen condition was submerged in an ethanol/dry ice slurry before being placed in the 80°C freezer. Frozen samples were thawed on ice, and the boiled sample was then subjected to 20 min at 99°C on a heat block before reactions were assembled.

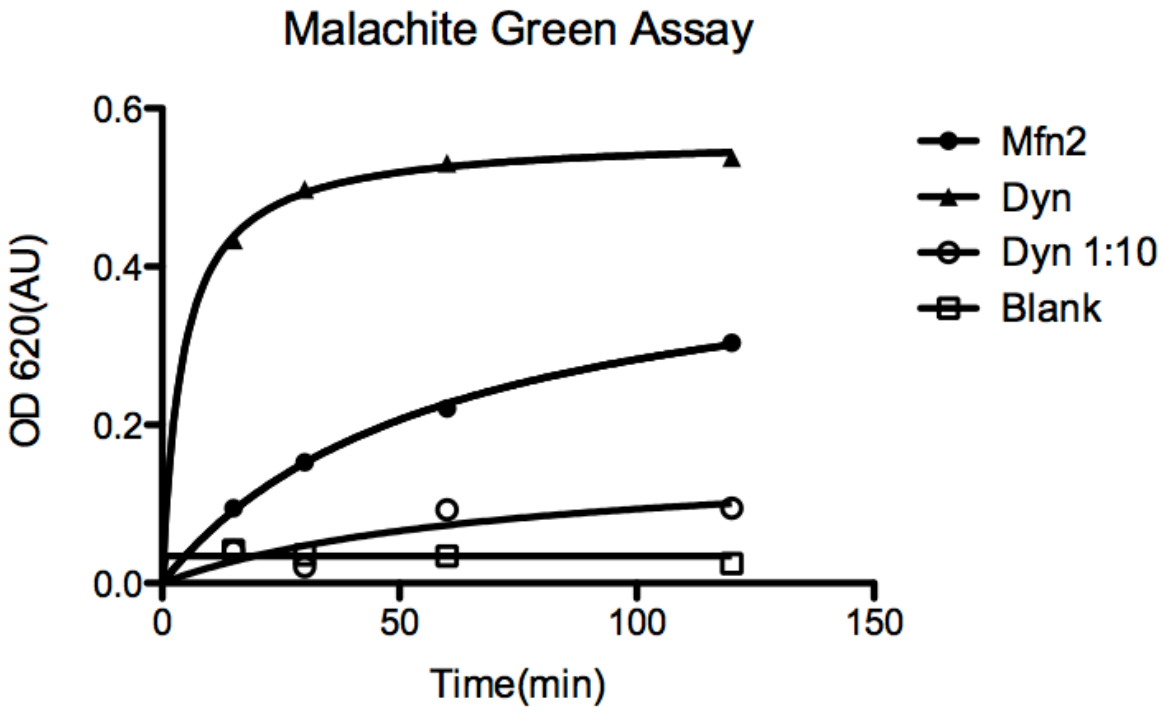

Figure 4: Malachite Green GTPase Assay Featuring a Positive Control. A sample of mitofusin 2 (Mfn2) was obtained from the Hoppins lab for use as a positive control because it was known to be an active GTPase. Each reaction contained 20 μl of the respective enzyme.

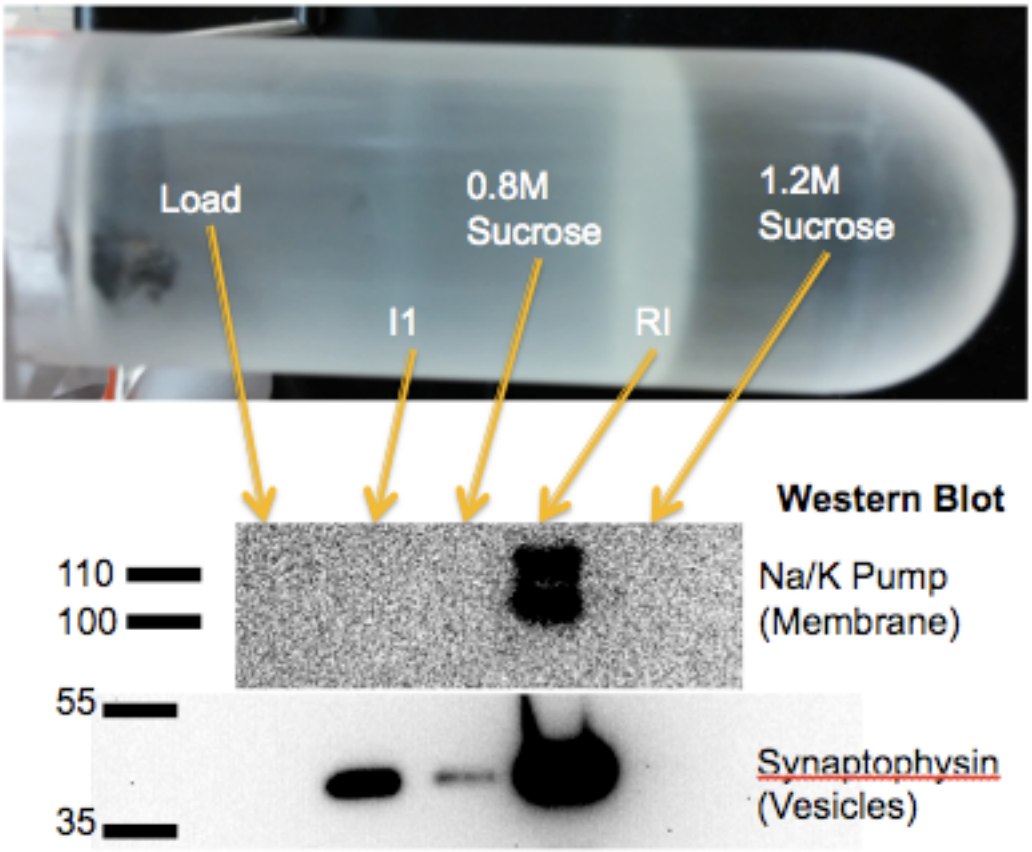

Figure 5: Sucrose Step Gradient to Separate Free Vesicles from Recycling Intermediates. Western blots performed on samples from the indicated fractions of the sucrose gradient show that recycling intermediates are present at the interface between 0.8 and 1.2 M sucrose (RI), based on the presence of plasma membrane and synaptic vesicle markers. In contrast, free vesicles, (lacking membrane marker) are found mostly at the interface between the loaded sample and 0.8 M sucrose (I1).

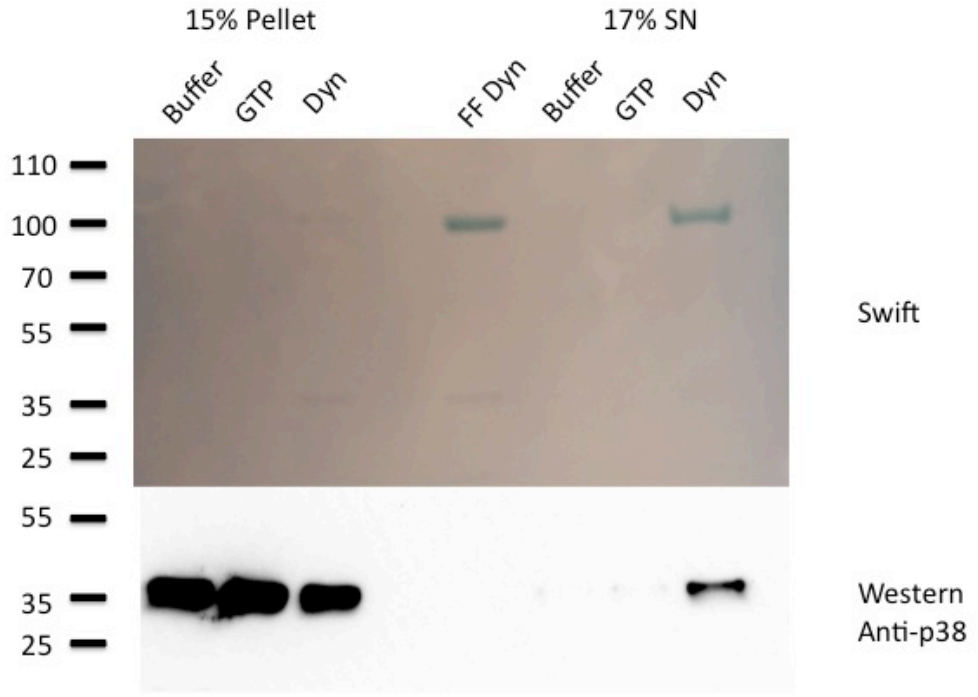

Figure 6: Western Blot Analysis of Dynamin Stimulated Vesicular Release. Top: SWIFT nonspecific protein stain (G-Biosciences) of the pellet and supernatant (SN) fractions resulting from dynamin stimulated vesicle release reactions, as well as an equivalent volume of purified, flash frozen dynamin (FF DYN) reveal the presence of dynamin at around 100 kD. Bands near 35 kD likely denote GST-Amph2-SH3. Bottom: Western blot analysis using an anti-synaptophysin antibody indicate that in the absence of dynamin, synaptophysin pellets out of the vesicle release reaction. In the presence of dynamin, however, synaptophysin is released. There is no synaptophysin contamination in the dynamin being added to the reactions.

into the -80°C freezer overnight was worse for the enzyme than storing it at 4°C overnight, and adding glycerol completely abolished its activity. The accuracy of the malachite green assay that I developed was borne out by its successful detection of the activity of mitofusin 2, and its ability to detect the difference in activity between 2 and 20 μl of concentrated dynamin (figure 4). This gave us further confidence that I was producing active dynamin that could be assayed for activity, stored and used to release trapped vesicles from recycling intermediates.

Purification of Recycling Intermediates: This protocol is well established now in the Bajjalieh lab. Evidence that the material collected from the interface between 0.8 and 1.2 M sucrose really contains recycling intermediates is indicated by the presence of synaptophysin and Na^+/K^+ ATPase, denoting vesicles and plasma membrane, respectively (figure 5). This material is separated from free vesicles released when synaptosomes are lysed (figure 5). The recycling intermediate nature of this material is further supported by the apparent release of synaptophysin in the dynamin stimulated release experiments (figure 6).

Dynamin Stimulated Vesicular Release: Figure 6 shows that synaptophysin, which in control conditions pellets with Na^+/K^+ ATPase and the plasma membrane, is released into the supernatant when recycling intermediates are incubated with dynamin. This is taken to indicate that trapped, newly recycled vesicles are being released from the plasma membrane under these conditions.

Discussion

The data presented here supports the hypothesis that active (figures 3, 4, and 6), recombinantly expressed dynamin can be purified HEK cells (figure 2). Our experiments indicate that the activity of the dynamin remains stable for at least 2 months. Figures 5 and 6

support the hypothesis that recycling intermediates can be separated from free vesicles and stimulated to release new vesicles using the aforementioned dynamin.

Studies of newly recycled synaptic vesicles will prove an invaluable tool for probing the processes whereby a fused, exocytotic synaptic vesicle is retrieved from the plasma membrane, re-sorted, re-formed and returned to the readily releasable vesicle pool in the synapse. Isolation of these materials will allow for proteomic analysis at an unprecedented resolution. Much of what is currently known about this recycling process has been garnered via electron micrographs, immunocytochemistry techniques, and membrane binding pH labile fluorescent dyes. Our technique improves upon past techniques by isolating vesicles that have just been freed from the plasma membrane, ostensibly before they have had the chance to undergo any other quality control, protein sorting or other potential processes of synaptic vesicle maturation.

Future avenues for study of this material should attempt to verify the characterization of the putative recycled vesicles. Do they represent fully mature and fusion competent synaptic vesicles or are there intermediate steps between vesicle fission and fusion competency? Are there elements that indicate that synaptosomal endosomes are present in this released material or would such endosomes pellet with the plasma membrane? Does the released material even form vesicles of the right size and shape? These questions remain to be answered, if this material is to be used as a way to probe the processes whereby vesicles are recycled.

Chapter 3: Levetiracetam Effect on Vesicle Composition

Introduction

Levetiracetam operates via an unknown mechanism of action, but it is known to bind to SV2A, and its effect depends on that binding. SV2A does not have a determined function at the synapse, but evidence indicates that its presence in synaptic vesicles is tightly regulated. It is also known to bind endocytic clathrin adapter machinery and it is involved in the trafficking of synaptotagmin into vesicles. Synaptotagmin is important for proper calcium responsivity at the synapse. Based on the activity requirement for Lev's mode of operation, combined with the observation that it's binding target is involved in protein sorting and trafficking during vesicle endocytosis, it is our hypothesis that Lev acts somewhere between the fusion of synaptic vesicles with the membrane and their return to the readily releasable pool. In fact, studies indicate that Lev decreases the size of the readily releasable pool.

Our research aims to show that Lev treatment alters the protein composition of newly recycled synaptic vesicles, specifically in terms of their stoichiometry.

Methods

Lev Treated Recycling Intermediate Preparation: Recycling intermediates are isolated from mouse brains using a protocol developed in the Bajjalieh lab and relying on a percoll-based protocol for the isolation of rat synaptosomes from the lab of Dr. Walter Volknandt and adapted by Kritstine Ciruelas and Jia Yao. Wild-type mice bred from SV2ABf12 KO heterozygous mice were raised and genotyped by Claire Schraeder. Mice were sacrificed using cervical dislocation and brains were immediately harvested and kept on ice for the remainder of the protocol unless otherwise specified. Brains were homogenized in a Teflon/Glass homogenizer in Homogenization Buffer (HEPES Buffered Saline (Salt, HEPES), 1x Roche Complete Protease Inhibitor, 1mM EGTA) for 8 strokes. The brain homogenate was cleared by spinning in the

Beckman Avanti centrifuge by spinning at 1000 x g for 10 minutes in a swinging bucket rotor at 4°C. The supernatant from this step was loaded onto a discontinuous Percoll gradient consisting of four 2ml steps of increasing Percoll density: 10%, 24%, 27% and 31% Percoll. This gradient step was centrifuged for 7 minutes at 15,700 rpm and 4°C, with the deceleration rate set at 4 in a Beckman Ultracentrifuge using an SW40Ti rotor. Synaptosomes, verified by a positive western blot for synaptophysin and Na^+/K^+ ATPase, were found in a visible band that formed at the interface between the 24 and 27% Percoll steps and collected by eye for washing with HBS + 1mM EGTA. Collected synaptosomes were diluted into 12 ml HBS + EGTA and spun in the Avanti at 17,000 x g for 15 minutes at 4°C. The pellet was resuspended in 1 ml of HBS + EGTA by trituration with a p1000 micropipet and diluted to 12 ml again. The centrifugation was repeated, resulting in a pellet that was washed of residual Percoll. This pellet was resuspended in 75ul HBS + 1mM Ca^{++} per brain by trituration with a p200 micropipet and allowed to recover for ten minutes at 30°C. Dynole 34-2 was added to a concentration of 100uM and Lev (or H2O control) was added to a concentration of 300uM. The synaptosomes were incubated at 30°C for 2 minutes before they were stimulated to release vesicles by adding 75 ul per brain High K+ Buffer(10 mM HEPES, 28 mM NaCl, 117.6 KCl, 1mM $MgCl_2$, 5mM d-glu pH 7.4) and incubated for 5 minutes at 30°C. Excitation was halted and hypotonic lysis initiated by addition of 9 volumes of ice cold H2O and Lev and Dynole 34-2 were added to maintain the concentrations at 100 and 300 uM, respectively. 1 M HEPES pH 7.4 was added to bring the concentration to 5mM. Synaptosomes were homogenized for 3 strokes in a Teflon/glass homogenizer and incubated 30 min at 4°C. Synaptosomal lysis solution divided into aliquots into 1.5 ml fractions and placed in 2ml Eppendorf tubes, then sonicated with the Branson Sonifier 450 on ice for 30 pulses at power level 6 and 30% duty cycle. Recycling intermediates were isolated by ultracentrifugation over a discontinuous sucrose grading consisting of five 2ml steps containing 0.4, 0.8, 0.9, 1.0, and 1.2M sucrose (5mM HEPES pH 7.4). A single 1.5 ml aliquot was added to

each gradient and the gradients were centrifuged for 1 hour at 23,700 rpm and 4°C in the Beckman L8-80M Ultracentrifuge using the SW40 Ti rotor. Recycling intermediates were collected from interfaces between 0.9 and 1.0M sucrose steps as well as 1.0 and 1.2M sucrose steps.

Vesicle Release Protocol: Previously described.

Trichloroacetic Acid Precipitation Protocol: Supernatants collected from vesicle release were brought to 10% Trichloroacetic Acid (TCA), vortexed and incubated on ice for 30 min, followed by a 20 min spin at 14,000xg and 4°C. Supernatant was thoroughly aspirated and pellets were resuspended in 2x SDS PAGE sample buffer. Residual TCA caused this solution to turn yellow, so the pH was adjusted back to normal with concentrated Tris base.

Results:

Production of Lev Treated Recycling Intermediates: Treatment with Lev did not detectably alter the recycling intermediate material collected from the protocol. Fractionation of the synaptosomal lysis material was accomplished with a 5-step gradient developed by Kristine Ciruelas for her experiments attempting to pull recycling intermediates out of synaptosomal lysis material using antibodies conjugated to magnetic beads. This was changed for consistency and comparability of out results, and not because of any defect in the previously described gradient. Other changes to the protocol to conform with her protocol were also made, including more disruptive sonication to increase lysis of the synaptosomes. These two changes account for the differences in the relative distribution of Na^+/K^+ ATPase and synaptophysin seen when comparing the blot in figure 5 to the blot in figure 7. Figure 7 does indicate the presence of recycling intermediates, similarly containing vesicular and plasma membrane markers in the denser steps of the gradient that separate from the lighter vesicular components that likely represent free vesicles.

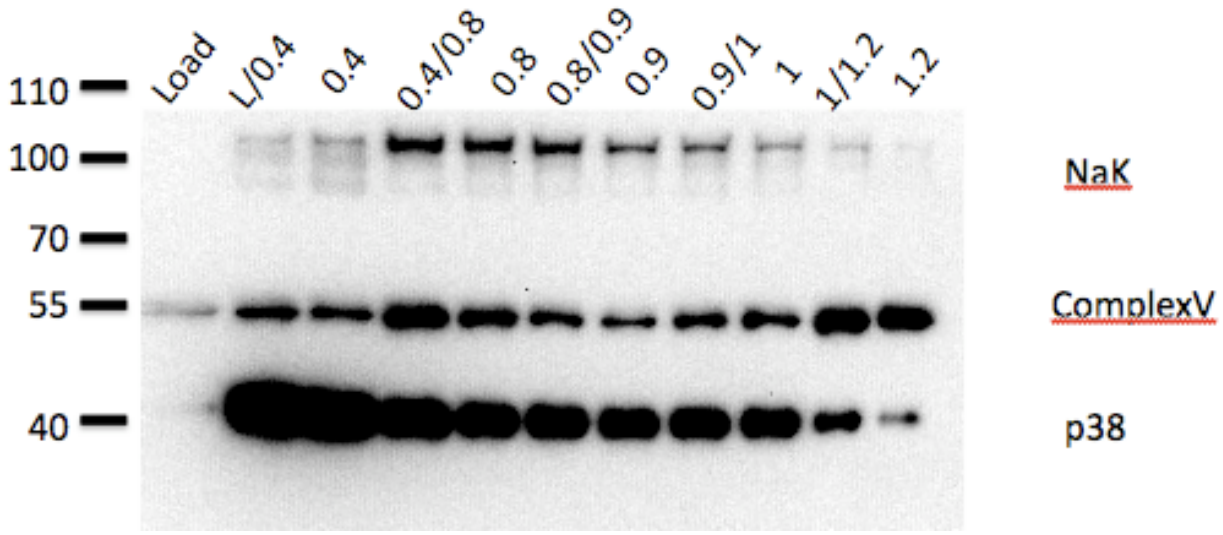

Figure 7: Five Step Sucrose Gradient for Recycling Intermediate Isolation. Fractions from the 5 step sucrose gradient were western blotted with antibodies for Na^+/K^+ ATPase, complex V and synaptophysin (p38) to detect the presence of plasma membrane, mitochondria and vesicular material, respectively. Fractions are labeled L for load, and then based on their molar sucrose concentration, with a '/' indicating an interface between two gradient steps. In contrast to the two step gradient, a greater proportion of the p38 is present in the form of free vesicles near the light end of the gradient. Regardless of this fact, Na^+/K^+ ATPase and p38 are co-represented at the interfaces between 0.9 and 1.0 M sucrose and 1.0 and 1.2 M sucrose, indicating the presence of recycling intermediates.

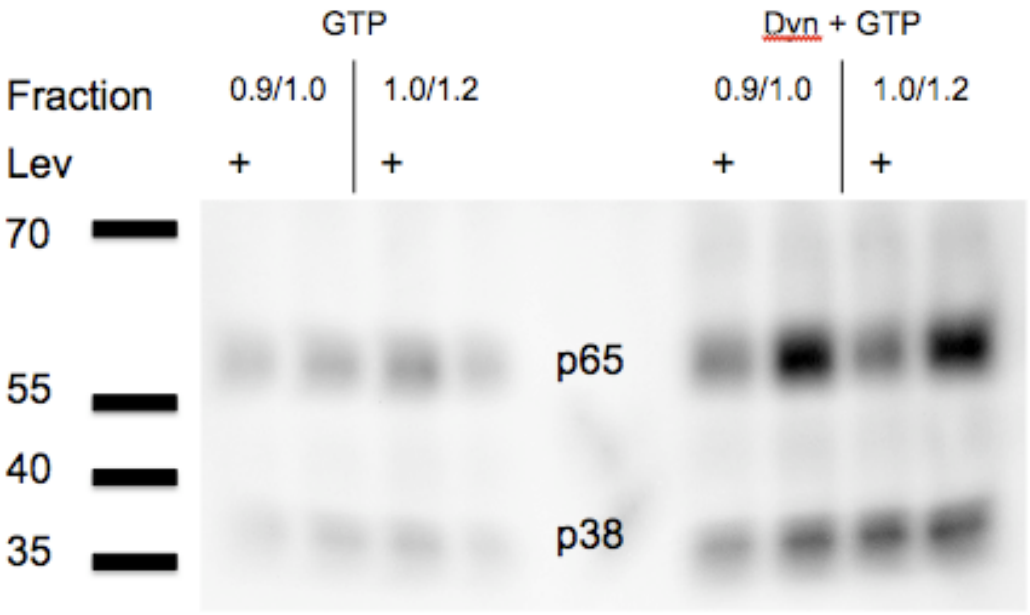

Figure 8: Representative Lev Effect Blot. Western blots were performed on TCA precipitated supernatant from dynamin release assays. These blots were analyzed for band intensity using Carestream MI software.

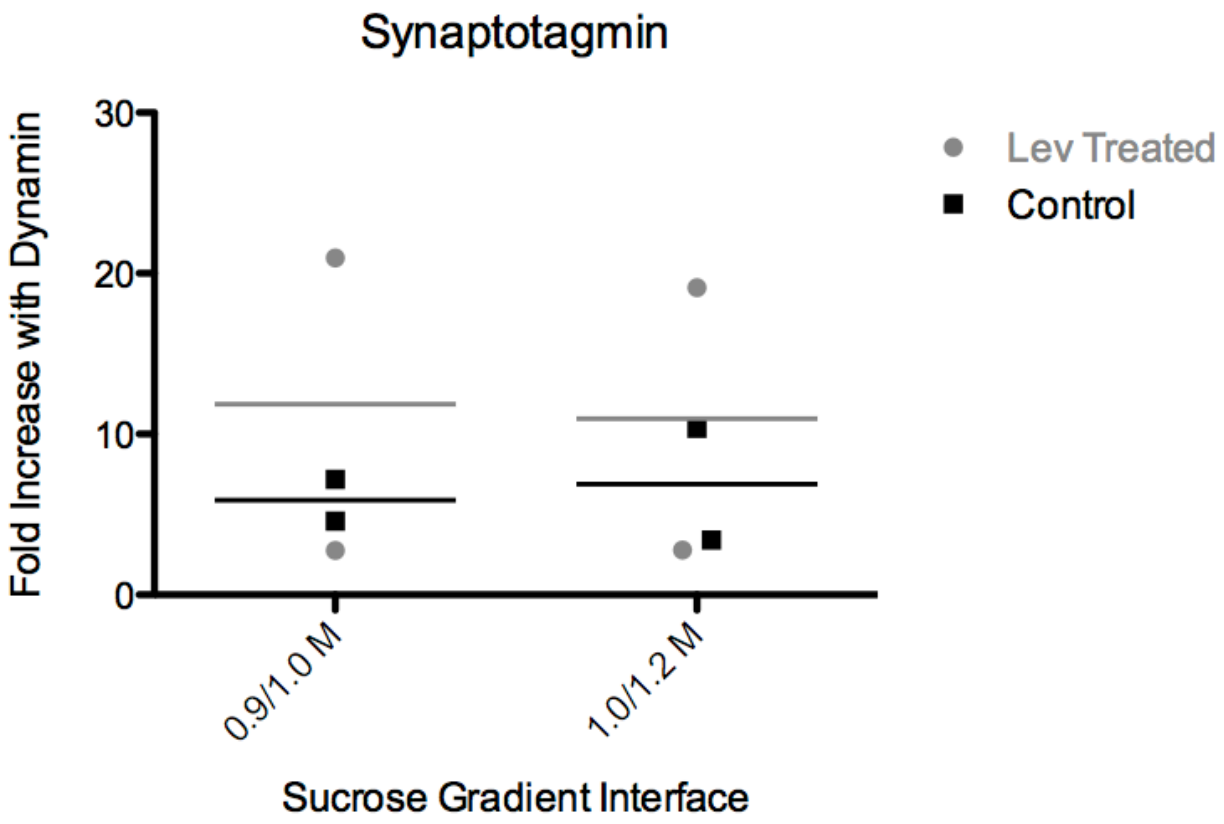

Figure 9: Synaptotagmin is Released by Incubation with Dynamin. In order to remove the effect of variability between individual western blots, all data points were created by dividing the intensity of the dynamin stimulated band by the intensity of the GTP stimulated band from the same blot and experiment. The results show that synaptotagmin is released from recycling intermediate material in greater quantities in the presence of dynamin, regardless of which interface the recycling intermediates were collected from and regardless of whether the material is treated with lev or not.

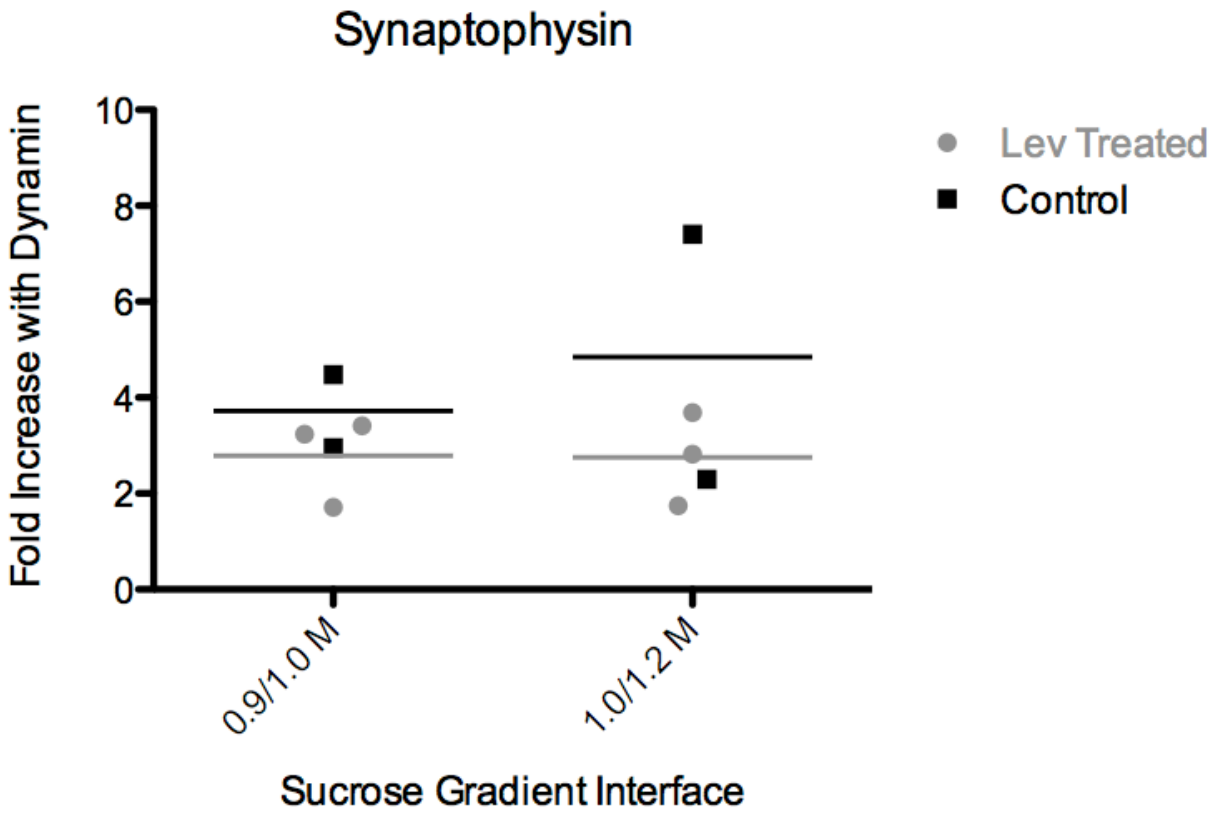

Figure 10: Synaptophysin is Released by Incubation with Dynamin. Western blots performed on TCA precipitated supernatant from dynamin release assays were analyzed for band intensity. In order to remove the effect of variability in raw intensity between individual western blots, all data points were created by dividing the intensity of the dynamin stimulated band by the intensity of the GTP stimulated band from the same blot and experiment. The results show that synaptophysin is released from recycling intermediate material in greater quantities in the presence of dynamin, regardless of which interface the recycling intermediates were collected from and regardless of whether the material is treated with lev or not.

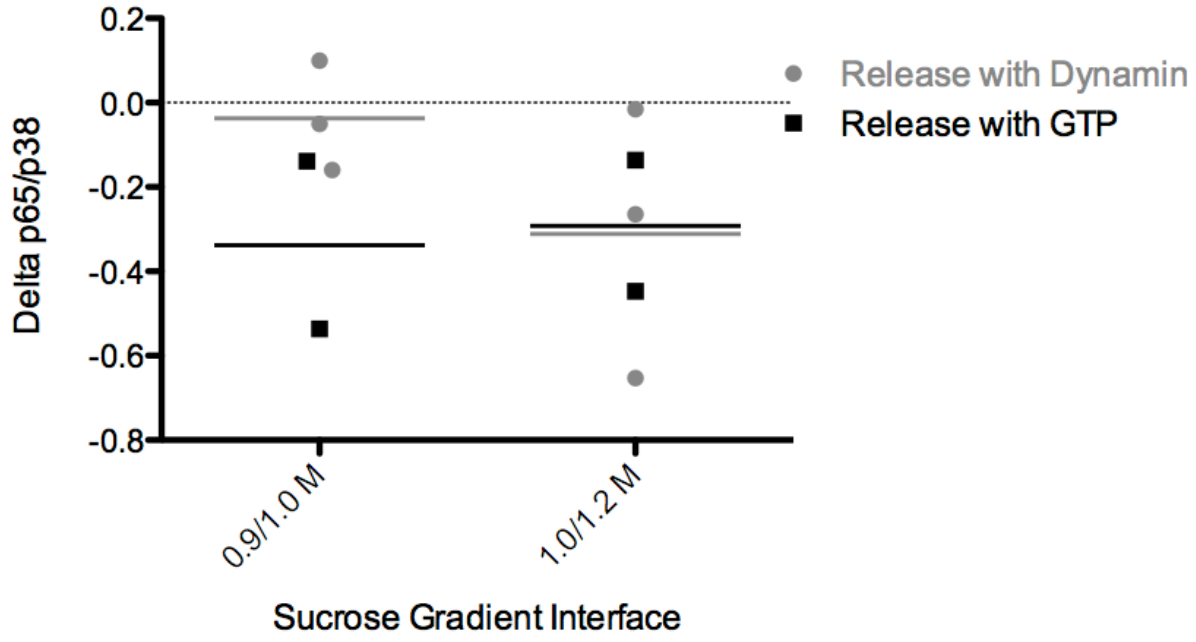

Figure 11: Lev Induced Changes in the Synaptotagmin:Synaptophysin Ratio. Western blots performed on TCA precipitated supernatant from dynamin release assays were analyzed for band intensity. In order to remove the effect of variability in raw intensity between individual western blots, all data points were created by dividing the intensity of the synaptagmin band (p65) by the intensity of the synaptophysin band (p38) and then subtraction this ratio in the control condition from the same ratio measured in the lev condition. The results show that synaptagmin is released from recycling intermediate material in smaller quantities relative to synaptophysin in the presence of lev, regardless of which interface the recycling intermediates were collected from and regardless of whether the material is released via dynamin and GTP or GTP alone.

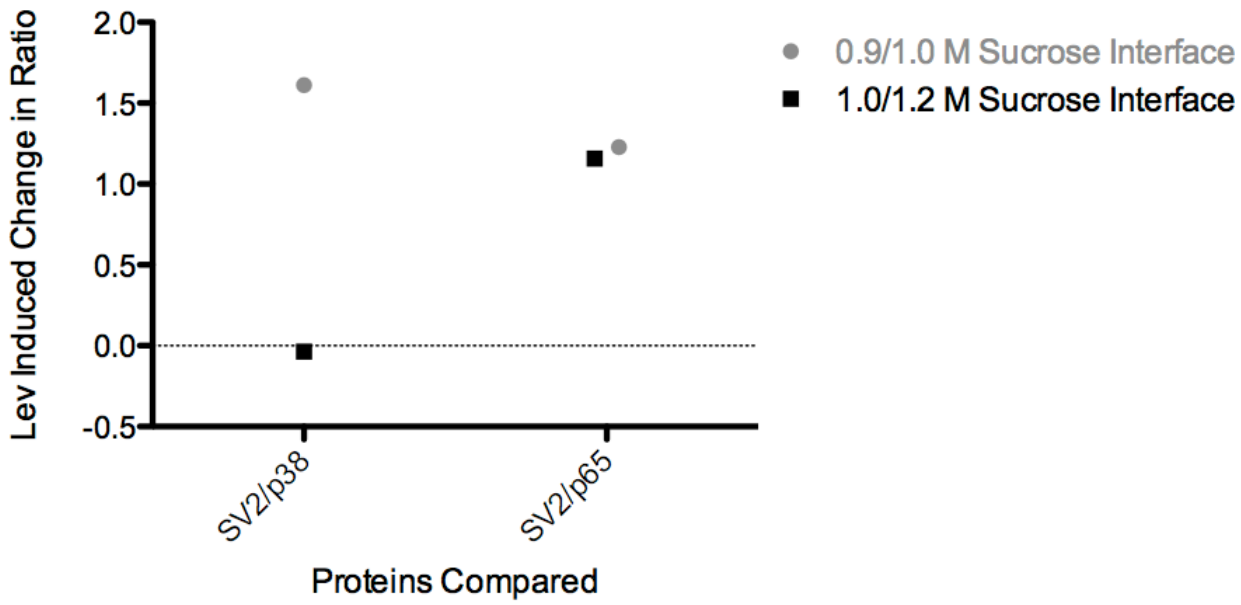

Figure 12: Lev Induced Changes in Vesicle Protein Stoichiometry. One western blot was performed on TCA precipitated supernatant from dynamin release assays using three antibodies. The blot was analyzed for band intensity, but no SV2 release was detected in the absence of dynamin. All data points were created by dividing band intensities as indicated and subtracting the control ratio from the lev treated ratio. The results show that SV2 might respond to lev in a different way at different interfaces.

Vesicle Release: Western blots for synaptotagmin and synaptophysin were analyzed by measuring the intensity of resultant bands detected in the supernatant from the release experiments using Carestream MI software. Figure 8 is a representative blot. In order to get the strongest signal from this released material, TCA precipitation was employed to concentrate it in order to fit on a single PAGE gel. Treatment with Lev did not alter the general effect that dynamin had on inducing release of vesicular proteins from recycling intermediates (figures 9 and 10). With only a handful of preliminary data points to analyze, no strong conclusions can be drawn about more subtle effects. In comparing figure 8 to figure 9, one can see that the relative amount of protein released in dynamin-stimulated conditions compared to GTP alone varies much more for synaptotagmin than for synaptophysin. No trend can be detected in comparing different segments of the gradient for either protein.

Stoichiometry Analysis: Results are preliminary, but they suggest that in the presence of lev, the synaptotagmin/synaptophysin ratio decreases, possibly indicating that less synaptotagmin is being sorted into recycling vesicles (figures 8 and 11). Figure 12 shows the result of one experiment with blotting for SV2. This protein was undetectable in the GTP only supernatants. This single experiment shows a striking effect of gradient fraction on the relationship between SV2 and synaptophysin, but it would be unwise to read much into that result without further confirmation.

Discussion:

It is known that SV2A plays a part in trafficking synaptotagmin into recycling synaptic vesicles. My results, though preliminary, seem to support the idea that Lev is altering this process, possibly by disrupting an interaction between SV2 and synaptotagmin that allows it to

be trafficked into the cell. Synaptophysin is a ubiquitous vesicular protein, but studies show that it is not trafficked into vesicles with high precision, in contrast to SV2 and synaptotagmin[38].

Future directions for this research include a full proteomic comparison of released material in the presence or absence of lev via mass spectrometry. MS is highly quantitative, and it will have the ability to accurately measure the full complement of proteins found in the supernatant of the vesicular release experiments. The full complement of proteins that change their internalization kinetics under lev treatment will help to elucidate its mechanism of action. These proteins are also likely to be clues to solving the puzzle of SV2As function in the synapse. Furthermore, this released material could be studied for its variability at the level of the individual vesicle (if it's indeed made up of vesicles), which could shed light on the processes whereby proteins are specifically and reproducibly sorted into vesicles.

Conclusions

In conclusion, the results presented here generally support the hypothesis that levetiracetam alters the way that proteins are trafficked into synaptic vesicles. While the evidence is not concrete, a solid groundwork has been laid for further inroads into this investigation. It remains to be seen whether the different densities of recycling intermediates have a different character in terms of the vesicular material that they release. It also remains to be seen whether the effect of lev is different at the different densities. Furthermore, the effect of lev on trafficking will not reveal lev's precise mechanism without a more nuanced understanding of the processes at play. However, the ability to closely study the vesicle recycling step in the process of neurotransmission will undoubtedly increase the understanding of the inner workings of the synapse, and hopefully this will eventually result in an enhanced ability to treat many neurological disorders, not just epilepsy.

References Cited

1. Poduri, A., *Expect the unexpected in epilepsy genetics: mutations in an epilepsy gene considered to be benign result in a severe phenotype.* Ann Neurol, 2012. **71**(1): p. 1-2.
2. Eadie, M.J., *Shortcomings in the current treatment of epilepsy.* Expert Rev Neurother, 2012. **12**(12): p. 1419-27.
3. George, M.S., et al., *Vagus nerve stimulation therapy: a research update.* Neurology, 2002. **59**(6 Suppl 4): p. S56-61.
4. Kimiskidis, V.K., A. Valentin, and R. Kalviainen, *Transcranial magnetic stimulation for the diagnosis and treatment of epilepsy.* Curr Opin Neurol, 2014. **27**(2): p. 236-41.
5. Lin, L.C., et al., *The long-term effect of listening to Mozart K.448 decreases epileptiform discharges in children with epilepsy.* Epilepsy Behav, 2011. **21**(4): p. 420-4.
6. Temkin, O., *The falling sickness; a history of epilepsy from the Greeks to the beginnings of modern neurology*. 2d ed. 1971, Baltimore,: Johns Hopkins Press. xv, 467 p.
7. Arzimanoglou, A., et al., *The evolution of antiepileptic drug development and regulation.* Epileptic Disord, 2010. **12**(1): p. 3-15.
8. Williams, S.W.D., *On the efficacy of the bromide of potassium in epilepsy and certain psychical affections*. 1865, London,: J. Churchill and sons; etc., etc. 35 p.
9. Friedlander, W.J., *The rise and fall of bromide therapy in epilepsy.* Arch Neurol, 2000. **57**(12): p. 1782-5.
10. Shorvon, S.D., *Drug treatment of epilepsy in the century of the ILAE: the first 50 years, 1909-1958.* Epilepsia, 2009. **50 Suppl 3**: p. 69-92.
11. Putnam, T.J. and H.H. Merritt, *Experimental Determination of the Anticonvulsant Properties of Some Phenyl Derivatives.* Science, 1937. **85**(2213): p. 525-6.
12. Mattson, R.H., et al., *Comparison of carbamazepine, phenobarbital, phenytoin, and primidone in partial and secondarily generalized tonic-clonic seizures.* N Engl J Med, 1985. **313**(3): p. 145-51.
13. Johnson, J.P., *Acquired craniofacial features associated with chronic phenytoin therapy.* Clin Pediatr (Phila), 1984. **23**(12): p. 671-4.
14. Richens, A., *Clinical pharmacokinetics of phenytoin.* Clin Pharmacokinet, 1979. **4**(3): p. 153-69.
15. Danielsson, B.R., A.C. Skold, and F. Azarbayjani, *Class III antiarrhythmics and phenytoin: teratogenicity due to embryonic cardiac dysrhythmia and reoxygenation damage.* Curr Pharm Des, 2001. **7**(9): p. 787-802.
16. Azarbayjani, F. and B.R. Danielsson, *Phenytoin-induced cleft palate: evidence for embryonic cardiac bradyarrhythmia due to inhibition of delayed rectifier K+ channels resulting in hypoxia-reoxygenation damage.* Teratology, 2001. **63**(3): p. 152-60.
17. Singh, G., P.H. Driever, and J.W. Sander, *Cancer risk in people with epilepsy: the role of antiepileptic drugs.* Brain, 2005. **128**(Pt 1): p. 7-17.
18. Farooq, M.U., et al., *Levetiracetam for managing neurologic and psychiatric disorders.* Am J Health Syst Pharm, 2009. **66**(6): p. 541-61.
19. UCB. *Keppra*. 2015 May 28, 2015]; Available from: http://www.ucb.com/investors/UCB-today/Keppra.

20. Fitzgerald, C.L. and M.P. Jacobson, *Generic substitution of levetiracetam resulting in increased incidence of breakthrough seizures.* Ann Pharmacother, 2011. **45**(5): p. e27.
21. Genton, P. and B. Van Vleymen, *Piracetam and levetiracetam: close structural similarities but different pharmacological and clinical profiles.* Epileptic Disord, 2000. **2**(2): p. 99-105.
22. Gower, A.J., et al., *ucb L059, a novel anti-convulsant drug: pharmacological profile in animals.* Eur J Pharmacol, 1992. **222**(2-3): p. 193-203.
23. Birnstiel, S., E. Wulfert, and S.G. Beck, *Levetiracetam (ucb LO59) affects in vitro models of epilepsy in CA3 pyramidal neurons without altering normal synaptic transmission.* Naunyn Schmiedebergs Arch Pharmacol, 1997. **356**(5): p. 611-8.
24. De Smedt, T., et al., *Levetiracetam: part II, the clinical profile of a novel anticonvulsant drug.* CNS Drug Rev, 2007. **13**(1): p. 57-78.
25. Rigo, J.M., et al., *The anti-epileptic drug levetiracetam reverses the inhibition by negative allosteric modulators of neuronal GABA- and glycine-gated currents.* Br J Pharmacol, 2002. **136**(5): p. 659-72.
26. Klitgaard, H., et al., *Pilocarpine-induced epileptogenesis in the rat: impact of initial duration of status epilepticus on electrophysiological and neuropathological alterations.* Epilepsy Res, 2002. **51**(1-2): p. 93-107.
27. Noyer, M., et al., *The novel antiepileptic drug levetiracetam (ucb L059) appears to act via a specific binding site in CNS membranes.* Eur J Pharmacol, 1995. **286**(2): p. 137-46.
28. Lynch, B.A., et al., *The synaptic vesicle protein SV2A is the binding site for the antiepileptic drug levetiracetam.* Proc Natl Acad Sci U S A, 2004. **101**(26): p. 9861-6.
29. Agrawal, N. and S. Govender, *Epilepsy and neuropsychiatric comorbidities.* Advances in Psychiatric Treatment, 2011. **17**(1): p. 44-53.
30. Sankar, R. and A. Mazarati, *Neurobiology of Depression as a Comorbidity of Epilepsy.* Epilepsia, 2010. **51**(s5): p. 81.
31. Sachdev, P., *Schizophrenia-like psychosis and epilepsy: the status of the association.* Am J Psychiatry, 1998. **155**(3): p. 325-36.
32. Krishnamoorthy, E.S., et al., *Forced normalization at the interface between epilepsy and psychiatry.* Epilepsy Behav, 2002. **3**(4): p. 303-308.
33. Helmstaedter, C., et al., *Positive and negative psychotropic effects of levetiracetam.* Epilepsy Behav, 2008. **13**(3): p. 535-41.
34. Mutch, S.A., et al., *Protein Quantification at the Single Vesicle Level Reveals That a Subset of Synaptic Vesicle Proteins Are Trafficked with High Precision.* Journal of Neuroscience, 2011. **31**(4): p. 1461-1470.
35. Wilhelm, B.G., et al., *Composition of isolated synaptic boutons reveals the amounts of vesicle trafficking proteins.* Science, 2014. **344**(6187): p. 1023-8.
36. Hill, T.A., et al., *Inhibition of dynamin mediated endocytosis by the dynoles--synthesis and functional activity of a family of indoles.* J Med Chem, 2009. **52**(12): p. 3762-73.
37. Quan, A. and P.J. Robinson, *Rapid purification of native dynamin I and colorimetric GTPase assay.* Methods Enzymol, 2005. **404**: p. 556-69.
38. Mutch, S.A., et al., *Protein quantification at the single vesicle level reveals that a subset of synaptic vesicle proteins are trafficked with high precision.* Journal of Neuroscience, 2011. **31**(4): p. 1461-70.