Elucidating Levetiracetam action at the presynaptic terminal

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Abstract

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Levetiracetam (Keppra™, **LEV**) is the first of a growing class of anti-epileptic drugs that shows great promise in the treatment of multiple neurological disorders. LEV has a unique receptor, Synaptic Vesicle Protein 2A (**SV2A**), which is both necessary and sufficient for binding. SV2A is the most widely expressed member of a three-protein family (SV2A, B, C) that is localized exclusively to vesicles that undergo calcium-stimulated secretion. Although multiple functions have been ascribed to SV2, it has one verified function. All three SV2s bind to and regulate the stability and trafficking of synaptotagmin, the calcium sensing protein that mediates fast, evoked transmitter release. Although LEV decreases synaptic transmission in *ex vivo* preparations, it has failed to show effects on isolated, autaptic (self-synapsing) neurons, complicating elucidation of its molecular mechanism of action. We asked if the absence of a LEV effect in autaptic neurons was due to the presence of non-LEV binding SV2 paralogs. While LEV had no effect on synaptic transmission in neurons from wild-type (**WT**) mice that express both SV2A and SV2B, it decreased synaptic depression in neurons cultured from SV2B knockout mice (**BKO)**. This suggests that LEV inhibits SV2 function at synapses that express primarily or exclusively SV2A. To test hypotheses of LEV's molecular action, we first focused on SV2's well-established role in regulating the stability and trafficking of synaptotagmin. LEV disrupted the

interaction between SV2A and synaptotagmin, resulting in decreased and slowed internalization of synaptotagmin in neurons from BKO but not WT mice. To determine if LEV impacted the trafficking of other proteins, we developed a technique to isolate newly recycled vesicles from synaptic terminals (synaptosomes). Proteomic analyses of this preparation revealed a LEV-dependent decrease in the amount of complexin1, a soluble protein that binds synaptotagmin and the fusion protein (SNARE) complex in vesicles isolated from BKO but not WT mice. This suggests LEV affects SV2A's protein-protein interactions and that further LEV studies should focus on synapses solely expressing SV2A. Two classes of neurons fit this category, most inhibitory neurons and the excitatory granule cells of the dentate gyrus. These two classes have also been implicated in epilepsy.

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CHAPTER 1: INTRODUCTION

Synaptic vesicles are self-contained organelles that play an essential role in neurotransmission. Because of this, studies have focused on understanding the molecular composition of vesicles and how vesicle proteins are sorted to ensure proper neurotransmitter release [2]. Of the many vesicle proteins, synaptic vesicle protein 2 (**SV2A**) became of interest because it is the receptor for an anti-epileptic drug, Levetiracetam (Keppra™, **LEV**) [3]. LEV demonstrates broad therapeutic potential but its mechanism of action is still unknown making it difficult to determine what other neurological disorders it can treat and how to develop better analogs [4]. Thus, one way to understand the pharmacological profile of LEV is to understand its receptor's function.

The SV2s are 12 transmembrane glycoproteins with cytoplasmic N and C-termini present in all synaptic vesicles [5, 6]. There are three SV2 paralogs in mammals, SV2A, SV2B and SV2C. One notable difference between the SV2s are their distribution in the brain. SV2A is the most widely distributed paralog and ubiquitously present throughout the brain and in endocrine cells. SV2B expression is more restricted to the brain and SV2C is only present in the basal forebrain and brainstem [6]. Although SV2A and SV2B are co-expressed in most synapses, SV2B is only found in glutamatergic synapses (**Figure 1**). SV2A is the only known paralog expressed in most GABAergic neurons and in glutamatergic neurons of dentate granule cells, two classes of neurons implicated in the development of epilepsy [7]. This suggests that loss of SV2A in these neurons could result in aberrant neurotransmission.

I. SV2A is essential

Of the three SV2 paralogs, SV2A is essential for survival. Mice lacking SV2A experience severe seizures and die by week three demonstrating that SV2A is required for normal neurotransmission and survival. But there was no observed change in the density or morphology of their synapses. These mice appear normal at birth but around day nine are

approximately half the size of their littermates. Compared to wild-type (WT) mice, their brains are smaller but in proportion to their smaller body size. Heterozygote mutants also experienced spontaneous seizures, but these mice were fertile and viable suggesting a single copy of SV2A is sufficient for growth [8, 9]. Further confirming SV2A is the essential paralog, $SV2A^{-/-}SV2B^{-/-}$ (**DKO**) mice had similar phenotypes to mice lacking SV2A suggesting that loss of SV2B does not aggravate the phenotype [8, 10].

In contrast to loss of the SV2A paralog, mice lacking SV2B (**BKO**) were fertile and viable. But there were some neurotransmission deficits in the retina due to SV2B being the primary paralog in these synapses [11]. Similarly, SV2C knockout mice were also fertile and viable but with neurotransmission deficits in dopaminergic synapses due to it being the primary paralog [12]. Interestingly, although SV2A is the essential paralog, each SV2 paralog can rescue normal neurotransmission when expressed in neurons lacking SV2 suggesting they have redundant functions [13].

II. SV2 is required for normal calcium-evoked neurotransmission

One of the main phenotypes in mice lacking SV2 is the neurotransmission deficits highlighting SV2's role in calcium-evoked neurotransmission. Briefly, during exocytosis, synaptic vesicles translocate to the active zone and dock to the plasma membrane. Docked synaptic vesicles undergo priming steps for calcium-dependent fusion. During priming, SNARE (soluble NSF (N-ethylmaleimide-sensitive fusion) attachment protein receptor) complexes are partially formed. An influx of calcium influx, facilitated by the calcium sensor synaptotagmin, then triggers neurotransmitter release. After exocytosis, synaptic vesicles are endocytosed and enter the recycling pool of vesicles [14]. Loss of SV2 had no effect in the frequency or amplitude of miniature neurotransmission events suggesting SV2 is not required for the uptake and storage of neurotransmitter nor vesicle fusion [8, 9]. But there was a reduction in preformed SNARE complexes with loss of SV2 suggesting SV2 affects priming of vesicles [15].

Autaptic recordings from neurons lacking SV2 showed decreased EPSC amplitudes but no reduction in synapse formation, no loss of functional synapses, or decreased quantal size. Neurons lacking SV2 also exhibited synaptic facilitation at the beginning of stimulus trains during evoked release indicating synapses had a lower release probability. This is consistent with the increase in paired pulse responses suggesting that loss of SV2 reduces the size of the readily releasable pool of vesicle (RRP) [10]. Thus, SV2's function may be to increase synaptic release probability by increasing the population of primed release competent vesicles.

III. SV2A can bind to endocytic proteins

In addition to SV2's role in calcium evoked neurotransmission, SV2 has also been implicated in endocytosis. Currently, there are four modes of endocytosis recognized: kiss and run, clathrin-mediated, clathrin-independent endocytosis and ultrafast endocytosis. During mild stimulation, clathrin-mediated endocytosis was thought to be the dominant form of endocytosis [16]. . Adaptor protein, AP2, binds to phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2) on the plasma membrane which triggers a conformational change. This reveals its binding sites to endocytic motifs (YXXΦ or D/EXXXLL) on cargo proteins. Peptides containing the endocytic motif in turn stimulate AP2 binding to calcium sensor, synaptotagmin1 [17].

In contrast to clathrin-mediated endocytosis, ultrafast endocytosis and clathrin-independent endocytosis form endosomes (large endocytic vesicles) at the plasma membrane [16]. Synaptic vesicles are then regenerated from these endosomes through clathrin-mediated endocytosis. Although membrane retrieval still occurs in the absence of clathrin and AP2, there is an accumulation of endosomes at the plasma membrane. This supports the idea that the primary function of clathrin-mediated endocytosis is to regenerate synaptic vesicles of proper size and composition, either directly from the plasma membrane or from endosomes [18].

SV2A's N-terminus contains two tyrosine-based endocytic motifs (YXXØ). The first motif is located in the cytoplasmic N terminus (46-49, YSRF) and shared with SV2C, while the second

motif is at the end of the cytoplasmic loop before the seventh transmembrane domain (443-446, YRRI) [17]. This motif has been found in other cargo proteins and is important for binding to clathrin-mediated endocytosis associated proteins such as AP2 [17, 19]. When AP2 binds to this motif, it stimulates it's recruitment to synaptotagmin to then facilitate clathrin coat pit formation [17]. Mutation of the first endocytic motif in SV2A resulted in reduced binding to endocytosis-related proteins such as AP2, EPS15, and Bin1 confirming it has a role in endocytosis [17, 19].

IV. SV2 is structurally similar to transporters

Although SV2 contains signature motifs of major facilitator transporters (**MFS**), a transporter function has not been confirmed. SV2A was reported to function as a galactose transporter when expressed in yeast [20] but there was no galactose uptake by purified synaptic vesicles (*R. Edwards, unpublished data*). To further understand the functional importance of conserved residues in SV2A, single and multi-residue mutations were made [13, 21] (**Fig. 2**). DXXGRR/K is a MF transporter signature motif present in SV2A in the cytoplasmic loop between transmembrane 2 and 3. Aspartate and the first arginine are important for transport activity in MF. Mutation of these residues in SV2A, (SV2A-D227A and R231Q) showed that SV2A-D227A did not traffic to synapses but SV2A-R231Q mutant did. SV2A-R231Q was also able to restore synaptic depression seen in neurons lacking SV2 suggesting that this residue was not crucial to SV2 function.

In addition to conserved residues in MFS transport activity, SV2A contains two conserved charged residues in its first transmembrane thought to be important for cation transport. Mutation of these sites produced lower levels of SV2A and failed to traffic to synapses. These mutants could not rescue the short-term plasticity seen in neurons lacking SV2. This suggests that these two residues are important for SV2A trafficking. SV2A contains two conserved tryptophans in the fifth (W300) and tenth (W666) transmembrane domain. These

residues are important for the transport activity in glucose transporter GLUT-1 and organic anion transporters. Expression of these mutants W300A and W666A in neurons lacking SV2 trafficked to synapses but could not restore synaptic depression. Although these mutants could not restore normal neurotransmission, they could restore internalization of synaptotagmin. This suggests that these residues are important for an undiscovered SV2 function [13].

SV2 contains three N-glycosylation sites on its intraluminal loop (N498, N548, and N573). N-glycosylation is a post-translation modification that involves the attachment of an oligosaccharide to the amino group of an asparagine residue in the endoplasmic reticulum. Single mutations of SV2A's N-glycosylation sites expressed in hippocampal neuron cultured lacking SV2 did not affect its synaptic location or SV2A protein expression levels. In contrast, mutations of two of the N-glycosylation sites (N548Q/N573Q) resulted in reduced SV2A expression levels did not target SV2A to recycling vesicles. This suggests that these sites are important for ER quality control and for protein sorting [13, 22].

Mapping studies of SV2A identified two Walker ATP-binding domains located in the cytoplasmic N-terminus (aa 129-143) and a region that spans the fourth transmembrane (amino acid residues 266-288). Similarly, to MFS transporters that contain nucleotide binding sites and can be regulated by ATP binding, all three SV2 paralogs can bind adenine nucleotides on both N- and C-termini. Expression of SV2 in HEK293 cell microsomes did not show any ATP transport activity indicating that SV2's function may be modulated by adenine nucleotides but SV2 does not transport them [23].

V. SV2 is important for synaptotagmin trafficking

Although SV2's function at the synapse has not been fully elucidated, it is known that all three SV2 paralogs can bind to synaptotagmin. Calcium inhibits SV2 binding to the C2B domain of synaptotagmin. Both SV2A and SV2C bind to synaptotagmin via its N-terminus cytoplasmic domain [24]. The SV2A and synaptotagmin interaction is regulated by Casein Kinase Family 1's

phosphorylation of threonine 84 of SV2A [25, 26]. SV2A phosphorylation is necessary for binding to the C2B domain of synaptotagmin and is important for synaptotagmin retrieval. An analogous threonine is also found in SV2B and SV2C consistent with all SV2's regulating synaptotagmin trafficking in a phosphorylation-dependent manner.

SV2's role in synaptotagmin trafficking overlaps with a specialized clathrin adaptor protein, stonin2 and together are considered intrinsic trafficking partners (iTRAP) [27]. Loss of either SV2 or stonin2 disrupts synaptotagmin retrieval at synapses, and loss of both proteins exacerbates the effect of disrupting just one of them. Interestingly, with loss of SV2, stonin2 levels are upregulated. This suggests that proteins required for sorting of crucial vesicle proteins, such as synaptotagmin, is tightly regulated to ensure proper neurotransmission in the brain [28].

In addition to its ability to bind to synaptotagmin, SV2 regulates synaptotagmin expression and trafficking. Mice lacking SV2 had reduced levels of synaptotagmin in brain homogenate and reduced levels in immunoisolated synaptic vesicles. Similarly, overexpression of SV2A in neurons led to increased synaptotagmin expression levels and trafficking at synapses [29].

Neurons lacking SV2 had decreased synaptotagmin levels, but this was rescued by expressing either wild-type SV2A or SV2A-Y46A. Although, SV2A-Y46A was able to restore synaptotagmin levels, synaptotagmin was not properly trafficked to synapses. This suggests that SV2's ability to regulate synaptotagmin expression levels independent of its trafficking function [15].

Synaptotagmin

Regulation of synaptotagmin1 trafficking and expression is important because synaptotagmin1 is a vesicle protein essential for survival and necessary for calcium-stimulated

neurotransmission. Loss of synaptotagmin1 in mice leads to death within 48 hours [30, 31]. Loss of synaptotagmin1 has shown to affect synchronous release, the fast component of evoked neurotransmitter release, but not the slow component, asynchronous release. Neurons lacking synaptotagmin1 also exhibited slower rates of endocytosis. Interestingly, its role in exocytosis was demonstrated to be independent of its role in exocytosis, [32, 33]

In addition to its ability to bind to SV2, synaptotagmin has other notable protein-protein interactions. Calcium binding to synaptotagmin induces its interactions with phospholipids and SNARE complexes [34]. As the structures of synaptic protein complexes are being solved, more studies have been interested in synaptotagmin1's interaction with complexin, a family of small soluble proteins that interact with SNARE complexes, that, like synaptotagmin, help mediate synchronized evoked release [1]. As for synaptotagmin's role in endocytosis, the C2B domain of synaptotagmin can bind to clathrin adaptor protein AP2 [17]. Disruption of this interaction inhibited calcium-dependent clathrin-mediated endocytosis but did not affect exocytosis [35] suggesting that this interaction is only important for endocytosis. Surprisingly, both C2A and C2B domains are capable of sensing calcium during endocytosis. It is hypothesized that although the C2A domain cannot directly bind to AP2, it can bind to another adaptor protein, stonin2, which then recruits AP2 to facilitate endocytosis [32].

VI. SV2 and disease

Due to the regulatory role presynaptic proteins have in neurotransmission, expression levels of these proteins are tightly regulated with alterations leading to neurological disorders. Studies from post-mortem brain tissue to cells *in vitro* show that alterations in SV2 levels are linked to etiology of epilepsy, Alzheimer's, Parkinson's and other neurological disorders. Further understanding how SV2 plays a role in these diseases could lead to the development of better SV2-directed therapeutic drugs.

Alteration in SV2A expression levels

Both increase and decrease in SV2A expression levels were found to be implicated in epilepsy. Post-mortem tissue from patients with temporal lobe epilepsy and hippocampal sclerosis had decreased SV2A expression [36]. Similarly, there were decreased levels of SV2A during epileptogenesis in rats correlating with increased seizure susceptibility and accelerated epileptogenesis [37]. Interestingly, SV2A expression levels were decreased particularly in the mossy fiber terminals in the latent and chronic epileptic phase. Loss of SV2A in these cells could lead to hyperexcitability in dentate granule cells and an imbalance between excitatory and inhibitory neurotransmission [38].

In addition to decreased SV2A levels playing a role in epilepsy, it was seen that overexpression of SV2A levels also contributed to the onset of seizures [39]. Both amygdala [40] and pentylenetrazole (PTZ) kindling [39] were found to have increased levels of SV2A in the hippocampus. Along with the increase in SV2A levels observed during amygdala kindling, there was an increase in SNARE complexes and a decrease in N-ethlymaleimide-sensitive factor (NSF) [41], the enzyme that mediates SNARE complex disassembly. PTZ kindling produced higher SV2A levels in the hilus of the dentate gyrus but not in the CA3 and CA1 regions. Because the hilar region of the dentate gyrus (DG) contains inhibitory GABAergic interneurons that negatively regulate the DG-CA3 excitatory region, the increase in SV2A levels may compensate for the increase in excitability by increasing inhibitory GABAergic neurotransmission [39]. Altogether, this suggests that maintaining SV2A levels is important for normal neurotransmission.

Mutations in SV2A are linked to epilepsy

Although changes in SV2A expression levels have been commonly implicated in epilepsy, mutations in SV2A have also been shown to be involved in the pathogenesis of epilepsy. Rats carrying the point mutation SV2A-L174Q demonstrate decreased synaptotagmin

expression levels and disrupted GABA release in the hippocampus. In addition, these rats are more susceptible to PTZ kindling [42]. Exome sequencing of a patient with intractable epilepsy revealed a homozygous arginine to glutamine mutation (R383Q) in exon 5 of SV2A [43]. This homozygous recessive mutation lies in the cytosolic loop before the seventh transmembrane domain which has been reported to be important in SV2A binding to adenine nucleotides. Further understanding SV2A's role in synapses could lead to the development of better anti-epileptic drugs that target SV2A.

SV2 and Alzheimer's disease

In addition to epilepsy, SV2 has also been implicated in Alzheimer's disease. SV2A was found in a proteomic screen identified for proteins that bind to FE65, a protein known to bind to amyloid precursor protein, APP, leading to APP cleavage and secretion. Co-expression of SV2A and FE65 in HEK293T cells suggest that SV2A interacts with FE65 and induces its relocalization from the nucleus to the cytoplasm where it alters APP processing [44]. Furthermore, in an Alzheimer's disease derived line overexpressing Presenilin1, there was decreased levels of SV2B and RAB3A [45]. Two Presenilin1-directed FDA approved drugs, BMS-708163 and Nilotinib, restored RAB3A and SV2B levels and recovered the abnormal sEPSC frequencies suggesting that normal levels of SV2B and RAB3A levels are key to recovering the sEPSC frequencies in this model. In contrast, wild-type mice injected with Aβ had deficits in cognitive behavioral tasks but mice lacking SV2B were protected from the deleterious effects of amyloid peptides. It is hypothesized that Aβ enters the presynaptic terminal via SV2B, making SV2B a likely drug target for preventing Aβ entry [45]. Further understanding of SV2A and SV2B's role in Alzheimer's could help the development of SV2 targeted therapeutic drugs.

SV2 and Parkinson's disease

Compared to SV2A and B, SV2C expression is more restricted in the brain. Regions with high levels of SV2C include the basal ganglia, where it is found primarily in the dopaminergic

projections from the substantia nigra. Consequently, decreased levels of SV2C were found to contribute to the development of Parkinson's disease. Proteomic analysis of post-mortem tissues of patients with Parkinson's disease found reduced levels of several proteins related to synaptic transmission including SV2C. Mice treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) displayed a loss of striatal dopamine terminals and reduced SV2C levels. In mice in which the vesicular monoamine transporter 2 (VMAT2) was reduced by 95%, striatal SV2C levels were also reduced [12]. In addition to loss of SV2C's contribution to the development of Parkinson's, overexpression of SV2C can also result in Parkinson's like phenotype. For example, mice overexpressing A53T α-synuclein had reduced dopamine release and loss of dopaminergic terminals but an increase in punctate SV2C staining [12].

To further elaborate on SV2C's role, SV2C knockout mice were generated. Loss of SV2C did not affect expression levels of SV2A, SV2B and other synaptic proteins. However, there was a 33% reduction in striatal dopamine content and a reduction in a dopamine metabolite, DOPAC, but no effect on the rate limiting enzyme in dopamine production, tyrosine hydroxylase [12]. Understanding SV2C's function will give insight into the treatment of Parkinson's disease.

SV2 and schizophrenia

While alterations in SV2 levels is a common underlying theme in neurological disorders, SV2 gene variations also play in the etiology of schizophrenia. In an mRNA sequencing approach to identify proteins with altered expression in post-mortem brains of schizophrenic patients, SV2A transcripts were found to be down-regulated in the cerebral cortex [46]. A HapMap-based candidate gene approach showed that variations at SV2A locus are risk factors for schizophrenia. Antipsychotic drugs were also shown to produce different responses depending on variations in the SV2C gene [47].

Altogether, while SV2A is the more widely studied SV2 paralog, SV2B and SV2C may also influence the development of neurological disorders. This raises the question whether the development of these diseases is due to changes in the ratio of SV2 paralog expression.

VII. SV2A is the receptor for Levetiracetam

Levetiracetam (Keppra™, **LEV)** is a second-generation antiepileptic drug (AED) [48, 49] [3][3][3]that was first discovered as an analog to potentially replace piracetam, a nootropic drug. Although LEV did not show any promising effects in treating cognitive impairment, it was found that LEV had anti-seizure effects and was approved by the FDA in 1999 to treat partial and generalized epilepsy [48, 49]. Its mechanism of action has not been fully elucidated but has been reported to inhibit N-type calcium channel function, inhibit calcium release from intracellular stores, reduce GABA release, and decrease calcium conductance [50].

Although its mechanism of action is unknown, it is known that LEV binds to SV2A [3]. Synaptic vesicle preparations lacking SV2A failed to bind LEV and expression of SV2A confers LEV binding to membranes from cultured fibroblasts [3]. Thus, SV2A is both necessary and sufficient for LEV binding. SV2's role as the receptor for LEV is further supported by the observation that the drug is less effective in mice lacking normal levels of SV2A [51]. In addtion, in humans carrying a mutation in SV2A LEV is ineffective [43].

A link between LEV action and SV2 expression was found in studies of protein expression following seizure induction via kindling. These studies confirmed increased expression of proteins such as GFAP, NPY, and TRH, which were previously reported to be increased in temporal lobe epilepsy. They also revealed an increase in SNARE complexes and SV2 expression. Treatment with LEV during the amygdala kindling process reduced expression of these genes [52]. Although the changes were not statistically significant, the relative changes were modest suggesting again that mild changes in this complex network have a big impact on synaptic activity [50]. LEV has no effect on SV2 levels in non-kindled rats supporting the idea

that LEV modulates SV2A function only in epileptic tissue. Similarly, LEV treatment restored synaptotagmin expression levels and rescued synaptic transmission in neurons overexpressing SV2A [29].

Because the SV2A structure has not been solved, researchers have relied on homology remodelling to identify LEV binding sites. It has been proposed that SV2A has two posible conformation states for LEV binding: an inward model in which the binding site is towards the cytosol and an outward model in which the binding site is inside the vesicle. These studies identified residue D670 in SV2A as a residue likely to affect drug binding with mutation of this residue in SV2A (D670A) abolishing LEV binding. Due to the location of this residue in the inward model, it suggests that's LEV binding site is positioned in the outward model [53].

Interestingly, LEV only affects the electrophysiology of epileptic tissue but has no effects in normal tissue [54, 55]. In wild-type hippocampal slice preparations, LEV treatment affects both inhibitory and excitatory synaptic transmission in an activity dependent manner suggesting that it needed synaptic vesicle turnover to modulate SV2A's activity [56, 57]. Further studies suggest LEV dampens synaptic activity by decreasing the readily releasable pool of vesicles [56] consistent with its effects on accelerating induction of supply rate depression [58] and increased short term depression [59].

Amygdala kindling of rats increased gene expression in proteins such as GFAP, NPY, and TRH similarly to those reported in temporal lobe epilepsy. Interestingly, there was also an increase in 7S SNARE complexes and SV2 expression. Treatment with LEV during the amygdala kindling process reduced expression of these genes [52]. Although the changes were not statistically significant, the relative changes were modest suggesting again that mild changes in this complex network have a big impact on synaptic activity [50]. LEV has no effect on SV2 levels in non-kindled rats supporting the idea that LEV modulates SV2A function only in epileptic tissue. Similarly, LEV treatment restored synaptotagmin expression levels and rescued

synaptic transmission in neurons overexpressing SV2A [29]. Further understanding of SV2A's role in the synapse will provide insight into LEV action.

IX. Conclusion

SV2A is the only synaptic vesicle protein targeted by a synthetic drug. Currently, other LEV analogs with higher affinity for SV2A such as Brivaracetam [60] and Seletracetam [61] are being developed, but their mechanism of action are also unknown. Because SV2A is ubiquitously expressed and found to have a role in neurological diseases, understanding LEV's mechanism of action is one way to gain further insight into SV2 function.

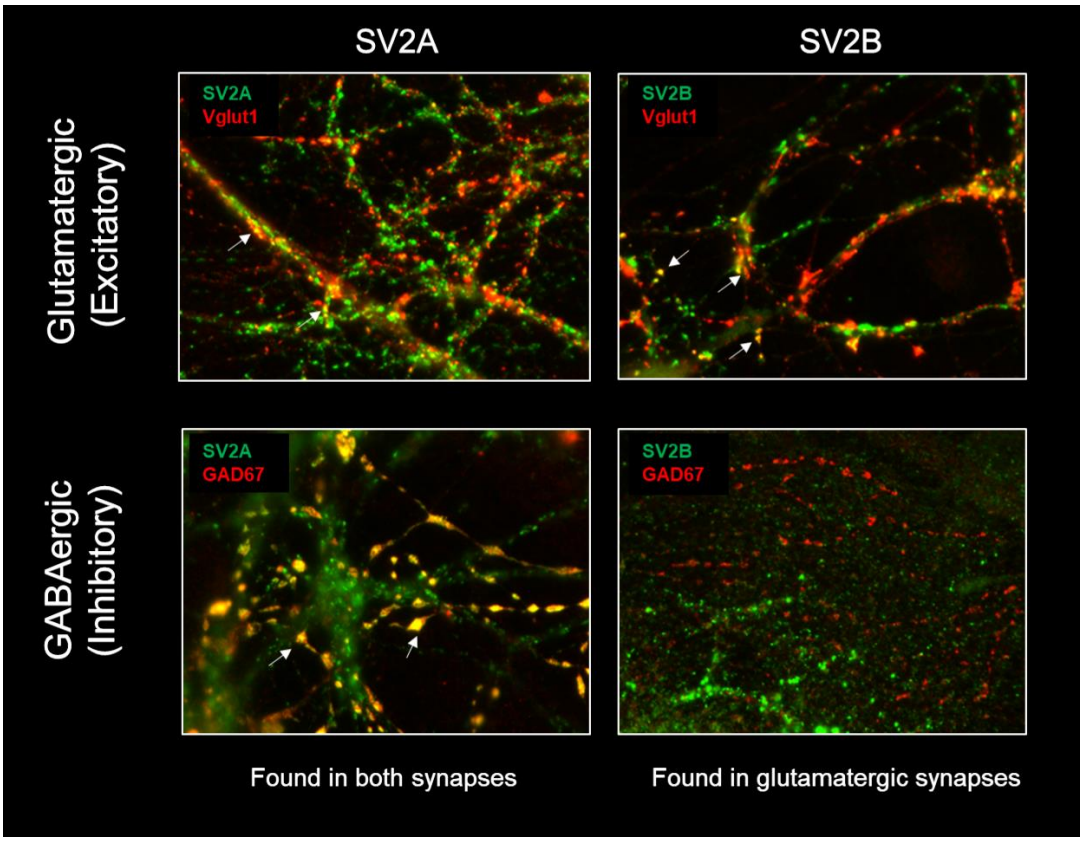


Figure 1: SV2A is found in both glutamatergic and GABAergic synapses, but SV2B is only found in glutamatergic synapses

Wild-type hippocampal neurons were immunolabeled with antibodies against SV2 proteins (green) and Vglut1 (red), a glutamatergic marker, or GAD67 (red), a GABAergic marker, as indicated. Although SV2A overlapped with both Vglut1 and GAD67, suggesting that SV2A is found in both excitatory and inhibitory neurons, SV2B was only found in excitatory neurons (indicated by white arrows).

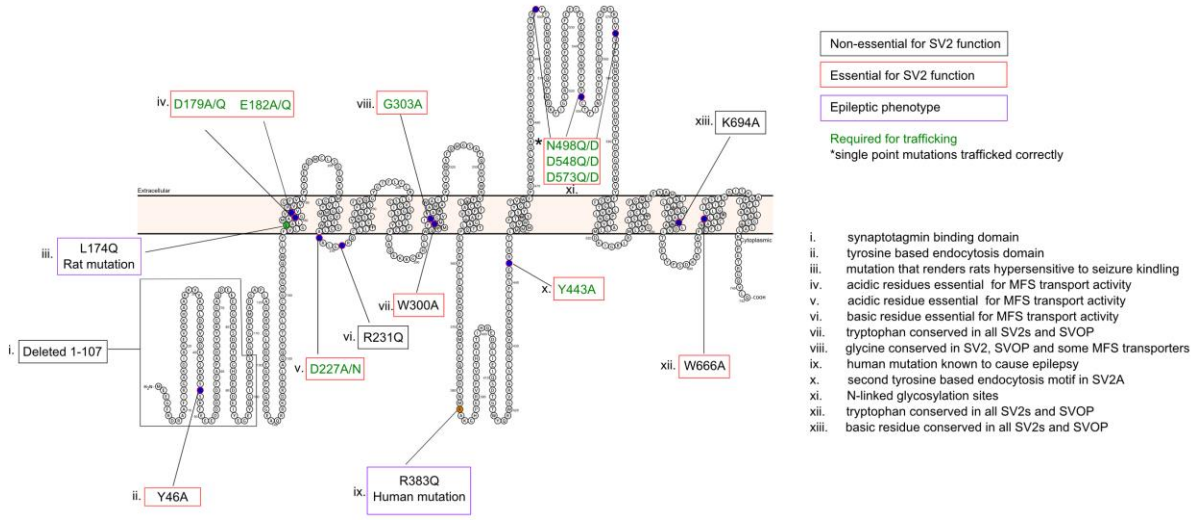


Figure 2: Mutational analyses of SV2A

Shown is a schematic of SV2A indicating residues that have been subjected to mutation. Each mutation is numbered and the feature of the corresponding residue is described in the key at the right. The effect of each mutation is indicated by the color of its outline. A black outline indicates mutants that were expressed and appropriately trafficked to synapses and restored normal neurotransmission, thus indicating that the residue is not essential to SV2 function. A red box indicates a mutation that trafficked to the synapse but did not restore normal neurotransmission, and thus is essential for SV2 actin at the synapse. A green box indicates a mutation that produced a protein that did not traffic to presynaptic terminals, indicating it is likely essential for proper SV2 folding. A purple box indicates a mutation linked to epilepsy. The illustration was generated using Protter [62]**.**

(Figure obtained from [63])

CHAPTER 2:

The SV2A-directed drug Levetiracetam disrupts interaction with synaptotagmin at synapses that lack SV2B

Introduction

Levetiracetam (Keppra™, **LEV**) is a second-generation anti-epileptic drug (**AED**) that lacks many of the adverse side effects of classic AEDs. In addition to its use for epilepsy, LEV is being used to treat a range of neurological disorders [64-68] and has recently been found to be a promising treatment for cognitive impairment [69]. Hindering its further development is the fact that LEV's molecular mechanism of action remains unknown.

Consistent with its unique pharmacological profile, LEV has a novel target, Synaptic Vesicle protein 2A, (**SV2A**). SV2A is one of three functionally redundant membrane proteins unique to secretory vesicles that undergo calcium-regulated secretion [5, 6, 13]. SV2A is both necessary and sufficient for LEV binding [3], and multiple observations suggest that LEV acts by affecting SV2A function. This includes the fact that SV2A hypomorphic ($SV2A^{+/-}$) mice are less responsive to LEV's anti-seizure effects [51]. Furthermore, the ability of structurally-related compounds to control seizures is directly correlated to affinity for SV2A [70]. Although SV2 has been proposed to have multiple functions [13, 20], its fully vetted action is calcium-inhibited binding to the calcium sensor protein synaptotagmin. This interaction has been shown to regulate synaptotagmin trafficking during endocytosis [19, 26, 28].

Of the three SV2 paralogs, SV2A is the most widely expressed. Notably, it is the only SV2 expressed in the majority of GABAergic neurons [6, 7]. Loss of SV2A results in severe seizures and early death [8, 9]. Interestingly, elevated SV2A is also associated with neural pathology. Seizure kindling in rodents is correlated with increased SV2A expression [39, 40, 52], and overexpression of SV2A in cultured neurons results in aberrant neurotransmission that is rescued by LEV [29]. Thus, it appears that both increases and decreases in SV2A may contribute to epilepsy.

LEV produces an activity-dependent decrease of both excitatory and inhibitory neurotransmission in hippocampal slice preparations [57, 71]. However, it has no effect on autaptic neurons [29]. This suggested that LEV action might not be cell autonomous, or, alternatively, that the presence of non-LEV binding SV2 paralogs masks drug effects. To begin to understand LEV's mechanism of action at the cellular level, we examined LEV effects in neurons in which SV2A was the sole SV2 paralog. We report that in preparations from SV2B knockout (**BKO**) mice, LEV reduces synaptic depression in autaptic neurons, reduces SV2A binding to synaptotagmin, and slows synaptotagmin endocytosis in conventionally cultured hippocampal neurons. Our findings suggest that LEV acts primarily at synapses that express only SV2A by disrupting SV2A-regulated synaptotagmin function.

Results

LEV decreases synaptic depression in hippocampal neurons from BKO mice

Previous studies examining LEV effects on synaptic transmission in autaptic hippocampal neurons found no measureable effect on neurotransmission [29]. The wild-type (**WT**) excitatory neurons used in those studies express SV2A and SV2B with low or non-existent levels of SV2C. Because all SV2 paralogs can rescue normal neurotransmission when expressed in neurons from $SV2A^{-/-}SV2B^{-/-}$ mice (**DKO**) mice [13], they are considered to be functionally redundant. Thus, the presence of SV2B, which does not bind LEV, might be masking measureable effects in WT excitatory neurons.

To assess LEV effects on neurotransmission in the absence of SV2B, we measured synaptic depression, an indicator of synaptic release probability [72]. Excitatory principal neurons from the CA region of the hippocampus were cultured from $SV2B^{-/-}$ (**BKO**) mice. Single neurons were grown on microislands of astrocytes allowing them to form autaptic synapses. Cultures were treated +/- 300uM LEV for 3-7 hours. This concentration and incubation time was chosen because it produced effects on synaptic transmission in hippocampal slices [73].

Excitatory postsynaptic currents (EPSCs) in response to 2 sec, 20 Hz stimulus trains were recorded.

Cultured excitatory hippocampal neurons normally demonstrate synaptic depression during 20Hz stimulus trains. LEV treatment reduced the extent of this depression in neurons from BKO mice (**Figure 3A**). Consistent with this, the paired-pulse ratio, a comparison of the first two EPSCs, was increased by LEV treatment (PPR_{Ctrl} = 0.87±0.06, PPR_{LEV} = 1.02±0.04, *P <0.05) (**Figure 3B**). These changes in the rate of synaptic depression suggest that LEV is decreasing the probability of neurotransmitter release from BKO synapses [74]. We note that this effect is similar to the decrease in synaptic depression observed in neurons lacking SV2 [10], suggesting that LEV impairs SV2 function. In contrast to its effects on neurons lacking SV2B, LEV had no significant effect on synaptic depression in neurons cultured from WT mice (PPR_{ctrl} = 0.82±0.05, PPR_{LEV} = 0.94±0.05) (**Figure 3C-D**). The selective effect of LEV on transmitter release from neurons expressing only SV2A suggests that the presence of other SV2 paralogs masks LEV effects in WT hippocampal neurons.

LEV decreases SV2A binding to synaptotagmin

SV2 binds the C2B domain of the calcium sensor protein synaptotagmin [24-26, 75, 76], domain essential to synaptotagmin's role in exocytosis [77-79] and endocytosis [32, 35]. To determine if LEV affects the interaction of SV2A with synaptotagmin, we assessed their binding *in vitro* in the presence and absence of 300uM LEV. Binding reactions included Triton X-100 extracts of mouse brain and resin coated with either recombinant glutathione-S-transferase (GST) or a glutathione-S-transferase–synaptotagmin 1 fusion protein (GST-Syt1) [75]. Because calcium inhibits SV2 - synaptotagmin binding, we assayed LEV action in both low calcium (10^{-9}M) and high (2mM) calcium. SV2A binding to the resin was then assessed by Western blot analysis.

LEV treatment reduced SV2A binding to GST-Syt1 in both high and low calcium conditions when the source of SV2A was brain extracts of BKO mice. At low calcium concentrations, LEV reduced binding by ~40%. Calcium alone decreased binding by 63%, and addition of LEV resulted in a further decrease to 14% of control (86% reduction) (**Figure 4A**). Somewhat surprisingly, LEV had no effect on SV2A binding to synaptotagmin when the source of SV2A was WT mouse brain (**Figure 4B**). The absence of LEV effects on SV2A when SV2B is present suggests that SV2s may form hetero-oligomers, precluding LEV action. Consistent with this, we find that SV2B co-immunoprecipitates with SV2A from extracts of WT mouse brain (**Figure 5**).

LEV slows synaptotagmin endocytosis at synapses

The interaction of SV2A and synaptotagmin plays a crucial role in synaptotagmin's ability to engage the proteins that mediate clathrin-dependent endocytosis [17]. Thus SV2A is essential to synaptotagmin trafficking to synaptic vesicles [19, 28]. To determine if LEV's inhibition of SV2A-synaptotagmin binding impacts synaptotagmin trafficking, we assessed its effects using two measures of synaptotagmin endocytosis in cultured hippocampal neurons.

First, we quantified the amount of surface stranded synaptotagmin in conventional hippocampal cultures treated with LEV. Cultures from WT or BKO mice were treated +/- 300uM LEV for 3 hours and then incubated with EZ-Link Sulfo-NHS-LC-LC-Biotin, a membrane-impermeant biotinylating reagent. After cultures were harvested and extracted in Triton X-100, surface stranded biotinylated proteins were isolated using streptavidin beads. The amount of biotinylated synaptotagmin and SV2A was quantified using Western blot analysis. Blots were probed for SV2A, synaptotagmin, and the plasma membrane protein sodium/potassium ATPase, which served as a loading control. Compared to untreated cells, LEV treatment increased the amount of synaptotagmin on the surface by ~60%, suggesting decreased internalization (**Figure 6A**). Interestingly, there was no significant effect on the amount of

biotinylated SV2A suggesting that SV2A's internalization is not affected by LEV. Consistent with its effects on neurotransmission and *in vitro* protein interaction, LEV had no effect on the amount of biotinylated synaptotagmin or SV2A in WT neurons (**Figure 6B**). To control for the possibility that increased surface synaptotagmin was due to increased expression levels, we compared the amount of synaptotagmin in brain post nuclear supernatant from WT and BKO mice. There was no difference (**Figure 7**). Thus, the most parsimonious interpretation is that LEV disrupts SV2A regulation of synaptotagmin trafficking.

To measure LEV effects on the rate of synaptotagmin retrieval, we tracked the exo- and endocytosis of synaptotagmin1-pHluorin (Syt1-pHluorin), a fusion protein consisting of synaptotagmin1 and pHluorin, a pH sensitive GFP variant [80]. When fused to the lumenal domain of a synaptic vesicle protein, pHluorin is quenched by the low pH of the vesicle interior. It becomes fluorescent upon vesicle fusion and exposure to the neutral pH of the synapse exterior. As pHluorin fusion proteins are recycled back into vesicles during endocytosis, fluorescence is quenched. The rate of fluorescence decline can be used to measure the endocytic time constant, tau.

Conventional hippocampal cultures from BKO mice were transfected with a construct driving the expression of Syt1-pHluorin under the control of the synapsin promoter. Prior to field stimulation, cultures were treated +/- 300uM LEV for 3-8 hours after which fluorescence was recorded at a rate of 2 Hz in response to a stimulus train of 200 action potentials delivered at 40Hz. LEV treatment slowed Syt1-pHluorin retrieval after the initial stimulus trai[81]n. LEV increased the endocytic time constant by ~60% (τ_{ctrl} = 53.9$\pm$5.4 s, τ_{Lev} = 86.7 $\pm$ 10.2 s) (**Figure 8A-C**). In contrast, LEV treatment had no significant effect on the time course of Syt1-pHluorin retrieval in cultures from WT mice (τ_{ctrl} = 49.69$\pm$5.18 s, τ_{LEV} = 55.6$\pm$7.44 s), consistent with it affecting only synapses that exclusively express SV2A (**Figure 8D-E**).

Because complete loss of SV2A results in early death, it has been impossible to test the requirement for SV2A in LEV action. But because the cultures used for these studies are generated from neonatal mice (Postnatal days 0-2), we were able to compare LEV action in cultures from BKO from DKO mice. Consistent with a requirement for SV2A, LEV had no effect on the time course of Syt1-pHluorin retrieval in cultures from DKO mice (τ_{ctrl} = 60.2±7.55 s, τ_{Lev} = 52.13±3.34 s) (**Figure 8F-H**).

Loss of SV2A leads to a selective effect on the trafficking of synaptotagmin. To assess the specificity of the LEV action on endocytosis, we measured retrieval of synaptophysin, a vesicle protein whose trafficking is not linked to SV2 [19, 28]. For these studies, we tracked the exo- and endocytosis of synaptophysin-pHluorin (SypHy) in cultures from both WT and BKO mice. In contrast to LEV's effects on Syt1-pHluorin trafficking, LEV had no significant effect on SypHy retrieval in either BKO (τ_{ctrl} = 61.17± 6.4 s, τ_{LEV} = 56.5±3.81 s) or WT (τ_{ctrl} = 55.45±6.92 s, τ_{LEV} = 45.92±3.92 s) cultures (**Figure 9).** This suggests that LEV has a selective effect on proteins that bind to SV2A.

Discussion

The work presented here provides two new insights into the mechanism LEV action. First, we show that LEV selectively affects synaptic transmission at synapses that lack SV2B. This suggests that LEV action is limited to synapses that express SV2A exclusively. This finding has important implications not only for understanding how LEV reduces seizure activity, but also for the development of compounds that target other SV2 paralogs. Second, we demonstrate that LEV acts at the molecular level by disrupting SV2A's interaction with the calcium sensor protein synaptotagmin. These findings provide further evidence that LEV action is unique among anti-epileptic medications.

LEV action predominates at synapses that express only SV2A

A standing puzzle in the search for LEV's cellular action has been the absence of any effect on neurotransmission at the cellular level [29]. In the absence of SV2B, we were able to detect LEV effects on neurotransmission. We show that LEV reduces synaptic depression in isolated hippocampal neurons from BKO but not WT mice allowing us to re-evaluate previous hypotheses of LEV action at the molecular level. The most parsimonious explanation of these findings is that LEV effects predominate at synapses that express primarily or only SV2A.

SV2A and SV2B are co-expressed in most brain synapses. The exceptions are the majority of GABAergic synapses and the glutamatergic synapses of the dentate granule neurons in the hippocampus, which contain only SV2A [6, 7]. Both of these classes of synapses have been implicated in the control of brain excitability [38, 82]. The targeted action of LEV at synapses that play a major role in the development and control of seizures may be a basis for its efficacy and high therapeutic index. Furthermore, because SV2A expression has been reported to increase in some forms of epilepsy [39, 83], determining whether LEV has expanded action in epileptic brain will be important to refining its therapeutic use.

Because our preparation utilizes neurons before the early death induced by loss of SV2A, we were able to rigorously test the requirement for SV2A in LEV action. The absence of a LEV effect in neurons from DKO mice is the first demonstration that loss of SV2A abolishes LEV action.

LEV inhibits SV2A binding to synaptotagmin

Although multiple functions have been ascribed to SV2 [20, 84-86], it has one verified function. SV2 binds to and regulates the stability and trafficking of synaptotagmin [19, 24, 28, 76, 87]. The fact that synaptotagmin expression levels and trafficking are disrupted with loss of SV2 suggests that SV2 plays an escort or chaperone-like function. Because synaptotagmin plays an essential role in both exocytosis and endocytosis, it suggests that LEV's ultimate mechanism of action may be the disruption of synaptotagmin function. This interpretation is

consistent with the previous finding that LEV restores normal synaptotagmin levels in synapses overexpressing SV2A [29]. It also supports the conclusion that regulation of synaptotagmin is a primary function of SV2 [27]. Thus, LEV-mediated inhibition of the SV2A-synaptotagmin interaction could have a direct effect on synaptotagmin's ability to engage with the vesicle fusion (SNARE) complex [79].

Synaptotagmin plays an essential role in regulating vesicle exocytosis by interacting with membrane lipids and the proteins that mediate vesicle fusion (SNAREs) [79]. By disrupting SV2A's chaperone or escort action, LEV could affect synaptotagmin's ability to contribute to exocytosis. Consistent with this, we note that loss of SV2 reduces synaptic vesicle priming and results in fewer assembled vesicle fusion (SNARE) complexes [10, 15].

Synaptotagmin has also been implicated in endocytosis [35]. SV2A promotes the binding of synaptotagmin to AP2, a component of the clathrin-dependent endocytosis machinery [17]. Our observation that LEV slows synaptotagmin endocytosis and decreases its internalization suggests that by disrupting the SV2A–synaptotagmin interaction, LEV's mechanism of action includes affecting synaptotagmin retrieval. This suggests that LEV could act by decreasing the amount of synaptotagmin in recycling vesicles.

One of the surprising findings reported here is that LEV does not decrease SV2A binding to synaptotagmin when SV2B is present. The SV2s have structural similarity to the Major Facilitator Superfamily of small solute transporters, many of which exist as oligomers [88]. Because all three SV2 paralogs bind to synaptotagmin [24-26, 75, 76], hetero-oligomers containing a non-LEV binding paralog could preclude LEV action. If true, this has important implications for the development of drugs that target SV2B or SV2C, which are, with few exceptions, always expressed with SV2A.

In summary, our results provide initial insights into the molecular action of LEV. Further analysis of how LEV impacts protein interactions in presynaptic terminals will be the next stage towards developing a structural approach to further drug development.

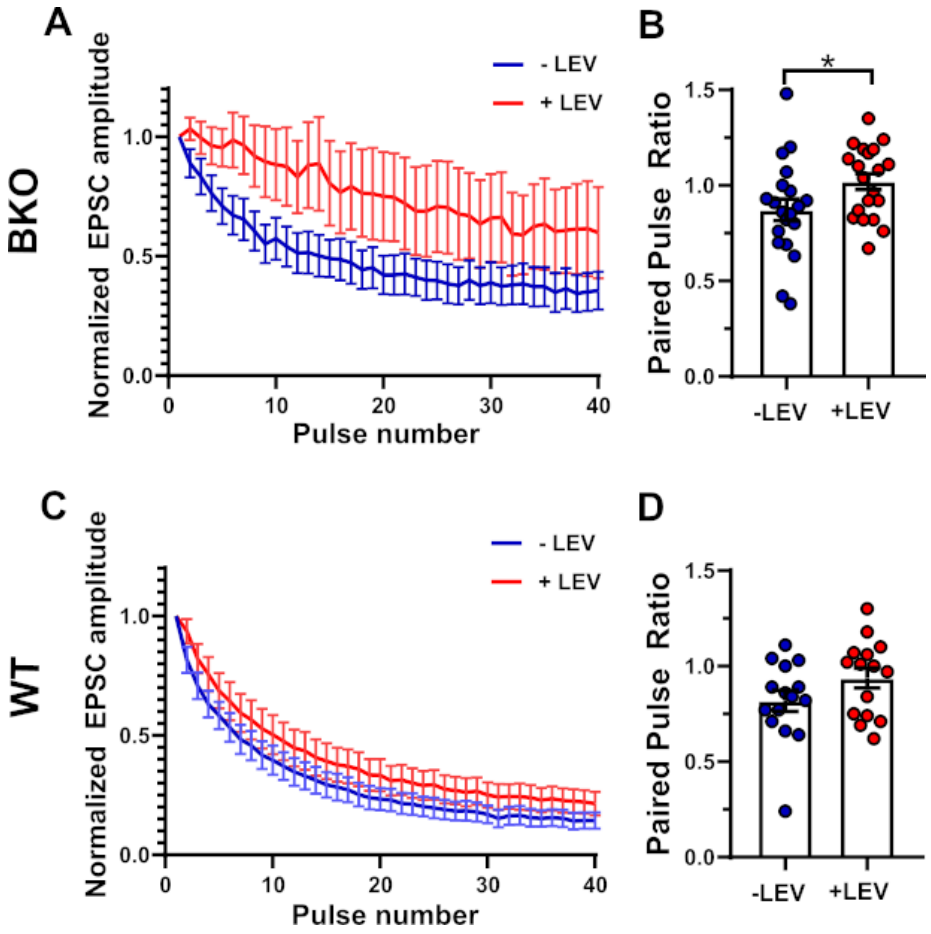


Figure 3: LEV decreases synaptic depression, an indicator of release probability, in isolated hippocampal neurons from SV2 BKO mice

Excitatory postsynaptic potentials (EPSCs) were measured using whole cell patch clamp stimulation and recording from isolated (autaptic) pyramidal neurons cultured on microislands of astrocytes.

(A,C) Shown are averaged EPSC amplitudes in response to a 2s, 20Hz train. Amplitudes were normalized to the initial response then averaged. (A) BKO and (C) WT neurons treated +/- 300uM LEV for 3-7 hours. LEV decreased synaptic depression only in BKO neurons, suggesting that it reduces synaptic release probability in synapses that express only SV2A.

(B,C) Paired pulse ratio, an index of synaptic release probability, was calculated as $EPSC_2/EPSC_1$. LEV (+LEV) increased the paired pulse ratio in neurons from BKO mice (PPR_{+LEV} =1.02 $\pm$ 0.04, student's t-test, *P<0.05, n= 20) compared to control (-LEV) (PPR_{ctrl}=0.87 $\pm$ 0.06, n=20). In contrast, LEV had no effect on the paired pulse ratio in neurons from WT (PPR_{+LEV} = 0.94 $\pm$ 0.05, n=15, PPR_{ctrl} = 0.82 $\pm$ 0.05, n=15). Data represent the mean $\pm$ SEM.

(Autaptic cultures plated by KC, Recordings performed by JM Sullivan)

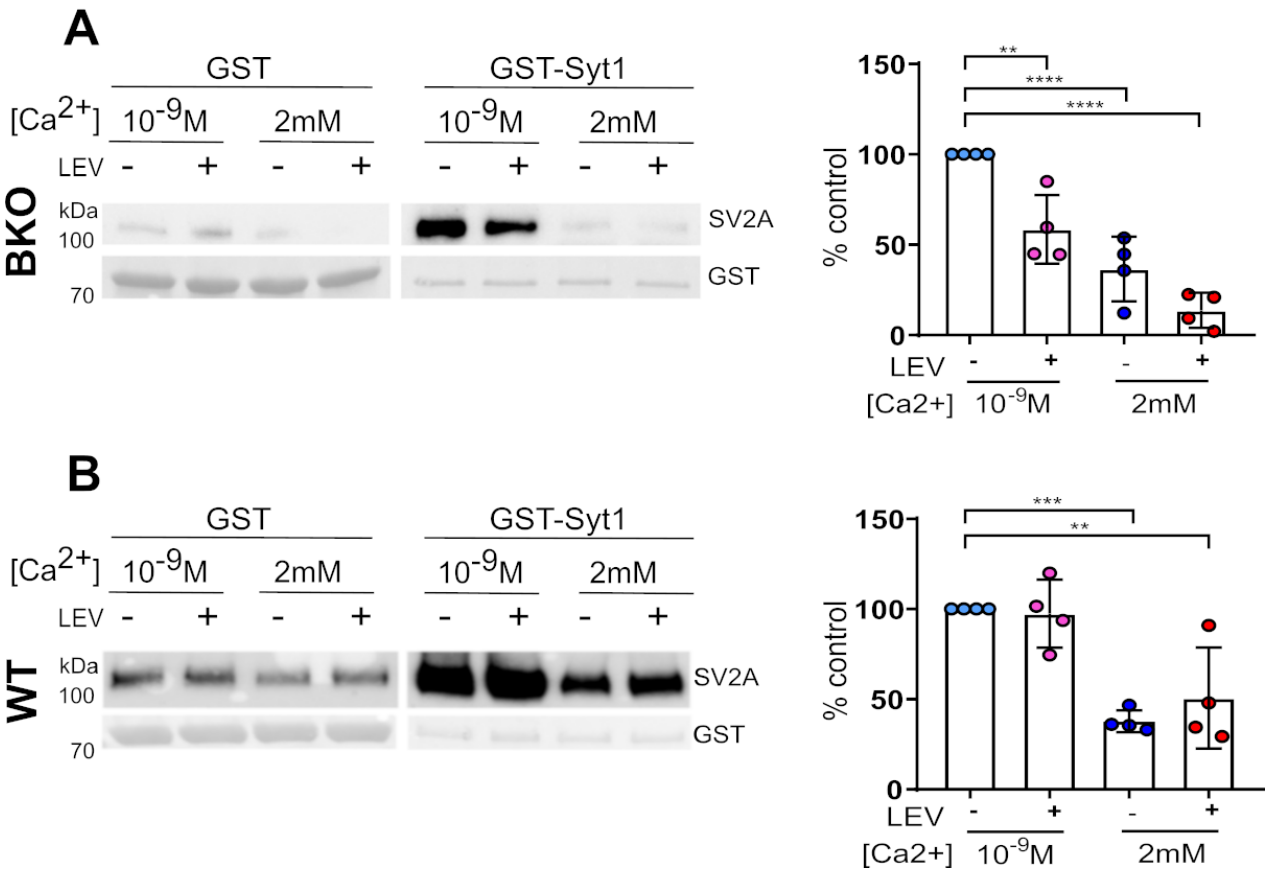


Figure 4: LEV decreases SV2A binding to synaptotagmin *in vitro* in the absence of SV2B
LEV effects of the SV2A-synaptotagmin interaction were assessed using a GST pull-down approach. The fusion protein GST- synaptotagmin 1 (GST-Syt1), or GST as a control, was attached to glutathione resin and incubated with extracts of mouse brain in the presence or absence of 300uM LEV and in low (10^{-9}M) or high (2mM) calcium. SV2A binding was assessed by Western blot analysis and normalized to the amount of GST-Syt1 or GST in the same lane. Binding was graphed as the percentage of the no LEV, low calcium (control) condition. (**A**) SV2A from extracts of BKO mouse brain shows reduced binding to GST-Syt1 in the presence of LEV. Consistent with previous studies, high calcium reduced binding, and addition of LEV in the presence of high calcium further reduced binding (n=4, One-way ANOVA ** P<0.01, *** P<0.0001). Blots from a representative experiment are shown at the left. (**B**) SV2A from extracts of WT mouse brain showed reduced binding to GST-Syt1 in the presence of high calcium. In contrast to extracts from BKO mice, LEV had no effect on the interaction (n=4, One-way ANOVA **P<0.01, ***P<0.0001, **** P<0.0001). Blots from a representative experiment are shown at the left. Bar graphs represent data ± SD.
(Experiments done with Daniele Marcotulli (DM))

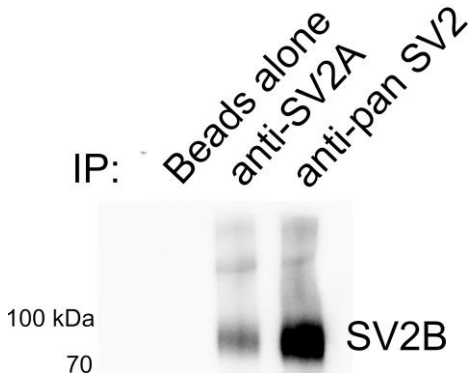


Figure 5: SV2A binds to SV2B

To determine if SV2A and SV2B bind and possibly hetero-oligomerize, SV2A was immunoprecipitated from brain post nuclear supernatant from wild-type mice using a polyclonal antibody that recognizes only SV2A. Western blot analyses showed that SV2B co-immunoprecipitates with SV2A suggesting that may form a complex. A monoclonal antibody that recognizes all SV2 paralogs was used as a positive control.

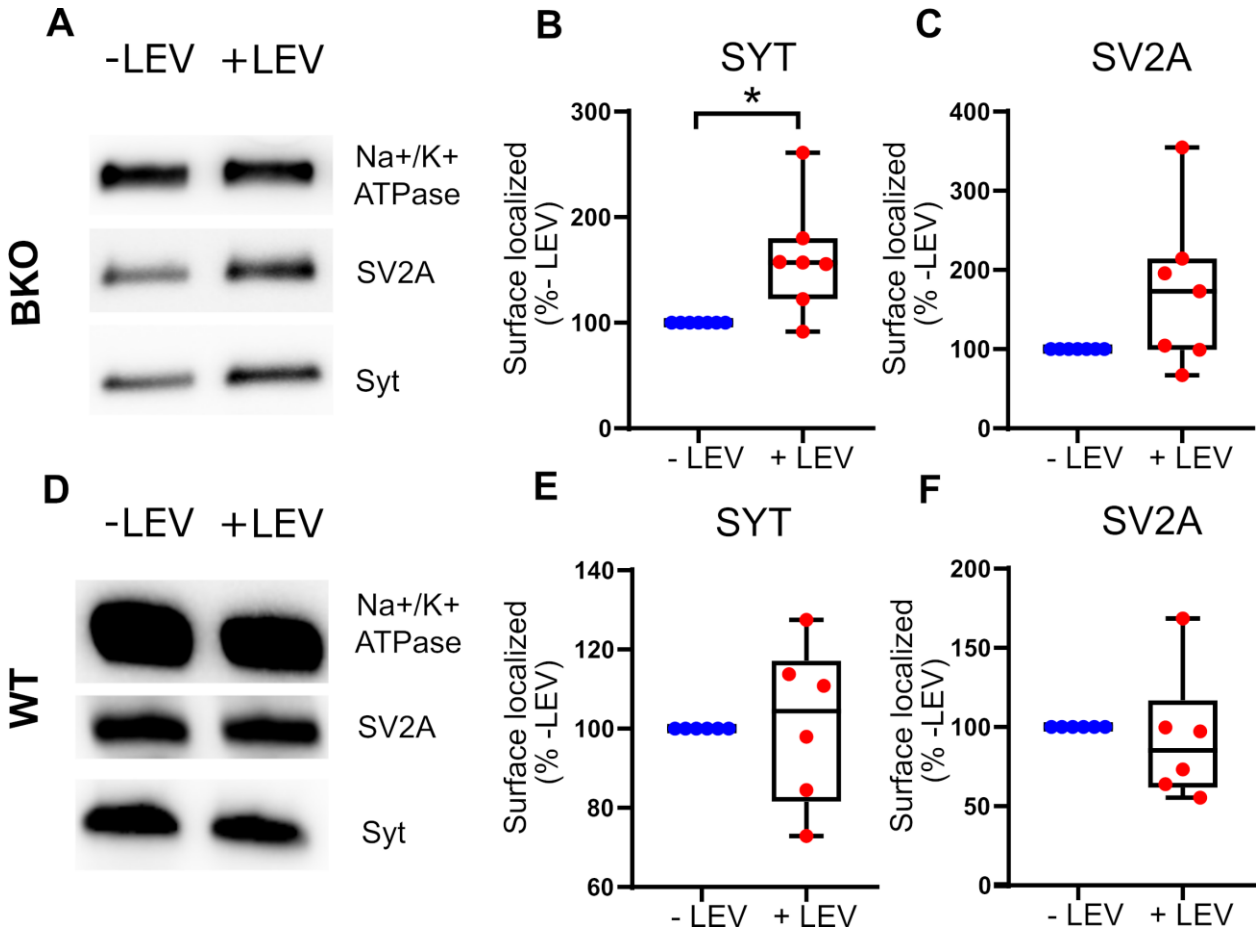


Figure 6: LEV increases surface localization of synaptotagmin in hippocampal neurons cultured from SV2 BKO mice

To assess LEV's effect on SV2's role in synaptotagmin trafficking, we compared the amount of surface localized synaptotagmin in hippocampal neurons treated +/-300uM LEV for three hours. Shown are representative Western blots (A, D) and quantification of biotinylated synaptotagmin (Syt1) (B, E) and SV2A (C, F) in BKO and WT hippocampal neurons. To control for protein loading, the amount of biotinylated Syt1 or SV2A was normalized to biotinylated sodium potassium ATPase (Na+/K+ ATPase). Values were expressed as the percentage of the no LEV condition. (B) LEV increased the amount of Syt1 on the surface in neurons from BKO mice (Mann-Whitney, **P*<0.05, n= 7) (C) LEV did not affect the amount of surface-localized SV2A. In neurons from WT mice, LEV had no significant effect on the amount of either Syt1 (E) or SV2A (F) in WT neurons (n=5). Each data point represents a different culture (n). (Experiments performed with DM)

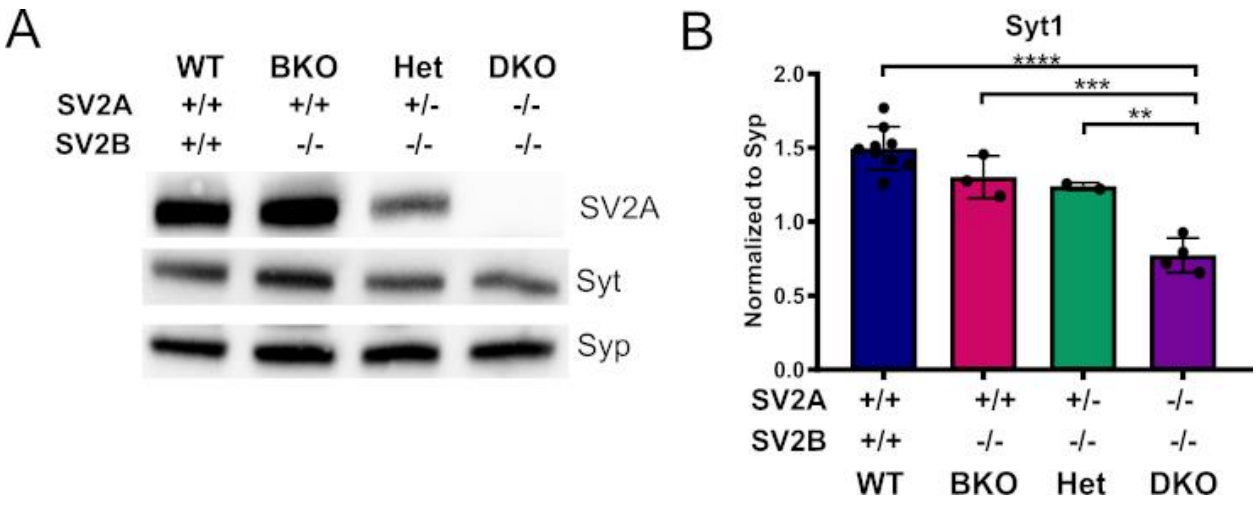


Figure 7: Loss of SV2B does not affect synaptotagmin expression
(A) Western blot analyses of brain post nuclear supernatant from P12-P14 mice with indicated genotypes. (B) Band intensities of synaptotagmin was normalized to the band intensity of synaptophysin (a vesicle protein known to not be regulated by SV2). As expected, we saw decreased levels of synaptotagmin with loss of SV2 (DKO) but no change in the amount of synaptotagmin in mice lacking SV2B (BKO and Het) suggesting that SV2B does not regulate synaptotagmin levels. (One-way ANOVA $^{**}P<0.01$, $^{***}P<0.0001$, **** $P<0.0001$) (WT n= 10, BKO n = 3, Het n = 2, DKO n = 4)

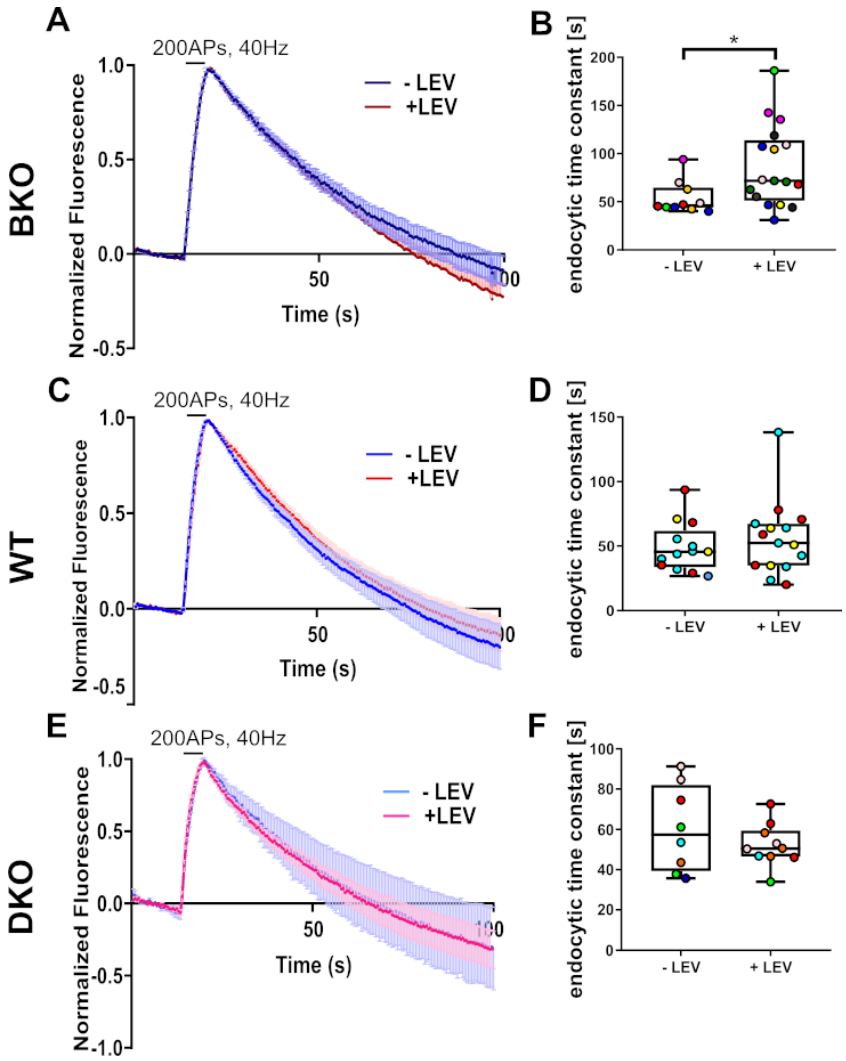


Figure 8: LEV slows Syt1-pHluorin retrieval in hippocampal neurons from SV2 BKO mice
To assess the effects of LEV on synaptotagmin trafficking, which is regulated by SV2A, we measured the fluorescence decay of Syt1-pHluorin in neurons from BKO, WT and DKO mice cultured on glass coverslips. Average time course (A, C, E) and endocytic time constants (B, D, F) of Syt1-pHluorin fluorescence in response to 200APs, delivered at 40Hz are shown.
(A, B) In neurons from BKO mice, LEV increased the endocytic time constant (t) indicating slower endocytosis of Syt1-pHluorin (τ_{ctrl} = 53.9 $\pm$ 5.4 s, n = 6, τ_{Lev} = 86.7 $\pm$ 10.2 s, n= 9, Mann-Whitney, *P <0.05).
(C, D) LEV had no effect on Syt1-pHluorin endocytosis in in WT neurons (τ_{ctrl} = 49.69$\pm$ 5.18 s, n=4, τ_{LEV} = 55.6 $\pm$ 7.44 s, n=3).
(E, F) In the absence of SV2A, LEV had no effect on Syt1-pHluorin retrieval, (τ_{ctrl} = 60.2 $\pm$ 7.55 s, n=6, τ_{Lev} = 52.13 $\pm$ 3.34 s, n= 5). Average time course are graphed as mean $\pm$ SEM. Endocytic time constants are graphed using box plots. Culture dates (n) are indicated by color. Each data point represents a separate coverslip.

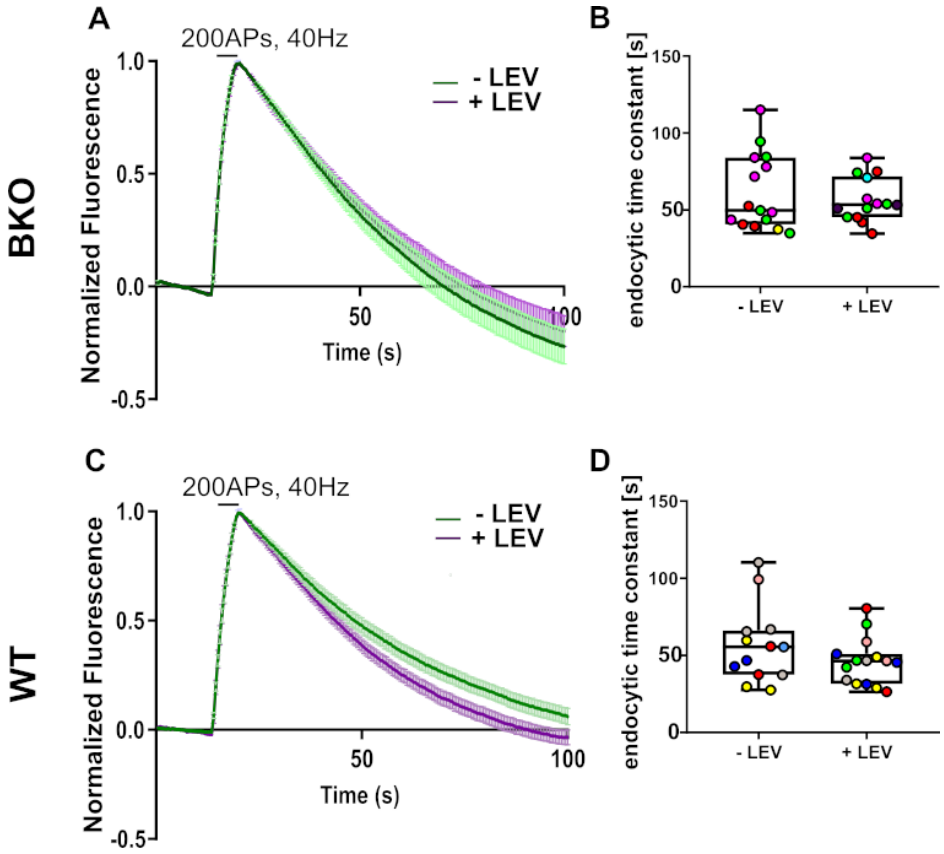


Figure 9: LEV does not affect SypHy retrieval in WT of BKO hippocampal neurons
To assess the effects of LEV on synaptophysin trafficking, which is not regulated by SV2A, we measured the fluorescence decay of synaptophysin-pHluorin (SypHy) in neurons from BKO, WT and DKO mice cultured on glass coverslips. Average time course (A, C) and endocytic time constants (B, D) of SypHy fluorescence in response to 200APs, delivered at 40Hz are shown. Average time course (A,C) and endocytic time constants (B,D) of synaptophysin (SypHy) in BKO (A,B) and WT (C,D) hippocampal neurons in response to 200APs, 40Hz. LEV did not affect SypHy endocytosis in BKO neurons (τ_{ctrl} = 61.17 $\pm$ 6.4 s, n= 4, τ_{LEV} = 56.5 $\pm$ 3.81 s, n = 5) or in WT neurons (τ_{ctrl} = 55.45 $\pm$ 6.92 s, n= 2, τ_{LEV} = 45.92 $\pm$ 3.92 s, n =3). Average time course are graphed as mean $\pm$ SEM. Endocytic time constants are graphed using box plots. Culture dates (n) are indicated by color. Each data point represents a separate coverslip.

Methods

Animals and reagents

The generation of $SV2A^{+/+}SV2B^{-/-}$ (BKO) and $SV2A^{-/-}SV2B^{-/-}$ (DKO) mice was reported previously [10]. Wild-type (WT) and BKO mice were kept as separate colonies generated from littermate heterozygous crosses. All animals were on a 99.99% C57BL/6 genetic background. DKO mice were generated by crossing $SV2A^{+/-}SV2B^{-/-}$ mice. Genotype was determined by PCR before culturing neurons. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Washington.

Levetiracetam (LEV) was from Sigma-Aldrich (L8668-100MG). Polyclonal anti-SV2A antibody was house generated and affinity purified from sera [6], rabbit polyclonal anti-synaptotagmin antibody was raised against the cytoplasmic domain, and anti-Na+/K+ ATPase was from Development Studies Hybridoma Bank (a6F). Antibody binding in Western blot analyses was detected using horse radish peroxidase-conjugated secondary antibodies and visualized using enhanced chemiluminescent chemistry (BioRad Clarity™) and visualized via Bio-Rad Chemidoc. Immunoreactive bands were quantified using ImageLab software (BioRad). Secondary antibodies were used at a 1: 5000 dilution (Invitrogen HRP-conjugated goat anti-rabbit G-21234 and HRP-conjugated goat anti-mouse G-21040).

Tissue Culture/Transfections

For electrophysiological recordings, neurons isolated from the CA region of the hippocampus of P0-P1 mice were cultured on small microislands of permissive substrate as previously described [10]. Neurons were plated on a feeder layer of astrocytes, then grown in neural medium without mitotic inhibitors and used for recordings after 12-16 days in vitro (DIV). For all other experiments, CA hippocampal neurons were cultured on 25mm coverslips coated with poly-D-lysine and rat collagen and layer of astrocytes as previously described [19].

Cultures were transfected using calcium phosphate at 6-8 DIV. Synaptotagmin1-pHluorin (Syt1-pHluorin) was obtained from Dr. Volker Haucke (Leibniz Institute of Molecular Pharmacology). Synaptophysin-pHluorin (SypHy) was obtained from Dr. Leon Lagnado (University of Sussex). For transfections, cultures were serum starved for 1.5 hours prior to transfection. A calcium phosphate/DNA precipitate was formed in HEPES-buffered saline (280mM NaCl, 10mM KCl, 1.5mM NaH_2PO_4, 12mM D-glucose, 50mM HEPES, pH 7.08) for 20-25 minutes. The precipitate (200uL) was added drop-wise to 2mL serum free media and incubated for 1.5 hrs. After transfection, the cultures were returned to their original culture medium.

Electrophysiology

Following treatment +/- 300 uM LEV for 3-7 hours, whole-cell voltage clamp recordings were made from excitatory neurons on single-neuron islands using an Axopatch 200A amplifier (Axon Instruments, Sunnyvale, CA). The extracellular recording solution contained 119 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, 30 mM glucose, 20 mM HEPES, and 1 uM glycine; 300 uM LEV or water (vehicle) was added to the external solution of cells during recording. The pipette solution contained 148.5 mM K-gluconate, 9 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, and 0.2 mM EGTA. Cells were held at −60 mV and stimulated with a 1 ms depolarization to +20 mV to evoke neurotransmitter release. Recording electrodes were 2.5-3.5 MΩ, and series resistance was compensated 70-85%. Cells with series resistance > 20 MΩ before series resistance compensation, or leak current >250 pA were excluded. All experiments were performed at room temperature.

Glutathione S-transferase (GST) pull-down assays

Protein binding (pull-down) assays were performed as previously described [75]. Brains from WT or BKO mice were homogenized in HBS buffer (10mM HEPES, 142mM NaCl, 2.4mM KCl, 1mM $MgCl_2$, 5mM D-glucose, 1mM EGTA, 1x protease inhibitor (Roche)). The brain

homogenate was centrifuged at 1000xg for 10 minutes to remove nuclei and undisrupted cells. One milligram of the resulting post nuclear supernatant was solubilized in an equal volume of solubilization buffer (20mM HEPES, 1mM EGTA, 95mM potassium acetate, 2% Triton X-100, with or without 3mM CaCl2 and 300µM LEV or water) for two hours on ice then centrifuged at 19,000xg for 30 minutes at 4°C. The resulting supernatant (extract) was incubated with bacterially-produced GST-synaptotagmin1 (GST-Syt1) bound to Pierce™ glutathione agarose (16100) for 1 hour at 4°C. After binding, beads were washed four times with 1mL wash buffer (10mM HEPES, 1mM EGTA, 47.5mM potassium acetate, 1% Triton X-100, with or without 3mM CaCl2 and 300µM LEV or water). Proteins were eluted with 8M urea dissolved in 100mM Tris-HCl, pH 8, or SDS-PAGE sample buffer, and subjected to Western analysis.

Immunoprecipitation

Brains were homogenized in HBS buffer (10mM HEPES, 142mM NaCl, 2.4mM KCl, 1mM $MgCl_2$, 5mM D-glucose, 1mM EGTA, 1x protease inhibitor (Roche)). The brain homogenate was centrifuged at 1000xg for 10 minutes to remove nuclei and undisrupted cells. One milligram of post nuclear supernatant was solubilized in 1% Triton X-100 for one hour at 4°C then centrifuged at 17000xg for 20 minutes at 4°C. The supernatant was incubated with polyclonal antibody against SV2A or a monoclonal antibody against SV2 conjugated to Protein A sepharose (CL-4B) beads for one hour at 4°C. Proteins were eluted with SDS-PAGE sample buffer and subjected to western blot analysis.

Surface Biotinylation

Hippocampal cultures (14-16 DIV) from WT or BKO mice were treated with 300uM LEV or water in neural media at 37°C for three hours. After incubation, cells were placed on ice and washed with cold phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 10mM NaH_2PO_4, 1.8mM $KH_2PO_{4)}$. To biotinylate surface stranded cells, cells were incubated with 2mM EZ-LINK Sulfo-NHS-LC-LC-Biotin (Thermo Scientific 21338) for 25 minutes at 4°C. Cells were washed

with 100mM glycine in PBS for 15 min to quench the reaction and then washed with PBS twice. Cells were harvested and extracted in lysis buffer (10mM HEPES, 140mM NaCl, 1% Triton X-100 and protease inhibitor) for 30 minutes at 4°C and spun at 19,000xg for 20 minutes at 4°C. Biotinylated proteins were isolated using Streptavidin Sepharose ® High Performance resin (GE 17-5113-01) and eluted with SDS-PAGE sample buffer. Eluted proteins were then subjected to Western blot analysis.

Imaging

Imaging of live cultures was performed at 14-16 DIV. Prior to imaging, neurons were incubated in neural medium with 300uM LEV or water for 3-8 hours at 37°C. Cultures were perfused in imaging buffer (170mM NaCl, 3.5mM KCl, 0.4mM KH_2PO_4, 20mM N-Tris(hydroxy-methyl)- methyl-2-aminoethane-sulphonic acid (TES), 5mM $NaHCO_3$, 5mM glucose, 1.2mM Na_2SO_4, 1.2mM $MgCl_2$, 1.3mM $CaCl_2$, 10μM CNQX, and 50μM AP-5, pH 7.4) +/- 300uM LEV prior to imaging. To evoke synaptic transmission, neurons were subjected to electrical field stimulation using an RC21-BRFS chamber (Warner Instruments). A train of 200 action potentials was delivered at 40Hz using a Grass SD9E Stimulator. Fluorescent images (100ms exposures) were captured using an Olympus IX-10 microscope equipped with a 60x water-immersion objective and an Andor scMOS camera. Images were acquired at 2 Hz using Micro-Manager software and analyzed using NIH ImageJ (with Time Series Analyzer V3 plugin). Responding boutons were identified and total fluorescence intensity monitored over time. Fluorescence intensity was corrected for background [89]. Endocytic time constants were determined by fitting the mono-exponential decay of fluorescence intensity using Prism (Graphpad) software.

Statistical Methods

All statistical analyses were performed using Prism (Graphpad) software. Data analysis was performed with the assistance from the Department of Biostatistics Consulting Service at the University of Washington.

CHAPTER 3:

Levetiracetam influences the protein composition of recycling endosomes in the absence of SV2B

Introduction

Exocytosis and endocytosis of synaptic vesicles are tightly coupled for efficient neurotransmission. During exocytosis, synaptic vesicles fuse to the plasma membrane and release neurotransmitters from presynaptic nerve terminals. To restore the plasma membrane and replenish the pool of vesicles, synaptic vesicles are reformed at the plasma membrane during endocytosis with fission catalyzed by the GTPase dynamin [14]. Because synaptic vesicles contain the machinery they need to fill with neurotransmitters and undergo regulated exocytosis again, understanding the molecular mechanisms that control protein sorting into vesicles is important for understanding how to maintain normal neurotransmission [27].

SV2 interacts with synaptotagmin, the calcium sensor protein that mediates calcium-dependent exocytosis. SV2 is important for sorting synaptotagmin to synaptic vesicles [19, 25, 28]. Our previous work shows that Levetiracetam (Keppra™, LEV), an antiepileptic drug with a novel target, SV2A, [3] disrupts SV2A and synaptotagmin interaction in synapses lacking SV2B (**BKO**). LEV treatment also resulted in decreased and slower synaptotagmin internalization suggesting LEV affects protein sorting and the protein composition of recycling vesicles.

To determine if LEV influences other protein-protein interactions, in addition to SV2A-synaptotagmin, we developed a new method to compare the proteome of newly recycled vesicles. Using this protocol, we show that LEV treatment increased Lypla1 and decreased complexin1 levels in recycling vesicles from BKO mice, but not wild-type mice. Preliminary data shows that LEV also disrupts the interaction between complexin1 and SV2A suggesting that SV2A may have a regulatory role in chaperoning complexin1 to vesicles.

Results

A protocol to isolate newly recycled vesicles

To determine if LEV's effects on vesicle protein trafficking are specific to synaptotagmin, we developed a protocol to assess the proteome of newly recycled vesicles. The synapse contains different types of vesicle pools, the non-cycling, reserve pool of vesicles make up 80-90% of the vesicles, and the recycling pool of vesicles make up 5-20% of the vesicles.[90] Because traditional synaptic vesicle preparations cannot distinguish between these vesicle pools, our lab developed a novel way to isolate the recycling pool of vesicles (**Figure 10A**) using a dynamin inhibitor, Dynole® 34-2. By allosterically modulating the GTP binding site of dynamin, we were able to trap vesicles undergoing endocytosis (recycling vesicles) at the plasma membrane.

Synaptosomes (pinched off nerve terminals) were purified using a Percoll density gradient from mouse brain homogenate. To confirm the presence of intact terminals, we used Western blot analyses and looked for enrichment of both the plasma membrane marker, Na+/K+ ATPase, and the vesicle protein, synaptophysin (**Figure 10B**). After isolation, exocytosis was induced with 60mM KCl, in the presence of 100uM Dynole® 34-2 to recycling vesicles at the plasma membrane. The synaptosomes were then ruptured with hypotonic shock and homogenization. Newly recycled vesicles were then released from the membrane by treating the recycling intermediates with recombinant dynamin (His-MBP-mDynamin) and 1mM GTP to allow completion of endocytosis and scission of vesicles. Endocytosed vesicles are separated from the plasma membrane by centrifugation. The supernatant containing endocytosed vesicles was collected and analyzed by mass spectrometry-based proteomics.

LEV significantly reduces the amount of complexin1 and increases the amount of Lypla1 associated with newly recycled membrane in synapses from SV2B KO mice.

To follow up our previous finding that LEV inhibits the SV2A-synaptotagmin interaction and decreases internalization of synaptotagmin, we isolated newly recycled vesicles to determine if the trafficking of other vesicle proteins is affected by LEV. Isolated synaptosomes were treated +/- 300uM LEV in the presence of Dynole® 34-2 for ten minutes before stimulation. Mass spectrometry analysis revealed de-enrichment of complexin1 and enrichment of Lypla1 in vesicles isolated from BKO mice (**Figure 11**). Consistent with previous studies, we did not see any LEV effects in vesicles isolated from WT mice, consistent with the conclusion that LEV exerts its effects only in the absence of other SV2 paralogs.

Both Lypla1 and complexin1 are implicated in presynaptic events and linked to synaptotagmin. Lypla1 removes the fatty acid palmitic acid from proteins, a modification that regulates membrane tethering at the synapse [91, 92]. Synaptotagmin is one of the synaptic proteins whose functioning has been linked to its palmitoylation [93]. Complexin1 binds both synaptotagmin and assembled fusion (SNARE) complexes [1]. It regulates vesicle fusion [1, 94] and has also been linked to vesicle endocytosis [33]. Because of its direct links to both vesicle exo/endocytosis and synaptotagmin, we focused our subsequent studies of LEV effects on complexin1.

SV2 does not regulate complexin1 levels

De-enrichment of complexin1 in recycling vesicles treated with LEV suggests that trafficking of complexin1 is directly or indirectly affected by SV2. To determine if SV2 regulates complexin1 expression levels similarly to its regulatory role of synaptotagmin expression, we analyzed protein expression levels in brain post nuclear supernatant from WT, SV2A$^{+/-}$ SV2B$^{-/-}$, BKO, and SV2A$^{-/-}$ SV2B$^{-/-}$ (**DKO**) mice (**Figure 12**). Because mice without SV2 do not survive past three weeks, we analyzed protein expression in P12-P14 mice. We normalized the amount

of complexin1 to synaptophysin, a vesicle protein whose expression is not regulated by SV2 expression. Loss of SV2 did not affect the amount of complexin1 in brain post nuclear supernatant (**Figure 12**) suggesting, that unlike synaptotagmin, SV2 does not regulate complexin1 expression complexin1 expression is not regulated by SV2.

Complexin1 co-immunoprecipitates with synaptotagmin

To determine how LEV might affect the amount of complexin1 associated with recycling membranes, we asked if complexin1 is in a complex with SV2A. A pan-SV2 antibody was used to immunoprecipitated SV2 protein complexes from a Triton X-100 extract of brain post nuclear supernatant. Western analyses of co-precipitated proteins revealed little to no complexin1 binding (**Figure 13A**), suggesting that complexin1 is not in a stable complex with SV2. In contrast, an immunoprecipitation performed with anti-synaptotagmin1 co-isolated complexin1 confirming that complexin1 interacts with synaptotagmin (**Figure 13B**).

Recombinant complexin1 binds to purified SV2A

The inability of complexin1 to co-immunoprecipitate with SV2 could reflect a transient interaction or low affinity binding. To address this, we used purified recombinant proteins to determine if there was a binary interaction between the proteins when they were incubated at relatively high concentrations. SV2A-FLAG purified from HEK293T cells was incubated with a glutathione-S- transferase-complexin1 fusion protein (GST-Cpx1) in low (10^{-9}M) calcium and high (2mM) calcium. Under these conditions, SV2A binds to complexin1 and the interaction is increased at least two-fold in higher calcium (**Figure 13C-D**).

To determine if LEV affects this interaction, we repeated the study with and without 300uM LEV. Our preliminary results suggest that LEV does not affect SV2A binding to GST-Cpx1 at low calcium conditions, but LEV decreases it binding at high calcium conditions (data

not shown). If verified, this outcome would be consistent with LEV influencing SV2A-dependent association of comlexin1 with endosomes.

Discussion

Consistent with previous findings, we find that LEV's effects only manifest in the absence of non-LEV binding SV2 paralogs. Our non-biased proteomic analysis revealed that LEV treatment leads to a decrease in the amount of complexin1 and an increase in Lypla1 associated with newly recycled vesicles from BKO mice but not WT mice. Surprisingly, we did not see changes in synaptotagmin levels suggesting that the effects were too small for mass spectrometry to detect, or that the intense stimulation used in the mass spectrometry study overcomes the effects of LEV on synaptotagmin recycling.

The effect of LEV on complexin1 association with endosomes suggests that, in addition to synaptotagmin, SV2 may also regulate the action of complexin1. Given complexin1's association with and stabilization of SNARE complexes [94] we note that neurons lacking SV2 have reduced proportion of SNARE protein in preassembled complexes [13].

Because our assays only assess the interaction between complexin1 and SV2A, we do not know how other vesicle proteins such as synaptotagmin or SNARE complex proteins affect these interactions. But based on the results we have collected, I am proposing a model in which the interaction between SV2A and complexin1 is calcium dependent and can be disrupted with LEV treatment.

In this model, SV2 interacts with both complexin1 and synaptotagmin. During priming, SV2A helps concentrate complexin1 to facilitate its interaction with partially formed SNARE complexes. Upon calcium entry, the complexin1, bound to SV2A, shifts away from the fusion pore to allow synaptotagmin to facilitate calcium-dependent fusion. During endocytosis, SV2A traffics both synaptotagmin and complexin1 to synaptic vesicles so that vesicles contain

complexin1 for the next round of fusion. But with LEV treatment, the SV2A-complexin1 interaction is disrupted and complexin1 retrieval is decreased. Thus, if less complexin1 is associated with vesicles, vesicle priming is affected, and vesicles are less competent for fusion (**Figure 14**). This is consistent with the decrease in synaptic release probability in LEV treated neurons and in complexin knock-out mice [95, 96].

As of now, we have only followed up on the observed LEV effects on complexin1, but palmitoylation has been implicated in synaptic plasticity and many vesicular proteins are known to be regulated by palmitoylation [91, 92]. Further studies will need to look at the implications of Lypla1 enrichment in vesicles and how it affects the function of other vesicle proteins.

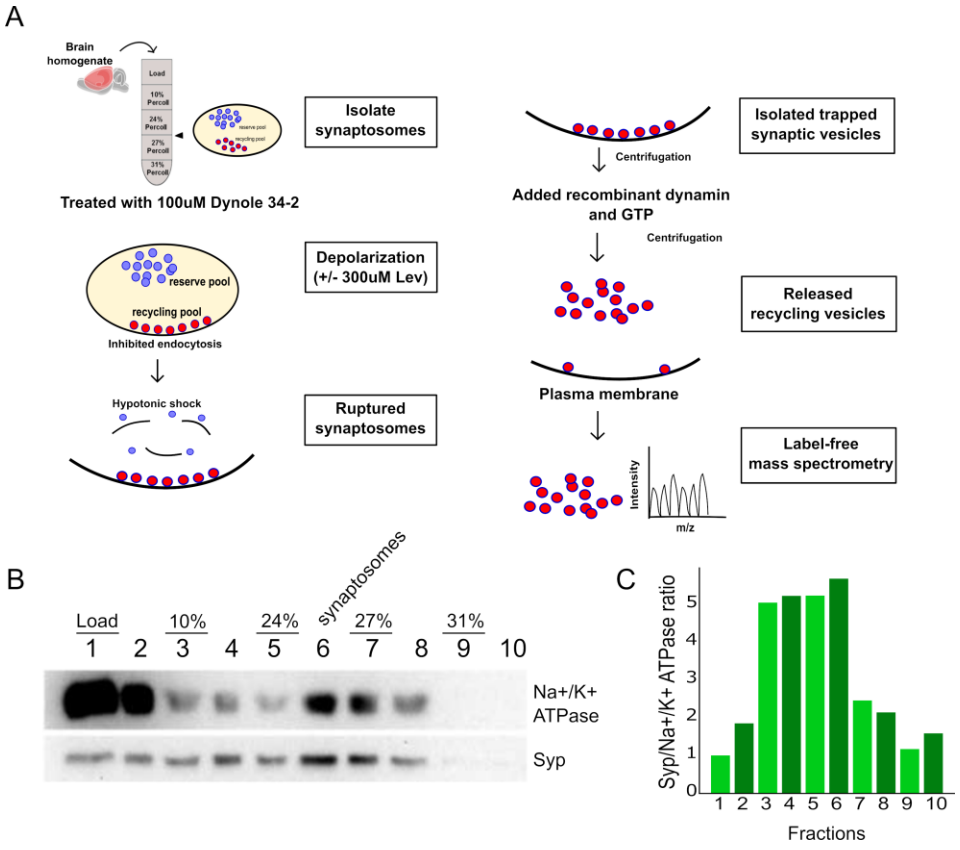
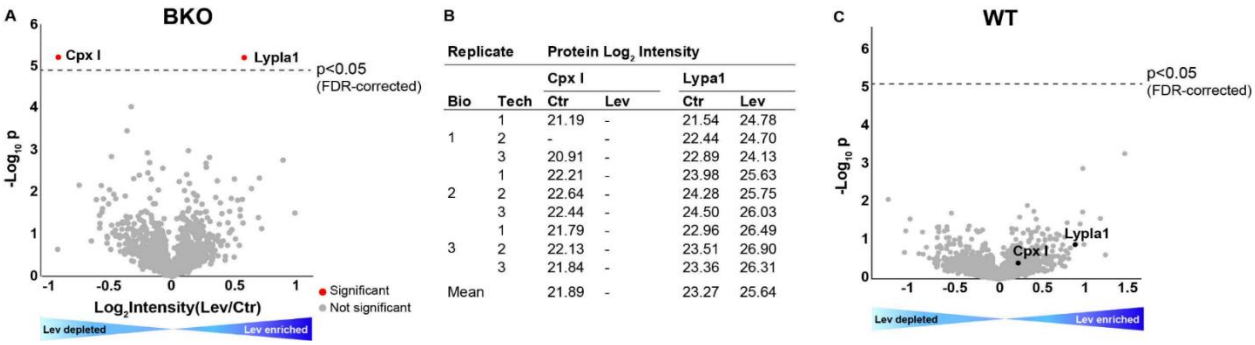


Figure 10: Protocol for isolating newly recycled vesicles

(A) Synaptosomes are purified from mouse brain using Percoll® density gradient ultracentrifugation. Purified synaptosomes are stimulated with 60mM KCl for five minutes in the presence of dynamin inhibitor, Dynole® 34-2. After a thirty-minute recovery in 1mM K^+ solution, synaptosomes are ruptured by hypotonic shock and homogenization. Recycling intermediates (plasma membrane with trapped recycling vesicles) are separated from membrane debris and the reserve vesicle pool by ultracentrifugation. Recycling intermediates are treated with recombinant dynamin and 1mM GTP to allow completion of endocytosis. Vesicles that underwent scission are isolated by centrifugation and analyzed by MS-based proteomics. (B) Using western blot analyses across the Percoll® gradient, we identified the fraction containing synaptosomes. (C) We looked for an enrichment of synaptophysin (syp), a vesicle protein marker, compared to Na+/K+ ATPase (ATPase), a plasma membrane marker, and found that Fraction 6 contains synaptosomes.

(Designed method with Jia Yao)



B

Replicate		Protein Log_2 Intensity			
		Cpx I		Lypa1	
Bio	Tech	Ctr	Lev	Ctr	Lev
	1	21.19	-	21.54	24.78
1	2	-	-	22.44	24.70
	3	20.91	-	22.89	24.13
	1	22.21	-	23.98	25.63
2	2	22.64	-	24.28	25.75
	3	22.44	-	24.50	26.03
	1	21.79	-	22.96	26.49
3	2	22.13	-	23.51	26.90
	3	21.84	-	23.36	26.31
Mean		21.89	-	23.27	25.64

Figure 11: LEV decreases the amount of complexin1 but increases the amount of Lypla1 in newly recycled vesicles

The protein composition of dynamin released membranes from control and LEV treated samples were compared using a label-free MS-based proteomics approach. Proteins were identified and quantified using MaxQuant [97]. (A) Volcano plot showing the magnitude (ratio of protein abundance in LEV condition vs control condition, *x-axis*) and significance ($-Log_{10}$ adjusted p-value, *y-axis*) of LEV effect on proteins associated with newly recycled synaptic vesicles in synaptosomes from BKO animals. The dashed horizontal line indicates statistical significance threshold ($P \leq 0.05$ after adjustment with permutation-based FDR correction); data represent 9 replicates (3 biological replicates, each analyzed 3 times). In total 1961 proteins were identified. LEV treatment decreased the amount of complexin1 and increased the amount of acyl-protein thioesterase 1 (Lypla1) associated to newly recycled vesicles
(B) Log_2 Intensity of LEV-regulated proteins complexin1 and Lypla1 in LEV treated samples (LEV) and untreated samples (Ctrl); 3 technical replicates for each biological replicate (n=3) are shown, dash indicates undetected protein. (C) Volcano plot showing the magnitude (ratio of protein abundance in LEV condition vs control condition, *x-axis*) and significance ($-Log_{10}$ adjusted p-value, *y-axis*) of LEV effect on proteins associated with newly recycled synaptic vesicles in synaptosomes from WT animals. The dashed horizontal line indicates statistical significance threshold ($P \leq 0.05$ after adjustment with permutation-based FDR correction); (n=3). LEV had no significant effect on the protein composition of newly recycled vesicles from WT mice. Thus, LEV affects the association of complexin1 and lypla1 to newly recycled vesicles only in the absence of SV2B.
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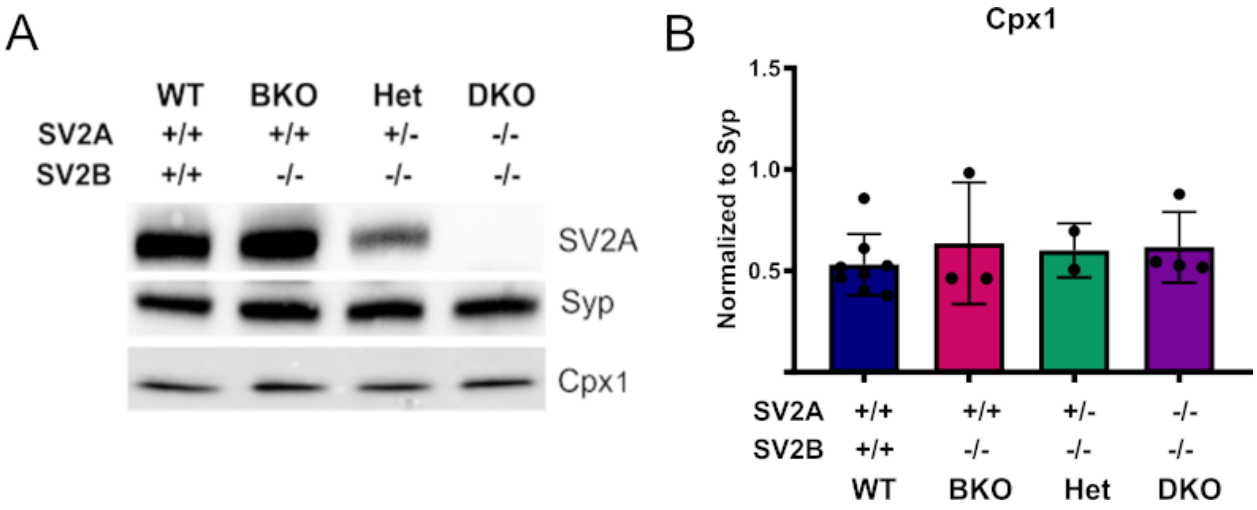

Figure 12: SV2 does not regulate complexin1 expression levels
(A) Western blot analyses of brain post nuclear supernatant from P12-P14 mice. (B) Band intensities of complexin1 was normalized to the band intensity of synaptophysin (a vesicle protein known to not be regulated by SV2). Loss of SV2 did not affect the amount of complexin1 suggesting SV2 does not regulate complexin1 levels.

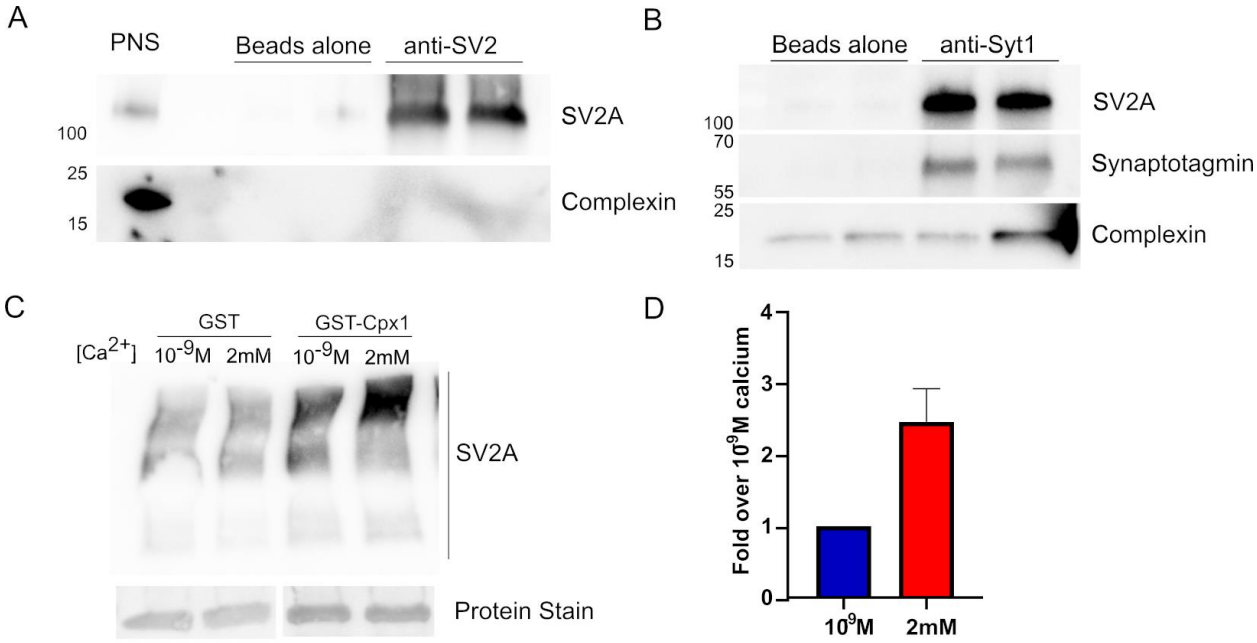

Figure 13: Complexin1 binds to synaptotagmin and SV2A

(A,B) Western blot of synaptotagmin (A) and SV2 (B) immunopurification. Antibody was bound to Protein A Sepharose beads and incubated with brain post nuclear supernatant from wild-type mice (PNS). Western blot shows that complexin1 binds to synaptotagmin but not SV2. (C) Western blot of GST-pull down assays and (D) quantification of SV2A binding to recombinant glutathione-S-transferase-complexin1 fusion protein (GST-Cpx1) or GST. Resin coated with GST-Cpx1 or GST was incubated purified SV2A-FLAG. SV2A binding was normalized to the amount of GST-Syt1 or GST in the same lane. Recombinant SV2A can bind to GST-Cpx1 in both low (10^{-9}M) calcium and high (2mM) calcium conditions in a calcium-dependent manner. There was increased SV2A binding with increased calcium levels (n=2).

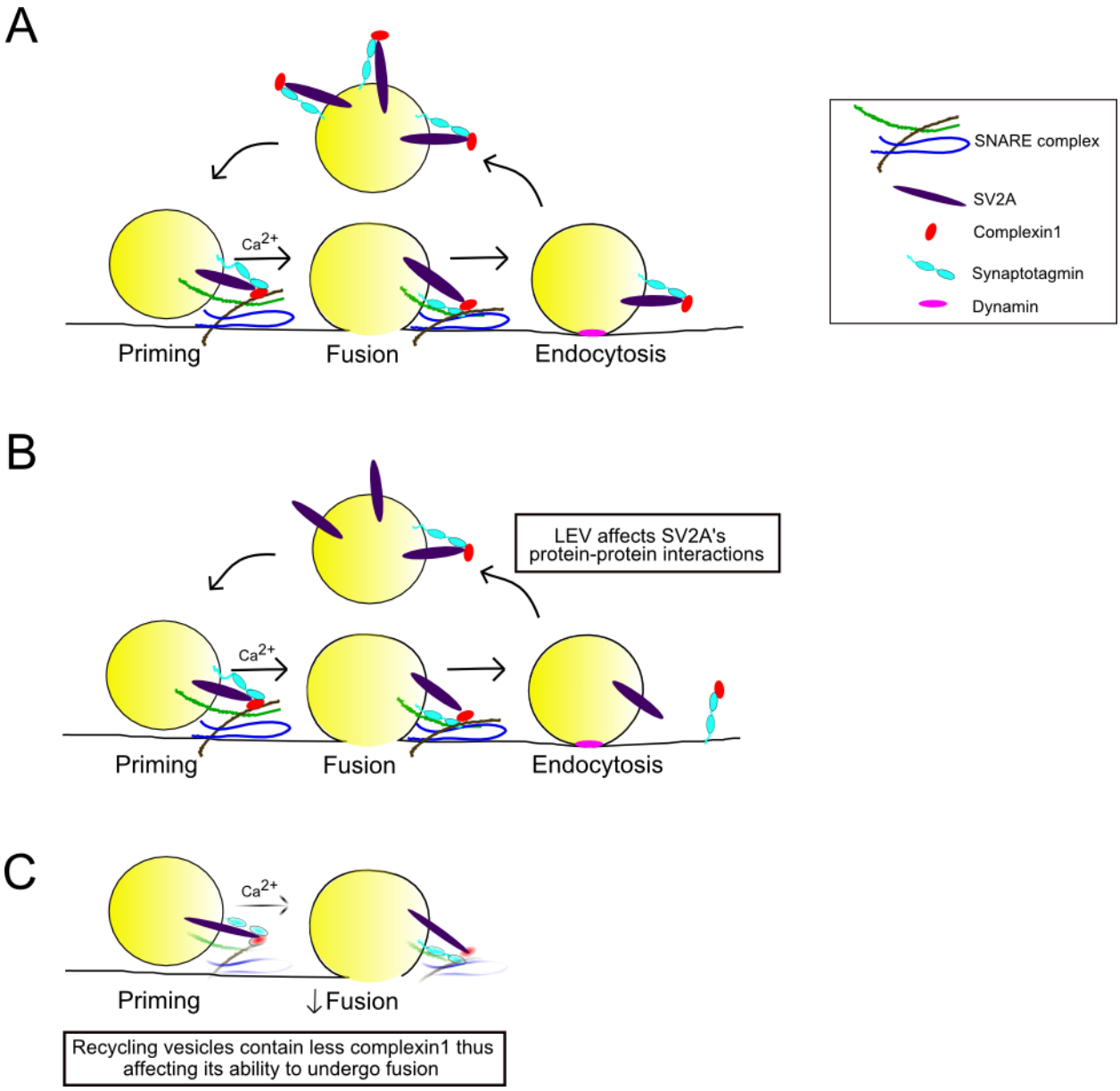

Figure 14: Proposed model for LEV's effect on SV2A-complexin1 interaction
(A) Schematic of vesicles undergoing exo/ endocytosis. In this model, SV2A interacts with both complexin1 and synaptotagmin. During priming, SV2A helps concentrate complexin1 to facilitate its interaction with partially formed SNARE complexes. Upon calcium entry, complexin1 binds to SV2A and shifts away from the fusion pore to allow synaptotagmin to facilitate calcium-dependent fusion. During endocytosis, SV2A traffics both synaptotagmin and complexin1 to synaptic vesicles so that vesicles contain complexin1 for the next round of fusion. (B) LEV treatment disrupts the interaction between SV2A and complexin1. (C) With less complexin1 associating with vesicles, the ability of vesicles to undergo fusion is affected and there are fewer primed vesicles available for fusion.

Methods

Animals and reagents

Refer to Chapter 2 methods. Primary antibody used: anti-CPLX1 (AB215066) and anti- synaptophysin (MAB525850UG and SySys 101 002). Secondary antibodies were used at a 1:3000-5000 dilution (Invitrogen goat anti-rabbit G-21234 and goat anti-mouse G-21040 and Jackson donkey anti-sheep 713-035-003).

Dynamin purification and activity

His-MBP-mDynamin1 was cloned into a pCDNA3 vector by amplifying mDynamin1 using primers: ATTCGTCGACGAGGCAACCGCGGCATGGAAGACC and ATTCAAGCTTTCATGGGTCACTGATAGTGATTCTGGGGACC. PCR product was digested with HindiIII and Sal1and inserted into His-MBP-TEV pRSF vector containing a cleavage site. His-MBP-mDynamin1 was then amplified with *A*TTCGCTAGCGGAGATATACCATGCATCACCACC and *A*TTCGCGGCCGC CCAAGCTTTCATGGGTCACTGATAG. After double digest with Nhe1 and Not1, it was inserted into a pCDNA3 vector.

Lenti-X™ HEK 293T cells were transfected by calcium phosphate transfection with His-MBP -mDynamin. Twenty-four hours post-transfection, the medium was replaced with fresh Dulbecco's Modified Eagle's Medium. After twenty-four hours, cells were harvested and resuspended in lysis buffer (20mM HEPES pH 7.4, 250mM NaCl, 1% Triton X-100, 1mM EDTA, 1mM EGTA with Roche™ protease inhibitor), sonicated and centrifuged at 15000xg for 20 min. The supernatant was incubated with amylose resin (1 ml; New England Biolabs) for 1 hour at 4°C. Resin was washed with wash buffer (20mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA) and incubated overnight with nickel resin purified recombinant 6x His-Tobacco Etch Virus (His-TEV) protease. After incubation with TEV, amylose beads were separated from supernatant

containing purified dynamin by centrifugation (1000xg). GTPase activity of recombinant dynamin-1 was demonstrated by its ability of dephosphorylating GTP, using a malachite-green based colorimetric assay.

Isolating Recycling Vesicles

Recycling vesicles were isolated from wild-type and SV2B knockout mice using differential centrifugation and Percoll® density separation. Brain tissue is homogenized in HBS buffer (10mM HEPES, 142mM NaCl, 2.4mM KCl, 1mM $MgCl_2$, 5mM D-Glucose pH 7.4) with 100mM EGTA and 1x Roche protease inhibitor. After homogenization, it is spun at 10,000xg for 10 minutes. The supernatant is loaded onto a Percoll™ step gradient containing (vol/vol) 10%, 24%, 27%, and 31% filtered Percoll diluted in 10x HBS. The gradient is then centrifuged in a Ti-40 rotor for 7 minutes. The interface between 24% and 27% is collected and diluted in HBS and centrifuged at 17,000xg for 15 minutes. The synaptosomes (pellet) are resuspended in HBS and centrifuged again at 17,000xg for 15 minutes. The synaptosomes are resuspended in HBS and 5mM K+ and treated with 300uM LEV or water. Exocytosis is induced by addition of 60mM KCl for 5 min in the presence of 100uM Dynole 34-2™ and +/- 300uM LEV. The high K+ solution was diluted to 1mM K+ with +/- 300uM LEV. Synaptosomes were incubated for 30 minutes at 30°C. Following the recovery step, synaptosomes are diluted in cold water to induce swelling and subjected to homogenization. Recycling intermediates (recycling vesicles trapped on the plasma membrane) are separated from membrane debris and reserve vesicle pool by ultracentrifugation in a JA25.50 rotor for 10,000xg for 10 minutes. The pellet is resuspended in HBS buffer and treated with recombinant dynamin and 1mM GTP to allow completion of endocytosis and scission of vesicles for 60 minutes at 35°C. Endocytosed vesicles are isolated by centrifugation at 14,000xg for 20 minutes at 4°C. The supernatant containing endocytosed vesicles are collected and analyzed by MS-based proteomics.

LC/MS mass spectrometry and data analysis

Proteins of newly recycled vesicles were acetone precipitated and resuspended in 8M Urea Buffer. Resuspended proteins were reduced and alkylated with 1mM tris(2-carboxyethyl)phosphine (TCEP) and 2mM chloroacetamide (CAM).

After reduction and alkylation, proteins were diluted with 100 µL (same as initial volume) of 50 mM Tris, pH 8.0 and digested with 1 µg of Lys-C (125-05061, Wako) for 2 hours at 37°C. The solution was further diluted by adding 200 µL (2 times of initial volume) of 50 mM Tris and subsequently digested with 1 µg of Pierce™ Trypsin Protease, MS Grade (90058, Thermo Fisher Scientific) overnight at 37°C. The digested proteins were acidified with trifluoroacetic acid (1% final concentration) and desalted with StageTip (Rappsilber, J. et al., 2007). Samples were subjected to LC/MS analysis.

Immunoprecipitation

Brains were homogenized in HBS buffer (10mM HEPES, 142mM NaCl, 2.4mM KCl, 1mM $MgCl_2$, 5mM D-glucose, 1mM EGTA, 1x protease inhibitor (Roche)). The brain homogenate was centrifuged at 1000xg for 10 minutes to remove nuclei and undisrupted cells. One milligram of post nuclear supernatant was solubilized in 1% Triton X-100 for one hour at 4°C then centrifuged at 17000xg for 20 minutes at 4°C. The supernatant was incubated with polyclonal antibody against the cytoplasmic domain of synaptotagmin or with a monoclonal antibody against SV2 conjugated to Protein A sepharose (CL-4B) beads for one hour at 4°C. Proteins were eluted with SDS-PAGE sample buffer and subjected to western blot analysis.

Purification of SV2A-FLAG

Lenti-X™ HEK 293T cells were transfected by calcium phosphate transfection with SV2A-FLAG in pie2. Twenty-four hours post-transfection, the medium was replaced with fresh Dulbecco's Modified Eagle's Medium. After twenty-four hours, cells were harvested and

resuspended in lysis buffer (10mM HEPES, 150mM potassium acetate, pH 7.4 Roche™ protease inhibitor), sonicated (duty cycle 40, power level 7, 10 pulses). After sonication, cells were extracted with equal volumes of extraction buffer (10mM HEPES, 150mM potassium acetate, 2% Triton x-100), incubated for 1 hour at 4°C, then centrifuged at 15000xg for 20 minutes. The lysate was then incubated with FLAG beads for at 4°C. After a two-hour incubation, the beads were washed with buffer (10mM HEPES, 150mM potassium acetate, 0.5% Triton x-100) four times. The second was contained 500mM potassium acetate. Bound SV2A-FLAG was eluted with 3x FLAG peptide for one hour in buffer containing 10mM HEPES, 150mM potassium acetate, 0.5% Triton x-100.

Purification of complexin1

Bacterially-produced glutathione-S-transferase-complexin (GST-Cpx1) was bound to Pierce™ glutathione agarose (16100) for one hour at 4°C. Resin was washed with phosphate based saline buffer with Tween-20 (PBS-T) three times. To remove the GST tag, resin was incubated with 6x His-Tobacco Etch Virus (His-TEV) protease in buffer containing 20mM Tris-HCl and 20mM NaCl, pH 7.4 overnight at 4°C. After centrifugation at 1000xg for one minute, the supernatant was incubated with Ni-Sepharose beads to remove His-TEV for two hours at 4°C. The sample was then centrifuged at 1000xg for one minute. Protein concentration of cleaved complexin1 was determined using a Bradford Assay with BSA as a standard and used for subsequent protein-protein interaction assays.

GST protein-protein interaction assay

Protein binding assays were performed with either GST-complexin1 (GST-cpx1), GST-synaptotagmin1 (GST-Syt1), or GST alone bound to Pierce™ glutathione agarose beads (16100). Purified proteins were incubated in buffer containing 10mM HEPES, 150mM potassium acetate, 1% Triton x-100, 1mM EGTA, +/-3mM $CaCl_2$, +/- 300uM LEV with coated glutathione

agarose beads for two hours at 4°C. Beads were washed four times with respective incubation buffers to remove non-specific binding and eluted with SDS-PAGE sample buffer.

CHAPTER 4:

Development of a method to isolate recycling intermediates

Introduction

Vesicle protein sorting requires assistance from endocytic adaptors such as AP2, AP180 and stonin2 to ensure proper vesicle protein retrieval. To date, it is thought that vesicles are retrieved through four different modes of endocytosis, kiss and run, clathrin-mediated endocytosis, ultrafast endocytosis and clathrin-independent endocytosis [16]. During moderate (physiological) stimulation, synaptic vesicles are retrieved through clathrin-mediated endocytosis facilitated by adaptor protein AP2 and recruitment of a clathrin coat. During high intense stimulation, ultrafast endocytosis and/or clathrin-independent endocytosis predominate, in which a large endosome forms for faster recovery of the plasma membrane [98, 99]. After clathrin-independent endocytosis, single synaptic vesicles bud off the endosomes via clathrin-mediated endocytosis to replenish the pool of vesicles [100].

One protein known to interact with endocytic adaptor proteins is synaptic vesicle 2 (**SV2**). SV2A contains tyrosine-based endocytic motifs (YXXΦ) that stimulate the binding of AP2 to synaptotagmin [17]. Mutation of one of SV2A's endocytic motifs (SV2A-Y46A) decreased binding to clathrin adaptor proteins such as AP2, Eps15 and amphiphysin. Mutation of this endocytic motif also affected SV2A's ability to traffic synaptotagmin to synaptic vesicles [19].

SV2A is a novel receptor for anti-epileptic drug Levetiracetam (Keppra™, **LEV**). Although LEV's mechanism of action is not fully known, we have shown that it affects the interactions between SV2A and synaptotagmin. We have also shown it may affect the interaction between SV2A, synaptotagmin, and complexin1. Thus, we wanted to know if LEV also affects SV2A's interactions with endocytic adaptor proteins and thus potentially influence the mode of endocytosis.

To address this possibility, we took advantage of the protocol designed to isolate recycling intermediates for a mass spectroscopy analysis of LEV effects on adaptor protein recruitment during endocytosis.

Results

A protocol to isolate recycling intermediates

A variant of the protocol designed to isolate recycling vesicles was developed to purify recycling intermediates from intact synaptosomes and the reserve pool of vesicles (**Figure 15**). After synaptosomes are ruptured, samples are loaded onto a sucrose gradient to isolate recycling intermediates (**Figure 16A**). Using western blot analyses, the fraction containing recycling intermediates was identified by an enrichment of vesicle protein, synaptophysin but also a de-enrichment of mitochondrial marker, complex V (**Figure 16B-C**). To further ensure there are no intact synaptosomes in this preparation, we immunopurified recycling intermediates using an antibody against the cytoplasmic region of SV2 conjugated to magnetic M270 Dynabeads® (Figure 16D-F).

The effects of LEV on the proteome of recycling intermediates were assessed using a dimethyl labeling mass spectroscopy analysis [101] of recycling intermediate fractions isolated from synaptosomes stimulated in the presence and absence of 300uM LEV as described under Methods (**Figure 15**)**.**

Mass spectrometry confirmed the presence of endocytic adaptor proteins such as AP2, clathrin heavy chain, and endophilin A-1 in the recycling intermediates. Gene ontology (GO) term enrichment analysis of pooled data confirmed the presence of proteins involved in the vesicle machinery such as regulation of synaptic vesicle cycle suggesting that this method can be used to isolate synaptic vesicles undergoing endocytosis and its endocytic machinery (**Figure 17**).

We found no significant differences between LEV treated and untreated recycling intermediates isolated from wild-type mice. While this may suggest that LEV does not affect SV2A's ability to bind to adaptor proteins, these studies need to be repeated with synaptosomes from mice lacking SV2B.

Discussion

Altogether, the development of this method has shown to be useful in two major ways. It can be used to isolate recycling vesicles, but it can also be used to isolate recycling intermediates. While most methods used to compare different modes of endocytosis rely on fluorescence microscopy and electron micrographs. But these studies lack information about the adaptor proteins recruited to vesicles to facilitate endocytosis. Using this method, we can gain insight into the endocytic machinery formed at these synapses and a further understanding of the molecular mechanisms involved in endocytosis.

The enrichment in adaptor proteins suggests that this method can be used to identify whether drug treatment is affecting adaptor protein recruitment and ultimately, endocytosis. While, LEV treatment did not affect recruitment of adaptor proteins to recycling vesicles from wild-type mice, it could have an effect in synaptosomes from mice lacking SV2B (**BKO**). It is possible that LEV affects the interaction between SV2A, synaptotagmin and AP2 thus affecting the endocytic machinery in synapses lacking SV2B. During high stimulation, such as in seizures, clathrin-independent endocytosis or ultrafast endocytosis, predominate for faster membrane retrieval. I hypothesize that in synapses lacking SV2B, if SV2A interaction with adaptor proteins is affected, LEV may be slowing neurotransmission by switching to the slower form of endocytosis (clathrin-mediated endocytosis) to slow vesicle pool replenishment. This is consistent with previous studies that found LEV affects replenishment of the readily releasable pool of vesicles [10].

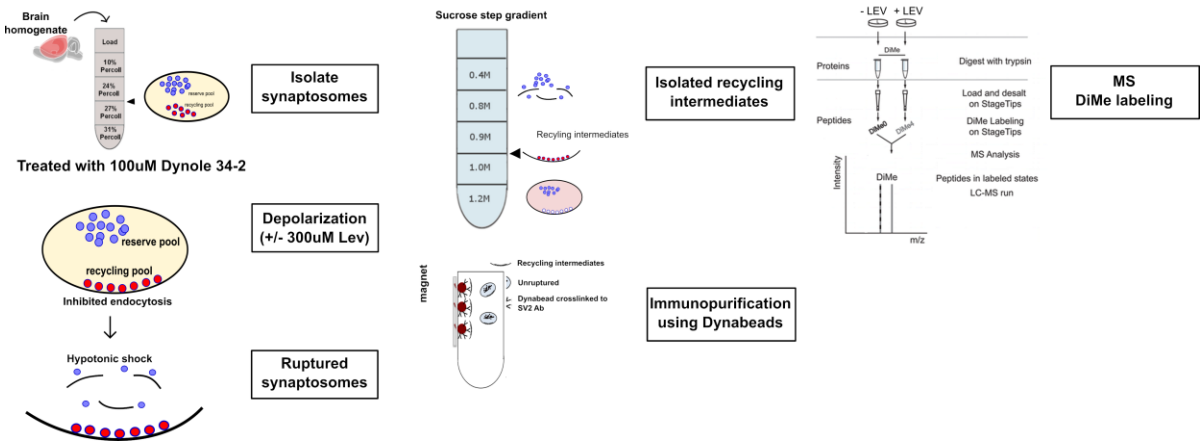

Figure 15: Protocol to identify endocytic adaptor proteins recruited to recycling vesicles
Left: Isolation of synaptosomes using density gradient fractionation. Mouse brain is homogenized and layered onto a step gradient of the density media Percoll®. Purified synaptosomes are stimulated with 60uM KCl for five minutes in the presence of a dynamin inhibitor, Dynole® 34-2 and +/- 300uM LEV. Synaptosomes are then ruptured using hypotonic swelling and shear force. Middle: Plasma membrane containing recycling intermediates (vesicles undergoing endocytosis that are trapped by Dynole® 34-2 is purified by sucrose gradient fractionation. To further ensure the fraction does not contain unruptured synaptosomes, recycling intermediates were immunopurified using an antibody against SV2 conjugated to M270 Dynabeads®. Right: Isolated recycling intermediates were then subjected to dimethyl labeling (heavy and light isotope) then analyzed with LC-mass spectrometry.

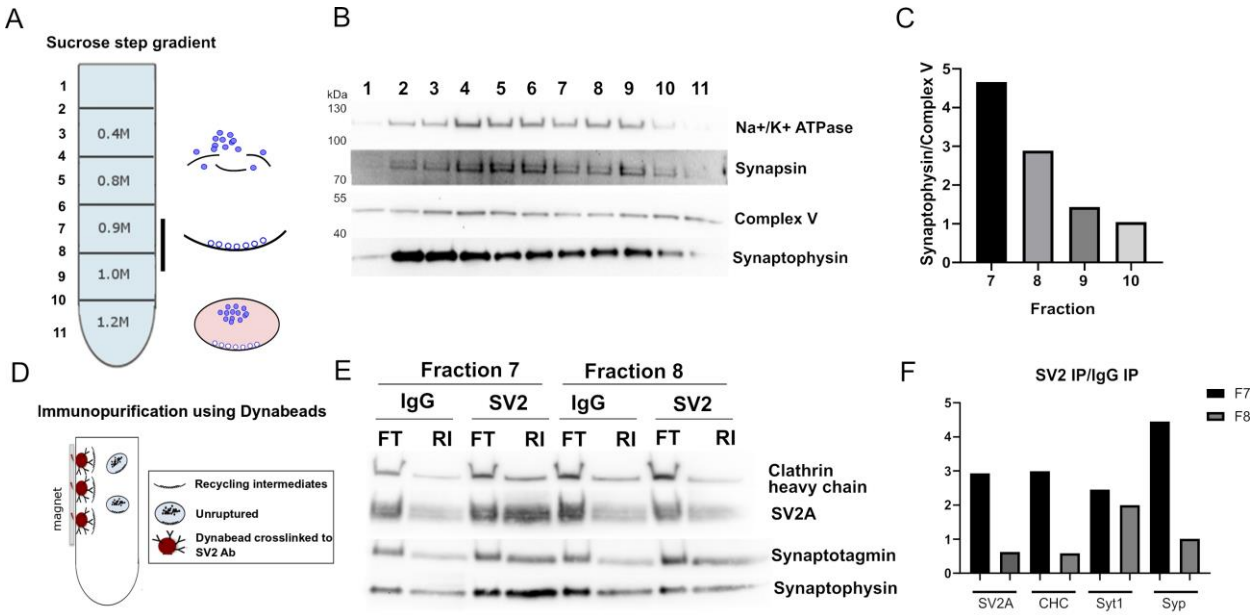

Figure 16: Validation of isolation of recycling intermediates and adaptor proteins
(A) After synaptosomes are ruptured with hypotonic shock, recycling intermediates are separated from intact synaptosomes and the recycling pool of vesicles using a sucrose gradient. (B) To identify the fraction containing recycling intermediates, western blot analysis of each fraction was done. (C) Band intensity of vesicle protein, synaptophysin (Syp) was compared to mitochondrial marker, complex V. Fraction 7 and 8 contained a higher synaptophysin to complex V ratio. (D) To ensure there were no intact synaptosomes, recycling intermediates (Fraction 7 and Fraction 8) were further immunopurified using an antibody against SV2 conjugated to M270 Dynabeads®. As a control, an antibody against immunoglobulin G (IgG) was used. (E) Vesicle protein binding was assessed using western blot analysis and normalized to proteins immunopurified with the control sample. (F) Quantification of the western blots showed SV2 antibody was specific to synaptic vesicles. Compared to Fraction 8, Fraction 7 contained more SV2A, clathrin heavy chain (CHC), synaptotagmin (Syt1) and synaptophysin.

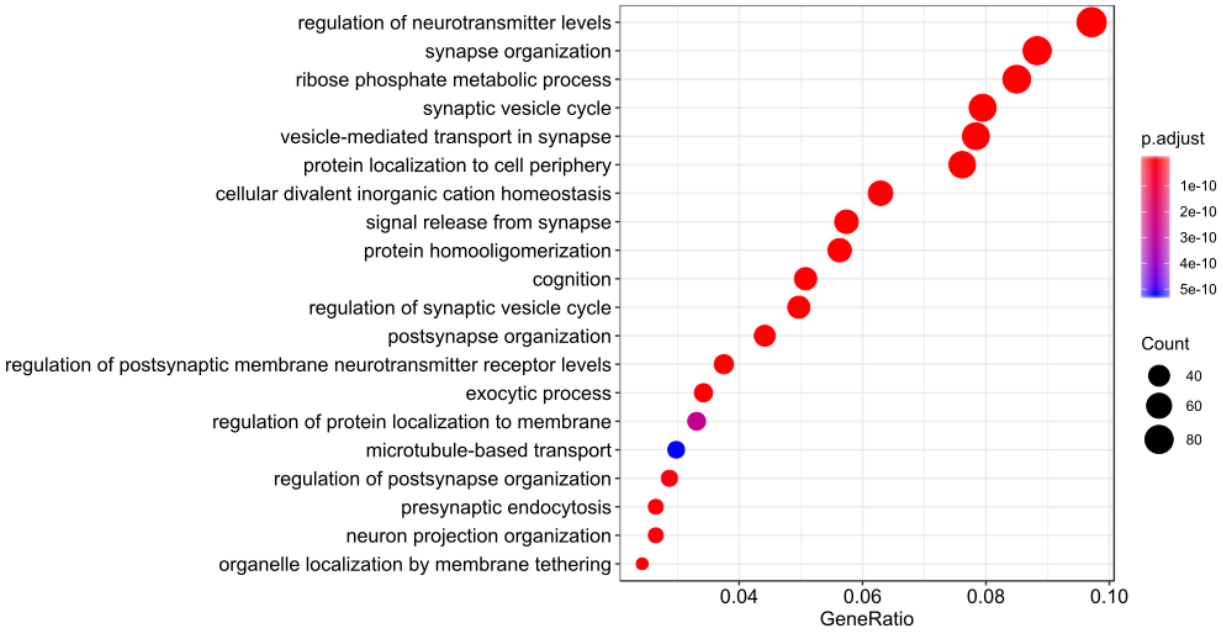

Figure 17: Gene Ontology (GO) over-representation test of proteins identified by MS-based proteomics

Recycling intermediates were subjected to dimethyl labeling and LC- mass spectrometry. Data from LEV-treated and untreated samples were combined. The twenty most represented GO terms are shown. Most notable are proteins part of the vesicle machinery such as those involved in the synaptic vesicle cycle, regulation of protein localization to membrane, and presynaptic endocytosis. Analysis was performed using the Bioconductor package ClusterProfiler; n=4. [102]

(Experiments done by me, figure made by DM)

Methods

Animals and reagents

Refer to Chapter 2 methods.

Isolating Recycling Intermediates

Recycling vesicles were isolated from wild-type mice using differential centrifugation and Percoll® density separation. Brain tissue is homogenized in HBS buffer (10mM HEPES, 142mM NaCl, 2.4mM KCl, 1mM $MgCl_2$, 5mM D-Glucose pH 7.4) with 100mM EGTA and 1x Roche protease inhibitor. After homogenization, it is spun at 10,000xg for 10 minutes. The supernatant is loaded onto a Percoll® step gradient containing (vol/vol) 10%, 24%, 27%, and 31% filtered Percoll ® diluted in 10x HBS. The gradient is then centrifuged in a JA25.50 rotor for 45 minutes. The interface between 27% and 31% is collected and diluted in HBS and centrifuged at 17,000xg for 15 minutes. The synaptosomes (pellet) are resuspended in HBS and centrifuged again at 17,000xg for 15 minutes. The synaptosomes are resuspended in HBS and 5mM K+ and treated with 300uM LEV or water. Exocytosis is induced by addition of 60mM KCl for 5 min in the presence of 100uM Dynole 34-2™ and +/- 300uM LEV. Synaptosomes are then diluted in cold water to induce swelling and subjected to homogenization. After thirty minutes at 4°C, sample was sonicated at 30% duty cycle, power level 6 for 30 pulses. Recycling intermediates (recycling vesicles trapped on the plasma membrane) are separated from membrane debris and reserve vesicle pool by sucrose gradient containing 0.4M, 0.8M, 0.9M 1.0M, and 1.2M sucrose. The gradient is centrifuged for in an SW-28 rotor for 55 minutes at 23, 200 RPM. Fraction 7 was incubated with monoclonal SV2 antibody coupled to M270 Dynabeads® (according to Life Technologies) for one hour at 4°C. Samples were washed with phosphate-based saline buffer (PBS), eluted with SDS-PAGE sample buffer, and ran on a gel for coommassie staining.

Dimethyl labeling

Gel was excised and destained in 50% ethanol/50% 50mM Triethylammonium Bicarbonate (TEAB). After destaining, gel slices were dehydrated in 100% ethanol until gel turned opaque. Samples were then reduced/alkylated by rehydrating the gel slices with 10mM TCEP for 20 minutes at 60°C, shaking at 1400 rpm. TCEP was aspirated off and then incubated in 60mM CAM with low vortexing. Sample was washed with 50% ethanol/ 50% 50mM TEAB then dehydrated in 100% ethanol. The gel slice was digested with trypsin and TEAB buffer and incubated at 37°C, shaking at 1400 rpm overnight.

The next day, added 33% (v/v) trypsin and incubated at 37°C for three hours. Added 20% (v/v) of 10% TFA (trifluoroacetic acid) and incubated for 10 minutes, shaking 1400 rpm. Digested peptides were desalted using C18 STAGEtips prepared as previously described. Stable isotope dimethyl labeling was performed as previously described [101].

CONCLUSION

Overall, we gained further insight into the molecular mechanism of the anti-epileptic drug, Levetiracetam (LEV). I have been able to show that not only does the LEV effect manifest in synapses predominantly expressing SV2A, but that LEV disrupts SV2A's protein-protein interactions. The absence of a LEV effect when other SV2 paralogs are present suggests that future LEV studies may want to focus on neurons solely expressing SV2A. These include inhibitory neurons and the excitatory neurons of the dentate granule cells, neurons that are implicated in the etiology of epilepsy.

In addition, I helped develop a new method to study the protein composition of recycling vesicles and a new method to isolate recycling intermediates. From these studies, it is seen that LEV modulates SV2's protein-protein interactions at the synapse, including SV2A's interaction with synaptotagmin and complexin1. Because both synaptotagmin and complexin1 pay a role in exocytosis, I proposed a model in which LEV treatment disrupts SV2A's protein-protein interactions which then disrupts SV2A's ability to traffic these two proteins to synaptic vesicles. The decreased association of synaptotagmin and complexin1 could then affect the synaptic vesicles competence, and its ability to undergo calcium-stimulated fusion. As more information about how these proteins interact and their role in vesicle priming and fusion, it will provide insight into how LEV's disruption of these interactions could affect neurotransmission. It will be interesting to see if other LEV analogs, such as Brivaracetam and Seletracetam, drugs with higher affinity for SV2A, also disrupt SV2A's protein-protein interactions and if their effect will also only be seen in synapses solely expressing Sv2A.

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