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Asymmetric Accumulation of Hippocampal 7S SNARE Complexes Occurs Regardless of Kindling Paradigm

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Abstract

Modifications of neurotransmission may contribute to the synchronization of neuronal networks that are a hallmark of epileptic seizures. In this study we examine the synaptosomal proteins involved in neurotransmitter release to determine if alterations in their interactions correlate with the chronic epileptic state. Using quantitative western blotting, we measured the levels of 7S SNARE complexes and SNARE effectors in the effected hippocampi from animals that were electrically kindled through stimulation from one of three different foci. All three kindling paradigms, amygdalar, entorhinal, and septal, were associated with an accumulation of 7S SNARE complexes in the ipsilateral hippocampus, measured one month after completion of kindling. Of the eight SNARE effectors examined (α -SNAP, NSF, SV2A/B, Munc18a/nSec1, Munc13-1, Complexin 1, 2, and synaptotagmin I), there was a statistically significant bihemispheric increase of hippocampal SV2 and decrease of NSF upon kindling; neither by itself would be expected to account for the asymmetry of SNARE complex distribution. These data suggest that an ipsilateral hippocampal accumulation of SNARE complexes is a permanent alteration of kindling-induced epilepsy, regardless of stimulation pathway. The significance of these findings toward a molecular understanding of epilepsy will be discussed.

Keywords

Epilepsy; Neurotransmission; NSF; SNARE; SV2

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1. Introduction

Calcium-dependent neurotransmitter (NT) release from synaptic vesicles is a specialized exocytic process that is the basis for inter-neuronal communication. The fusion of vesicle and plasma membranes, which mediates NT release, is initiated by the formation of a stable, ternary complex of Soluble N-ethylmaleimide Sensitive Factor Attachment Protein Receptors (SNAREs) that spans the two bilayers (reviewed in (Brunger, 2005; Jahn and Scheller, 2006; Sudhof, 2004)). This 7S SNARE complex is composed of synaptobrevin/VAMP-2 from the synaptic vesicle and syntaxin 1 and SNAP-25 from the neuronal active zone, a specialized region of the presynaptic bouton where synaptic vesicles dock and fuse with the plasma membrane. These three proteins form a four-helical bundle that is minimally required for membrane fusion (Weber *et al.*, 1998). Formation and disassembly of this complex represent two key steps in which control over NT release can be exerted. General factors such as N-ethylmaleimide Sensitive Factor (NSF) and Soluble N-ethylmaleimide Sensitive Factor Attachment Protein (SNAP) are required for SNARE complex disassembly and subsequent recycling of SNARE monomers. Dysfunction of NSF or α -SNAP leads to cessation of NT release and paralysis (reviewed in (Whiteheart *et al.*, 2001)). Formation of SNARE complexes is less understood and appears to require a number of SNARE-specific, interacting proteins such as members of the Sec1/Munc18 family (reviewed in (Jahn, 2000; Toonen and Verhage, 2003)). Several other proteins such as Munc13s, Complexins, and SV2s are thought to further affect how SNAREs are made accessible to the other SNAREs prior to complex formation and membrane fusion (Ashery *et al.*, 2000; Hu *et al.*, 2002; Xu and Bajjalieh, 2001). Epilepsy is associated with hyper-synchronous activation of large populations of neurons. This may occur through hyper-secretion of excitatory NT or hypo-secretion of inhibitory NT, leading to disinhibition (Engel *et al.*, 1997). Furthermore, *in vivo* electrophysiologic studies indicate enhanced inhibition, perhaps reflecting natural protective mechanisms, as well as enhanced excitation in local areas of human epileptogenic hippocampus (Colder *et al.*, 1996; Engel and Wilson, 1986). Hence, the elements of the neuronal secretory machinery represent potential steps where molecular dysfunction could play a role in epileptogenesis.

Kindling, a model of complex partial epilepsy and epileptogenesis (Sato *et al.*, 1990; Sutula, 1990), is a process of progressive and permanent intensification of epileptiform after-discharges culminating in a generalized seizure in response to repeated subconvulsive electrical stimulation. Kindling can be induced from many sites, usually within the limbic system, including amygdala, entorhinal cortex, and septal region. Development of kindling in the rat is characterized by electrographic and behavioral stages (Racine, 1972). The behavior in stages 1–2 mimics human complex partial seizures; behavior in later stages 3–5 is consistent with evolution to secondarily generalized motor seizures. Once the fully kindled state has been achieved, spontaneous generalized convulsions may continue to be seen throughout the lifespan of a kindled animal. Typically, however, an animal must experience additional stimulation-induced Stage V seizures for the development of spontaneous seizures. This permanently enhanced excitability is thought to result from changes both at the cellular level, through altered synaptic neurotransmission, and at a network level (McNamara, 1995; Mody, 1993). Electrical stimulation of the entorhinal cortex/perforant pathway, the medial septum/septohippocampal pathway, and the amygdala all stimulate the hippocampal trisynaptic excitatory circuit which is an important component of the kindling phenomenon (Dasheiff and McNamara, 1980; Savage *et al.*, 1985; Yoshida, 1984). This circuit is directly stimulated by cholinergic-septal and glutamatergic-entorhinal afferents; the route from amygdala is polysynaptic.

We have previously shown that kindling *via* entorhinal electrical stimulation is associated with an accumulation of 7S SNARE complexes in the ipsilateral hippocampus (Matveeva *et al.*,

2003). This increase of 7S SNARE complexes appears to begin early in the kindling process, achieves a peak with full kindling, and remains at this level for at least a month following cessation of further kindling stimuli. The present study was carried out to determine if change in the levels of hippocampal 7S SNARE complex formation is anatomically specific to sites involved in the kindling process and universal to kindling, independent of the site of initiation. We also sought to determine if alterations in the synaptosomal levels of specific exocytic machinery regulators correlate with increased 7S SNARE complex formation.

2 Methods

2.1 Kindling

Fourteen-week-old male Sprague–Dawley rats had stimulating electrodes surgically implanted in the right brain as previously described (Slevin and Ferrara, 1985). Coordinates for the amygdala (from bregma: AP −2.8; ML +4.8; DV −8.5; nose bar −3.3 [flat skull]), entorhinal cortex in the region of origin of the perforant pathway (from lambda: AP +1.2; ML +4.0; DV −7.0; nose bar −1.0), and medial septal area (AP +0.5; ML +0.7; DV −5.2; nose bar −3.3) were determined using the atlas of Paxinos and Watson (Paxinos and Watson, 1982). Electrode placement was histologically confirmed in one animal from each kindling group. The three groups received electrical stimulation resulting in amygdalar, entorhinal, or septal kindled seizures; control groups received sham operations but no electrical stimulation, two electroconvulsive shocks (ECS; 50 mA for 0.8 s through ear clips), or were left untreated (naïve). All animal procedures were approved by the Lexington VA IACUC. Animals were housed individually and maintained on a 12 h light/dark cycle with food and water available *ad libitum*.

Kindled animals were stimulated once daily, five days/week until they experienced two consecutive Racine stage 5 seizures (tonic-clonic activity with loss of postural control/falling). ECS animals were stimulated on each of the two days a kindled animal experienced a stage 5 seizure. Both groups were then given no further stimulations and were euthanatized 30 days later. None of the animals used for quantification experienced a spontaneous seizure within at least 1 week prior to sacrifice. The mean stimulus current necessary to produce an after discharge and the mean number of stimulations required to cause a stage 5 seizure in these studies are shown in Table 1 and are typical for the three kindling models.

2.2 Analysis of SNARE Complexes

Animals were euthanatized by decapitation after completion of kindling. Their brains were rapidly removed and tissues of interest were dissected on ice. Percoll gradient purified synaptosomes were prepared from individual hippocampi, occipital cortices and cerebella as previously described (Dunkley *et al.*, 1988; Dunkley *et al.*, 1986). Extracts were prepared by incubating equal quantities of synaptosomal protein in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer for 30 min at 37°C. Under these conditions, monomeric SNAREs are denatured but the thermally stable 7S SNARE complexes fail to disassemble (Fig. 1A and (Matveeva *et al.*, 2003)). After electrophoresis and transfer to PVDF membranes (Millipore), these complexes were probed by western blotting using antibodies against the t-SNARE, syntaxin 1 (HCP-1; (Inoue and Akagawa, 1992)) as previously described (Matveeva *et al.*, 2003). Parallel samples were boiled for 10 min to denature 7S SNARE complexes. Bands that were detected by antibody and were sensitive to boiling were considered authentic 7S SNARE complexes. Western blot based detection of 7S SNARE complexes was performed using alkaline phosphatase coupled secondary antibodies with Vistra ECFTM for visualization and images were obtained using a Typhoon 9400 imager (GE Healthcare BioSciences, Piscataway, NJ). The raw data were the integrated fluorescence intensity for all pixels in a given immuno-decorated protein band and expressed by the ImageQuant 5.2

software (GE Healthcare Bio-Sciences, Piscataway, NJ) as arbitrary units (AU). To normalize for protein loading, the fluorescent intensity of all SNARE complex bands was standardized to the intensity of the syntaxin 1 monomer and/or to actin (see below).

2.3 Analysis of Secretory Machinery Protein Levels

Analysis of other secretory machinery proteins was performed by western blotting using the methods above. NSF was detected with the 2E5 monoclonal antibody (Tagaya *et al.*, 1993; Whiteheart *et al.*, 1994) and α -SNARE was detected with the 4E4 monoclonal antibody (Gamma One Laboratory Inc., Lexington, KY). Complexin 1 and 2, Munc18a, and Munc13-1 were detected with commercially obtained antibodies (Synaptic Systems, Gottingen, Germany). Antibody to SV2 (appears to detect both A and B isoforms) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, (Iowa City, IA). The antibody to actin was from Sigma. Fluorescent intensities of the bands in each lane were normalized to the intensity of the actin band in the same lane (see method above). To normalize measurements taken on different days, the actin-normalized, average fluorescence intensity of an immuno-decorated protein from the contralateral hippocampi of two surgical controls (in a gel set) was used. To determine "Total Value", normalized ipsilateral and contralateral measurements from either experimental or control animals were summed, divided by two, and compared to the averaged surgical control (Total Value = $[(\text{Contralateral}/\text{Act} + \text{Ipsilateral}/\text{Act})/2]/[\text{Contralateral}_{\text{control}}/\text{Act}]$). If no changes in protein level occur, then the ratio should be unity.

2.4 Statistical Analyses

The ipsilateral/contralateral 7S SNARE band intensity ratios determined from hippocampus of surgical controls and hippocampus, cerebellum, and occipital cortex of kindled animals for each kindling paradigm were analyzed by ANOVA and *post hoc* t-tests using Fisher's protected least significant differences procedure. Quantified secretory machinery proteins from hippocampus of kindled and surgical control animals were compared by t-tests.

3. Results

3.1 Kindling Increases 7S SNARE Complexes in Hippocampal Synaptosomes Regardless of Stimulation Site

As previously demonstrated (Matveeva *et al.*, 2003), several high molecular weight SNARE complexes are detected in synaptosomes using the anti-syntaxin 1 antibody, HCP-1 (Fig. 1A). These bands are stable in SDS-PAGE buffer at 37°C but not at 100°C (Hayashi *et al.*, 1994) and they are co-stained with anti-v-SNARE (anti-synaptobrevin/VAMP-2) antibodies (data not shown and (Matveeva *et al.*, 2003)). The presence of SNAP-25 in the complexes is inferred since synaptobrevin/VAMP-2 and syntaxin 1 do not interact strongly in its absence (Hayashi *et al.*, 1994). The five bands representing 7S SNARE complexes (bands indicated in Fig. 1A) may be SNARE oligomers, as suggested by (Tokumaru *et al.*, 2001). These bands were quantified and their sum was considered the total 7S SNARE complex in that synaptosomal preparation. This quantification was normalized to the actin (Fig. 1B) detected in the same lane. These normalized values for 7S SNARE complexes were then used for the comparisons discussed below.

One month following the cessation of stimulation of animals kindled to stage 5, there was a significant ($P < 0.001$ by ANOVA; all three stimulation paradigms, 12 groups compared), asymmetric accumulation of 7S SNARE complexes in the hippocampus ipsilateral to the stimulus site, regardless of stimulation loci (Fig. 2, solid bars). This was not seen when the levels of 7S SNARE complex in synaptosomes prepared from left (contralateral) and right

(ipsilateral) hippocampi were compared in naïve animals; the ratio of right vs. left was near unity (0.97 ± 0.03 , $n = 14$). The same ratio was near unity for surgical control animals (Fig. 2, open bars) suggesting that electrode implantation does not adversely affect SNARE complex levels. Finally, this asymmetric accumulation is not a result of an acute generalized seizure since the ratio does not change in ECS animals (ratio of 1.0 ± 0.0 , $n = 4$). Asymmetric accumulation was specific to the hippocampus; it was not seen at synaptically distant sites (occipital cortex and cerebellum, hatched bars) that cannot serve as stimulus foci and are not thought to be directly involved in the neural network of kindling (McNamara *et al.*, 1980). In these distant regions, despite stable kindling, the ratio of right vs. left 7S SNARE complex levels was near unity (Fig. 2, hatched bars), equivalent to that seen in naïve animals. All of these controls demonstrate a 7S SNARE ratio near unity for each of the three kindling paradigms used in our study. Similar asymmetric accumulation of SNARE complexes was seen in all three kindling paradigms (Fig. 2). As previously reported, kindling by entorhinal cortical stimulation causes a significant accumulation of SNARE complexes in the ipsilateral hippocampus (Matveeva *et al.*, 2003). Kindling *via* the amygdala is more rapid and requires fewer stimuli (see Table 1), but also leads to an increased distribution of 7S SNARE complexes in the ipsilateral hippocampus. The effect was highly statistically significant but not as robust as seen in the entorhinal cortex kindled rats. Finally, kindling induced by medial septal stimulation also showed a significant asymmetric accumulation of SNARE complexes. The monosynaptic septohippocampal pathway is distinct from polysynaptic routes from amygdala and includes a large cholinergic influence in contrast to the monosynaptic, glutamatergic perforant pathway from entorhinal cortex, however the effect is similar. Taken together, these data demonstrate that, despite different kindling stimulation sites, there is a consistent and stable increase in ipsilateral 7S SNARE complex accumulation in the hippocampus.

3.2 Analysis of Other Secretory Machinery Proteins

Previous studies (Matveeva *et al.*, 2003), indicated that SNARE levels did not significantly change after kindling *via* the perforant pathway. Given the asymmetric accumulation of 7S SNARE complexes seen in each kindling paradigm above, we focused on elements of the secretory machinery known to directly affect SNARE complexes. α -SNAP serves as an adaptor mediating the binding of NSF to the SNARE complex and ultimately triggering NSF's ATPase activity to disassemble SNARE complexes (reviewed in (Whiteheart *et al.*, 2001)). Generally, there was no difference in α -SNAP and NSF proteins levels between ipsilateral and contralateral hippocampi (Laterality, Table 2), though there was an upward tendency in entorhinally kindled hippocampi. Interestingly, there were some differences when absolute levels (Total Value, Table 2) of the two proteins were compared between control and kindled animals. One month after establishment of the kindled state, α -SNAP levels showed an upward tendency (16%) with amygdala stimulation. However, combining the data from all three kindling models failed to show a significant change in α -SNAP levels in kindled vs. control animals. The levels of NSF appeared to decrease upon amygdala (7%) and septal kindling (11%). While the decrease was only a tendency in amygdala, it was statistically significant in the septally kindled animals. However, when all data from all three models were combined, there was no significant decrease in NSF protein levels in kindled animals compared to controls.

Other SNARE effector proteins control how SNAREs form complexes and thus how they facilitate exocytosis. Munc18 proteins are syntaxin chaperones holding the SNARE in a closed configuration until an appropriate trigger is sensed (Toonen and Verhage, 2003). Munc13 proteins are priming factors that promote the formation of the “readily releasable pool” of secretory vesicles (Ashery *et al.*, 2000). Complexins compete with α -SNAP for SNARE complex binding and affect SNARE complex stability (Hu *et al.*, 2002; Tokumaru *et al.*, 2001). Synaptotagmins are potential calcium sensors that regulate the final steps of membrane fusion (Yoshihara *et al.*, 2003). SV2's role is relatively unclear yet transgenic strains of mice

lacking it have spontaneous seizures and at least one anti-epileptic drug, levetiracetam (LEV), specifically binds to this membrane protein (Lynch *et al.*, 2004). The ipsilateral and contralateral levels of these potential SNARE regulators were examined in the hippocampi from amygdalar kindled animals (Table 3). None of the proteins showed chronic, kindling-induced changes in either ipsilateral or contralateral hippocampus. Strikingly, there was a statistically significant increase in the level of total SV2 (A and B isoforms combined) in the kindled hippocampi relative to controls (36%; $p = 0.004$). While the data in Table 2 and 3 show that α -SNAP, NSF and SV2 all change in hippocampal synaptosomes from amygdalar kindled rats, only the increase in SV2 was significant. Despite these alterations, none of the proteins show the same asymmetric changes as seen for the 7S SNARE complexes.

4. Discussion

In this manuscript, we demonstrate an ipsilateral accumulation of 7S SNARE complexes in the hippocampi of kindled animals. This accumulation was not due to a generalized seizure *per se* and was present at one month, post kindling. It is possible that the asymmetric accumulation could have been due merely to chronic, intermittent unilateral low frequency electrical stimulation and would have occurred even in the absence of a kindling response. No accumulation was found outside of limbic structures, specifically occipital cortex and cerebellum, where no functional changes have been reported in kindling (McNamara *et al.*, 1980). Although other limbic sites may demonstrate a similar accumulation, they were not the focus of this study. Asymmetric SNARE complex accumulation was seen in hippocampal synaptosomes regardless of the kindling stimulation site and the neuronal circuitry involved (Fig. 2). Based on these data, it would appear that accumulation of 7S SNARE complexes does correlate with a stable, kindling-induced, epileptic state.

Amygdalar and entorhinal kindling has been associated with hippocampal mossy fiber sprouting and dentate granule cell neuronal loss (Cavazos *et al.*, 1991). (Osawa *et al.*, 2001) have demonstrated that in the case of amygdalar stimulation, kindling develops without evidence of mossy fiber sprouting until after repeated kindled convulsions, following death of granule cells in the dentate gyrus. In their study, animals experienced 29 ADs and 2 kindled convulsions before these histopathological changes were seen. In contrast, amygdala kindled animals in the present study experienced 7 ADs (Figure 1) and 2 kindled convulsions. Furthermore, we previously demonstrated that 7S SNARE complexes begin to accumulate as early as behavioral Stage 1 with entorhinal kindling (Matveeva *et al.*, 2003). Thus, it is unlikely that the alterations in 7S SNARE complexes are merely a consequence of sprouting or neuronal loss.

To examine potential molecular explanations for the asymmetric complex accumulation, we measured the levels of known SNARE regulators, which could promote either SNARE complex formation (Munc18a, Munc13-1, Complexins, SV2) or disassembly (α -SNAP and NSF).

Despite some tendencies, there were no statistically significant alterations in these proteins that could account for the asymmetric increase in SNARE complexes (Table 2 and 3). There were however two mechanistically significant changes when total levels were examined. NSF showed a decrease (7% in amygdalar and 11% in septal) and SV2 showed an increase (36% in amygdalar) in kindled versus control animals. The potential significance of these alterations is not clear, yet these two molecules have been shown to affect SNARE complexes (see discussion below).

We examined three major routes by which epilepsy can be induced by kindling. The major monosynaptic excitatory afferent of the hippocampal formation, the perforant pathway or

angular bundle, arises from the entorhinal cortex (Andersen *et al.*, 1966). In the rat, this glutamatergic projection forms terminal and *en passant* synaptic connections on dendrites of both dentate granule cells and hippocampal pyramidal cells (Hjorth-Simonsen and Jeune, 1972; Steward, 1976). A smaller monosynaptic excitatory input to the ipsilateral hippocampal formation projects from the septal region through fornix/fimbria (Crutcher *et al.*, 1981; Swanson and Cowan, 1979). The cell bodies of this largely cholinergic septohippocampal pathway reside in the medial septum with contributions from both the vertical and horizontal limbs of the diagonal band of Broca (Amaral and Kurz, 1985); the bulk of lateral septal efferents projects to medial septal nuclei with little or no input to the hippocampal formation (Colom, 2006). These cells project to the supra- and infragranular layers of the dentate gyrus (Chandler and Crutcher, 1983) and the hippocampus (Amaral and Kurz, 1985). A monosynaptic connection between the amygdala and hippocampus has been described only for projections to the temporal aspect of CA1 near the subiculum. In the rat, the amygdala projects to ventral subiculum, parasubiculum and lateral entorhinal cortex (Krettek and Price, 1977). Furthermore, (Kajiwaru *et al.*, 2003) have shown that amygdalar stimulation promotes the spread of excitatory neural activity from perirhinal cortex to the entorhinal-hippocampal circuit (the hippocampal formation circuitry is extensively reviewed in (Schwartzkroin and McIntyre, 1997; Witter and Amaral, 2004)). Thus, electrical stimulation of the entorhinal cortex/perforant pathway, medial septum/septohippocampal pathway and the amygdala all stimulate the hippocampal trisynaptic excitatory circuit thought to be an important component of the kindling phenomenon (Dasheiff and McNamara, 1980; Savage *et al.*, 1985; Yoshida, 1984). This circuit is directly stimulated by septal and entorhinal afferents; the route from amygdala is polysynaptic. Regardless of how the ipsilateral hippocampus is accessed, our data suggest that the high frequency synchronous discharges responsible for development of the kindled state and that pass through it are associated with a molecular alteration in presynaptic mechanisms involved in neurotransmitter release. At this stage, our data cannot distinguish at what type of synapses (*i.e.*, excitatory or inhibitory) this alteration occurs.

The molecular changes underlying the kindling process, including any presynaptic mechanisms, remain largely unknown. However, increased excitatory L-glutamate neurotransmitter release from the presynaptic nerve terminals has been demonstrated in the hippocampus of amygdalar (Minamoto *et al.*, 1992) and entorhinal cortical (Geula *et al.*, 1988; Jarvie *et al.*, 1990) kindled rats implying some dysfunction in the exocytosis machinery. Similar results are seen in rat hippocampal slices 1 month after Schaffer collateral-commissural kindling (Kamphuis *et al.*, 1991) and in cerebral cortical synaptosomes two weeks after septal kindling (Yamagata *et al.*, 1995). Extracellular hippocampal glutamate overflow induced by repeated short-term high potassium stimuli and measured by *in vivo* microdialysis has been correlated to kindling phenomena (Ueda *et al.*, 2000). These reports suggest that the high frequency network discharges passing through the hippocampus and associated with kindled epilepsy involve changes in the regulation of elements of the presynaptic secretory machinery at excitatory synapses. Consistently, our work shows that there is accumulation of SNARE complexes in the ipsilateral hippocampus regardless of kindling paradigm.

SNARE proteins mediate the membrane fusion events, needed for exocytosis, by forming *trans*-bilayer complexes that bridge the target and vesicle membranes and facilitate lipid mixing and subsequent membrane fusion (Weber *et al.*, 1998). An accumulation of such *trans* complexes could indicate that, during kindling, there is an increase in SNARE complex formation which would imply that more vesicles are primed for release. Munc18a and Munc13-1 have been shown to be “priming” factors that increase the release potential for a given population of vesicles (Ashery *et al.*, 2000; Voets *et al.*, 2001). Complexins are also purported to play a role in SNARE complex assembly and stabilization (Reim *et al.*, 2001; Tokumaru *et al.*, 2001). Though a dysfunction in any of these proteins could lead to an increase in SNARE complexes, no alterations in their levels were detected in the amygdalar kindled

animals (Table 3). In addition, despite several studies, no evidence supports an increased level of morphologically docked vesicles in kindled synapses (Hovorka *et al.*, 1989; Hovorka *et al.*, 1997). Therefore it is not clear if the SNARE complex accumulation we measured is indicative of an increase in vesicle priming.

Once fusion occurs, the SNAREs are in a *cis* configuration in the same membrane. To continue secretion this complex must be disassembled and the individual SNAREs recycled. An accumulation of *cis*-complexes would suggest a dysfunction in the disassembly machinery, specifically α -SNAP or NSF. Though no differences in α -SNAP were detected, there was a statistically significant, albeit small, decrease in NSF (Table 2). A decrease in NSF protein also correlates with kainic acid-induced epilepsy (Guan *et al.*, 2001; Yu *et al.*, 2002). Such a decrease, especially in the synapse, could affect the flux of SNARE complex disassembly; the effect would be expected to cause a decrease in NT release. A decrease in inhibitory NT release, disinhibition, would be consistent with epileptogenesis (discussed in (MacDonald, 1977)). The accumulation of SNARE complexes is reproducible and correlates with the kindled phenotype regardless of stimulation route. At this stage, it is not possible to distinguish between *trans*- and *cis*-SNARE complexes.

It is difficult to point to a single molecular explanation for the SNARE complex accumulation observed. Several possible molecular scenarios could lead to increased SNARE complex (discussed in (Jahn and Scheller, 2006)). Our analysis focuses on some of the better characterized SNARE regulators which could affect complex assembly (Munc18a, Munc13-1, Complexins, Synaptotagmin, *etc.*) and/or disassembly (SNAP and NSF). Other proteins (*e.g.* Tomosyn and Synaptophysin) and other factors (*e.g.* calcium, phosphatidylinositides, and presynaptic energetics) could affect SNARE complex dynamics but were not directly examined in this study. While the data in Table 1 and 2 perhaps eliminate some of possible scenarios further analysis will be needed to determine which proteins or perhaps combinations of components are affected during epileptogenesis to account for our observations.

One specific finding to note is the increased level of SV2 associated with amygdalar kindling (Table 3). Although the specific molecular role(s) of the SV2s is debated (Brose and Rosenmund, 1999), direct measures of exocytosis in neurons and adrenal chromaffin cells show that SV2A is a key control element for neurotransmitter (Crowder *et al.*, 1999; Janz *et al.*, 1999) and neuroendocrine release (Xu and Bajjalieh, 2001). In chromaffin cells, the calcium-induced exocytotic burst (operationally defining the “ready release” pool of vesicles) is significantly reduced in mice lacking SV2A (Xu and Bajjalieh, 2001). This suggests a role for SV2A in controlling assembly of SNARE complexes but not in membrane fusion. Consistently, analyses of brain tissue from SV2A null animals show a 50% decrease in SNARE complexes. These data suggest that SV2A could maintain SNARE complexes but is perhaps not essential for complex formation.

Levetiracetam (LEV) is a potent anticonvulsant in fully amygdala-kindled rats (Loscher and Honack, 1993). Recently, (Lynch *et al.*, 2004) demonstrated that LEV binds to SV2A. Derivative compounds with increasing affinity for SV2A appeared to have better anti-seizure potency. These data suggest that the anti-epileptic/epileptogenic effects of LEV may be through the modulation of SV2A; but, since LEV does not affect the electrophysiology of normal tissue (Klitgaard *et al.*, 1998; Klitgaard and Pitkanen, 2003), it must have a specific effect on SV2A in epileptic tissues. Our data indicate that SV2 (A and B isoforms combined) is increased in kindled animals. A possible mechanism that would unite these two observations is that LEV attenuates the effect of increased SV2A in kindled animals, leading to a reduction, toward normal, of SNARE complex formation. This could explain the antiepileptic effect of LEV while offering a molecular mechanism to explain an aspect of the kindling phenomenon.

Further experimentation, currently in progress, will be needed to directly address this possibility.

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Fig. 1. 7S SNARE Complexes in Rat Hippocampi

A) 50 μ g of synaptosomal protein was treated with 2% SDS (SDS-PAGE Sample Buffer) for 30 min at 37°C or for 10 min at 100°C (**Boiled**). Samples were fractionated by SDS-PAGE on a standard 10% acrylamide gel and then probed by western blotting with anti-syntaxin 1 antibody. Bracketed bands, which disappeared upon boiling, were considered authentic 7S SNARE complexes. Bands indicated by the star (*) denote position of monomeric syntaxin 1. **B)** Sample western blots of synaptosomal proteins using the indicated antibodies to the secretory machinery discussed in the text. The relative migration of indicated molecular weight markers is given.

Fig. 2. Asymmetric Accumulation of 7S SNARE Complex Differences in Different Kindling Paradigms

All SNARE complex bands (bracketed bands in Fig. 1), detected with the HPC-1 antibody to syntaxin 1, were quantified by enhanced chemifluorescence and normalized to monomeric syntaxin 1 or actin intensity. The solid bars represent the ipsilateral/contralateral ratio of the normalized band intensities in the hippocampal synaptosomes from rats kindled via amygdalar (n=10), entorhinal cortex (n=10), or septal (n=9) routes as indicated. The ratio in kindled animals was greater regardless of kindling route; * = P value by ANOVA. Open bars represent the values for surgical control animals (4–5/group) and the hatched bars represent the ratios of 7S SNARE complexes seen in distant brain regions (occipital cortex, horizontal hatching; cerebellum, diagonal hatching; n=4–8/group) of kindled animals.

Table 1

Kindling electrophysiological parameters

Kindling Paradigm	Afterdischarge (μ A)		Stimulations	
	Mean	SD	Mean	SD
Amygdala (n= 40)	248	64	7.0	2.2
Entorhinal (n= 18)	561	217	14.1	3.9
Septal (n= 10)	210	160	18.8	5.8

Table 2
Quantification of Hippocampal Synaptosomal α -SNAP and NSF in Kindling Models.

<u>Amygdala</u>	Control	<u>α-SNAP</u> Kindled	p	Control	<u>NSF</u> Kindled	p
Laterality difference*	0.93 $\pm$ 0.11	1.19 $\pm$ 0.14	NS	1.01 $\pm$ 0.02	1.06 $\pm$ 0.04	NS
Total Value[#]	0.96 $\pm$ 0.05	1.12 $\pm$ 0.06	0.07	1.01 $\pm$ 0.01	0.93 $\pm$ 0.02	0.08
Animals	8	20		8	19	
<u>Entorhinal</u>	Control	Kindled	p	Control	Kindled	p
Laterality difference*	0.88 $\pm$ 0.04	1.05 $\pm$ 0.07	0.06	0.95 $\pm$ 0.04	1.00 $\pm$ 0.03	NS
Total Value[#]	0.94 $\pm$ 0.02	0.94 $\pm$ 0.07	NS	1.05 $\pm$ 0.04	1.04 $\pm$ 0.02	NS
Animals	6	10		5	11	
<u>Septal</u>	Control	Kindled	p	Control	Kindled	p
Laterality difference*	1.10 $\pm$ 0.18	1.15 $\pm$ 0.06	NS	1.01 $\pm$ 0.02	0.95 $\pm$ 0.07	NS
Total Value[#]	1.05 $\pm$ 0.09	1.03 $\pm$ 0.12	NS	1.00 $\pm$ 0.01	0.89 $\pm$ 0.04	0.02
Animals	9	9		8	8	

Fluorescence intensity of the protein band of interest was normalized to the intensity of the actin band in the same lane, hence quantities have no units. P value determined by Student's t-test.

* Laterality = [Ipsilateral/Actin]/[Contralateral/Actin] where unity is expected

[#] Total Value = [(Contra/Actin + Ipsi/Actin)/2]/(Contra_{control}/Actin). If no changes in protein level occur, then the ratio should be unity.

Table 3
Effect of Amygdalar Kindling on Proteins of the Synaptic Secretory Machinery.

	Control	<u>SV2</u> Kindled	p	Control	<u>Munc18a</u> Kindled	p
Laterality *	1.01± 0.11	1.09± 0.12	NS	1.06± 0.21	0.92± 0.08	NS
Total Value #	0.99± 0.05	1.36± 0.09	0.004	1.03± 0.11	0.95± 0.04	NS
Animals	11	25		5	10	

	Control	<u>Munc13-1</u> Kindled	p	Control	<u>Complexin 1</u> Kindled	p
Laterality *	0.97± 0.15	1.02± 0.15	NS	0.98± 0.11	0.87± 0.13	NS
Total Value #	0.98± 0.03	0.98± 0.05	NS	0.99± 0.06	1.17± 0.22	NS
Animals	6	10		5	10	

	Control	<u>Complexin 2</u> Kindled	p	Control	<u>Synaptotagmin I</u> Kindled	p
Laterality *	1.18± 0.23	0.95± 0.10	NS	1.07± 0.04	0.94± 0.05	NS
Total Value #	1.09± 0.12	0.97± 0.17	NS	1.03± 0.02	1.01± 0.05	NS
Animals	5	10		6	10	

Fluorescence intensity of the protein band of interest was normalized to the intensity of the actin band in the same lane, hence quantities have no units. P value determined by Student's t-test.

* Laterality = [Ipsilateral/Actin]/[Contralateral/Actin] where unity is expected

Total Value = [(Contra/Actin + Ipsi/Actin)/2]/(Contra_{control}/Actin)). If no changes in protein level occur, then the ratio should be unity.