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Levetiracetam Prevents Kindling-Induced Asymmetric Accumulation of Hippocampal 7S SNARE Complexes

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Summary

Purpose—Understanding the molecular mechanisms underlying epilepsy is crucial to designing novel therapeutic regimens. This report focuses on alterations in the secretory machinery responsible for neurotransmitter (NT) release. Soluble N-ethylmaleimide Sensitive Factor (NSF) Attachment Protein Receptor (SNARE) complexes mediate the fusion of synaptic vesicle and active zone membranes thus mediating NT secretion. SNARE regulators control where and when SNARE complexes are formed. Previous studies showed an asymmetric accumulation of SNARE complexes (7SC) in the ipsilateral hippocampus of kindled animals. The present studies probe the persistence of 7SC accumulation and the effect of the anticonvulsant, levetiracetam (LEV), on 7SC and SNARE regulators.

Method—Quantitative western blotting was used to monitor levels of 7SC and SNARE regulators in hippocampal synaptosomes from kindled animals both before and after LEV treatment.

Results—The asymmetric accumulation of 7SC is present one year post-amygdalar kindling. The synaptic vesicle protein, SV2, a primary LEV-binding protein, and the SNARE regulator Tomosyn increase whereas NSF decreases in association with this accumulation. Treatment with LEV prevented kindling-induced accumulation of SV2, but did not affect the transient increase of Tomosyn or the long-term decrease of NSF. LEV treatment retarded the electrical and behavioral concomitants of amygdalar kindling coincident with a decrease in accumulation of 7SC.

Conclusions—The ipsilateral hippocampal accumulation of SNARE complexes is an altered molecular process associated with kindling that appears permanent. Kindling epileptogenesis alters synaptosomal levels of the SNARE regulators, NSF, SV2, and Tomosyn. Concomitant treatment with LEV reverses the kindling-induced 7SC accumulation and increase of SV2.

Keywords

kindling; epileptogenesis; hippocampus; levetiracetam; SNARE Complexes

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1. Introduction

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 generalized seizures in response to repeated subconvulsive electrical stimulation. Kindling can be induced from many sites, usually within the limbic system. The development of kindling in the rat is characterized by electrographic and behavioral stages (Racine, 1972). Stages 1−2 mimic human complex partial seizures; behaviors in Stages 3−5 are consistent with evolution to secondarily generalized motor seizures. Once the fully kindled state is achieved, spontaneous generalized convulsions may continue throughout the lifespan of the animal. However, an animal must usually experience additional stimulus-induced Stage 5 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). Stimulus-induced release of neurotransmitter (NT) from synaptic vesicles is facilitated by docking to and fusion of the vesicle membrane with the presynaptic plasma membrane at a specialized region of the presynaptic bouton called the neuronal active zone. This membrane fusion is mediated by integral membrane proteins called SNAREs, as well as a host of regulatory proteins that control how and when the SNARE proteins interact. Cognate SNAREs, from the synaptic vesicle (v-SNARE) and the plasma membrane (t-SNARE) form a stable, *trans* bilayer complex that promotes membrane fusion and NT release (for reviews see (Sudhof, 2000; Sudhof, 2004)). The 7S SNARE complex (7SC) in neurons, a four-helical bundle composed of synaptobrevin/VAMP-2 from the synaptic vesicle and syntaxin 1 and SNAP-25 from the neuronal active zone, is the minimal requirement for vesicle/plasma membrane fusion (Weber *et al*., 1998). Formation of stable 7SC is believed to represent one of the last steps before membrane fusion and is thus a hallmark of vesicles in a “ready-release” state. SNARE regulators such as Munc13, Tomosyn, and SV2, which influence SNARE complex assembly, are thought to affect the dynamics of this “ready release” pool (Yizhar *et al*., 2004; Custer *et al*., 2006; Ashery *et al*., 2000). Equally important is the disassembly of SNARE complexes for recycling, which is controlled by other SNARE regulators: N-ethylmaleimide Sensitive Factor (NSF) and α-Soluble NSF Attachment Protein (α-SNAP) (Whiteheart *et al*., 2001). Several of these assembly/disassembly regulators appear to be responsive to different factors (*e.g.* calcium, diacylglycerol, phosphorylation) thus accounting for the dynamic nature of neurotransmission.

Our work has shown that 7SC accumulate in hippocampal synapses of kindled rats and are maintained for at least 1 month following cessation of kindling stimuli (Matveeva *et al*., 2003). This accumulation occurs ipsilateral to the stimulus and evolves during the epileptogenesis of kindling in response to entorhinal cortical, amygdalar, and septal kindling (Matveeva *et al*., 2007). It appears to be associated with a bihemispheric increase of hippocampal SV2 and decrease of NSF. One focus of the present study is to better define the long term durability and specificity of the asymmetric accumulation of 7SC as well as the other biochemical changes in SNARE regulator levels associated with kindling.

Levetiracetam (LEV) is a highly effective and well-tolerated antiepileptic drug (AED) indicated for use as adjunctive therapy in adults with partial-onset seizures. LEV markedly suppresses kindling development (Klitgaard and Pitkanen, 2003; Loscher *et al*., 1998), suggesting that it may be the prototype of a new class of AEDs which not only suppress seizures, but block epileptogenesis. It was recently shown that LEV inhibits presynaptic NT release in a use-dependent fashion, possibly through selective binding to a presynaptic protein (Yang *et al*., 2007). Lynch *et al*. (2004) previously demonstrated that LEV binds to synaptic vesicle protein 2A (SV2A) in a stereospecific, saturable, and reversible manner.

Derivative compounds with increasing affinity for SV2A appear to have better anti-seizure potency. These data suggest that LEV's anti-epileptic effects may, in part, be through the modulation of SV2A, which itself is a key control element for neurotransmitter (Crowder *et al*., 1999; Janz *et al*., 1999) and neuroendocrine (Xu and Bajjalieh, 2001) release. SV2 appears to play a role in 7SC formation since SV2A null animals show a 50% decrease in 7SC accumulation (Crowder *et al*., 1999). LEV, however does not acutely affect normal neurotransmitter release (Birnstiel *et al*., 1997), and thus may exert its effect on SV2 in neurons already made susceptible to epileptogenesis. The second focus of this study is to determine if LEV perturbs the biochemical changes that we have shown to occur with kindling, specifically the asymmetric accumulation of 7SC and the increase in SV2.

2 Methods

2.1 Kindling

Fourteen-week-old male Sprague–Dawley rats had stimulating electrodes surgically implanted in the right amygdala (from bregma: AP −2.8; ML +4.8; DV −8.5; nose bar −3.3 [flat skull]) as previously described (Matveeva *et al*., 2007); some animals had implantation into the homologous left amygdala (ML −4.8). Animals received either electrical stimulation (1 second train of biphasic square wave pulses of 1 msec duration) delivered at 60 Hz (Slevin and Ferrara, 1985) resulting in kindled seizures, no electrical stimulation following sham operations, or were left untreated (naïve). To control for shock-burst effects, some animals received the same kindling current but at a different frequency (at 6 Hz for 1 sec). All animal procedures were approved by the Lexington VA IACUC. Animals were housed individually in an enriched environment and maintained on a 12 hr 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), operationally defined as “fully kindled”. Non-kindled, electrically stimulated animals had their after-discharge threshold (ADT) determined but then received that amperage, typically 200 μA, at 6 Hz. They continued to receive daily stimulations until the last animal in their group receiving 60 Hz kindling stimuli was fully kindled. Fully kindled and control animals receiving no drug were given no further stimuli and were euthanized at 2, 6, or 12 months post-kindling. No animals in this chronic kindled group experienced a spontaneous generalized seizure within at least 1 week prior to sacrifice as verified by daily inspection of cages for evidence of excessive defecation, blood, or other sign of bodily injury and by direct visual and auditory scrutiny for up to 1 hr daily. Animals did not undergo continuous split screen video electroencephalographic (SSV-EEG) surveillance during this time and so it is possible a seizure may have gone undetected. However, we have previously demonstrated that a generalized seizure *per se* does not affect the 7SC ratio (Matveeva et al., 2003). During the kindling process, animals treated with LEV or its inactive enantiomer, were euthanized 24 hr after the last kindling stimulus.

2.2 Levetiracetam Treatment

At the initiation of the kindling procedure (after the ADT was determined) some animals were treated once daily with 108 mg/kg LEV or 108 mg/kg of its inactive enantiomer, delivered *i.p*. 1 hr prior to stimulation (Loscher and Honack, 2000) [levetiracetam (UCB L059) and its inactive enantiomer (UCB L060) provided courtesy of UCB Pharma]. Enantiomer - and LEV-treated animals were yoked and each was stimulated at its individually-determined ADT. Pairs were euthanized 18−24 hr after having received 3, 6, 9, 12, or 15 days of kindling stimuli. All animals were staged based on behavior (Racine classification) at the time of each stimulus. To determine if LEV directly affected secretory

machinery proteins independent of kindling, a set of naïve animals was treated once daily with 108 mg/kg LEV for either 3 or 15 days.

2.3 Quantification of Levetiracetam Levels

We developed a micro-analysis assay for LEV based on previous publications (Doheny *et al*., 1999; Ratnaraj *et al*., 1996). Those protocols, which involve only a single organic solvent extraction, gave 40−70 % recoveries, in our hands. Our modified protocol, below, routinely gave recoveries of 90−95% of internal standard and LEV derivatives. We found chloroform/methanol: 9/1 (v/v), to be superior to dichloromethane as an extraction solvent and acidification with HCl rather than alkalinization with NaOH to provide superior recovery. As with all published procedures, levels of LEV determined in serum were corrected for the recovery of the internal standard in the extracted sample relative to an identical amount of internal standard determined in a parallel sample by direct HPLC analysis without extraction. This protocol was also used to determine LEV or enantiomer accumulation in hippocampus.

Fifty μl of sera or 75 μl of brain homogenate (see below) were used for extraction. To this was added 10 μl of 1 mM internal standard [UCB 17025] in methanol and 12.5 μl of 8 N HCL. The sample was mixed, then extracted twice with 500 μl of chloroform/methanol: 9/1 (v/v). The combined extracts were dried under a stream of N_2 and dissolved in 100 μl of column buffer (85 % 50 mM NaPi, pH 5.6 and 15% acetonitrile (v/v)). Twenty μl aliquots were analyzed by HPLC on RP-B as described previously (Ratnaraj *et al*., 1996).

For analysis of LEV in hippocampus, tissue was homogenized in a volume of deionized H_2O equal to twice the tissue weight and the homogenates were clarified by centrifugation at 100,000 × g, 4°C for 30 min. An aliquot (75 μl) of the resulting supernatant fraction was subjected to chloroform/methanol extraction and the extract analyzed by HPLC exactly as above. LEV values determined in these samples were normalized to mM concentrations in brain assuming that initial tissue weight in grams is 78% water (Vachon and Moreau, 2003). Aliquots (1 μl) of homogenate supernatants were analyzed for protein concentration (Bradford, 1976) to verify that they were comparable.

2.4 Analysis of 7S SNARE Complexes (7SC)

Animals were euthanatized by decapitation, their brains were rapidly removed and the hippocampi excised on ice. Percoll-gradient-purified synaptosomes were prepared from individual hippocampi 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 7SC fail to disassemble (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). Western-blot-based detection of 7SC was performed using alkaline phosphatase coupled secondary antibodies with Vistra ECF™ for visualization and images were obtained using a Typhoon 9400 imager (GE Healthcare Bio-Sciences, Piscataway, NJ). The raw data were the integrated fluorescence intensities for all pixels in a given immuno-decorated protein band as determined by ImageQuant 5.2 software (GE Healthcare Bio-Sciences, Piscataway, NJ) as arbitrary units (AU). To normalize for protein loading, the fluorescence intensity of all 7SC bands was standardized to the intensity of the syntaxin 1 monomer and/or to actin (see below).

2.5 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). Mouse monoclonal antibody to SV2 (which detects 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 mouse monoclonal antibody to actin was from Sigma and the antibody to Tomosyn was from BD Biosciences (San Jose, CA). Fluorescence intensities of the bands in each lane were normalized to the intensity of the actin band in the same lane. To standardize 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 Relative 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 = [(Contralateral/Actin + Ipsilateral/Actin)/2]/ [$\text{Contralateral}_{\text{control}}$/Actin]). If no changes in protein level occur, then the ratio should be unity.

2.6 Statistical Analyses

The ipsilateral/contralateral 7SC band intensity ratios determined from hippocampi of LEV-treated *vs*. inactive enantiomer controls and of chronically kindled (≥ 1 month) *vs*. surgical, electrical, and naïve controls were analyzed by ANOVA and *post hoc* t-tests using Fisher's protected least significant differences procedure. Quantified secretory machinery proteins from hippocampi of chronically kindled (≥ 1 month) *vs*. surgical, electrical, and naïve controls as well as quantified secretory machinery proteins from hippocampi of LEV-treated *vs*. inactive enantiomer controls were analyzed by ANOVA and *post hoc* t-tests using Fisher's protected least significant differences procedure. These immunoblotting measurements are quite accurate and reproducible, suggesting that there is little variability introduced by the methodology. Hippocampal 7SC symmetry measured in naïve controls from two randomly selected experiments performed in 2001 (0.979 ± 0.017, n=13) and 2006 (0.979 ± 0.020, n=9) generated nearly identical results.

3. Results

3.1 The Kindling-Associated Increase of 7S SNARE Complexes in Hippocampal Synaptosomes Persists to One Year

In our previous two reports (Matveeva *et al*., 2007; Matveeva *et al*., 2003), several high molecular weight SNARE complexes were detected when western blotting synaptosomal proteins with the anti-syntaxin 1 antibody, HCP-1. Our work, showed a stable, kindling-dependent, asymmetric accumulation of 7SC in the ipsilateral hippocampus that was not present in naïve animals, surgical control animals, or in animals receiving acute electroconvulsive seizures. To further define the asymmetric accumulation of SNARE complexes, two additional control experiments are reported in this study. First, chronic electrical stimulation at the same current and voltage, but at the non-kindling frequency of 6 Hz (instead of 60 Hz), was used. The ipsilateral/contralateral ratio in these animals was essentially unity (right/left: 0.98 ± 0.03; n=4) suggesting that current alone is insufficient to induce the observed accumulation. Second, standard stimulation of the left, rather than right, amygdala does lead to an asymmetric accumulation of 7SC in the left hippocampus one month post kindling (left/right: 1.10 ± 0.03; n=6), indicating that complex accumulation is stimulation-dependent and not due to some unrecognized, inherent hippocampal asymmetry.

A time course study was undertaken to examine the persistence of this asymmetric accumulation. One day or two months following the cessation of right amygdalar stimulation of animals kindled to Stage 5, there was a significant ($p < 0.001$ by t-test), asymmetric accumulation of 7SC in the ipsilateral hippocampus, as indicated by ratios greater than unity (Table 1). This asymmetric accumulation was maintained at 6 months and 1 year in animals that received only two consecutive Stage 5 kindling-induced seizures. Typically, a larger number of kindled seizures per animal are required for a population of animals to reliably develop a stable kindled state, including the evolution of spontaneous seizures. In this regard, closer analysis of the 11 animals evaluated at 12 months is revealing (Figure 1). Six of them demonstrated a 7SC ratio of unity, whereas five maintained a ratio similar to that observed at 2 months (1.20 ± 0.02; $p<0.001$ by t-test). These data are striking because they indicate persistence in the 7SC asymmetry, despite the small number of stimulations given to these animals.

3.2 Analysis of Other Secretory Machinery Proteins 12 Months Post-Kindling

Given that the asymmetric accumulation of 7SC persists at 12 months, we focused on elements of the secretory machinery known to directly affect SNARE complexes. At 12 months, α-SNAP levels in kindled animals (all values = 1.0, n = 11) were not different from controls (all values = 1.0, n= 8). NSF levels were significantly lower in those animals that maintained an asymmetry of 7SC compared to those that did not (0.84 ± 0.02, n = 11 vs. 0.95 ± 0.02, n= 8; p=0.009, 9 df, two-tailed t-test).

We have demonstrated a statistically significant increase in the level of total SV2 (A and B isoforms combined) in hippocampi one month after establishment of the amygdala-kindled state (Matveeva *et al*., 2007). This increase persists at 12 months, but only in those animals in which the 7SC asymmetry persists (1.16 ± 0.02, n = 11 *vs.* 1.03 ± 0.02, n = 8; p=0.003, 9 df, two-tailed t-test). There was no difference in SV2 protein levels between ipsilateral and contralateral hippocampi.

3.3 Effects of Levetiracetam

The relationships between LEV and SV2A (Loscher *et al*., 1998; Lynch *et al*., 2004) and the effect of SV2A on SNARE complex levels seen in $SV2A^{-/-}$ mice (Crowder *et al*., 1999) suggest that LEV might affect 7SC accumulation in kindled animals. To test this, initial experiments were needed to determine the clearance rates of LEV from blood and hippocampus. Rats receiving injections of 108 mg/kg LEV were harvested at increasing post-injection times and the plasma and hippocampal concentrations of LEV were measured by HPLC (Figure 2). The average half-life of levetiracetam was approximately 5 hr in the bloodstream and 7.5 hr in the hippocampus. The greater hippocampal half-life most likely reflects its lipid partition coefficient and low serum protein binding of 10%. In addition, the plasma concentration of either LEV or its inactive enantiomer at 1 hr when animals received a kindling stimulus ranged between 0.41 and 0.71 mM (average 0.57 mM ± 0.01 S.D.). Kindling did not significantly alter these levels (data not shown).

3.4 Effect of Levetiracetam on the Evolution of Kindling and Hippocampal 7S Complex Accumulation

In animals treated 1 hr prior to stimulation with the inactive LEV enantiomer, asymmetric 7SC accumulation begins early in amygdala kindling, gradually attaining a maximum by Stage 5, where it reaches a plateau (Figure 3B, closed squares). This is similar to our observations using the entorhinal kindling paradigm where 7SC accumulation correlated with Racine staging progression (Matveeva *et al*., 2003). In animals treated with LEV, ipsilateral hippocampal 7SC accumulation is similar to control until the 3^{rd} to 6^{th} stimulation, when the right to left ratio drifts back towards unity (Figure 3B, open squares).

This dissociation becomes significant at Stage 4 (LEV: 1.02 ± 0.04, n=5; enantiomer: 1.18 ± 0.02, n=4; p=0.01) and Stage 5 (1.04 ± 0.01, n=9; 1.16 ± 0.01, n=16; p=0.01). As reported by Loscher *et al.* (1998), we also observed that LEV treatment retards the development of kindling as measured by the temporal progression to Stage 5 (Figure 3A). In Figure 3A, both LEV- and inactive enantiomer-treated animals reach Stages 1 (LEV: 1.04 ± 0.04 [stimuli ± SEM], enantiomer: 1.10 ± 0.05; p=0.38) and 2 (2.86 ± 0.49 *vs*. 1.85 ± 0.16; p=0.06) with similar numbers of stimuli. However, at Stage 3 (4.57 ± 0.72 *vs*. 2.88 ± 0.26; p=0.02) the number of stimuli required for each group begins to separate and this difference maintains through Stage 4 (5.61 ± 0.75 *vs*. 3.48 ± 0.34; p=0.02) and 5 (9.31 ± 0.72 *vs*. 6.57 ± 0.45; p=0.004). The behavioral and electrographic inhibition appears to begin after Stage 3 and is greatest at Stages 4 and 5, the same stages during which the reversal of ipsilateral 7SC accumulation is most dramatic. During the window between 3 and 9 days LEV treatment delays kindling progression and dampens the asymmetric accumulation of 7SC (Figure 3B). Once 7SC accumulation is returned to unity with LEV treatment, it does not appear to increase again, though the animals do attain behavioral Stage 5 seizures.

3.5 Effect of Levetiracetam During Amygdalar Kindling on Selected Secretory Machinery Proteins

The levels of α-SNAP were not affected in animals treated with either LEV or its inactive enantiomer (Figure 4). Early in the kindling process there was a transient increase in Tomosyn (ANOVA with *post hoc* t-tests, $p < 0.001$ for both LEV- and enantiomer-treated animals, 0 *vs*. 3 Days, Figure 4), whether animals received LEV or inactive enantiomer. This was already diminishing at day 6; at one month following the cessation of amygdalar stimulation of animals kindled to Stage 5, Tomosyn levels were equal to surgical controls (data not shown), as suggested by the curves in Figure 4. Total NSF levels showed a tendency to diminish late in kindling development (≥ 12 days), whether animals were treated with LEV or the inactive enantiomer (Figure 4). Analysis demonstrated a significant decrease (ANOVA with *post hoc* t-test, $p = 0.001$) of total hippocampal synaptosomal NSF in the groups of animals euthanatized after receiving ≥ 12 days of stimulation (0.87 ± 0.003) compared to non-kindled controls and animals stimulated <12 days (0.95 ± 0.01). There was no asymmetry of hippocampal synaptosomal α-SNAP, Tomosyn or NSF at any time point or with any treatment.

We previously reported (Matveeva *et al*., 2007) an increase of total synaptosomal SV2 at 30 days post amygdalar kindling that appears to persist to 12 months post-kindling (discussed above). This increase is also seen and emerges early in kindling of animals treated with the inactive enantiomer (Figure 4). SV2 levels do appear to initially increase in LEV-treated animals, but, by day 12, they decrease, approaching non-kindling control values by stimulation day 15. There was no asymmetry of hippocampal synaptosomal SV2 at any time point or with any treatment. LEV has no effect on NSF, α-SNAP, SV2 or Tomosyn in non-kindled animals. There was no difference from untreated controls in hippocampal concentrations of NSF (all values at day 3 [n = 3] and day 15 [n = 3] were 1.0), α-SNAP (all values at day 3 [n = 3] and day 15 [n = 3] were 1.0), Tomosyn (all values at day 3 [n = 3] and day 15 [n = 3] were 1.0), and SV2 (all values at day 3 [n = 3] were 1.0; the values ranged from 0.9 to 1.1 at day 15 [n = 3]) in naïve animals treated with LEV for either 3 or 15 days. The effect of LEV treatment on SV2 is apparently mediated through an influence on amygdalar kindling.

4. Discussion

In this study, we further characterize the asymmetric accumulation of 7S SNARE complexes that occurs during kindling-induced epileptogenesis. Past studies showed that 7SC accumulation was not due to electrode implantation or electroshock-induced seizure and did

not occur outside the limbic system (Matveeva *et al*., 2003; Matveeva et al., 2007). 7SC accumulation did correlate with behavioral stage (Racine staging) and did occur regardless of the stimulation route: amygdalar, entorhinal, or septal. The present study shows that 7SC accumulation occurs in the ipsilateral hippocampus, regardless of stimulation in left or right hemisphere. Accumulation is only seen when a kindling-inducing frequency is used for stimulation and is not merely an effect of current or an applied potential difference. 7SC accumulation is robust and persists out to ≥ 12 months post-kindling. Thus this ipsilateral accumulation of 7SC appears to reflect stable biochemical changes occurring in hippocampal neurons during kindling.

As denoted in Table 1 and Figure 1, the ipsilateral accumulation of 7SC in the hippocampi of kindled animals persists, on average, for at least 12 months. We are not aware of any prior reports of a biochemical marker that changes with kindling and persists for at least one year. Interestingly, when one examines the 7SC accumulation in the individual animals there is a bimodal distribution. As delineated in Figure 1, 7SC accumulation persists in a significant subset of animals at a level similar to earlier time points, while that in others returns to naïve levels. This could reflect animal-to-animal variability, though such variation is not as obvious at the earlier time points (Figure 1). Alternatively, this divergence could reflect the minimal criteria used to define “fully kindled”: only one tonic-clonic, Stage 5 seizure on two sequential days was required. This was generally attained after fewer that 10 stimuli (Table 1) and no further stimuli were administered until the synaptosomes were harvested for analysis. In other reports, animals typically require many more seizure-invoked stimuli to have interictal discharges and stable spontaneous seizures months after cessation of kindling stimuli ((Bertram, 2007) for review). It seems possible that our kindling protocol is only sufficient to bring animals to a threshold but not sufficient to produce the robust effects seen in other studies where stimulation is continued after reaching Stage 5. Future analysis will be needed to determine if long-term maintenance of 7SC asymmetry directly correlates with sustained behavioral seizure activity. At this stage our data are only suggestive of this being the case.

Another unique finding of this study is that early in the kindling process there appears a bilateral spike in hippocampal Tomosyn levels. Tomosyn is a 130 kDa protein, originally purified from brain (Fujita *et al*., 1998), which contains a C-terminal domain that mimics the coiled-coil, SNARE motif of v-SNAREs (Hattendorf *et al*., 2007). The SNARE motif allows Tomosyn to interact with t-SNARE heterodimers, forming a heterotrimer very similar to the 7S SNARE complex (Hatsuzawa *et al*., 2003; Widberg *et al*., 2003). Tomosyn is needed for secretion in neurons (Okabayashi *et al*., 1988) and yeast (Mauxion *et al*., 1996), but its over-expression inhibits release (Yizhar *et al*., 2004). In their model, McEwen *et al*. (2006) suggest that Tomosyn is a place-holder for the v-SNARE and is displaced by activated Munc13. Thus, secretion is controlled by balancing Tomosyn binding of the t-SNARE heterodimer, and Munc13 activation of the SNAREs for membrane fusion. The kindling-induced increase in Tomosyn could shift the balance dampening neurotransmitter release by blocking t-SNARE complexes and preventing 7SC formation. This would imply that kindling provokes an early compensatory increase in Tomosyn via either stabilization of the protein or increased production. Further analysis will be required to assess which molecular mechanisms are relevant.

LEV is a potent anticonvulsant in fully amygdala-kindled rats (Loscher and Honack, 1993). It has been proposed to be antiepileptogenic based on the observation that treatment with LEV delays kindling (Figure 3A and Loscher *et al*., 1998). The antiepileptic/epileptogenic effect of acute LEV treatment does not appear to derive from any interaction with known mechanisms involved in inhibitory and excitatory neurotransmission (Klitgaard *et al*., 1998; Noyer *et al*., 1995; Rigo *et al*., 2002). Asymmetric 7SC accumulation correlates with

kindling stage in inactive enantiomer-treated animals and reaches an apparent plateau once the animal reaches Stage 5 (Figure 3B). LEV treatment has a significant effect on this process and leads to a decrease in 7SC asymmetry (relative to enantiomer-treated controls) from day 3 onward, reaching close to unity (control levels) by day 9. It has recently been demonstrated that 3 hr exposure of hippocampal slices to LEV resulted in significantly reduced synaptic vesicle NT release (Yang *et al*., 2007). Such a response would be predicted if LEV were to exert its anticonvulsant effect at excitatory synapses through an action on 7SC formation, perhaps through its putative interaction with SV2A (Lynch *et al*., 2004). If the enhanced hippocampal glutamatergic release associated with kindling development (Jarvie *et al*., 1990; Minamoto *et al*., 1992; Ueda *et al*., 2000) occurs, at least in part, at the individual cell level, a LEV effect on 7SC formation would provide an explanation for its observed antiepileptogenic effect. To directly address this point, techniques to assess 7SC levels *in situ* will have to be developed.

Treatment with LEV delays kindling but does not prevent it. This discordance between kindling progression and the reversal of asymmetric 7SC accumulation is of note because it is the first indication that the two can be separated. This may simply demonstrate that multiple semi-independent processes are associated with the complexity of kindling epileptogenesis, *e.g*., altered 7SC activity with increased excitatory NT release at the cellular level and enhanced synchronization at the network level. These particular events at the cellular level participate in kindling induction and persist with its maintenance, but their suppression alone is not sufficient to abort the process, particularly if abnormal stimulus input persists. This is supported by the finding of Brandt *et al.* (2007) that LEV is neither antiepileptogenic nor neuroprotective when administered chronically after *status epilepticus* (SE) induced in rats by sustained electrical stimulation of the amygdala. SE initiates a sequence of pathological morphological changes that themselves promote epilepsy. Focal epilepsy in humans seldom begins with SE and one may speculate that altering one aberrant molecular event of many early in the process, before the onset of potential morphologic changes, may be sufficient to alter the outcome.

SV2A provides a potential locus where LEV treatment may uncouple the mechanistic connections between kindling and 7SC accumulation. This must mediate through some alteration of SV2 activity during kindling because LEV does not induce 7SC asymmetry in non-kindled brain (data not shown). Lynch *et al.* (2004) demonstrated that LEV binds to SV2A and that derivative compounds with increasing affinity for SV2A had 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 acutely 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. It should be noted that brain tissue from SV2A null animals shows a 50% decrease in total 7SC suggesting that active SV2A is important for maintaining 7SC but is perhaps not essential for its formation. Our previous data indicate that SV2 (A and B isoforms combined) is increased in kindled animals and a similar increase in seen in enantiomer-treated animals (Figure 4). Whether the kindling-associated increases of SV2 and 7SC are themselves mechanistically associated is unknown. However, LEV treatment appears to dampen the SV2 increase which might be expected to cause a reduction of 7SC levels, toward normal. While this mechanism is a plausible explanation for the LEV-induced decrease in 7SC asymmetry it does not yet explain the effects of LEV on kindling. LEV does not appear to mitigate kindling through an effect that includes Tomosyn since its early kindling-associated increase is unaffected by LEV treatment.

Given that the observed asymmetry in 7SC reflects some basic change in the neurosecretory machinery of hippocampal neurons that is a consequence of kindling, the challenge is to

understand the molecular mechanisms underlying this alteration to determine what it reveals about the molecular changes that are occurring during epileptogenesis. This understanding will also elucidate the molecular mechanism of LEV and perhaps point the way to better anti-epileptogenic therapeutics.

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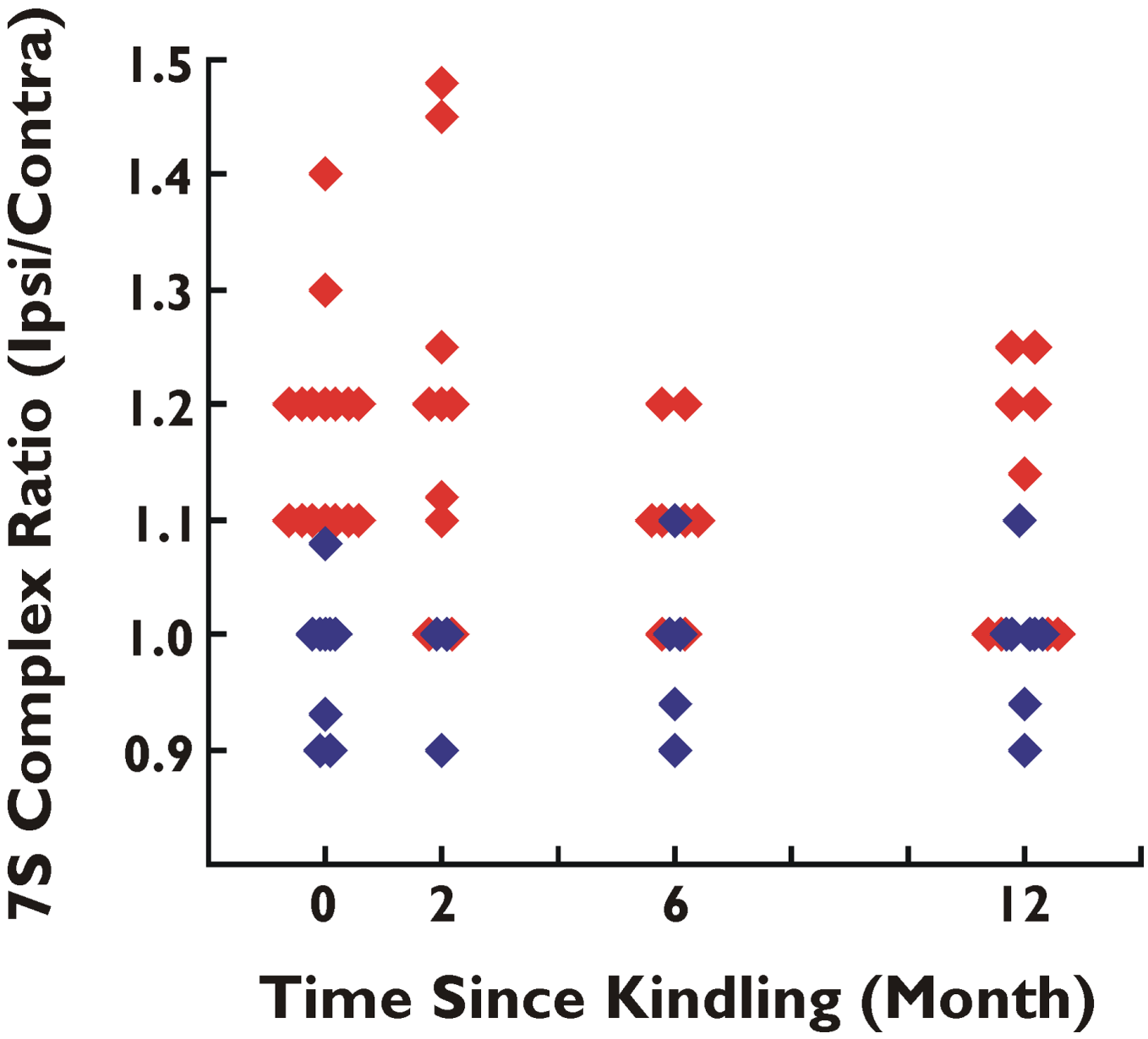

Figure 1.
Ipsilateral Accumulation of 7S SNARE Complexes Persists. Animals were amygdalar kindled until they displayed two Stage 5 seizures on subsequent days and then they were left untreated. At the indicated times, animals were euthanatized, synaptosomes were prepared and 7SC measurements were made as described in Methods. The hippocampal 7SC ipsilateral/contralateral ratio measured for each animal used in the 12-month time study is presented as a single point (kindled = red diamonds, control = blue diamonds).

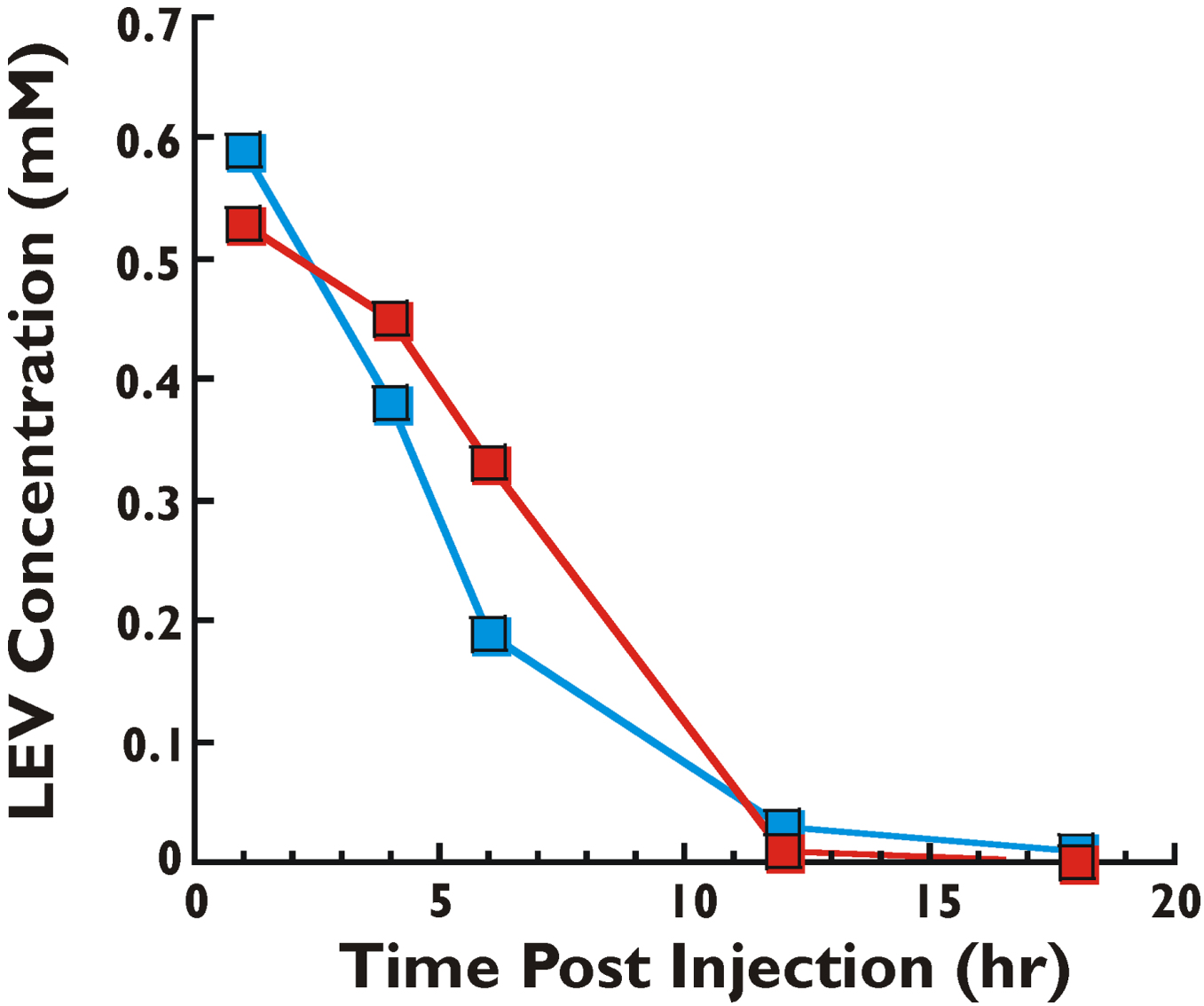

Figure 2.
Pharmacokinetics of LEV in Rat Blood and Brain. Rats were injected *i.p.* (108 mg/kg) and LEV (UCB L059) levels were measured as described in Methods in sera (blue squares) and in hippocampus (red squares) at the indicated time points, post injection. LEV values determined in brain were normalized to mM assuming that initial tissue weight in grams is 78% water (Vachon and Moreau, 2003). Each point represents the mean of two determinations differing by ± 1.5%.

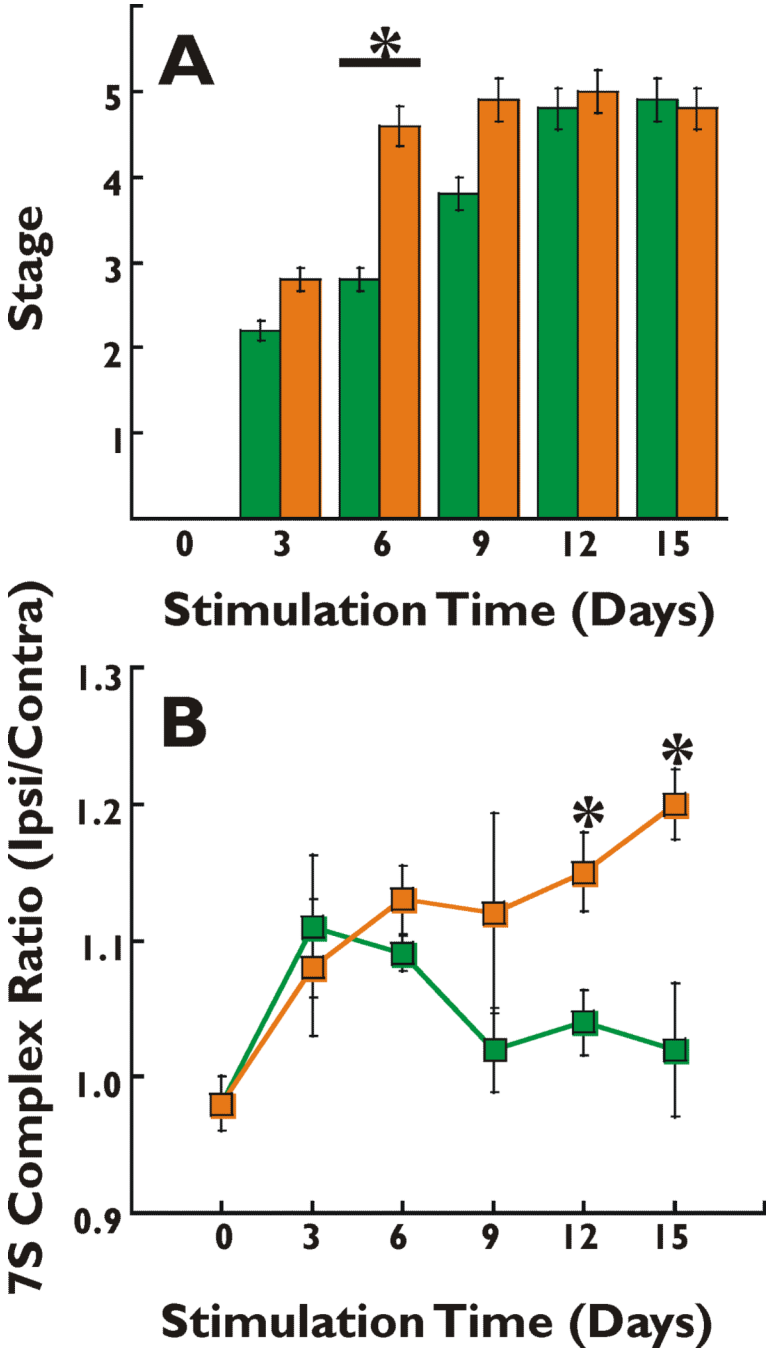

Figure 3.

LEV Treatment Delays Kindling Progression and Dampens 7S SNARE Complex Accumulation. Rats were injected *i.p.* with LEV (UCB L059, 108 mg/kg, green bars/squares) or an inactive enantiomer of LEV (UCB L060, 108 mg/kg, orange bars/squares) 1 hr prior to electrical stimulation. On the indicated days, the Racine stage of the animal was evaluated immediately post-stimulation (A) and hippocampal synaptosomes were prepared for 7SC measurements as described in Methods (B). Data presented are the mean ±SEM and * indicates $p < 0.05$ for the indicated pairs.

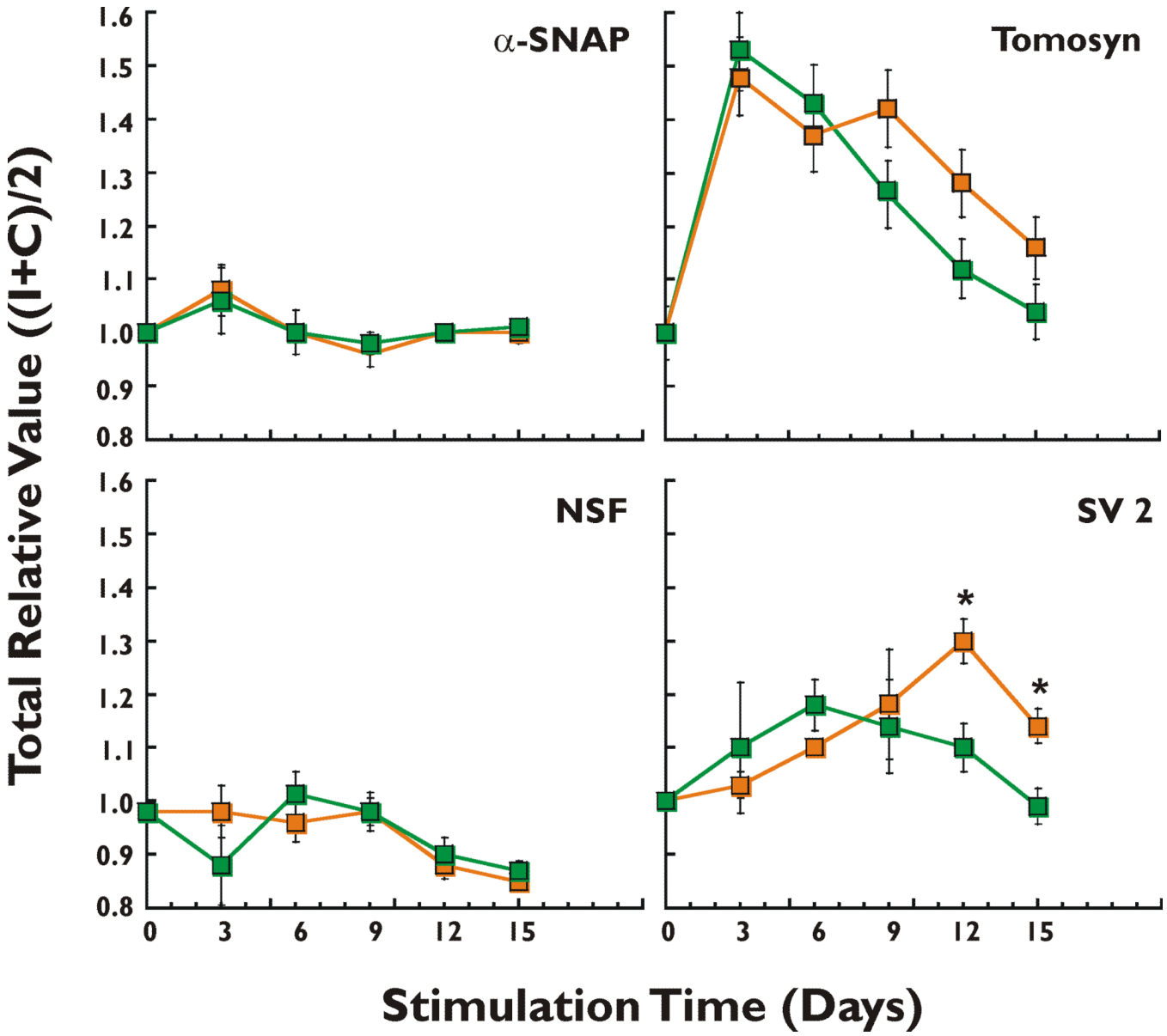

Figure 4.

SNARE Effectors Tomosyn and SV2 Show Kindling- and LEV-Dependent Changes in Expression Levels. Rats were injected *i.p.* with LEV (UCB L059, 108mg/kg, green squares) or an inactive enantiomer of LEV (UCB L060, 108mg/kg, orange squares) 1 hr prior to daily amygdalar kindling-inducing electrical stimulation. At the indicated times, hippocampal tissue was prepared and synaptosomal proteins were probed for α-SNAP, NSF, Tomosyn, and SV2 (as indicated) by quantitative western blotting and compared and "Total Relative Value" was calculated as described in Methods. Each square represents the mean ±SEM of protein determinations from 4−10 animals; * indicates $p < 0.05$ for the indicated pairs. For SV2 determinations at Day 12, n=5 for both LEV and enantiomer; at Day 15, n=7 for LEV and n=8 for enantiomer.

Table I

Durability of 7S Complex (7SC) asymmetry following amygdalar kindling

	7S SNARE Complex Ratios			Stimulation Parameters	
Post Kindling Time	**Kindled[$] Mean ±SEM (n)**	**Control[$] Mean ±SEM (n)**	**Stimuli[#] Mean ± SEM**	**Current Mean ± SEM μA**	**Days[@] Mean ± SEM**
Newly Kindled[*]	1.08 ± 0.03 (6)	0.99 ± 0.03 (6)	7.5 ± 0.7	217 ± 40	1 ± 0
2 months	1.20 ± 0.05 (10)	0.96 ± 0.03 (15)	7.7 ± 0.3	274 ± 13	53 ± 4
6 months	1.10 ± 0.03 (8)	0.99 ± 0.03 (5)	8.0 ± 1.0	200 ± 19	200 ± 3
12 months	1.09 ± 0.03 (11)	0.99 ± 0.01 (8)	6.3 ± 0.8	245 ± 21	370 ± 2

[$]The ipsilateral/contralateral ratio of 7S Complex in the rat hippocampus with SEM and sample size (n) indicated.

[*]Newly Kindled are rats 24 hr after achievement of full kindling defined as two Racine Stage 5 seizures occurring on two consecutive days;

[#]number of kindling stimuli;

[@]the time interval between achievement of full kindling and 7SC determination.