Received: 24 February 2017 | Revised: 31 May 2017 | Accepted: 29 June 2017

DOI: 10.1002/hipo.22761

RESEARCH ARTICLE

WILEY

# The calcium sensor synaptotagmin 1 is expressed and regulated in hippocampal postsynaptic spines

Suleman Hussain[1] | Daniel Lawer Egbenya[1] | Yi-Chen Lai[2] | Zita J. Dosa[3] | Jakob B. Sørensen[3] | Anne E. Anderson[2] | Svend Davanger*[,1]

[1]Division of Anatomy, Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo, Norway

[2]Jan and Dan Duncan Neurological Research Institute, Baylor College of Medicine, Houston, Texas, USA

[3]Department of Neuroscience and Pharmacology, Faculty of Health and Medical Sciences, Lundbeck Foundation Center for Biomembranes in Nanomedicine, University of Copenhagen, Denmark

**Correspondence**
*Prof. Svend Davanger, Division of Anatomy, Department of Molecular Medicine, Institute of Basic Medical Science, University of Oslo, Oslo, Norway.
Email: svend.davanger@medisin.uio.no

**Funding information**
The University of Oslo; Norsk Epilepsiforbunds forskningsfond; the European Union Projects, Grant/Award Numbers: QLG3-CT-2001-02089 (KAR-TRAP) and LSCHM-CT-2005-005320 (GRIPANNT)

## Abstract

Synaptotagmin 1 is a presynaptic calcium sensor, regulating SNARE-mediated vesicle exocytosis of transmitter. Increasing evidence indicate roles of SNARE proteins in postsynaptic glutamate receptor trafficking. However, a possible postsynaptic expression of synaptotagmin 1 has not been demonstrated previously. Here, we used postembedding immunogold electron microscopy to determine the subsynaptic localization of synaptotagmin 1 in rat hippocampal CA1 Schaffer collateral synapses. We report for the first time that synaptotagmin 1 is present in rat hippocampal postsynaptic spines, both on cytoplasmic vesicles and at the postsynaptic density. We further investigated whether postsynaptic synaptotagmin 1 is regulated during synaptic plasticity. In a rat model of chronic temporal lobe epilepsy, we found that presynaptic and postsynaptic concentrations of the protein are reduced compared to control animals. This downregulation may possibly be an adaptive measure to decrease both presynaptic and postsynaptic calcium sensitivity in excitotoxic conditions.

**KEYWORDS**
calcium sensor, electron microscopy, epilepsy, hippocampus, synaptic plasticity

## 1 | INTRODUCTION

Postsynaptic dendritic spines on the brain's principal neurons are biological nodes for receiving, integrating, and diverging information transmitted from presynaptic terminals (Pozo and Goda, 2010). A crucial function is also the ability to change these computing properties with time, known as synaptic plasticity (Kessels and Malinow, 2009; Sprengel, 2006). Synaptotagmins constitute a family of membrane-trafficking proteins that can act as calcium sensors (Sudhof, 2002). Calcium sensors play important roles in regulating synaptic plasticity in a multitude of different physiological and pathological conditions (Burgoyne and Haynes, 2015). Modifying the synaptic expression of calcium sensors could be a possible mechanism to reduce the effects of postsynaptic calcium overload in excitotoxic diseases (Kerrigan, Daniel, Regan, & Cho, 2012; Rao et al., 2015). In brains, following seizure activity, the concentration of synaptotagmin 1 is reduced both at mRNA and protein levels (Tocco, Bi, Vician, Lim, Herschman, & Baudry, 1996; Zhang et al., 2014). The synaptotagmin 1 hypothalamic knockdown ensures almost complete prevention of seizure-induced damage of hippocampal neurons (Kobayashi, Ohno, Iwakuma, Kaneda, & Saji, 2002). In glutamatergic presynaptic terminals, the calcium sensor synaptotagmin 1 regulates SNARE-mediated exocytosis of neurotransmitter-containing vesicles at the active zone (AZ) (Hussain and Davanger 2011; Sudhof, 2013). Increasing evidence supports a role of SNARE proteins in facilitating vesicular insertion of glutamate receptors in the plasma membrane of the postsynaptic spine (Hussain and Davanger, 2015; Hussain, Ringsevjen, Egbenya, Skjervold, & Davanger, 2016; Jurado, 2014; Jurado et al. 2013). However, a possible postsynaptic expression of synaptotagmin 1 remains unclear. The aim of this study was to determine the ultrastructural localization of synaptotagmin 1 in hippocampal synapses, and to evaluate functional dynamics of the protein by analyzing changes in synaptic synaptotagmin 1

concentrations after chronic temporal lobe epilepsy. We hypothesized, firstly, that synaptotagmin 1 is present in postsynaptic spines, and, secondly, that the postsynaptic concentrations of this calcium sensor during epilepsy may be reduced to adapt to the increased calcium loads in epilepsy.

## 2 | MATERIAL AND METHODS

### 2.1 | Antibodies

#### 2.1.1 | Primary antibodies

Anti-synaptotagmin 1 was raised in rabbit, immunized with recombinant synaptotagmin 1 protein fixed in 1.25% glutaraldehyde (GA) and mixed with Freund's adjuvant. The synaptotagmin 1 construct (pGEX-KG vector) was a generous gift from Richard Scheller. Crude antiserum was affinity-purified with recombinant synaptotagmin 1 protein prefixed with GA (affi-gel column). In-house anti-synaptotagmin 1 was used at 1:5,000 for western blotting (WB), 1:10–1:50 for electron microscopy (EM), 1:50 for light microscopy (LM), and 1:100 for immunofluorescence (IF). Anti-synaptotagmin 1 (Millipore, MA, Cat#AB5600) was used at 1:20–1:200 for EM. Anti-TUJ 1 (Covance, CA, Cat#MMS-435P) was used at 1:100 for IF. Anti-PSD-95 (Novus Biologics, ON, Canada, Cat#NB300–556) was used at 1:100 for IF.

#### 2.1.2 | Secondary antibodies

Goat anti-rabbit alkaline phosphatase (Sigma, MO, Cat#A3687) was used at 1:10,000 for WB. Donkey anti-rabbit Cy3 (Jackson Immuno, MD, Cat#711–165-152) and donkey anti-mouse A488 (Invitrogen, CA, Cat#A21202) were used at 1:1,000 for IF. IgG coupled to 10 nm colloidal gold (British BioCell International, Cardiff, UK, Cat#R14007) and (Abcam, Cambridge, UK, Cat#ab27234) were used at 1:20 for EM. Biotinylated goat anti-rabbit (Abcam, Cambridge, UK, Cat#Ab64256) was used at 1:100 together with a streptavidin biotinylated horseradish peroxidase complex (GE Healthcare, Buckinghamshire, UK, Cat#RPN1051V) at 1:100 for LM.

### 2.2 | Animals

Wistar male rats weighing 250–300 g ($n = 3$) were used for basic EM experiments, PVG male rats weighing 200–250 g ($n = 10$) for WB and PVG male rats weighing 200–250 g ($n = 5$) for LM and 1–4 day-old Wistar rats ($n = 6$) for primary hippocampal cultures. The animals used in epilepsy experiments were male juvenile Sprague-Dawley rats (120–180 g), treated in the animal facility at Baylor College of Medicine, TX. For EM studies, these consisted of 3 rats in the control group and 3 rats in the kainate-treated group. For WB, 7 control rats and 8 kainate-treated rats were used. Animals in the control group were injected with only normal saline while the animals in chronic temporal lobe epilepsy group were injected with kainate. Intraperitoneal kainate administration (18 mg/kg i.p.) was used to induce status epilepticus in the Sprague-Dawley rats. Behavioral seizures and status epilepticus were monitored using the Racine Scale. A continual limbic seizure with a score of 4 or 5 was defined as status epilepticus. Following 1 hr of status epilepticus, seizures were terminated using intraperitoneal administration of pentobarbital (20 mg/kg). Eight weeks after the seizure induction, the rats were sacrificed; their hippocampi were quickly dissected and frozen immediately. All experiments involving the induction of status epilepticus were done in conformity to the Guidelines of the National Institute of Health for the Care and Use of Laboratory Animals and were accordingly approved by the Institutional Committee on Animal Care, Baylor College of Medicine. Every effort was made to minimize the suffering and number of animals used.

Generation of synaptotagmin 1 knockout (KO) embryos (Geppert et al., 1994). Since the KO animals were not viable, the heterozygous animals were crossed to generate KO mice. The mother was killed by cervical dislocation around embryonic day 18 (plus/minus 1 day), followed by Cesarian section to recover KOs, heterozygous and wild type (WT) animals. Embryos were killed by decapitation. Left and right hippocampi were dissected from pups of embryonic day 18 (E18) synaptotagmin-1-null mutant ($-/-$) mice and control littermates ($+/+$) of either sex in HBSS (Sigma), buffered with 7 mM HEPES, and collected in fixative solution [0.1% GA, 4.0% formaldehyde (FA) in sodium phosphate]. The mice were kept in an AAALAC-accredited stable at the Panum Institute, University of Copenhagen and all required permissions were obtained from the Danish Animal Health Inspectorate. The Institutional Animal Care and Use Committee (IACUC) oversaw procedures.

### 2.3 | Subcellular fractionation

Preparation of brain homogenate, synaptosome and vesicle membrane fractions: Ten rats were decapitated, the brains were dissected out and submerged in ice cold Hepes-buffered sucrose (0.32 M sucrose, 4 mM Hepes, pH 7.4) containing a protease inhibitor cocktail (Promega, Winconsin). The tissue was homogenized in Hepes buffer with a glass-Teflon homogenizer (900 rpm, 10–15 strokes) and centrifuged (800–1,000g, 10 min, 4 °C). The postnuclear supernatant, S1, was centrifuged (10,000g, 15 min), and the pellet containing crude synaptosomes was resuspended in 10 volumes of Hepes-buffered sucrose and centrifuged (10,000g, 15 min) to yield washed crude synaptosomal fraction. The synaptosomal fraction was resuspended in 10 mM sucrose, layered onto 1.2 M sucrose and centrifuged (161,000g, 25 min). The gradient interphase was collected and diluted in Hepes-buffered sucrose layered on 0.8 M sucrose and centrifuged again (161,000g, 25 min) to get a pellet containing pure synaptosomes. To extract synaptic vesicles, the pure synaptosome pellet was lysed by hypo-osmotic shock in nine volumes of ice-cold $H_2O$ plus protease/phosphatase inhibitors and three strokes with a glass-Teflon homogenizer, this solution was adjusted to 4 mM Hepes and mixed at 4 °C for 30 min to ensure complete lysis. The lysate was centrifuged (161,000g, 25 min), before the resulting supernatant again was centrifuged (165,000g for 2 hr) and then resuspended in 50 mM Hepes, 2 mM EDTA plus protease/phosphatase inhibitors.

## 2.4 | Immunoblotting

The homogenates were run on 4–20% SDS-acrylamide, electroblotted onto PVDF membrane (Hoefer Scientific Instruments, San Francisco, CA) and immunostained with primary antibodies and alkaline phosphatase linked secondary antibodies. The signal was detected by fluorescence using ECF substrate (Amersham Biosciences, UK). The fluorescence signals were visualized by a fluorescence digital camera detection system (Typhoon scanner). Hippocampi from rats in kainate-treated and control groups were used to make crude synaptosomes as described above.

## 2.5 | Bright field microscopic studies

Free floating vibratome sections from rat brain (50 μm) were treated with 1 M ethanolamine-HCL (pH 7.4), blocked with 3% (v/v) normal calf serum in 0.1 M sodium phosphate buffer (NaPi), pH 7.4, and incubated with primary antibodies, overnight at room temperature, followed by incubation with secondary antibodies for 1 hr at room temperature and development with the biotin-streptavidin-peroxidase system and 3,3-diaminobenzidine.

## 2.6 | Preparation of hippocampal neuronal cultures

Primary hippocampal cultures containing both neurons and glial cells of 1–4 day-old rats (Wistar) were prepared as previously described (Vik-Mo et al., 2003). Briefly, cultures were prepared from 1 to 4 day-old rats. They were maintained in cell medium (Gibcos MEM with the addition of 30 mg/100 mL glutamine; 2.5 mg/100 mL insulin; 5–10% fetal calf serum; 2 mL/100 mL B27 and 2–10 μL/100 mL ARA-C) in 5% $CO_2$, 95% air incubator at 37 °C. The cells were fixed in 4.0% FA by the following procedure: freshly prepared fixative (2.0% FA in 0.1 M sodium phosphate buffer) was heated to 37 °C before adding it to the culture medium (equal volumes). After 30 min, this mixture was substituted with 0.4% FA in 0.1 M sodium phosphate buffer ON. The cells were stored in 0.4 FA in 0,1 M sodium phosphate buffer. The sections were labeled with primary antibodies in 2% (v/v) normal calf serum and 1% bovine serum albumin in NaPi (ON/RT). The sections were rinsed in NaPi, incubated for 2 hr with secondary antibodies at RT and rinsed again in NaPi. The cultures were mounted with fluoromount mounting media (Southern Biotech), and examined with an Axioplan 2 equipped with a LSM 5 Pascal scanner head (Carl Zeiss, Heidelberg, Germany).

## 2.7 | Quantitative postembedding immunocytochemistry

### 2.7.1 | Perfusion fixation

For EM studies, the rats were deeply anesthetized with Equithesin (0.4 mL/100 g body weight) followed by intracardiac perfusion with 10–15 s flush of 4.0% Dextran-T70 in sodium phosphate buffer (pH 7.4) followed by a mixture of 4.0% FA (freshly prepared) and 0.1% GA in the same buffer.

### 2.7.2 | EM samples

Small (0.5–1.0 mm) blocks from CA1 were freeze substituted, sectioned, and immunolabeled as described previously (Mathiisen et al., 2006). The sections were examined with Philips Fei Tecnai 12 electron microscopes at 60 kV.

### 2.7.3 | EM quantification and statistical analysis

Electron micrographs were obtained at random from the middle layer of stratum radiatum of the CA1 region of the hippocampus. Synaptotagmin 1 immunolabeling was quantified as number of gold particles/μm of plasma membrane length and as number of gold particles/$\mu m^2$ for region of interests in the intracellular compartments, in asymmetric synapses. Specific plasma membrane and cytoplasmic compartments were defined as regions of interest (ROIs) and used for quantifications. The ROIs were: the postsynaptic density (PSD); the AZ; the lateral or perisynaptic membranes, that is, on each side of the PSD (PoL) or the AZ (PreL); the postsynaptic cytoplasm (PoCy); the presynaptic cytoplasm (PreCy); the dendritic membrane (DM); the dendritic cytoplasm (DCy). The synaptic lateral membranes were defined for convenience of measurement as equal to the length of the PSD, on both sides of the PSD or AZ, for all synapses. Only synaptic profiles with clearly visible synaptic membranes and PSD were selected for quantitative analysis. An in-house extension to analySIS connected with SPSS (SPSS, Chicago, IL) was used to evaluate the gold particle labeling of the specific plasma membrane and cytoplasmic compartments. The software calculated area particle density (number per unit area) over cytoplasmic compartments and linear particle density (number per unit length of curve) over membrane domains. In the latter case, it measured the distance from each particle center to the membrane and included only those particles that were within an operator-defined distance of +/− 21 nm (negative signifying an intracellular location) from the curve segment. Data for particles were collected in ASCII files as flat tables and exported to SPSS for further statistical and graphical analysis.

# 3 | RESULTS

## 3.1 | Hippocampus contains the highest concentration of synaptotagmin 1 in the brain

To test the first hypothesis, we examined synaptotagmin 1 expression in different brain fractions and regions using synaptotagmin 1 specific antibody. Consistent with the synaptic localization of the protein, we observed strong immunoreactivity in brain fractions enriched with synaptosome and synaptic vesicles (Figure 1a). Quantitative WB showed that the hippocampus contained the highest amount of this protein in the brain (Figure 1b,c), the lowest concentration was observed in the spinal cord. The cortex, thalamus, and cerebellum showed intermediate levels of synaptotagmin 1. To use the antibodies also for postembedding immunogold EM, we compared the sensitivity on embedded tissue of KO and WT animals. Quantitative analysis of gold particle densities over presynaptic terminals from the hippocampus CA1 region showed almost 5 times lower labeling intensity in KO mice compared with WT group (WT: 40.1 ± 3.9, KO: 8.7 ± 1.6, $n = 20$ synaptic profiles in both

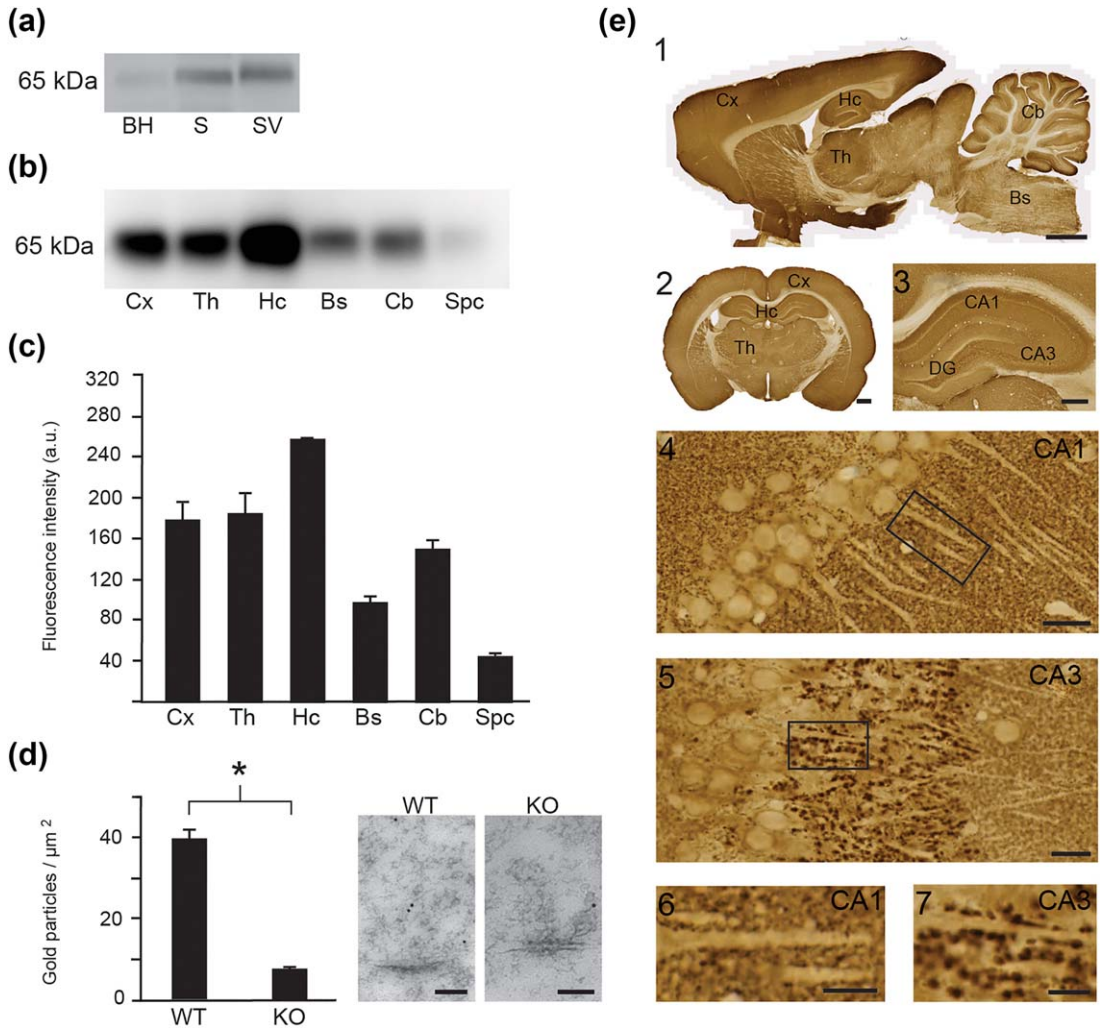


**FIGURE 1** Specificity of in-house synaptotagmin 1 antibody and regional expression of synaptotagmin 1 in the brain. (a) Western blot of subcellular fractions from WT rat brain labeled with synaptotagmin 1 antibody. Abbreviations: BH, brain homogenate; S, synaptosomes; SV, synaptic vesicles. (b) Western blot of homogenates from different brain regions stained with synaptotagmin 1 antibody, that is, cortex (Cx), thalamus (Th), hippocampus (Hc), brain stem (Bs), cerebellum (Cb), and spinal cord (SpC). (c) Quantitation of band intensities seen above. (d) Quantitative analysis of synaptotagmin 1 immunogold labeling over presynaptic terminal cytoplasm at synapses in the hippocampus of synaptotagmin 1 KO and WT mice. Asterisk denotes statistical significant difference ($p < .001$). EM image samples also shown. (e) Vibratome sections of rat brain immunostained for synaptotagmin 1. (E1–E3) Staining of neurons in the hippocampus, cerebellum, cerebral cortex, and thalamus. (E4, E6) CA1. (E5, E7) CA3. Error bars denote SEM. Scale bars: (d) 100 nm. (E1) 2,000 μm. (E2) 1,000 μm. (E3) 500 μm. (E4 and E5) 20 μm. (E6 and E7) 10 μm. [Color figure can be viewed at wileyonlinelibrary.com]

groups, $p < .001$, Mann-Whitney U test; Figure 1d). The remaining staining in KO synapses is regarded as background levels over synaptic areas very rich in protein. The embryonic hippocampi did not show structurally defined spines, so the antibody specificity was only tested through the use of presynaptic terminals. At the cellular level, LM showed strong immunostaining of synaptotagmin 1 in the hippocampus, cortex, striatum, and thalamus (Figure 1e). CA1 and CA3 of the hippocampus displayed characteristic punctate staining, as would be expected of a synaptic protein (Figure 1e). On the basis of these analyses, we chose the CA1 region of the hippocampus for an ultra-high resolution investigation of synaptotagmin 1 in excitatory synapses (Schaffer collateral axons from CA3 pyramidal neurons terminating on CA1 pyramidal apical dendrites).

### 3.2 | Synaptotagmin 1 is expressed in postsynaptic spines

Ultrastructural examination by immunogold EM revealed presynaptic localization of synaptotagmin 1, predominantly on presynaptic vesicles in the CA1 Schaffer collateral terminals (Figure 2a). Some gold particles were located over the AZ and the presynaptic lateral membranes. However, confirming our hypothesis, synaptotagmin 1 was also expressed in the postsynaptic spines. A large share of the postsynaptic gold particles was associated with vesicles (Figure 2b). Some gold particles were associated with the PSD and the postsynaptic lateral membrane, indicating that postsynaptic vesicles have fused with the plasma membrane. In dendrites, synaptotagmin 1 was located on DM and on

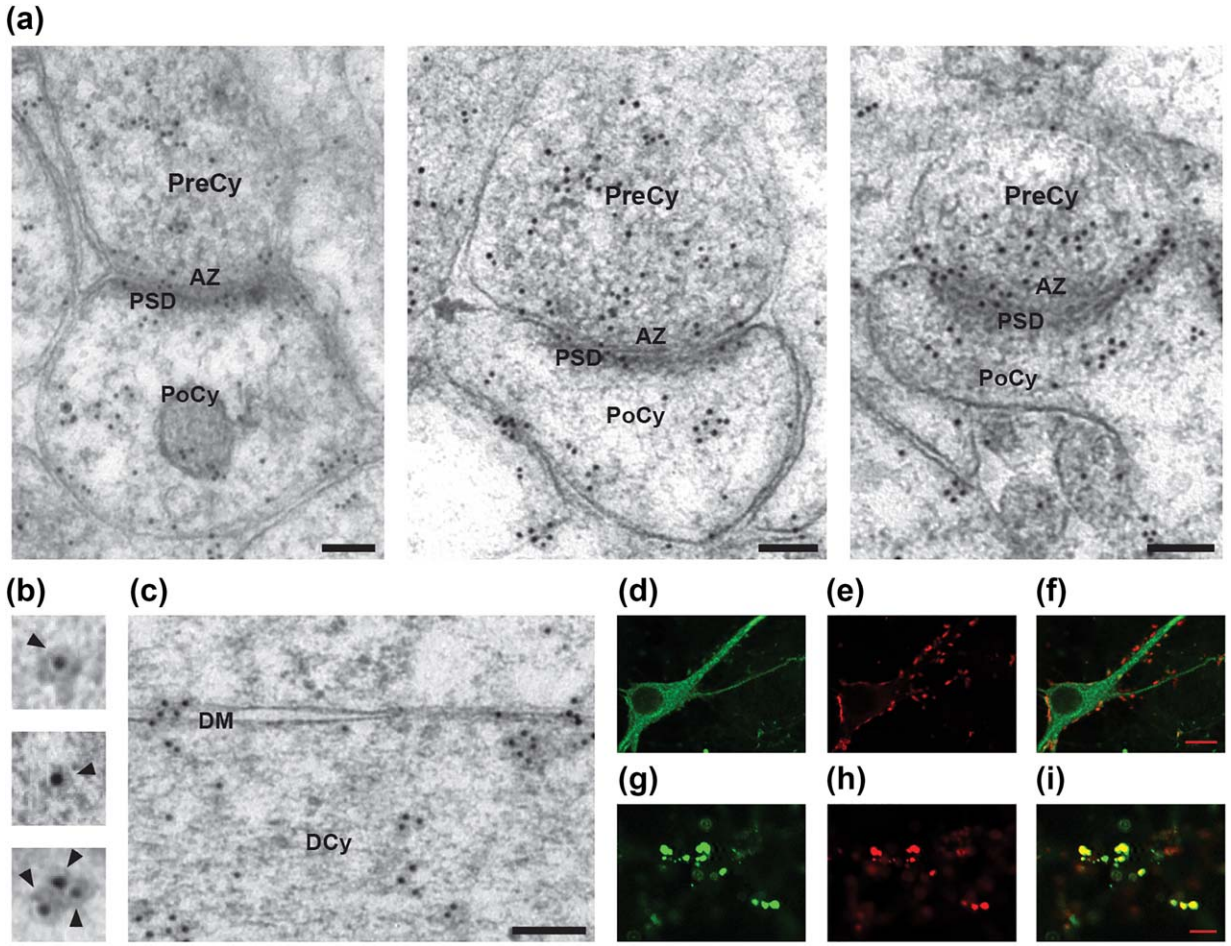


**FIGURE 2** Synaptotagmin 1 immunoreactivity in hippocampal synapses. (a) Electron micrographs displaying synaptotagmin 1 immunogold labeling of synapses in the rat hippocampus. Gold particles are located over both presynaptic and postsynaptic plasma membrane. Many gold particles are associated with presynaptic and postsynaptic vesicles. Abbreviations: PSD, the postsynaptic density; AZ, the active zone; PoCy, the postsynaptic cytoplasm; PreCy, the presynaptic cytoplasm. (b) Selected postsynaptic vesicles labeled for synaptotagmin 1 (arrowheads) at higher magnification. (c) Electron micrograph showing immunogold labeling of synaptotagmin 1 in dendrite. Note gold particles on small vesicles in cytoplasm and close to plasma membrane. Abbreviations: DM, dendritic membrane; DCy, dendritic cytoplasm. (d–i) IF labeling of dissociated hippocampal cultures: Double labeling with beta-tubulin (d) and synaptotagmin 1 (e) demonstrates characteristic synaptic punctate staining of synaptotagmin 1 (red) along the dendrite (green) (f). Double labeling with postsynaptic marker PSD-95 (green; g) and synaptotagmin 1 (red; h) shows colocalization (yellow) of the proteins in postsynaptic spines (i). Scale bars: (a, c) 100 nm. (f, i) 5 μm. [Color figure can be viewed at wileyonlinelibrary.com]

DCy vesicles (Figure 2c). To confirm postsynaptic localization of synaptotagmin 1, we performed double IF labeling of dissociated hippocampal cultures. Immunolabeling of synaptotagmin 1 showed punctate staining of synaptic terminals along neuronal dendrites (Figure 2d–f). Colocalization of the protein with the postsynaptic marker PSD-95 verified the expression of synaptotagmin 1 in postsynaptic sites (Figure 2g–i).

To determine the relative concentration of synaptotagmin 1 in different compartments of the synapse, we quantified the mean number of immunogold particles over different cytoplasmic and membrane ROIs (Figure 3a). Analysis of cytoplasmic immunogold labeling showed that the highest concentration of synaptotagmin 1 was in the presynaptic terminal, peaking at about 50–100 nm from the presynaptic AZ (Figure 3b), consistent with a lineup of primed vesicles ready for exocytosis. The protein was significantly expressed also in the postsynaptic spine cytoplasm, however, at about 74% of the presynaptic levels, but still about 40% higher than the concentration in DCy (Figure 3c), which points to a high capacity for exocytosis also in spines. Some or most of the dendritic synaptotagmin 1 protein may be there only in transit to the spines. Analysis of plasma membrane labeling (Figure 3d) revealed that the postsynaptic synaptotagmin 1 plasma membrane concentrations were about 80% of the corresponding presynaptic membranes. The dendritic plasma membranes, however, expressed only about 50% of the synaptotagmin 1 concentrations found at the PSD, again pointing to the spines as specialized sites for exocytosis. The protein was not concentrated at the AZ or PSD relative to the corresponding perisynaptic membranes, so we were not able to determine whether exocytosis occurs through the PSD or at the perisynaptic

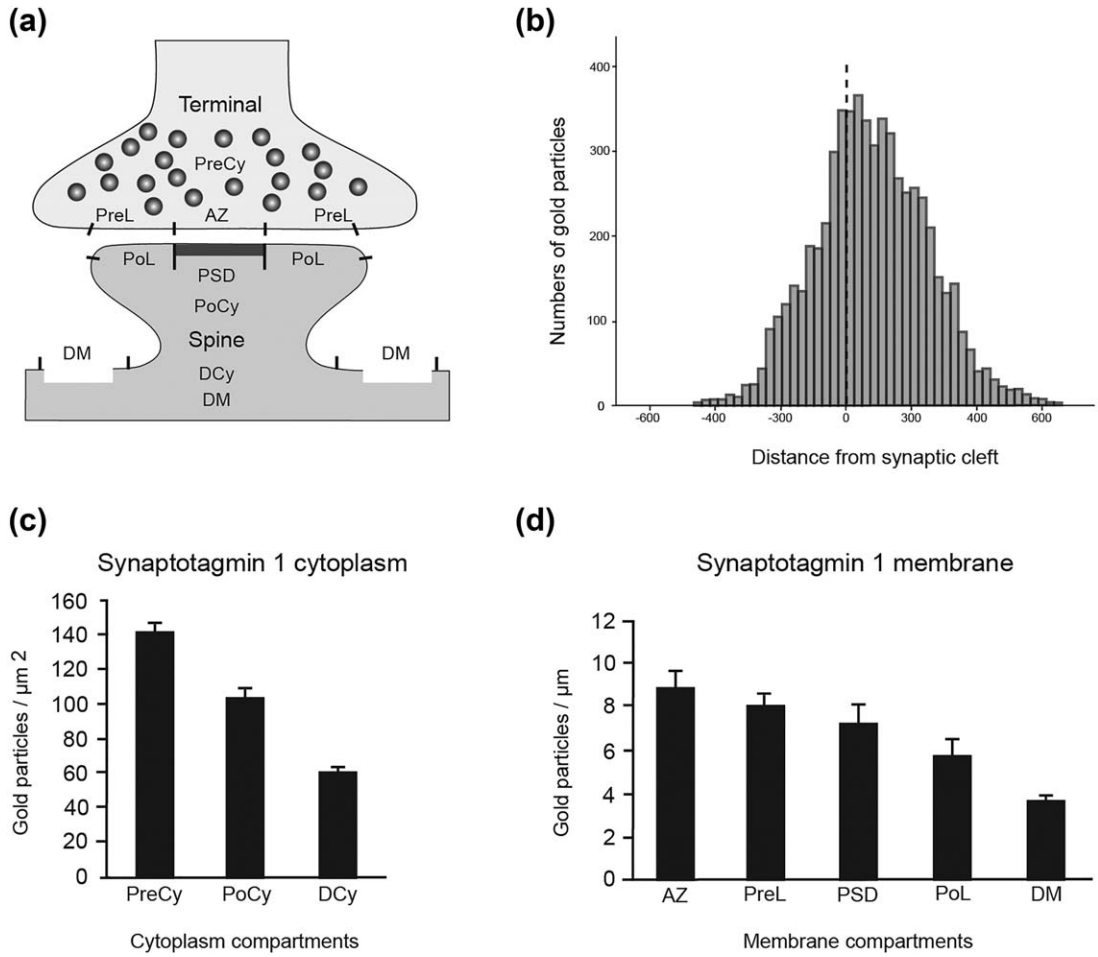


**FIGURE 3** Quantitative EM of synaptotagmin 1 immunogold labeling at the synapse. (a) Schematic drawing showing ROI used for quantification: The PSD; the AZ; the lateral or perisynaptic membranes, that is, on each side of the PSD (PoL) or the AZ (PreL); the PoCy; the PreCy; the DM; the DCy. (b) Transverse histogram depicting the mean numbers of gold particles at every 50 nm from the center of synaptic cleft, negative values are postsynaptic, positive values are presynaptic. (c) Mean immunogold labeling over cytoplasmic ROI. (d) Mean immunogold labeling over plasma membrane ROI. Error bars denote SEM.

membrane. In fact, our results indicate that both alternatives may be possible.

### 3.3 | Synaptotagmin 1 is downregulated in chronic temporal lobe epilepsy

Because of its role as a synaptic calcium sensor, we hypothesized that synaptotagmin 1 would be dynamically regulated during synaptic plasticity. Calcium plays a key role in the pathophysiology of epilepsy. Thus, in synapses on neurons that have survived chronic epilepsy, we expected to find an adaptive downregulation of synaptotagmin 1. To address this question, we investigated protein levels and subcellular localization of synaptotagmin 1 using a model of kainate-induced status epilepticus and acquired temporal lobe epilepsy. Compared with the control animals, hippocampal synaptosomes of kainate-treated rats exhibited decreased synaptotagmin 1 immunoreactivity (Figure 4a). Mean fluorescence intensity of synaptosomes from control rats was 15.1 (a.u.) while intensity in kainate-treated rats was 11.6 (a.u.). The T-test between the groups gave a $p$-value of .002 ($n = 7$ hippocampi in the control group and $n = 8$ hippocampi in the epilepsy group).

To further determine this overall downregulation of synaptotagmin 1 within presynaptic and postsynaptic ROIs of a hippocampal synapse, we conducted quantitative EM of CA1 Schaffer collateral synapses from kainate-treated and control rats (Figure 4b). Quantitative analysis of immunogold labeling over presynaptic terminal cytoplasm revealed significant reduction of synaptotagmin 1 in epileptic animals (ctrl: $33.8 \pm 2.0$, epilepsy: $31.3 \pm 2.7$, $n = 130$ synaptic profiles in both groups, $p$-value: .029, Mann-Whitney U test; Figure 4c). Quantification of gold particles over postsynaptic spine cytoplasm showed a trend of reduction of the protein in kainate-treated rats, however this reduction did not reach statistical significance (ctrl: $40.4 \pm 3.0$, epilepsy: $39.4 \pm 3.4$, $n = 130$ synaptic profiles in both groups, $p$-value: .105, Mann-Whitney U test, Figure 4c). The plasma membrane density of synaptotagmin 1 was significantly reduced in epileptic animals at both the

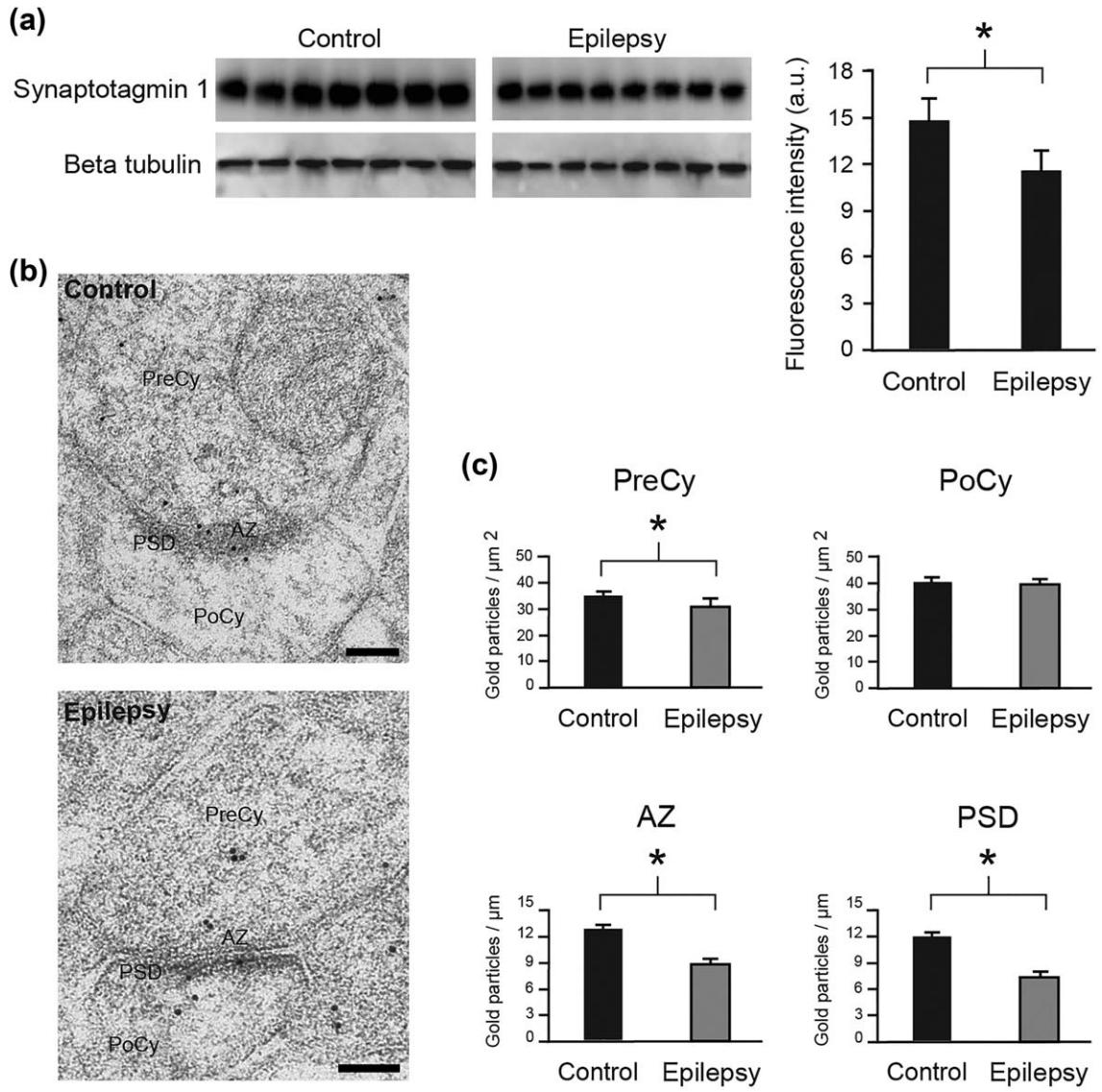


**FIGURE 4** Synaptotagmin 1 is downregulated in chronic temporal lobe epilepsy. (a) Quantitative western blot analysis of hippocampal synaptosomes from kainate-treated rats and control rats immunolabeled with anti-synaptotagmin 1. Beta-tubulin was used as loading control. Asterisk denotes statistical significant difference ($p$-value: .002). (b) Electron micrographs showing synaptotagmin 1 immunogold labeling of CA1 hippocampal synapse from control and epilepsy rats. (c) Mean immunogold labeling over cytoplasmic and plasma membrane ROI in hippocampal CA1 area synapses from kainite-treated and control rats. Error bars show SEM. Asterisks denote statistical significant difference. PreCy ($p$-value: .029). AZ and PSD ($p$ value $< .001$). Scale bars: (b) 100 nm.

presynaptic AZ (ctrl: $12.7 \pm 1.0$, epilepsy: $8.9 \pm 1.2$, $n = 130$ synaptic profiles in both groups, $p < .001$, Mann-Whitney U test) and the PSD (ctrl: $12.2 \pm 1.0$, epilepsy: $7.7 \pm 1.0$, $n = 130$ synaptic profiles in both groups, $p < .001$, Mann-Whitney U test, Figure 4c).

## 4 | DISCUSSION

Our two main conclusions are (1) Synaptotagmin 1 is present in postsynaptic spines in glutamatergic synapses at high concentrations only slightly less than in presynaptic terminals. (2) Synaptotagmin 1 is down regulated in surviving hippocampal synapses in chronic epilepsy. In view of recent reports that SNARE proteins facilitate insertion of glutamate receptors in postsynaptic spines (Hussain and Davanger, 2015; Hussain et al., 2016; Jurado, 2014; Jurado et al., 2013; Maher, Maher, Mackinnon, Bai, Chapman, & Kelly, 2005), it seems likely that a main function of postsynaptic synaptotagmin 1 is to regulate the exocytotic insertion of one or more types of these receptors.

The presynaptic and postsynaptic vesicle labeling patterns shown in the present study were very similar to what we recently reported for the vesicle SNARE protein VAMP2 (Hussain and Davanger, 2015). The VAMP2 positive vesicles were shown to be necessary for insertion of AMPA receptors into the synaptic plasma membrane. Because synaptotagmin 1 is necessary for the completion of presynaptic SNARE-mediated exocytosis, our findings strongly indicate that this calcium

sensor also may be a regulating step in insertion of AMPA receptors in the postsynaptic spine membrane.

We assume that postsynaptic spines will need more than one set of SNARE complexes to be able to differentially regulate plasma membrane insertion of different types of glutamate receptors. The classical presynaptic SNARE complex consisting of VAMP2, syntaxin 1, and SNAP-25 may thus be supplemented with other SNARE complexes in postsynaptic spines. In line with this, different SNARE complexes may be regulated by different synaptotagmin homologs, even within the same spines. Of the synaptotagmins, synaptotagmin 1 is by far the one with the strongest expression in the brain (Mittelsteadt et al., 2009). However, two other synaptotagmins, that is, synaptotagmin 2 and synaptotagmin 4, have calculated molecular weights close to synaptotagmin 1. Potentially, our synaptotagmin 1 antibody could then cross-react with synaptotagmin 2 or 4 without giving more than a single band on western blots. A comparative BLAST analysis of the mRNA sequence of synaptotagmin 1 versus 2 gave 80% total similarity and 46% overlap, and for synaptotagmin 1 versus 4 we found no significant similarity. Thus, crossreactivity with synaptotagmin 4 is unlikely. Both synaptotagmin 2 and 4 are expressed in the hippocampus at levels that are much lower than synaptotagmin 1 (Mittelsteadt et al., 2009). Our KO control of immunogold EM labeling showed a five-fold decrease in labeling intensity compared to the WT. The KO labeling thus most likely represents only background levels, but we cannot in principle exclude minor crossreactivity with synaptotagmin 2. Such a crossreactivity, though, is not by far sufficient to give the significant levels of spine labeling compared to presynaptic terminals, given the low expression levels of synaptotagmin 2 (and 4) in the hippocampus. Lastly, the relative expression levels we have observed of synaptotagmin 1 in the cortex, hippocampus, and cerebellum, closely mimic the mRNA expression pattern that has been described for synaptotagmin 1 (Mittelsteadt et al., 2009), but it is strikingly different from the patterns of both synaptotagmins 2 and 4.

Further studies will be needed to investigate which of the many different AMPA and/or NMDA receptors that are dependent on synaptotagmin 1 for plasma membrane insertion in spines. The postsynaptic and presynaptic pools of this molecule are anatomically and biochemically sequestered in strictly isolated compartments and may thus have different molecular tasks in the two locations. It is likely, however, that given the various types of receptors that need to be differentially trafficked and inserted in the postsynaptic spine, there will be a number of distinct SNARE complexes and synaptotagmins working in parallel within a single postsynaptic spine.

A few previous studies have found support for a downregulation of synaptotagmin 1 after epilepsy. These studies have been conducted under the assumption that synaptotagmin 1 has a function in presynaptic terminals only. Thus, Tocco et al. (1996) found that synaptotagmin 1 mRNA was decreased in adult rat brains, particularly in dentate granule cells, after kainic acid induced seizures. Similar observations have been made in spontaneously epileptic rats (Hanaya et al., 2012) and rats with spontaneous recurrent seizures after kainic acid treatment (Zhang et al., 2014). The reduction in synaptotagmin 1 expression has been interpreted as either a cause of epileptogenesis and seizures, or an adaptation to these.

Our study is the first, however, to show that synaptotagmin 1 is reduced not only in presynaptic terminals or in neurons in general, but specifically in postsynaptic spines in surviving, anatomically healthy, glutamatergic terminals. Although synaptotagmin 1 primarily is a vesicle protein, it exerts its function in initiating synaptic vesicle fusion only of the vesicles that are already docked to the plasma membrane. The reduced concentration at both the AZ and PSD in epilepsy, points to a reduced capacity for vesicle fusion with the plasma membrane both pre- and postsynaptically.

In this study, we wanted to investigate the status of synaptotagmin 1 in surviving, healthy neurons in chronic epilepsy, eight weeks after the first seizure. It would be highly relevant, however, also to elucidate the role of synaptotagmin 1 in the initial stages of epilepsy, immediately after induction. Such a study could shed light on possible adverse or adaptive regulations of this $Ca^{2+}$-sensor, also, during epileptogenesis.

One study (Xiao et al., 2009) has shown that synaptotagmin 1 expression is increased in the anterior temporal lobe from refractory epilepsy patients, compared to non-refractory epilepsy patients and normal trauma patients. The difference in synaptotagmin 1 between the refractory and non-refractory patients indicates that higher levels of synaptotagmin 1 are detrimental in epilepsy compared to more moderate levels, well in line with our results. This is perfectly understandable from a presynaptic perspective. A reduction in the molecular machinery responsible for vesicular exocytosis of excitotoxic glutamate into synapses makes sense as an adaptive or protective measure to counteract the detrimental effects of hyperactive glutamatergic neurons in epilepsy. Our results add a postsynaptic perspective to this: By reducing also the postsynaptic concentration of synaptotagmin 1, the $Ca^{2+}$-dependent insertion of glutamate receptors may be decreased. We hypothesize then, that experimental knock down of synaptotagmin 1 will increase the survival of hippocampal neurons after seizures by both presynaptic and postsynaptic mechanisms. We have previously found evidence for VAMP2 and syntaxin 1 regulating glutamate receptor insertion in postsynaptic spines (Hussain and Davanger, 2015; Hussain et al., 2016). These are the SNARE partners synaptotagmin 1 is known to interact with in presynaptic terminals, indicating that the classical SNARE complex of VAMP2, syntaxin 1 and SNAP-25 is functionally active also in postsynaptic spines and may be regulated by synaptotagmin 1.

Synaptotagmin 1 KOs, however, have shown normal mEPSC amplitudes (Geppert et al., 1994; Liu, Dean, Arthur, Dong, & Chapman, 2009), which have been taken to mean that there are no major postsynaptic changes in AMPA receptor concentrations. However, synaptotagmin 1 KO leads to strong increases in mEPSC frequency in larger cultures or in brain slices (Broadie, Bellen, DiAntonio, Littleton, & Schwarz, 1994; Chicka, Hui, Liu, & Chapman, 2008; Kochubey and Schneggenburger, 2011; Littleton, Stern, Perin, & Bellen, 1994; Pang, Sun, Rizo, Maximov, & Sudhof, 2006).

Synaptotagmin 1 KO (but not point mutations) has also been shown to massively increase spontaneous release (Xu et al., 2009). This

increased spontaneous release remains calcium dependent, but it is activated at lower calcium concentrations than synaptotagmin-driven spontaneous release. This was interpreted as evidence that in addition to serving as a calcium sensor for spontaneous and evoked release, synaptotagmin 1 also clamps a second, more sensitive calcium sensor for spontaneous release. However, homozygote synaptotagmin 1 KO animals die soon after birth, so these mechanisms have been studied in immature brain slices or cultured cells, which may probably, partly, use molecular mechanisms that are different from those of the intact, mature, adult brain. Most likely, the reduction in synaptotagmin 1 in chronic epilepsy that we have observed has specific effects on plasticity that may be difficult to study in vitro.

In conclusion, we demonstrate for the first time, the postsynaptic localization of synaptotagmin 1, at concentrations moderately lower than, but comparable to presynaptic concentrations. It is present in significant concentrations also at the PSD, pointing to the likelihood of insertion of glutamate receptors directly into the synaptic plasma membrane. Finally, synaptotagmin 1 is reduced in postsynaptic spines after eight weeks of kainite-induced epilepsy, pointing to a possible adaptive mechanism against calcium overload in excitotoxic conditions.

## ACKNOWLEDGMENTS

The authors thank Karen Marie Gujord, Jorunn Knutsen, Bjørg Riber, Johannes Helm, and Bashir Hakim for their expert technical assistance. They thank Finn-Mogens S. Haug for assistance with immunogold quantification and statistics. The University of Oslo, Norsk Epilepsiforbunds forskningsfond, and the European Union Projects QLG3-CT-2001–02089 (KARTRAP) and LSCHM-CT-2005–005320 (GRIPANNT) supported this work. The authors declare that they have no competing interests. Five weeks after the submission of this article to Hippocampus, another paper was published online providing evidence of synaptotagmin 1 and synaptotagmin 7 acting together as $Ca^{2+}$-sensors for $Ca^{2+}$-dependent exocytosis of postsynaptic AMPA receptors during LTP (Wu et al., 2017).

## REFERENCES

Broadie, K., Bellen, H. J., DiAntonio, A., Littleton, J. T., & Schwarz, T. L. (1994). Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission. *Proceedings of the National Academy of Sciences of the United States of America*, *91*, 10727–10731.

Burgoyne, R. D., & Haynes, L. P. (2015). Sense and specificity in neuronal calcium signalling. *Biochimica et Biophysica Acta*, *1853*, 1921–1932.

Chicka, M. C., Hui, E., Liu, H., & Chapman, E. R. (2008). Synaptotagmin arrests the SNARE complex before triggering fast, efficient membrane fusion in response to Ca2+. *Nature Structural & Molecuar Biology*, *15*, 827–835.

Geppert, M., Goda, Y., Hammer, R. E., Li, C., Rosahl, T. W., Stevens, C. F., & Sudhof, T. C. (1994). Synaptotagmin I: A major Ca2+ sensor for transmitter release at a central synapse. *Cell*, *79*, 717–727.

Hanaya, R. Hosoyama, H., Sugata, S., Tokudome, M., Hirano, H., Tokimura, H. . . . Arita, K. (2012). Low distribution of synaptic vesicle protein 2A and synaptotagimin-1 in the cerebral cortex and hippocampus of spontaneously epileptic rats exhibiting both tonic convulsion and absence seizure. *Neuroscience*, *221*, 12–20.

Hussain, S., & Davanger, S. (2011). The discovery of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex and the molecular regulation of synaptic vesicle transmitter release: the 2010 Kavli prize in neuroscience. *Neuroscience*, *190*, 12–20. 10.1016/j.neuroscience.2011.05.057

Hussain, S., & Davanger, S. (2015). Postsynaptic VAMP/Synaptobrevin Facilitates Differential Vesicle Trafficking of GluA1 and GluA2 AMPA Receptor Subunits. *PLoS One*, *10*, e0140868. doi:10.1371/journal.pone.0140868

Hussain, S., Ringsevjen, H., Egbenya, D. L., Skjervold, T. L., & Davanger, S. (2016). SNARE Protein Syntaxin-1 Colocalizes Closely with NMDA Receptor Subunit NR2B in Postsynaptic Spines in the Hippocampus. *Frontiers in Molecular Neuroscience*, *9*, 10.

Jurado, S. (2014). The dendritic SNARE fusion machinery involved in AMPARs insertion during long-term potentiation. *Frontiers in Molecular Neuroscience*, *8*, 407.

Jurado, S., Goswami, D., Zhang, Y., Molina, A. J., Sudhof, T. C., & Malenka, R. C. (2013). LTP requires a unique postsynaptic SNARE fusion machinery. *Neuron*, *77*, 542–558.

Kerrigan, T. L., Daniel, J. W., Regan, P. L., & Cho, K. (2012). The role of neuronal calcium sensors in balancing synaptic plasticity and synaptic dysfunction. *Frontiers in Molecular Neuroscience*, *5*, 57.

Kessels, H. W., & Malinow, R. (2009). Synaptic AMPA receptor plasticity and behavior. *Neuron*, *61*, 340–350.

Kobayashi, S., Ohno, K., Iwakuma, M., Kaneda, Y., & Saji, M. (2002). Synaptotagmin I hypothalamic knockdown prevents amygdaloid seizure-induced damage of hippocampal neurons but not of entorhinal neurons. *Neuroscience Research*, *44*, 455–465.

Kochubey, O., & Schneggenburger, R. (2011). Synaptotagmin increases the dynamic range of synapses by driving Ca(2)+-evoked release and by clamping a near-linear remaining Ca(2)+ sensor. *Neuron*, *69*, 736–748.

Littleton, J. T., Stern, M., Perin, M., & Bellen, H. J. (1994). Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in Drosophila synaptotagmin mutants. *Proceedings of the National Academy of Sciences of the United States of America*, *91*, 10888–10892.

Liu, H., Dean, C., Arthur, C. P., Dong, M., & Chapman, E. R. (2009). Autapses and networks of hippocampal neurons exhibit distinct synaptic transmission phenotypes in the absence of synaptotagmin I. *Journal of Neuroscience*, *29*, 7395–7403.

Maher, B. J., Mackinnon, R. L., II, Bai, J., Chapman, E. R., & Kelly, P. T. (2005). Activation of postsynaptic Ca(2+) stores modulates glutamate receptor cycling in hippocampal neurons. *Journal of Neurophysiology*, *93*, 178–188.

Mathiisen, T. M., Nagelhus, E. A., Jouleh, B., Torp, T., Frydenlund, D. S., Mylonakou, M. N., . . . Ottersen, O. P. (2006). Postembedding immunogold cytochemistry of membrane molecules and amino acid transmitters in the central nervous system. In L. Zaborszky, F. G. Wouterlood & J. L. Lanciego (Eds.), *Neuroanatomical Tract-Tracing 3: Molecules, Neurons, and Systems* (pp. 72–108). New York: Springer.

Mittelsteadt, T., Seifert, G., Alvarez-Baron, E., Steinhauser, C., Becker, A. J., & Schoch, S. (2009). Differential mRNA expression patterns of the synaptotagmin gene family in the rodent brain. *Journal of Comparative Neurology*, *512*, 514–528.

Pang, Z. P., Sun, J., Rizo, J., Maximov, A., & Sudhof, T. C. (2006). Genetic analysis of synaptotagmin 2 in spontaneous and Ca2+-triggered neurotransmitter release. *EMBO Journal*, *25*, 2039–2050.

Pozo, K., & Goda, Y. (2010). Unraveling mechanisms of homeostatic synaptic plasticity. *Neuron*, *66*, 337–351.

Rao, W., Zhang, L., Peng, C., Hui, H., Wang, K., Su, N., ... Fei Z. (2015). Downregulation of STIM2 improves neuronal survival after traumatic brain injury by alleviating calcium overload and mitochondrial dysfunction. *Biochimica et Biophysica Acta*, *1852*, 2402–2413.

Sprengel, R. (2006). Role of AMPA receptors in synaptic plasticity. *Cell and Tissue Research*, *326*, 447–455.

Sudhof, T. C. (2002). Synaptotagmins: Why so many? *Journal of Biological Chemistry*, *277*, 7629–7632.

Sudhof, T. C. (2013). Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. *Neuron*, *80*, 675–690.

Tocco, G., Bi, X., Vician, L., Lim, I. K., Herschman, H., & Baudry, M. (1996). Two synaptotagmin genes, Syt1 and Syt4, are differentially regulated in adult brain and during postnatal development following kainic acid-induced seizures. *Brain Research. Molecular Brain Research*, *40*, 229–239.

Vik-Mo, E. O., Oltedal, L., Hoivik, E. A., Kleivdal, H., Eidet, J., & Davanger, S. (2003). Sec6 is localized to the plasma membrane of mature synaptic terminals and is transported with secretogranin II-containing vesicles. *Neuroscience*, *119*, 73–85. doi:S0306452203000654 [pii]

Wu, D., Bacaj, T., Morishita, W., Goswami, D., Arendt, K. L., Xu, W., ... Sudhof, T. C. (2017). Postsynaptic synaptotagmins mediate AMPA receptor exocytosis during LTP. *Nature*, *544*, 316–321.

Xiao, Z., Gong, Y., Wang, X. F., Xiao, F., Xi, Z. Q., Lu, Y., & Sun, H. B. (2009). Altered expression of synaptotagmin I in temporal lobe tissue of patients with refractory epilepsy. *Journal of Molecular Neuroscience*, *38*, 193–200.

Zhang, F. X., Sun, Q. J., Zheng, X. Y., Lin, Y. T., Shang, W., Wang, A. H., ... Chi Z. F. (2014). Abnormal expression of synaptophysin, SNAP-25, and synaptotagmin 1 in the hippocampus of kainic acid-exposed rats with behavioral deficits. *Cellular and Molecular Neurobiology*, *34*, 813–824.

**How to cite this article:** Hussain S, Egbenya DL, Lai Y-C, et al. The calcium sensor synaptotagmin 1 is expressed and regulated in hippocampal postsynaptic spines. *Hippocampus*. 2017;27:1168–1177. https://doi.org/10.1002/hipo.22761