Down-regulation Synaptic Vesicle Protein 2A in the Anterior Temporal Neocortex of Patients with Intractable Epilepsy

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Abstract Synaptic vesicle protein 2A (SV2A) involvement has been reported in the animal models of epilepsy. The aim of this study was to investigate the expression of SV2A in human intractable epilepsy (IE) brain tissue. Using immunohistochemistry, immunofluorescence, and Western blot, we detected SV2A expression in tissue samples from the anterior temporal neocortex of 33 patients who had been surgically treated for IE. We compared these tissues with nine histologically normal anterior temporal lobe samples from controls. SV2A immunoreactive staining was 0.1651 ± 0.0564 in patient group and 0.2347 ± 0.0187 in the control group ($p < 0.05$) using immunohistochemistry, and this finding was consistently observed with Western blot analysis (0.1727 ± 0.0471 versus 0.3976 ± 0.0983, $p < 0.05$). Immunofluorescence staining showed that SV2A was mainly accumulated in neurons. Our findings demonstrate that down-regulation of SV2A is present in patients with temporal lobe epilepsy.

Keywords Epilepsy · Synaptic vesicle protein 2A (SV2A) · Temporal lobe

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Introduction

Epilepsy, a group of diseases characterized by recurrent spontaneous seizures, is a prevalent chronic neurological disorder (Scheuer and Pedley 1990). Approximately 30% of epilepsy cases are medically intractable even when treated with one or more antiepileptic drugs (AEDs) at maximal dosages (Regesta and Tanganelli 1999; Sisodiya 2003; Rogawski and Loscher 2004). Such epilepsy cases are thus referred to as intractable epilepsy (IE). This intractability is even higher (50–70%) in patients with temporal lobe epilepsy (TLE; Kwan and Brodie 2000; Schmidt and Loscher 2005). To date, the mechanisms of underlying pathogenesis of epilepsy are still unclear.

Synaptic vesicle protein 2A (SV2A) is a highly glycosylated membrane protein (Buckley and Kelly 1985) that is localized in all presynaptic nerve terminals and is required for normal neurotransmission (Buckley and Kelly 1985; Bajjalieh et al. 1994). SV2A knockout mice have spontaneous seizures and die within 3 weeks of birth (Crowder et al. 1999; Janz et al. 1999). Moreover, SV2A has been identified as the binding site for the AED levetiracetam both in animals and in humans (Lynch et al. 2004; Gillard et al. 2006). These findings suggest that SV2A may play a role in epileptogenesis.

SV2A expression has been investigated in different rat brain structures (Bajjalieh et al. 1994; Janz and Sudhof 1999) and recently in the hippocampus of human patients with epilepsy (van Vliet et al. 2009; Toering et al. 2009) There are, however, no data regarding the expression of SV2A in the temporal lobe of patients with IE. Here we used immunohistochemistry, immunofluorescence, and Western blot to examine whether the level of SV2A protein expression is abnormal in the anterior temporal neocortex of patients with intractable TLE.

Table 1 Clinical characteristics of patients with intractable TLE

Characteristic	Value
Age (years)	28.24±10.44
Gender (n)	
Male	17
Female	16
Onset of seizures (n)	
<18 years	21
≥18 years	12
Course of epilepsy (years)	
<10 years	10
>10 years	23
Frequency of seizures per month (n)	
3–4/month	6
4–8/month	15
>8/month	12

Values are means±SEM, or numbers

Materials and Methods

Patient Selection

Thirty-three patients (ages 15 to 58 years; mean 28.24± 10.44 years) who had undergone resection of the anterior temporal neocortex for medically IE were chosen randomly from our epilepsy center. Pre-surgical assessment consisted of obtaining a detailed history and neurological examination and carrying out interictal and ictal electroencephalogram recordings, neuropsychological testing, and a neuroimaging test. For resection of the anterior temporal neocortex, the anterior 3.5–4.0 cm of the lateral temporal lobe was resected en bloc from the superior temporal gyrus to the collateral fissure. Clinical characteristics of the patients are shown in Table 1.

Control temporal lobe samples were obtained from nine patients who had undergone neurosurgical intervention in the Neurosurgical Department of The First Affiliated Hospital of Chongqing Medical University because of brain trauma. All control tissues were from patients diagnosed with brain trauma, and neuropathologists found no abnormalities upon examination of slides of these specimens. The mean age of the control patients was 34.55±12.55 years (range, 14 to 51 years). The time interval from the trauma to surgery ranged from 5 to 24 h. These subjects had no history of epilepsy or exposure to AEDs and had no other neurological diseases.

This protocol was approved by the ethics committee on human research at Chongqing Medical University. Informed consent was obtained from the patients or their relatives for the use of any data and tissues for research, which was performed in accordance with The Declaration of Helsinki.

Tissue Preparation

Samples were collected from the patients in the operating room. Part of each sample was immediately placed in a cryovial that had been soaked in buffered diethylpyrocarbonate (1:1,000) for 24 h. Those sample portions were stored in liquid nitrogen for later Western blotting. The other portion of each sample was fixed in 10% buffered formalin. After fixation for 24 h, tissues were embedded in paraffin and sectioned at a thickness of 5 μm for immunohistochemistry or 10 μm for immunofluorescence; sections were then mounted onto poly-L-lysine-coated slides. Two sections from each specimen were processed for hematoxylin and eosin staining. Control samples were prepared in the same manner. Neuropathological examination with hematoxylin and eosin staining showed no histological abnormalities in control specimens.
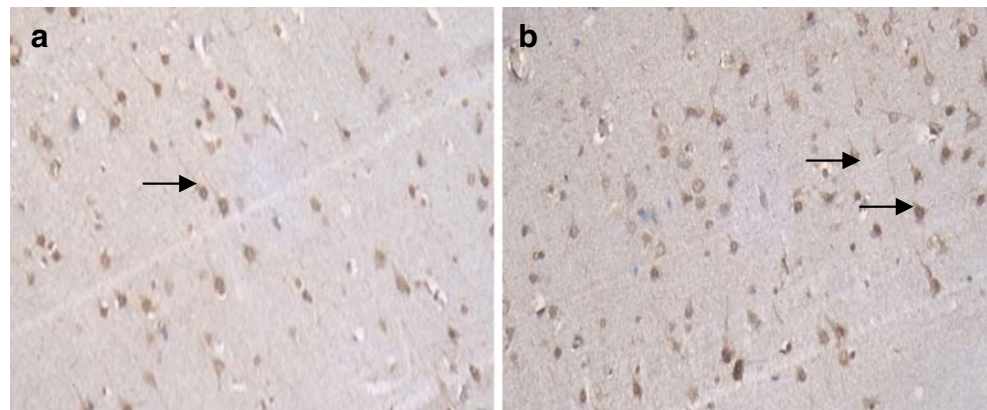
Immunohistochemistry

Sections were deparaffinized in xylene and rehydrated in a graded series of ethanol before staining. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 10 min. The sections were heated in 10 mmol/l sodium citrate buffer (pH6.0) for 10 min at 92–98°C for antigen retrieval. A blocking solution of 10% normal goat serum was then added to the sections at room temperature (20–26°C) for 30 min. The sections were incubated in primary rabbit anti-human SV2A (1:100; SC-28955, Santa Cruz Biotechnology,

Table 2 Clinical characteristics of the subjects

	Epilepsy (n=33)	Control (n=9)	Results
Age(years)	28.24±10.44	34.55±12.55	t=1.5408; p=0.1312
Gender			
Female	16 (48.5%)	5 (55.5%)	χ^2=0.1414; p=0.7069
Male	17 (51.5%)	4 (44.5%)	
Topography of studied tissue			
Left	17 (51.5%)	5 (55.5%)	χ^2=0.0260; p=0.8710
Right	16 (48.5%)	4 (44.5%)	

Santa Cruz, CA, USA) overnight at 4°C. After extensive washing with PBS, sections were incubated with biotinylated secondary goat anti-rabbit for 30 min at 37°C. Sections were then incubated in avidin–biotin peroxidase complex (Zhongshan Golden Bridge Inc, Beijing, China) for 30 min. The immunoreactivity signal was developed with 3,3′-diaminobenzidine (Zhongshan Golden Bridge Inc.). Hematoxylin was used to counterstain nuclei. IE and control sections were processed alongside each other in the same immunohistochemistry experiment. As a negative control, primary or secondary antibodies were replaced with PBS for some sections, and this technique was mainly used to evaluate the reagent quality and the staining technique.

An OLYMPUS PM20 automatic microscope (Olympus, Osaka, Japan) and TC-FY-2050 pathology system (Yuancheng Inc., Beijing, China) were used to collect the images. Four visual field images were analyzed with the Motic Med 6.0 CMIAS pathology image analysis system. The computer software determined the intensity of labeling, which gave a gray value ranging from 0 (black) to 1.0 (white). Immunohistochemical parameters were assessed using the mean optical density (mean OD), which indicated the mean intensity of the staining for each pixel and the mean amount of stained material in that area. Positive cells were stained yellowish-brown under the microscope, and negative cells were unstained.

Double Immunofluorescence and Confocal Microscopy

Sections were deparaffinized, and antigen retrieval was performed as described above. Tissue sections were incubated in calf serum for 1 h and then in normal goat serum (Zhongshan Golden Bridge Inc.) for 30 min. The sections were incubated with polyclonal rabbit anti-human SV2A (1:100; Santa Cruz Biotechnology) and monoclonal mouse anti-neuron-specific enolase (NSE; 1:100; Zhongshan Golden Bridge Inc.) overnight at 4°C. After washing in PBS, the sections were incubated in the dark with anti-rabbit fluorescein isothiocyanate (FITC) and anti-mouse tetramethylrhodamine isothiocyanate (TRITC; Zhongshan

Golden Bridge Inc.) at a dilution of 1:100 for 1 h at room temperature. After washing with PBS, sections were mounted in 50% glycerol and 50% PBS, sealed, and dried overnight. Fluorescently stained sections were examined by confocal laser scanning microscopy (Leica Microsystems Heidelberg GmbH, Germany), and the images were collected and processed using Olympus Micro image software (version 4.0).

Western Blot

Protein was extracted from the samples and assayed quantitatively using the Bradford method. The samples were cut into small pieces and homogenized in buffer containing protease inhibitors (l5 μg/ml aprotinin and 1 mmol/l phenylmethylsulfonyl fluoride), followed by centrifugation at $16,000 \times g$ for 5 min at 4°C. The protein concentration of each lysate was determined using a Coomassie blue G-250 kit (Sigma, St. Louis, MO, USA). The protein extracts (50 μg) were resolved with 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (DuPont, Wilmington, DE, USA). The PVDF membranes were blocked with 3% BSA (Sigma) in PBS (pH 7.2) and incubated for 2 h at room temperature. After extensive washing with PBS, the membranes were incubated with rabbit anti-human SV2A (1:100; Santa Cruz Biotechnology)
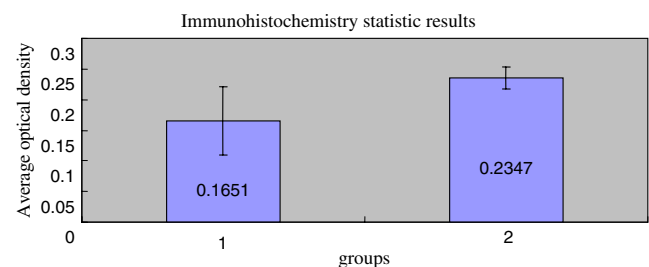

Figure 2 The mean OD value comparison indicates the significantly lower expression of SV2A in IE group ($p<0.05$). *X axis* Group 1: epileptic tissue; group 2: control tissue. *Y axis* Average optical densities

Figure 3 a to **f** Double-labeled immunofluorescence for SV2A (*green*) and NSE (*red*) in neurons. **c**, **f** SV2A and NSE co-localization. FITC staining (**a**, **d**); TRITC staining (**b**, **e**); IE (**a**, **b**, **c**); normal neocortex (**d**, **e**, **f**); *arrows* indicate positive neurons; original magnification, ×400
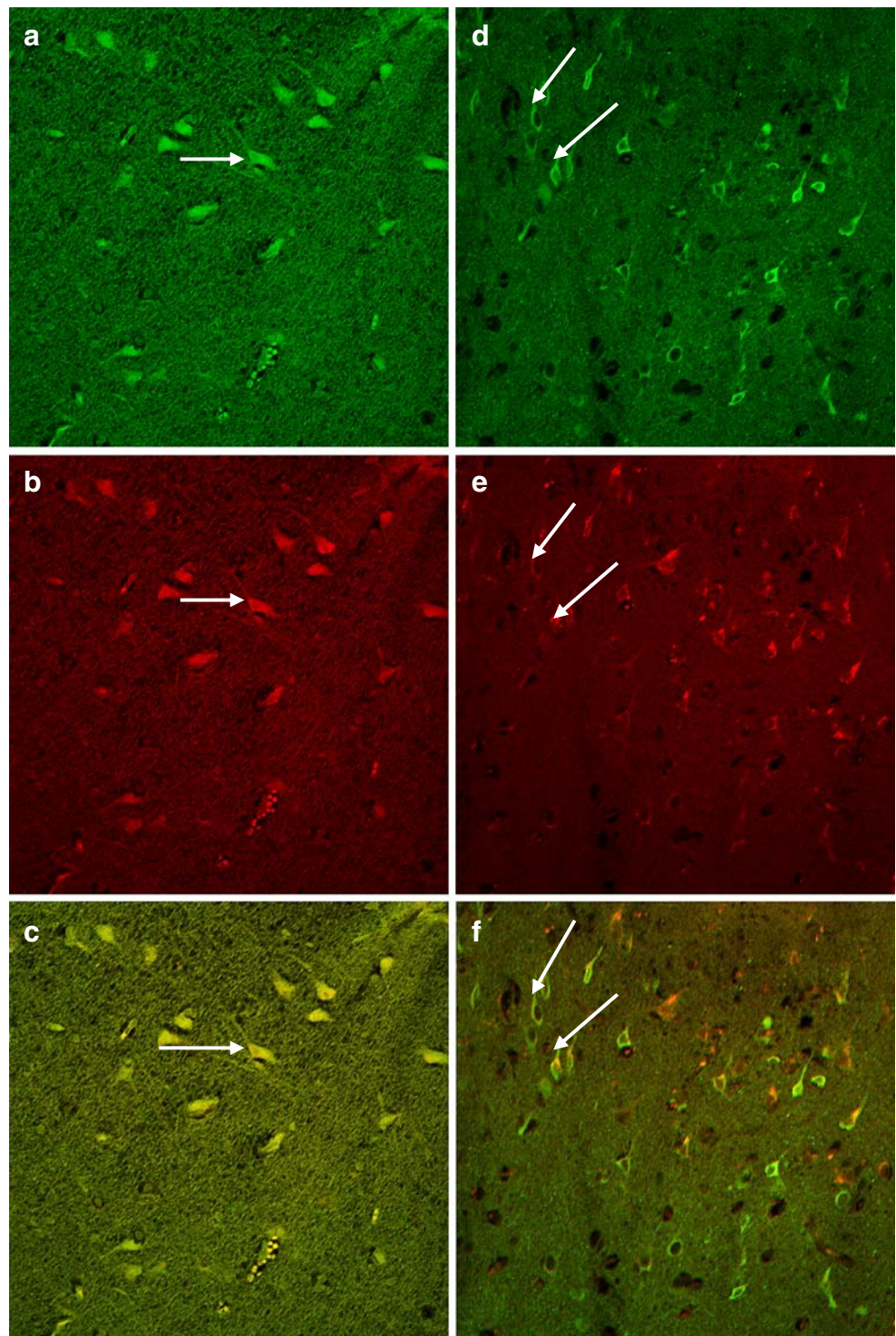
or mouse monoclonal anti-β-actin (1:1,000; Santa Cruz Biotechnology) for 1 h. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000 goat anti-rabbit or goat anti-mouse; Santa Cruz Biotechnology) for 1 h at 37°C. Protein bands were visualized by enhanced chemiluminescence (Amersham Biosciences) after exposure to film. Films were scanned, and the pixel density of the images was quantified using

Labworks Analysis Software (UVP, Upland, CA, USA). The band intensity ratio of SV2A to β-actin (SV2A/β-actin) was analyzed.

Statistical Analysis

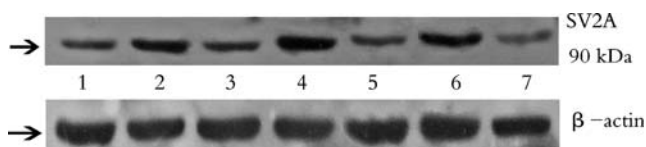Data are represented as means±standard error of mean (SEM). Student's *t* test was used for statistical analysis

Figure 4 Semi-quantitative Western blot analysis of SV2A in the temporal neocortex of TLE patients. *Cases 1, 3, 5,* and *7* show SV2A in tissue from TLE patients; and *cases 2, 4,* and *6* show SV2A expression in control tissue. The SV2A protein bands in epileptic tissues were fainter than those in control tissues

between epileptic tissues and the control tissues. A *p* value of <0.05 was considered significant.

Results

Patients with IE were deemed suitable candidates for surgery because of their intractability and the concordance of conventional pre-surgical evaluation procedures, including clinical, neurophysiological, and qualitative imaging modalities. Table 2 summarizes the demographic and clinical characteristics of the subjects who participated in this study. There were no significant differences as in age, sex, and topography of tissue studied between IE and control subjects.

SV2A immunoreactivity was consistently observed in all cases and was confined mainly to neurons. In the samples from TLE patients (Fig. 1a), faint SV2A immunoreactivity was observed, whereas strong staining for SV2A was present in sections from controls (Fig. 1b). No immunoreactivity was observed in negative controls for temporal tissue in which the primary antibody has been omitted. The mean optical density value of SV2A expression was 0.1651 ± 0.0564 in TLE patients and 0.2347 ± 0.0187 in the control group ($p < 0.05$). Figure 2 shows a histogram of the optical density values derived from the immunohistochemical comparison of IE and control samples. Moreover, SV2A (green) and NSE (red) were co-expressed within neurons in TLE patients and controls as observed with confocal microscopy (Fig. 3c, f).

To further confirm the decrease in SV2A in TLE brain tissue, Western blotting was performed. The SV2A protein was detected as a 90-kDa band, consistent with the molecular weight of SV2A (Lynch et al. 2004). Strongly stained bands were present in all samples from control tissue, whereas relatively faint expression of SV2A was observed in samples from the epileptic group (Fig. 4). A band of 42 kDa that corresponds to β-actin (positive control) was observed in all samples. The relative optical density of SV2A was significantly decreased in the temporal neocortex of TLE patients as compared with that from control patients (0.1727 ± 0.0471 versus 0.3976 ± 0.0983, $p < 0.05$). Figure 5 shows a histogram of the optical density ratio between the epilepsy and control groups.

Discussion

In the present study, for the first time, we put forward that SV2A expression is significantly decreased in the anterior temporal neocortex of patients with IE when compared with age-matched control subjects.

Because of the practical difficulty in obtaining normal human brain specimens, we used structurally normal brain tissue obtained from temporal lobectomies performed for the treatment of traumatic brain injuries as controls. In adults with TLE, pathology in the hippocampus and temporal lobe is the most common findings. Pathological changes such as aberrant neurogenesis and aberrant synaptogenesis are often found in the temporal lobe of patients with IE.

SV2A is expressed ubiquitously throughout the brain (Bajjalieh et al. 1994). Although the function of SV2A at the molecular level is not completely understood, it is required for synaptic vesicles' normal folding and trafficking (Chang and Sudhof 2009). The publication by Chang and Sudhof (2009) suggested that SV2 functions in a maturation step of primed vesicles that converts the vesicles into a Ca^{2+}- and synaptotagmin-responsive state. In our previous study, synaptotagmin I was up-regulated in temporal lobe tissues of patients with IE (Xiao et al. 2009).The observation that SV2A knockout mice develop lethal seizures soon after birth (Crowder et al. 1999), along with evidence that SV2A is the binding site for the AED levetiracetam and its analogs (brivaracetam and seletracetam; Lynch et al. 2004; Gillard et al. 2006; Bennett et al. 2007; von Rosenstiel 2007), indicates a role for SV2A in the antiepileptic properties of AEDs. Therefore, decreased SV2A expression may contribute to the progression of epilepsy.

In this study, the experimental samples from patients with TLE varied in gender, course of epilepsy, frequency of seizures, and age of onset of seizures, all of which may influence SV2A expression. Our conclusions would be stronger if our results could have been compared among
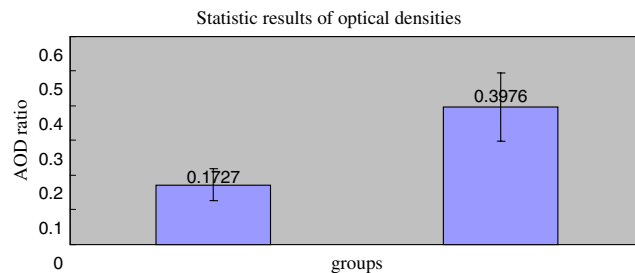

Figure 5 The statistic results of optical densities of Western blot (OD ratio: the ratio of optical densities). It indicates the significantly lower expression of SV2A in IE group ($p < 0.05$). *X axis* Group l: epileptic tissue; group 2:control tissue. *Y axis* Average optical densities ratio

subgroups; however, because of the small sample size of each subgroup, we could not carry out this comparison.

In conclusion, we observed abnormally decreased SV2A expression in the anterior temporal neocortex of patients with IE. The underlying mechanism of SV2A in the formation of TLE was not tested in this study because of the limitations of performing the study in humans. Based on our results, along with the known physiological roles of SV2A, we hypothesize that altered expression of SV2A may play a pathogenic role in the progression of IE. Further studies using animal models and in vitro experiments are required to elucidate the role of SV2A in the temporal neocortex of IE patients.

References

Bajjalieh SM, Frantz GD, Weimann JM, McConnell SK, Scheller RH (1994) Differential expression of synaptic vesicle protein 2 (SV2) isoforms. J Neurosci 14:5223–5235

Bennett B, Matagne A, Michel P et al (2007) Seletracetam (UCB 44212). Neurotherapeutics 4:117–122

Buckley K, Kelly RB (1985) Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. J Cell Biol 100:1284–1294

Chang WP, Sudhof TC (2009) SV2 renders primed synaptic vesicles competent for Ca^{2+}-induced exocytosis. J Neurosci 29(4):883–897

Crowder KM, Gunther JM, Jones TA et al (1999) Abnormal neurotransmission in mice lacking synaptic vesicle protein 2A (SV2A). Proc Natl Acad Sci 96:15268–15273

Gillard M, Chatelain P, Fuks B (2006) Binding characteristics of levetiracetam to synaptic vesicle protein 2A (SV2A) in human brain and in CHO cells expressing the human recombinant protein. Eur J Pharmacol 536:102–108

Janz R, Sudhof TC (1999) SV2C is a synaptic vesicle protein with an unusually restricted localization: anatomy of a synaptic vesicle protein family. Neuroscience 94:1279–1290

Janz R, Goda Y, Geppert M, Missler M, Sudhof TC (1999) SV2A and SV2B function as redundant Ca^{2+} regulators in neurotransmitter release. Neuron 24:1003–1016

Kwan P, Brodie MJ (2000) Early identification of refractory epilepsy. N Engl J Med 342:314–319

Lynch BA, Lambeng N, Nocka K et al (2004) The synaptic vesicle protein SV2A is the binding site for the antiepileptic drug levetiracetam. Proc Natl Acad Sci 101:9861–9866

Regesta G, Tanganelli P (1999) Clinical aspects and biological bases of drug-resistant epilepsies. Epilepsy Res 34:109–122

Rogawski MA, Loscher W (2004) The neurobiology of antiepileptic drugs. Nat Rev Neurosci 5:553–564

Scheuer ML, Pedley TA (1990) The evaluation and treatment of seizures. N Engl J Med 323:1468–1474

Schmidt D, Loscher W (2005) Drug resistance in epilepsy: putative neurobiologic and clinical mechanisms. Epilepsia 46:858–877

Sisodiya SM (2003) Mechanisms of antiepileptic drug resistance. Curr Opin Neurol 16:197–201

Toering ST, Boer K, de Groot M et al (2009) Expression patterns of synaptic vesicle protein 2A in focal cortical dysplasia and TSC-cortical tubers. Epilepsia 50(6):1409–1418

van Vliet EA, Aronica E, Redeker S, Boer K, Gorter JA (2009) Decreased expression of synaptic vesicle protein 2A, the binding site for levetiracetam, during epileptogenesis and chronic epilepsy. Epilepsia 50(3):422–33

von Rosenstiel P (2007) Brivaracetam (UCB 34714). Neurotherapeutics 4:84–87

Xiao Z, Gong Y, Wang X-F et al (2009) Altered expression of synaptotagmin I in temporal lobe tissue of patients with refractory epilepsy. J Mol Neurosci 38(2):193–200