Altered vesicular glutamate transporter expression in human temporal lobe epilepsy with hippocampal sclerosis

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Altered vesicular glutamate transporter expression in human temporal lobe epilepsy with hippocampal sclerosis

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HIGHLIGHTS

- VGLUTs mRNA remains unaffected in human sclerotic hippocampus ILAE type1.
- Unaltered total VGLUT1 protein level in hippocampus of human TLE with HS ILAE type1.
- Decreased VGLUT2 protein expression in hippocampus of human TLE with HS ILAE type1.
- Increased VGLUT3 protein expression in hippocampus of human TLE with HS ILAE type1.

ARTICLE INFO

ABSTRACT

Vesicular glutamate transporters (VGLUTs) are responsible for loading glutamate into synaptic vesicles. Altered VGLUT protein expression has been suggested to affect quantal size and glutamate release under both physiological and pathological conditions. In this study, we investigated mRNA and protein expression levels of the three VGLUT subtypes in hippocampal tissue of patients suffering from temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS), International League Against Epilepsy type1 (ILAE type1) compared to autopsy controls, using quantitative polymerase chain reaction and semi-quantitative western blotting.

mRNA expression levels of the VGLUTs are unaffected in hippocampal epileptic tissue compared to autopsy controls. At the protein level, VGLUT1 expression remains unaltered, while VGLUT2 is significantly decreased and VGLUT3 protein is significantly increased in hippocampal biopsies from TLE patients compared to controls. Our findings at the protein level can be explained by previously described histopathological changes observed in HS.

Although VGLUTs have been repeatedly investigated in distinct rodent epilepsy models, their expression levels were hitherto not fully unraveled in the most difficult-to-treat form of epilepsy: TLE with HS ILAE type1. We here, demonstrate for the first time that VGLUT2 protein expression is significantly decreased and VGLUT3 protein is significantly increased in the hippocampus of patients suffering from TLE with HS ILAE type1 compared to autopsy controls.

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1. Introduction

The vesicular glutamate transporters (VGLUTs) mediate the import of glutamate, the most abundant and major excitatory neurotransmitter of the brain, into synaptic vesicles. In response to neuronal activity, glutamate is released into the synaptic cleft by calcium (Ca^{2+})-dependent exocytosis [31].

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Three VGLUT subtypes have been molecularly identified and functionally characterized in mammals; VGLUT1-3, all members of the solute carrier 17 (*SLC17*) gene family [1,6,7,8,9,13,22,31]. Recently, van der Hel et al. [33] determined VGLUT1 and VGLUT2 (but not VGLUT3) distribution in human hippocampus. The highest VGLUT1-IR was observed in glutamatergic terminals in the subiculum and stratum pyramidale of all hippocampal cornu ammonis (CA) fields. Contrary to VGLUT1, no VGLUT2-immunoreactivity (IR) was detected in human hippocampal tissue using immunohistochemistry. However, in rat hippocampus examined in the same study, VGLUT2-IR was very low but detectable in glutamatergic afferents to the dentate gyrus (DG) [33], as described in literature [13]. Finally, VGLUT3 displays a complementary distribution to VGLUT1 and VGLUT2 in rat hippocampus, with the highest expression levels found in the pyramidal and granular cell layers of CA2 and CA3 [10].

Changes in expression levels of VGLUTs have been observed to modulate the efficacy of glutamatergic neurotransmission and contribute to presynaptic plasticity. Overexpression of VGLUTs, for example, increases the amount of glutamate released per vesicle. Subsequently, altered VGLUT expression can affect quantal size and glutamate release under both physiological and pathological conditions [6,26,31,35,36]. Excessive glutamatergic neurotransmission and subsequent glutamate excitotoxicity have been observed in various neurological diseases, including temporal lobe epilepsy (TLE) [31].

TLE is characterized by spontaneous, recurrent seizures (SRS), caused by abnormal synchronized, high frequency neuronal discharges with hippocampal sclerosis (HS), being the most common neuropathological co-morbidity in patients undergoing surgery for intractable TLE [32]. In 60–80% of all TLE–HS cases, HS International League Against Epilepsy (ILAE) type1 is observed. This type of HS is marked by significant neuronal cell loss and gliosis predominantly pronounced in CA1 and CA4, with more variable damage to CA3 and DG subregions and sparing of CA2 [3,32].

Several studies have investigated, VGLUT1 and VGLUT2 mRNA and/or protein levels in distinct animal models of epilepsy (for review see [34]). In addition, the involvement of VGLUT2 and VGLUT3 in, respectively, generalized and absence seizures has been proposed [23,25]. Only recently, the distribution and expression of VGLUT1 were described in patients suffering from TLE with HS ILAE type1 and without HS [33].

In this study, we investigated for the first time the hippocampal expression levels of the three VGLUT subtypes in human tissue resected from patients with TLE and HS ILAE type1, compared to autopsy controls with no history of seizures or neurological disorders.

2. Material and methods

2.1. Human samples

The human cases investigated in this study, were obtained from the files of the Departments of Neuropathology of the Academic Medical Center (AMC, University of Amsterdam) and the VU Medical Center (VUMC). Patients (*n* = 5) underwent resection of the complete hippocampus for medically intractable TLE and were neuropathologically diagnosed to have HS ILAE type1 [3]. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. All samples were obtained and used in a manner compliant with the declaration of Helsinki. Control hippocampal tissue was obtained at autopsy from persons without a history of seizures or neurological diseases (*n* = 5). All autopsies were performed within 12 h after death. There were no significant differences in sex distribution or age between the two groups (Supplementary Table S2 in [17]). Autopsy hippocampal tissue from controls and surgically resected hippocampal tissue from

patients with TLE and HS were snap frozen in liquid nitrogen and stored at −80 °C, until further use in qPCR and western blotting experiments.

2.2. Quantitative polymerase chain reaction

Total RNA was isolated from human hippocampal cryosections using the TRIzol® (Life Technologies™) extraction method. Between 4 and 15 mg of tissue was collected from cryosections of each human sample, yielding between 0.11 and 3.2 ug of total RNA. The Taqman reverse transcription kit (Life Technologies™) was used to generate cDNA. VGLUT1 and VGLUT2 gene expression was analyzed using SYBR® Green Real-Time-PCR Master Mix (Applied Biosystems®, Life Technologies™) and primers designed using the Primer Express 3 Software (Applied Biosystems®, Life Technologies TM): VGLUT1 (forward 5′-GGTGCAGAAAGCCCAGTTCA-3′, reverse 5′-GAGTGACAATGTAGCCCCAGAAA-3′), VGLUT2 (forward 5′-AGAGAGGAGTAGACTGGCAACCA-3′, reverse 5′-GCCAGCTAAAGGCATTGCA-3′) and GAPDH (forward 5′-TGCACCACCAACTGCTTAGC-3′, reverse 5′-GGCATGGACTGTGGTCATGAG-3′). All primer sets had efficiencies between 98% and 100% over 5 log changes, and amplified a single band as determined by melt analysis. For VGLUT3, self-designed primer sets did not meet specifications and so RNA expression was assessed using predesigned TaqMan® (Life Technologies™) expression assays for VGLUT3 (Hs00900423_m1; Life Technolgies™) and GAPDH (Hs02758991_g1; Life Technologies™). VGLUT gene expression was calculated as fold expression and normalized to reference gene GAPDH. Standard Life-Technologies, Real-Time Master Mix cycling conditions were used. No amplification was detectable in the no-template (water) controls or in controls, where the reverse transcriptase was omitted during cDNA generation. All samples were analyzed in duplicate.

2.3. Western blotting

Protein extraction and blotting was conducted as described previously [19]. Primary antibodies comprised guinea pig antibody to VGLUT1 (1:7500; AB5905; Millipore), mouse antibody to VGLUT2 (1:500; MAB5504; Millipore), guinea pig antibody to VGLUT3 (1:500; AB5421; Millipore) and mouse antibody to β-actin (1:8000; A3853; Sigma–Aldrich). Immunoreactive bands were visualized using ImageQuant LAS 4000 imager (GE Healthcare). Optical densities of protein bands were normalized to the density of β-actin bands visualized on the same membrane. Western blotting experiments were done in triplicate.

2.4. Statistics

Statistical analysis was performed using GraphPad Prism 4.0 Software. The Mann–Whitney test was used for all experiments and the α value was set at 0.05.

3. Results

3.1. No changes in hippocampal VGLUT mRNA expression in TLE with HS

To quantify hippocampal VGLUTs mRNA levels, qPCR was performed on resected tissue from patients with HS and autopsy controls. Although a trend toward an increased VGLUT3 mRNA expression level could be observed, no significant differences in VGLUTs mRNA levels were detected between both groups (VGLUT1: $p = 0.690$; VGLUT2: $p = 0.841$; VGLUT3: $p = 0.095$) (Fig. 1C, F, and I).

3.2. Altered hippocampal VGLUT2 and VGLUT3 protein expression in TLE with HS

Hippocampal protein expression level of VGLUT1 was not different between both groups ($p = 0.690$) (Fig. 1A and B). VGLUT2 protein level was significantly decreased ($p = 0.032$) (Fig. 1D and E), whereas VGLUT3 protein expression was significantly increased in epileptic hippocampal tissue compared to autopsy controls ($p = 0.016$) (Fig. 1G and H).

4. Discussion

Although we could not detect any significant effect at mRNA level, significant alterations in VGLUT2 and VGLUT3 protein expression were observed in hippocampal tissue from patients suffering from TLE with HS ILAE type1 compared to autopsy control tissue. The discrepancy between VGLUT mRNA and protein expression levels might be explained by both intra-hippocampal post-transcriptional alterations and up- or downregulation of VGLUT mRNA in afferent, extra-hippocampal presynaptic neurons [33]. Indeed, afferents from the cortical and thalamic regions are, respectively, VGLUT1 and VGLUT2 positive [16]. As such, changes in VGLUT expression can be reflected in their projection region (i.e., hippocampus) at the protein level, but not necessarily at the mRNA level. This kind of discrepancy between protein and mRNA expression levels has already been described for VGLUT1

in both human and animal pathological tissue [4,11]. Noteworthy, a strong trend toward increased VGLUT3 mRNA expression levels is observed. Possibly significance is not reached due to the small sample size.

No difference could be detected in hippocampal VGLUT1 mRNA and protein levels between TLE tissue with HS and control tissue. The unaltered total hippocampal VGLUT1 expression level in patients suffering from TLE, is in contrast to previous observations of van der Hel et al. [33]. Possibly, this discrepancy results from a slight difference in severity of HS together with the fact that, we have used total hippocampus. Although the so-called classical and severe cell loss patterns in HS [32], are now internationally classified as HS ILAE type1 [3], the hippocampal sclerotic tissue used by Wyler et al. was, at that time, diagnosed and classified as the most severe form, Wyler grade 4 [37]. A significant decrease of VGLUT1 protein was described in subfields with neuronal loss and a significant increase in the DG, marked by mossy fiber sprouting [33]. In our hippocampal homogenates from patients, diagnosed and classified as the classical form, Wyler grade 3 [17,37], the unaltered hippocampal VGLUT1 protein expression could be the result of increased VGLUT1 expression in the DG that compensates the more moderate loss of VGLUT1 in other hippocampal subregions.

Our results, showing a decreased protein expression level of VGLUT2 in patients with TLE and HS, are in line with the loss of mossy cells of the ventral DG observed in both epileptic animals [14,29] as well as in patients suffering from mesial temporal lobe
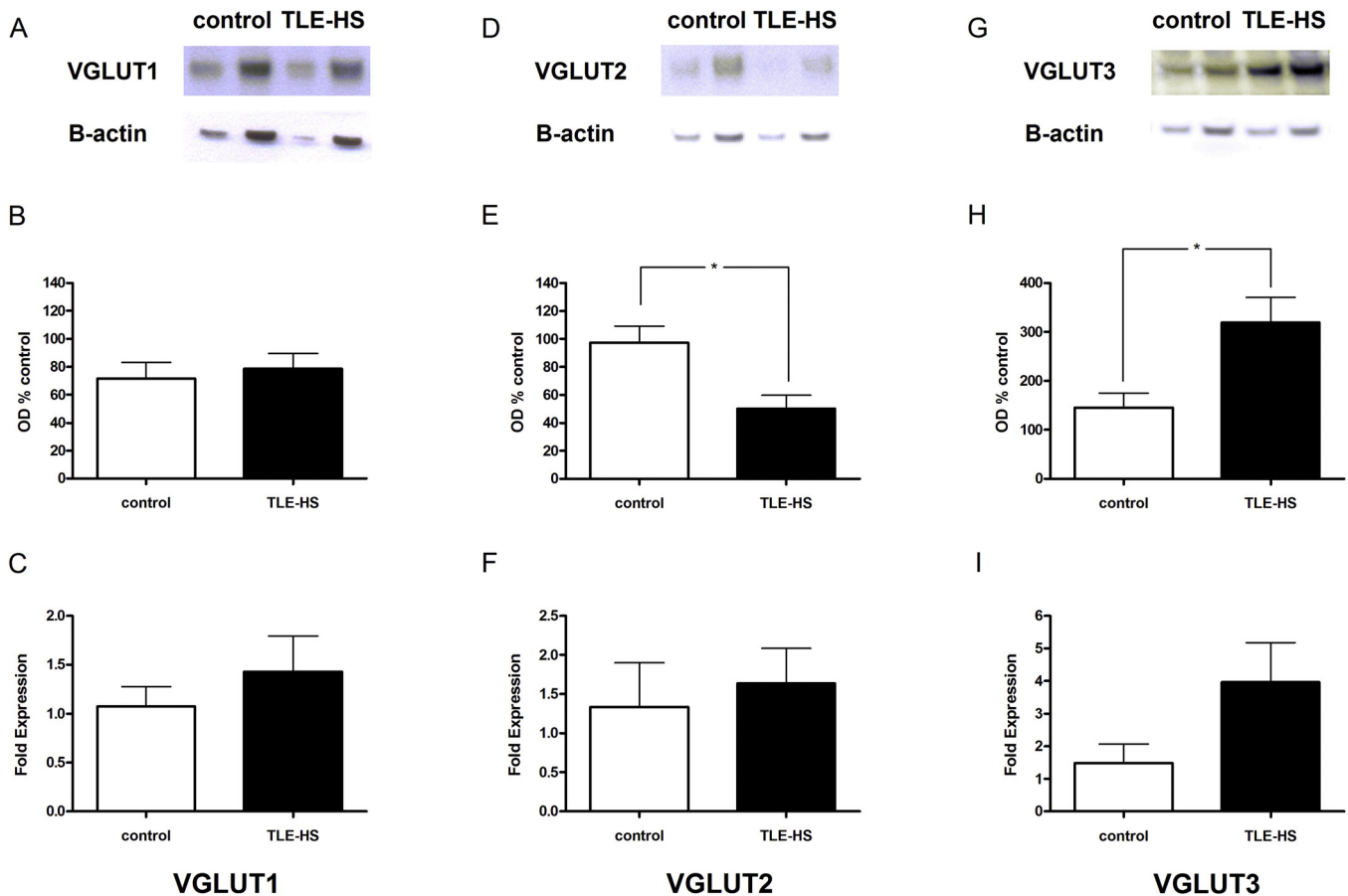

Fig. 1. Quantification of vesicular glutamate transporter (VGLUT) expression levels by qPCR and western blotting in hippocampus of temporal lobe epilepsy (TLE) patients with hippocampal sclerosis (HS) International League Against Epilepsy type1 (ILAE type1) compared to autopsy controls (A, D, and G). A representative example of a western blotting experiment (2 concentrations of each sample were loaded; lane 1–2: control samples; lane 3–4: TLE–HS samples). Upper bands correspond to (A) VGLUT1 (~55 kDa), (D) VGLUT2 (~55 kDa), (G) VGLUT3 (~55 kDa), and lower bands to β-actin (~42 kDa) (B, E, and H). Protein expression levels of (B) VGLUT1, (E) VGLUT2 and (H) VGLUT3 in human hippocampus of autopsy controls ($n = 5$) and patients with HS ($n = 5$). Bars represent relative optical density ±SEM as measured from a western blotting experiment, normalized to β-actin. Mann–Whitney test, *$p < 0.05$ (C, F, I). Hippocampal (C) VGLUT1, (F) VGLUT2 and (I) VGLUT3 mRNA expression levels of patients with HS ($n = 5$) compared to autopsy controls ($n = 5$). Bars represent fold expression ±SEM, normalized to GAPDH.

epilepsy [2]. This mossy cell degeneration and concomitant loss of the excitatory VGLUT2-rich synapses on translamellar interneurons ("basket cells") could make these inhibitory interneurons hypoactive ("dormant"), resulting in granule cell hyperexcitability and a seizure-prone dentate network [12,27,28]. Moreover, we previously showed that VGLUT2$^{+/-}$ mice, compared to their wild-type littermates, are more susceptible to generalized seizures induced by pentylenetetrazole [23], suggesting that, the loss of VGLUT2 might possibly play a role in recurrent seizure generation. Recently, increased hippocampal excitability was also observed in conditional VGLUT2 knockout mice [20].

The process of glial activation and proliferation in all CA regions of sclerotic hippocampi might be a possible explanation for the observed increase in VGLUT3 protein levels. In rat hippocampus, VGLUT3 is found in astrocytes and postulated to be crucial for Ca^{2+}-dependent astroglial glutamate exocytosis [21]. Moreover, intracellular Ca^{2+}-release and Ca^{2+}-oscillations were observed in sclerotic hippocampal astrocytes, along with upregulation of the synaptic vesicle protein SNAP23. In addition to two other astrocytic targets currently investigated in new anti-epileptic drug development, i.e., aquaporin 4 (AQP4) and inwardly rectifying potassium (Kir) channels [5], VGLUT3 might play a major role in the sustained hyperexcitability of the seizure focus. Interestingly, we recently showed increased xCT levels in the same human sclerotic hippocampal samples compared to autopsy controls [17]. Moreover, we already reported the joint upregulation of xCT and VGLUT3 in the cortex of 18-month-old AβPP23 mice, a model for Alzheimer's disease marked by plaque formation and gliosis, coupled with a strong tendency toward increased cortical extracellular glutamate levels [24]. These findings suggest an important role of both glial transporters in neurological diseases involving gliosis and glutamate excitotoxicity.

Noteworthy, Li et al. did not observe expression of VGLUT3 by gray matter protoplasmic astrocytes in mouse hippocampus [18]. This contradictory finding is well founded with experiments on VGLUT3 knockout mouse tissue, however, extrapolation to other species requires caution. Indeed, considerable species differences in the hippocampal expression of VGLUT2 mRNA have been observed in the past [16]. Nonetheless, the increased VGLUT3 protein levels observed in the human samples of TLE HS type1 will not solely depend on VGLUT3 expression in hippocampal GABAergic interneurons [30], as these neurons are severely reduced in sclerotic hippocampi [5]. However, since the expression of VGLUT3 in distinct subcortical projection regions to the hippocampus, such as septohippocampal, mesolimbic dopaminergic and raphe serotonergic neurons is still matter of debate [16], it remains impossible to draw unambiguous conclusions concerning the role of VGLUT3 in hippocampal sclerosis.

Collectively, additional evidence has been found to support a possible involvement of VGLUTs in TLE, but still more and better experimental tools, such as specific inhibitors, are required (for review see [34]). Finally, it should be mentioned that antiepileptic drug treatment might affect VGLUT expression. Treatment with valproate and riluzole has been observed to reduce VGLUT1-IR in a chronic limbic and absence seizure rat model [15]. In our study, patients treated with valproate did not display aberrant VGLUT1 protein levels. In addition, these patients suffered from seizures for many years. Subsequently, it is hard to speculate whether or not altered VGLUT expression might be cause or consequence of the disease. However, these limitations are inevitable when working with human epileptic tissue.

Conflicts of interest

None of the authors has any conflict of interest to disclose.

Acknowledgements

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