Hippocampal Characteristics and Invariant Sequence Elements Distribution of *GLRA2* and *GLRA3* C-to-U Editing

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Key Words

Glycine receptor alpha subunit · GlyR · Mooring · RNA editing · Spacer · Temporal lobe epilepsy

Abstract

Glycine receptor $\alpha 2$ and $\alpha 3$ subunit (*GLRA2/GLRA3*) high-affinity variants, of which the subjacent amino acid substitutions issue from C-to-U RNA editing, are thought to influence tonic inhibition and pathophysiology. In light of the detection of *GLRA3* NM_006529:r.1157C>U and *GLRA2* NM_002063:r.1416C>U exchanges in hippocampus explants of temporal lobe epilepsy patients, we now examine the healthy situation and relate it to the epileptic situation by ascertaining controls in a legitimate reanalysis. The *GLRA2* and *GLRA3* editing events that would ultimately result in a glycine receptor with increased affinity occur in the post-mortem nonepileptic hippocampus. Most notably, their relative amounts do not significantly differ from those in increased damaged hippocampus explants, whereas curbed relative amounts in epileptic explants without cell loss come out statistically significant. Local sequence alignment reveals invariant sequence stretches consistent in *GLRA2/GLRA3* and other edited transcripts that coincide with known *APOB* sequence elements. Concerning the essential mooring element, *GLRA2/GLRA3* comply strictly only with the motif's 5' part. While this lack of canonical mooring elements and

uncertain action of the famous deaminase APOBEC1 suggest a specific regulation of *GLRA2/GLRA3* editing, its reduction in the less-damaged epileptic hippocampus could be attributed to anomalous epileptic neurogenesis.

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Epilepsy, a major neurological disorder, is associated with a large number of syndromes all involving unpredictable seizures. About 36% of epileptic patients have inborn or acquired resistances to antiepileptic pharmaceuticals. Neurosurgery of an identified epileptic focus can provide remedy from seizures for highly affected patients, but novel medications are still sought for [Kwan and Brodie, 2000; Mohanraj and Brodie, 2006; Wood, 2012].

Typically, epileptogenic activities at the hippocampus would be balanced by its inhibitory neurons. A disruption of hippocampus interneurons can contribute to excitatory-inhibitory imbalance within the epileptic network [Sloviter, 1987; Nadler, 2003; Fröhlich et al., 2006; Reyes, 2006; Dudek and Sutula, 2007]. The glycine receptor (GlyR) is an inhibitory ligand-gated ion channel, like the GABA_A receptor, although less abundant. It consists of an extracellular ligand-binding domain, a transmembrane chloride pore, formed by a pentameric setup of ligand-binding alpha subunits (*GLRA1*, *GLRA2*, *GLRA3*, also referred to as: GlyR $\alpha 1$, $\alpha 2$, $\alpha 3$) with the structuring beta

subunit, and a rather variable intracellular domain [Langosch et al., 1990; Betz et al., 1999; Chattipakorn and McMahon, 2002; Keck et al., 2008]. GlyR is located inter alia in the hippocampus, in interneurons, at synaptic sites as well as extrasynaptically [Avila et al., 2013]. Modulation of excitatory postsynaptic potentials and inhibition in GABAergic interneurons via GlyR [Song et al., 2006] contributes to glycinergic tonic inhibition [Zhang et al., 2008; Xu and Gong, 2010].

Previously, a variant cDNA of GLRA3 (*Gla3* NM_053724:r.701C>U) that encodes a certain proline-to-leucine exchange (GLRA3 NP_446176:p.Pro234Leu; colloquially referred to as: GlyR α 3 P185L) was described. The exchange alters a loop at the bottom of the ligand-binding domain adjacent to the brink of the pore. The variant subunit displays an increased apparent affinity for its ligand, likely enabling a high-affinity GlyR that responds to ambient glycine [Sherwin, 1999; Meier et al., 2005; Ogino et al., 2007; Du et al., 2015]. The corresponding substitution *Gla2* NM_012568:r.1207C>U causes another high-affinity alpha subunit (GLRA2 NP_036700:p.Pro219Leu; colloquially referred to as: GlyR α 2 P192L). In an analysis of hippocampus explants of pharmacoresistant temporal lobe epilepsy (TLE) patients, it was reported that the relative amounts of the same variations in humans, *GLRA2* NM_002063:r.1416C>U and *GLRA3* NM_006529:r.1157C>U (referred to as: GlyR α 2 C575U and GlyR α 3 C554U), increase under severe sclerotic cell loss and secondary generalized tonic-clonic seizure (grand mal) frequency. However, no information on the nonepileptic state was given. Genomic *GLRA2* DNA of patients carried no corresponding point mutation or single nucleotide polymorphism (SNP) [Eichler et al., 2008]. This supported the assumption of C-to-U RNA editing being responsible for the variants' origin, deduced from a sequence stretch similar to a spacer sequence downstream of *Gla3* NM_053724:r.701C>U [Meier et al., 2005].

In C-to-U-edited apolipoprotein B mRNA (*APOB*), the spacer and the mooring sequence elements are constituents of a stem loop that exposes a cytidine to enzymatic deamination. Several additional sequence elements, most important for *APOB* editing, flank the deaminated position from 60 nucleotides upstream to 160 nucleotides downstream [Shah et al., 1991; Backus and Smith, 1992; Driscoll et al., 1993; Hersberger and Innerarity, 1998; Sowden et al., 1998; Hersberger et al., 1999; Maris et al., 2005]. Two prominent proteins bind the *APOB* transcript: the cytidine deaminase apolipoprotein B editing complex (APOBEC1) and the RNA-binding protein APOBEC complementary factor. In the liver,

APOBEC1 deaminates a cytidine residue in the *APOB* transcript to a uridine residue, resulting in 2 protein variants, both physiologically relevant in energy metabolism [Teng et al., 1993; Chester et al., 2000; Lellek et al., 2000; Mehta et al., 2000; Henderson et al., 2001]. Until recently, few further C-to-U-edited transcripts had been characterized [Skuse et al., 1996; Wang et al., 2004]. Nowadays, numerous murine targets of APOBEC1 have been described, leading to further characterization of the mooring motif [Rosenberg et al., 2011].

In our study, postmortem hippocampus material is used to display the healthy situation of C-to-U edited human *GLRA2* and *GLRA3*. By doing this, control groups are generated that augment the study mentioned above on epileptic hippocampus explants [Eichler et al., 2008]. Consequently, these results are presented alongside a legitimate reanalysis of the former series of experiments, adjusting the previous explanatory view on the relation of editing levels and course of disease. In addition, we describe the situation of several sequence elements and features most crucial to RNA editing in this matter.

Materials and Methods

Quantitative Cloning Analysis

Postmortem hippocampus tissue was obtained at autopsy from 3 individuals whose deaths had occurred out of hospital and were attributable to different natural and unnatural causes. Pre-existing pathological conditions of the central nervous system were ruled out by histology, and underlying intoxications were ruled out by toxicological analysis. Specimens were comparable to the TLE neurosurgery explant series in terms of their local origin, age, and fixationless procession [Eichler et al., 2008], except for their postmortem delays that exceeded the surgery explants for up to 5 h. RNA was isolated with Trizol (Thermo Fisher, Waltham, MA, USA). Additionally, a commercially available pooled hippocampus RNA (provided by R.J. Harvey, London) was used [Rees et al., 2006]. Cloning assays to quantify *GLRA2* NM_002063:r.1416C>U and *GLRA3* NM_006529:r.1157C>U were performed in the identical setup described previously [Meier et al., 2005; Eichler et al., 2008]. Briefly, PCR used degenerated mismatch primers that introduce restriction sites only into templates with the respective nucleotide exchanges. After restriction endonuclease digestion, insertion into custom vectors, transformation, and determination of confirmed-positive colony numbers, the percentage of clones with a variant insert proportionate to the total amount of control clones reflected the relative amount of edited *GLRA2/GLRA3* mRNA for each sample.

Instead of averaging triplicate measurements for one specimen and grouping the averages, all individual data points of each condition were grouped collectively. In postmortem *GLRA2* measurements, 13/15 individual data points represent the 3 matched individuals, while 2/15 data points represent the 20 pooled individuals. In the postmortem *GLRA3*, 6/8 data points represent the 3 matched

Fig. 1. The occurrence of *GLRA2/GLRA3* C-to-U RNA editing in the healthy postmortem hippocampus is compared with reanalyzed corresponding data obtained from surgical explants of pharmacoresistant epilepsy patients [Eichler et al., 2008]. The relative amounts of edited *GLRA2/GLRA3* (% edited) in the healthy post-mortem hippocampus versus the epileptic situation with increased damage (attributable to sclerotic cell loss and frequent grand mal) do not differ significantly. By contrast, the low relative amounts in epileptic explants without hippocampal cell loss and marked sclerosis are significant.

individuals, while 2/8 data points represent the 20 pooled individuals. The approach allowed reanalyzing the dataset from formerly examined series of epilepsy surgery explants from pharmacoresistant TLE [Eichler et al., 2008], also including several specimens that had not yielded triplicate data. For comparability, general data grouping was retained. Seven individuals of the group of patients' explants without marked hippocampal cell loss and sclerosis are represented by 29 data points in *GLRA2* measurements and by 18 data points in *GLRA3*. In the group with increased damages attributable to sclerosis, cell loss, and frequent grand mal, the 18 individuals are represented by 57 data points in *GLRA2* measurements and by 53 data points in *GLRA3*. The Mann-Whitney test was performed with OriginPro 8G (OriginLab, Northampton, MA, USA), with a *p* value less than 0.05 considered as statistically significant.

Database Coexpression Approach

Conserved domain database entries [Marchler-Bauer et al., 2015] with cytidine deaminase domains were examined for coexpression with GlyR in the hippocampus. GeneAtlas datasets in BioGPS gene reports were used to check for human brain expression of any genes of interest [Wu et al., 2009]. Murine in situ hybridization (ISH) data publicly available from the Allen Brain Atlas [Lein et al., 2007] were used to compare the hippocampal expression patterns of the candidate deaminase *CDADC1* as well as *GLRA2* and *GLRA3*. *GLRA4* expression data served as negative control. Of each set of images (antisense probe, sagittal plane, experiment numbers: 70525807, *CDADC1*; 71587739, *GLRA2*; 70723453, *GLRA3*; 70724744, *GLRA4*) consisting of 19–20 sections, approximately the sixth (for the parasagittal plane) and thirteenth section (for the midsagittal plane) were selected, depending on pattern/plane visibility. To visualize the expression data in grayscale, the ISH and expression layers were transferred into the GIMP 2.8 raster graphics editor. Relevant expression domains were cropped. ISH layers were grayscaled, and brightness was set to +50. The expression layers were inverted; the white color was replaced with transparent color, and all remaining colors were changed to black. Finally, both layers were merged.

Sequence Comparison

Nucleotide sequences of C-to-U-edited transcripts were traced from their respective GenBank entries. Sequence conservation not revealed by conventional comparison matrices was exposed via manual alignment with GeneDoc [Nicholas and Nicholas, 1997]. The hyphens indicate introduced gaps. The shading mode was set to conserved; primary conservation (black shading, uppercase consensus line) was set to 100%; secondary conservation (dark gray shading, lowercase consensus line) was set to 75%, and tertiary conservation (light gray shading, not marked in consensus line) was set to 40%.

Results

GLRA2 and GLRA3 Editing in the Hippocampus

Pronounced relative amounts of the C-to-U editing events *GLRA2* NM_002063:r.1416C>U and *GLRA3* NM_006529:r.1157C>U occur in postmortem hippocampus RNA (Fig. 1), detected via quantitative cloning assays targeting the respective cDNA nucleotide exchanges.

Conjoint with equivalently reanalyzed epilepsy surgery explants, the postmortem series serves as nonepileptic controls. These data show comparatively smaller relative amounts of edited *GLRA2/GLRA3* in explants without hippocampal cell loss than in both the postmortem control group and the group of explants with marked cell loss and grand mal frequency. The relative amounts of the 2 latter groups do not show significant differences (see n.s. in Fig. 1). The low of editing in the former group is statistically significant (asterisks in Fig. 1), attenuated in *GLRA2*, and more intensive in *GLRA3*.

Consistent Stretches of Invariant Nucleotides, Shared in GLRA2 and GLRA3 and Other C-to-U-Edited Transcripts

Using manual alignment of sequence subsections flanking the edited nucleotide, consistent sequence stretches of invariant and highly invariant nucleotides, shared between *GLRA2/GLRA3* as well as in other C-to-U-edited transcripts and distributed in an interlaced pattern, were identified (Fig. 2a: viewed from 5' to 3', these consistent stretches appear in the "A....ug.....AA.....UG" stretch located at marker position 1–23, the "cAa.....UG....a" stretch located at position 48–66, the "Ga.u.a" stretch at position 80–85, and the "C.....a.uu.....uga" stretch located around position 101–118), each formed of up to 7 shared nucleotides. Another, more weakly shared stretch, that is not consistent in each inspected transcript, is "c.ga..tata", located at alignment position 63–80. Within the consistent stretches, variant or unique nucleotides appear, up to 5 or 9 in number. Outside of the consistent stretches, variant and unique nucleotides are present more often and in longer succession (see alignment positions 24–47, 86–100, and 130–154 in Fig. 2a).

The consistencies agree with sequence elements that have been demonstrated to be highly relevant for *APOB* editing: the "A....ug.....AA.....UG" stretch resides directly at the 5' border of the upstream A-rich element determined in *APOB*; the "Ga.u.a" stretch encloses the regulator sequence element; the "C.....a.uu.....uga" stretch comprises the well-known spacer and, in parts, the mooring element. *GLRA2* and *GLRA3* transcript sequences lack most of the 3' and mid parts of the mooring motif, and they supplant it at position 120–124. In the 5' part, they conform very well to the motif. Downstream of the moor-

Fig. 2. a Invariant nucleotides of *GLRA2/GLRA3* shared with other C-to-U edited transcripts agree with known sequence elements. Rectangular bars indicate consistent stretches of invariant nucleotides. Hatched bars indicate weakly shared stretches, which are not consistent with all inspected transcripts (main diagonal hatching for consistencies in *GLRA2/GLRA3* and few other transcripts, but not with *APOB*; anti-diagonal hatching indicates consistencies in *APOB* and other transcripts, but not with *GLRA2/GLRA3*, and checkered hatching for consistencies in *GLRA2/GLRA3* and *APOB*, but not with every single one of the others). *APOB* sequence elements [Shah et al., 1991; Backus and Smith, 1992; Driscoll et al., 1993; Hersberger and Innerarity, 1998; Sowden et al., 1998; Hersberger et al., 1999] are indicated with lines at the bottom; mooring elements are indicated with hooked lines. Transcript prefixes indicate species. Deaminated cytidine at alignment position 100. **b** The 3' UTR of rat *Gla3* (genebank entry M55250) contains a sequence nearly identical to the mooring element of *Apob*. Motif sequence from Rosenberg et al. [2011].

ing, another A-rich stretch is shared in all inspected transcripts. The 3' untranslated region (UTR) of rat *Gla3* features an almost exact mooring sequence located 1,121 nucleotides downstream of the edited cytidine (Fig. 2b).

Concurrent Features of CDADC1

Apparent from database queries, CDADC1 possesses 2 cl00269 cytidine deaminase-like superfamily domains that resemble the catalytic domain of APOBEC1 (Fig. 3a, b). In murine hippocampus, there are overlaps in the gene expression patterns of *Cdad1* in the dentate gyrus and in CA1-CA3 with those of *Gla2* as well as in hilus with *Gla3* (Fig. 4).

Discussion

Lately, one could follow a controversial debate on the physiologic extent of RNA editing, once again emphasizing the high level of detail necessary for its examination [Chakravarti, 2011; Li et al., 2011; Kleinman and Majewski, 2012; Wang et al., 2014]. Also the case of C-to-U-editing of GlyR alpha subunit RNA deserves thorough interpretation. After the original description, another study fit quite well to the feasible accuracy of detecting *Gla3* NM_053724:r.701C>U, whereas it was omitted to detect native edited *Gla3* transcripts [Meier et al., 2005; Nakae

et al., 2008]. Later on, it was assumed that increased levels of editing were a specific attribute of the diseased epileptic state [Eichler et al., 2008; Winkelmann et al., 2014].

For the first time, this study describes distinct *GLRA2* NM_002063:r.1416C>U and *GLRA3* NM_006529:r.1157C>U RNA editing events in the healthy hippocampus using heterogenous postmortem material. This finding enabled our legitimate reanalysis of the previous series of experiments with the postmortem material integrated as necessary nonepileptic controls, showing the particular decline of the relative amounts of edited *GLRA2* and *GLRA3* in the epileptic hippocampus with lesser degrees of damage. This is a relevant result and refines accurate assessment of how epilepsy damages and editing levels are associated in pharmacoresistant TLE.

Ectopic granule neurons or anomalous latency phase neurogenesis [Parent et al., 1997, 2006; Scharfman and Pierce, 2012] may provide one explanation for the pathological decrease in editing and its different extent in the 2 subunit transcripts. Newly generated cells in the epileptic hippocampus can arise from more than one source area and can migrate in varying amounts towards DG and CA1/CA2 (the main expression domains of *Gla2*) as well as towards CA4 (the main region of *Gla3* gene expression) [Crespel et al., 2005]. In general, young neurons show less GlyR gene expression [Aroeira et al., 2011], and rodent prenatal hippocampal neurons seldom display

Fig. 3. a Domain structure of CDADC1 compared with those of 2 paradigmatic Apobec family members. CDADC1 contains 2 domains that match the zinc-binding region of the cl00269 cytidine deaminase-like superfamily, to which the catalytic domain of the C-to-U deaminase APOBEC1 also belongs. Modified after Marchler-Bauer et al. [2015]. **b** Sequence alignment of the similarities between CDADC1 and APOBEC1, within the cl00269 superfamily of cytidine and deoxycytidylate deaminases. The 2 deaminase domains of CDADC1 are abbreviated as dom1 and dom2. cd01286, deoxycytidylate (dCMP) deaminase domain; pfam08210, APOBEC-like N-terminal (catalytic) domain; cd01283, cytidine deaminase zinc-binding domain. Modified after Marchler-Bauer et al. [2015].

Fig. 4. Murine hippocampus gene expression patterns of *Cdadc1* notably overlap with those of *Gla2* in pyramidal layer of CA1–3 and in granule cell layer of dentate gyrus, with those of *Gla3* in hilus or polymorph layer of dentate gyrus. *Gla4* as negative control. Scale bars, parasagittal 500 μ m; midsagittal 1000 μ m. Modified after Lein et al. [2007].

pronounced *Gla2/Gla3* C-to-U events (unpubl. observation). Accordingly, a “dilution” of hippocampal cells by aberrant immature cells could be one possible cause of the apparent drop in editing. Sclerotic cell loss [Zhu et al., 1997; Blümcke et al., 2000, 2009] in the increased damaged explants group is not accompanied by significant reductions of C-to-U levels, compared to the nonepileptic postmortem situation, yet they could still play a role in the very slight reduction visible in this group.

Detecting editing events in PCR amplicons does not directly prove the existence of functional high-affinity

GlyR, but it tracks the activity of its deaminase, whose identity is another open problem. We present properties of CDADC1 that resemble those of the important cytidine deaminase APOBEC1 and of the concerned GLRA subunits in the hippocampus. In the brain, gene expression of *APOBEC1* is relatively meagre (publicly available microarray data), and recent studies reported only very vaguely on its native expression in brain tissue [Gee et al., 2011; Guo et al., 2011; Papavasiliou et al., 2014]. Despite this apparent absence, silencing of glycinergic currents following steric inhibition of Apobec cytidine deaminas-

es with a cytidine analogue had been reported [Meier et al., 2005]. Although the drug indeed can affect APOBEC-family deaminases, it should be argued that administering the cytidine analogue without sequence context could have influenced channel gating in different ways [Rausch et al., 2009; Seifert et al., 2011; Wedekind and Smith, 2012; Zong et al., 2012].

To determine the extent of the common sequence basis of well-characterized APOBEC1-mediated C-to-U editing with that of *GLRA2/GLRA3*, manual alignment of various cDNA sequences with documented C-to-U editing events was performed. The consistent stretches of invariant nucleotides found accord with the upstream A-rich, the regulator, and the spacer elements, which are very well characterized in *ApoB* [Shah et al., 1991; Backus and Smith, 1992; Driscoll et al., 1993; Hersberger and Innerarity, 1998; Sowden et al., 1998; Hersberger et al., 1999]. The discovery of additional murine targets of APOBEC1 had led to further characterization of the mooring element [Rosenberg et al., 2011], but GlyR $\alpha 2$ and $\alpha 3$ transcript sequences fit this motif only in its 5' part (it should be noted that the seemingly merged spacer and mooring elements of *Serinc1* also deviate from the motif). Whether the conventional mooring in the 3' UTR of rat *Gla3* corresponds to a respective C-to-U event is not known. Many of the additional murine transcripts carry their edited positions and their mooring elements in the 3' UTR [Rosenberg et al., 2011], whereas in transcripts encoding *GLRA2/GLRA3*, the edited positions and their adjacent 3' deviant mooring elements are present in the open reading frame (ORF). A mooring sequence in a UTR can understandably comply with the motif more

closely than in an ORF, where mandatory compliance to codon information restricts sequence variability.

In this regard, 2 known synonymous SNPs in the inspected sequence section of *GLRA3* should be mentioned: Rs140655344 shifts a glutamate codon from GAG to GAA, making the A-rich region more A-rich. Rs41279513 can appear in the spacer, changing a glutamine codon from CAG to CAA, creating a spacer element identical to that of *APOB*. While invariant nucleotides could participate in secondary structures that capacitate binding or efficacy of a deaminase complex [Hersberger et al., 1999; Maris et al., 2005], deviations from the established elements could possibly even condition an unusual deaminase setup.

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Statement of Ethics

Ethical approval for the conducted experiments was under Charité EA 1/142/05.

Disclosure Statement

The authors have no conflicts of interest to declare.

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