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Expression of the Hippocampal NMDA Receptor GluN1 Subunit and Its Splicing Isoforms in Schizophrenia: Postmortem Study

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Abstract There is accumulating evidence that disturbances in *N*-methyl-D-aspartate receptor (NMDA-R) functioning are associated with the pathogenesis of schizophrenia. To assess actual changes in the expression of the GluN1 subunit and its isoforms, we measured absolute differences in the levels of mRNA/protein for panGluN1 (eight isoforms altogether) as well as the mRNA individual isoforms in the postmortem left/right hippocampus of patients with schizophrenia in comparison with non-psychiatric subjects. There were no significant differences in the panGluN1 subunit mRNA expression, but the absolute left/right differences were much more pronounced in the patients with schizophrenia. Protein levels of the GluN1 subunit in the left hippocampus in male schizophrenic patients were lower than controls. The expression of the NR1-4b isoform was attenuated in the left, whereas the NR1-2b was reduced in the right hippocampus of schizophrenic patients. Isoforms associated with the efficiency of NMDA-induced gene expression and with phosphorylation occurred more commonly in schizophrenic hippocampi. In summary, our study suggests that NMDA-R hypofunction in schizophrenia might be selectively dependent on the dysregulation of GluN1 subunit expression, which exhibits a somewhat different expression in the left/right hippocampus of psychotic patients.

Keywords Schizophrenia · Hippocampus ·
GluN1 subunit of NMDA receptor · Splice variants ·
Laterality

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Introduction

Schizophrenia is a progressive brain disorder [1] with high etiological heterogeneity employing a set of candidate genes and their interactions with various pre- and perinatal risk factors [2]. Through clinical and biochemical observation, the strength of abnormal glutamatergic neurotransmission has been linked to the pathophysiology of this illness. Recent evidence suggests that the decreased functioning of the *N*-methyl-D-aspartate receptor (NMDA-R) is related to the etiology of schizophrenia.

At the molecular level, NMDA-Rs are heteromeric proteins consisting of two ubiquitous GluN1 subunits and two regulatory GluN2 subunits. The GluN1 subunit occupies the glycine binding site and the GluNR2A-D subunits provide the binding site with glutamate. The family of four GluN2 subunits arises from separate genes, whereas the GluN1 subunit is encoded by a single *GRIN1* gene that undergoes alternative splicing to generate eight different

splice variants (isoforms) that differ in function and regional distribution [3, 4]. Their differential expression throughout the human central nervous system suggests that NMDA-R composition is unique to each brain region [5, 6] with one of the highest receptor densities across the hippocampal formation.

Molecular cloning of the human GluN1 subunit has revealed that the human gene is composed of 21 exons [7, 8]. Exon 4 encodes 21 amino acid sequences in the N terminus domain (cassette N1), the expression of which makes NMDA-Rs less sensitive to proton inhibition, and modifies glycine binding, whereas exons 20 and 21 encode the C terminus domain (C1 and C2 cassettes, respectively). The C1 cassette is important in interactions with several intracellular proteins. It contains the major phosphorylation sites of NR1 [9]. Their functioning can be regulated by the phosphorylation of NMDA-Rs [10] and might play a role in many forms of synaptic plasticity [11]. By contrast, the C2 cassette participates in the NMDA-R insertion into the plasma membrane. In addition to the C2, the C2′ variant interacts with postsynaptic density protein [12].

All eight functionally different GluN1 subunit isoforms (Table 1) exhibit unbalanced distribution in autopsied human brain regions with a selective modification in pathologically vulnerable brain regions in schizophrenia [13, 14]. However, the studies aimed at the GluN1 mRNA and protein abnormalities in schizophrenic hippocampi arrived at inconsistent results [15–19]. This could have been caused, at least partially, by different splicing in the C terminus of the GluN1.

Previous studies have shown that oestrogen's mediated expression and activity changes in NMDA receptors in rats [20, 21]. Reduced circulating levels of oestrogen have also since been observed in both men and women with schizophrenia compared with controls, suggesting oestrogen neuropathology in schizophrenia [22]. Because the hippocampus is an oestrogen-sensitive brain region, we are interested in GluN1 gender comparisons. Changes in brain asymmetry have been observed in neurodevelopmental disorders such as schizophrenia [23], so the next factor of interest is the laterality of GluN1 expression. A left side decrease in the hippocampus has been reported in schizophrenic patients [15, 17] with a significant reduction in NMDA receptor binding on the left side taking place in vivo [24].

To further elucidate the hippocampal abnormalities of the NMDA receptor in schizophrenia, we will focus on the panGluN1 expression at both a protein and mRNA level. The second aim is to characterise the role of GluN1 alternative splicing in hippocampal NMDA changes. Thus, we will compare schizophrenic patient and control expression of panGluN1 as an mRNA/protein and splice variants of GluN1 as an mRNA in the hippocampus.

Subjects and Methods

Subjects and Hippocampal Tissue Sampling

A study was carried out on human postmortem tissue from subjects diagnosed with schizophrenia (DSM-IV; $n = 13$) and from deceased elderly persons ($n = 8$). For splice variant determinations of mRNA and the protein, tissue samples were collected from 30 and 31 subjects, respectively. All cases and controls were recruited from social care houses for the elderly or from chronic ill patients from Prague and neighbouring districts of central Bohemia. Most patients suffered from chronic or residual forms of schizophrenia, and there was a subgroup of patients who did not take antipsychotic medication for ∼2 months before death (Table 2). The control group consisted of donors who died without any history of neuropsychiatric disease (Table 2). There was no significant difference in age and postmortem intervals (PMIs) between both groups. Brain autopsies of accurately dissected left and right hippocampi frozen at −78°C generated recommendations for human autopsy brain tissue [25]. We used a fast-freeze protocol, and our samples were used for protein and RNA assessment. The tissue storage time at −78°C before assay did not differ between the schizophrenia and control group. We avoided repeated freezing and thawing because it is considered an important degradation factor for autopsy [25]. Because of the heterogeneity of the dorsal and ventral parts of the hippocampal formation (hippocampus proper and dentate gyrus), transversal tissue slices (∼50 mg wet weight) were dissected from the central parts of the frozen hippocampus proper. All experiments were in accordance with the declaration of Helsinki. The Ethical Committee of Prague Psychiatric Center approved this study.

Table 1 Nomenclature of GluN1 splice variants (isoforms)

Splice variants	Insert cassettes			
	N(4)	C1(20)	C2(21)	C2′(21)
NR1-1a	−	+	+	−
NR1-1b	+	+	+	−
NR1-2a	−	−	+	−
NR1-2b	+	−	+	−
NR1-3a	−	+	−	+
NR1-3b	+	+	−	+
NR1-4a	−	−	−	+
NR1-4b	+	−	−	+

Table 2 Summary of subject characteristics

Analysis	n	Sex	Age (years)	PMI (h)	Diagnosis	Medication/non
panGluN1						
RT-qPCR (±SEM)	21	11 M/10 F	66.9 (±3.26)	35.62 (±4.7)	8 CONT/13 SCZ	4 Med/9 non
GluN1 isoforms						
RT-qPCR (±SEM)	31	15 M/16 F	67.53 (±2.85)	30.7 (±4.14)	14 CONT/17 SCZ	6 Med/11 non
panGluN1						
WB (±SEM)	30	15 M/15 F	66.32 (±2.93)	29.29 (±4.01)	13 CONT/17 SCZ	7 Med/10 non

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WB, western blots; F, female; M, male; PMI, post-mortem interval; CONT, control group; SCZ, schizophrenia; med, antipsychotic medication before death; non, without antipsychotic medication for ~ 2 months before death

Quantitative Real Time-Polymerase Chain Reaction (RT-PCR) of the panGluN1 Subunit

RNA from frozen hippocampal samples was isolated using TriZol (Invitrogen, USA); cDNA was prepared using a Reverse-IT RTase Blend kit (Abgene, UK) according to the recommendations of the manufacturer. Based on the preliminary results, β_2-microglobulin (primers: For GAGGCT ATC CAGCGTACTCC; Rev GATAGAAAGACCAGTC CTTGCTG) was chosen as the most suitable reference gene for the human hippocampus, the expression of which remains stable throughout all tested samples (data not shown).

To ensure the reproducibility of the quantification, plasmid standards were prepared using a standard T/A cloning approach. As the cloning vector, pCR2.1 (Invitrogen, USA) was used. Inserts were prepared by the PCR amplification of a fragment of the β_2-microglobulin gene as well as a fragment of the *GRIN1* gene. Primers used for the PCR amplification of the *GRIN1* gene (For TCTACA ATGGCACCC ACGTCATC; Rev GGTGCAGATCACCT TCTTGAC) were chosen to match a sequence shared by all eight isoforms of mRNA for the *GRIN1* gene; thus, panGluN1 expression was measured. Sequences of the TaqMan probes (Promega, USA) are for the β_2-microglobulin gene: AGGTTTACTCACGTCATCCAGCAG and for the *panGRIN1* gene: TGACAGGAAGATCATCT GGCCAGGC. Plasmids were purified using the Qiagen Plasmid Midi Kit (Qiagen, Germany) according to the manufacturer's recommendations.

To quantify the amount of panGluN1 mRNA expressed, the cDNAs from hippocampal samples were prepared as described above. Plasmids harbouring a fragment of the β_2-microglobulin gene as well as a fragment of the *GRIN1* gene were serially diluted by a factor of 10 starting from 1 ng to 1 pg per microlitre (μl). The hippocampal cDNA samples and diluted plasmids were subjected to quantitative RT-PCR amplification in duplicates. The relative expression of the panGluN1 transcripts was represented as a ratio of the quantifications of *GRIN1* and the control

β_2-microglobulin genes. Then, the relative quantification of panGluN1: $\beta2$-microglobulin expression was done by comparative Ct method, using the formula $2^{-\Delta\Delta Ct}$, because the reaction efficiency of both the *GRIN1* and β_2-microglobulin were comparable.

Quantification of Individual Splice Variants by Quantitative RT-PCR

The isolation of mRNA from frozen tissue was accomplished by using the chemagic mRNA Direct Kit (Chemagen, Germany) as directed in the instructions manual. Based on published cDNA sequences of the human *GRIN1* gene for the GluN1 subunit (hGluN1) (NCBI access numbers: NM_021569.1, NM_007327.1, NM_000832.4), four primers (SRT1-4) for reverse transcription were designed by the Primer3 program [26]. These primers were able to distinguish between four different N-terminal splice variants of GluN1. In addition, a primer for the reverse transcription of β-actin (GCGCAAGTTAGGTTTGCT) was included in all reverse transcription reactions. The sequences of primers are: STR1 GGTGCTCGTGTC TTTGGA; STR2 GGTGCTCTGCAGGTTCTT; STR3 GGATGGTACTGCGTGTCTT; STR4 GATGGTACTGC TGCAGGTT. Each reverse transcription was carried out in the reaction mixture (15 μl) with ImProm-II^{TM} Reverse Transcriptase (Promega, USA). First, four different mixtures were prepared containing 20 pmol of individual reverse transcription primers SRT1-4 and 20 pmol of primer for β-actin. To each mix, 4.4 μl of mRNA was then added.

Individual splice variants of GluN1 were quantified by RT-PCR on ABI7300 (Applied Biosystems), and detections with TaqMan probes and reporter dyes FAM (for GluN1) and/or VIC (for β-actin) were carried out. Two primer–probe sets were designed by the Primer Express 2.0 software. The first primers–probe set detected that exon 19 of the *GRIN1* gene was presented in all splice variants. This allowed the separate quantities of each of the four different N-terminal splice variants of the GluN1 gene to

be distinguished from previous reverse transcription reactions. The next primers–probe set detected exon 4 of the *GRIN1* gene and revealed the presence or the absence of this exon in the individual N-terminal splice variants. All reverse transcription reactions for the quantification of β-actin were undertaken as well. The sequences and the localisation of primers and probes were: exon 4—For CAGTTTGACCCAGGGACCAA, Rev CAGTTTGACCC AGGGACCAA, Probe AGCTGGAGGCCCGGGTCAT CAT; exon 19—For GTACCGGCATAGGAGAAGCA, Rev AAGGCATGCAGCTTGTTGTCT, Probe ACTACG AGAGTGCGGCGGAGGC; β-actin—For CGCGAGAAG ATGACCCAGAT, Rev CCCTCGTAGATGGGCACAGT, Probe ACGCCTCTGGCCGTACCACTGG.

The calculation of relative quantification was done by comparative Ct method and all reactions were performed in triplicate. Before performing statistical analyses, the relative gene expression values corresponding to individual variants were transformed into percentages of the sums over all variants.

Western Blotting

Samples dissected from the central parts of the hippocampi were homogenised in 1.0 ml of lysis buffer (320 mM sucrose; 10 mM Tris, pH 7.4; 0.2 mM EDTA; 2 mM PMSF; 1 mM 2-mercaptoethanol and a cocktail of protease inhibitors, Sigma). Crude synaptosomal (P_2) fractions were isolated from hippocampal homogenates and resuspended in a loading buffer (63 mM Tris; 10% glycerol; 2% SDS; 5% 2-mercaptoethanol and 0.01% bromophenol blue). The protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as a standard (Bio-Rad, USA). The resuspended material was used for the electrophoresis in the 7.5% polyacrylamide gel (Criterion Cell, Bio-Rad, USA) followed by electroblotting in the criterion blotter (Bio-Rad, USA). Nonspecific binding was blocked with 3% BSA dissolved in TBS-T buffer. Blots were incubated overnight with anti-NMDAR1 as primary antibodies (1:100; Chemicon, USA) and anti-NMDAR1 C1 splice variant (1:800; Chemicon, USA) and the loading control with an anti-α-tubulin antibody (1:1,000; Exbio, CZ) for 1 h. Then, the blots were washed in TBS-T buffer and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (1:3,000; Dako, Denmark). Detections were performed with a chemiluminescent substrate (Pierce, USA) and evaluated by the Gel Doc Analysis system (Bio-Rad, USA).

Statistics

All data are given as means ± SEM (standard error of the mean) unless stated otherwise. The quantitative RT-PCR data were analysed by two-way ANOVA with a between-group factor of diagnosis and a within-subject factor of laterality. The quantitative RT-PCR data (splice variants) were analysed by (1) four-way ANOVA with two between-group factors of sex and diagnosis and two within-subject factors of laterality and variant, and (2) a simplified three-way ANOVA with the sex factor eliminated. Moreover, a two-way ANOVA with one between-group factor of diagnosis and one within-subject factor of variant was applied to the data subsets from the left or right hippocampi as well as to the individual left/right differences. Protein expression data were subjected to analysis by two-way ANOVA with one between-group factor of diagnosis and one within-subject factor of laterality. The two-sample student's *t*-test was used as a post-hoc test. The dependence of expression of mRNA panGluN1, panGluN1 protein and mRNA of GluN1 splice variants on age and PMI was tested using the Spearman rank correlation coefficient.

Results

Quantitative RT-PCR of the panGluN1 Subunit

In our study, we tested hippocampal tissue samples from autopsies of 13 patients with diagnosis of schizophrenia and eight non-psychiatric controls. Before statistical analysis of diagnosis effects, correlation analyses were conducted and no significant association between panGluN1 expression and age or PMI was found. Relative quantification of the panGluN1 form revealed no significant differences in the mRNA levels comparing control and the schizophrenic groups (Fig. 1a). However, 12 of 13 psychotic patients exhibited considerably high absolute difference between the values ascertained in the left and right hippocampi; in seven patients the mRNA levels in the left hippocampus were about half of the value in the right hippocampus whereas in remaining five patients the values in the left hippocampus were about 25% higher than in opposite hippocampus. Consequently, the absolute left/right differences in the amount of mRNA for panGluN1 subunit were significantly greater (T [19] = 2.67, $P = 0.0152$) in the patients with schizophrenia (Fig. 1b). For summary of statistical results, see Table 3.

Protein Expression of panGluN1

Semi-quantification of the panGluN1 protein, expressed as a protein ratio of panGluN1 and α-tubulin as a loading control (Fig. 2), showed significant sex differences given by the interaction of laterality and sex (F [1, 26] = 11.74, $P = 0.0020$) between schizophrenic patients and controls.
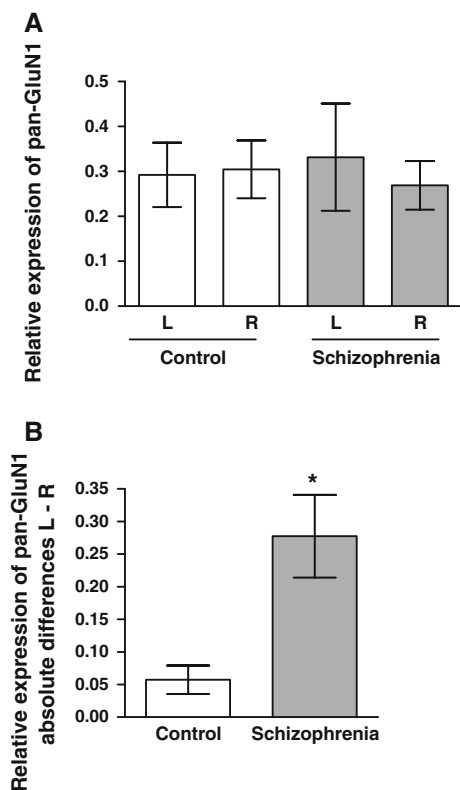
Fig. 1 a, b RT-PCR quantification of the panGluN1 subunit in schizophrenics and controls. Values of mRNA for the GluN1 subunit in the left and right hippocampus (**a**) and the absolute left/right difference (**b**) are given as mean ± SEM. Significance: * $P < 0.05$. Designations: left hippocampus, *L*; right hippocampus, *R*

Similarly, statistically significant interactions of sex and diagnosis (F [1, 26] = 5.82, $P = 0.0232$) and of sex, laterality and diagnosis (F [1, 26] = 4.63, $P = 0.0409$) were found. Difference between laterality in women and men was more pronounced in schizophrenic patients than in controls. Separate analysis of the group of males (schizophrenia vs. control) revealed statistical significance for the laterality (F [1, 13] = 4.78, $P = 0.0477$) as well as for the difference between groups of patients and controls (F [1, 13] = 8.91, $P = 0.0105$). In post-hoc analyses (*t*-test) we have found a significant change of panGluN1 protein in left hippocampi when the groups of schizophrenic and control male persons (T [13] = 2.935, $P = 0.0116$) were compared (Fig. 2). The laterality of panGluN1 expression in the group of female patients was different from controls with the panGluN1 protein prevalence in the left hippocampus (Fig. 2). Generally, schizophrenic women had higher levels of the panGluN1 protein in left hippocampi than control groups and schizophrenic males had lower levels of panGluN1 than corresponding male controls.

Despite different protocols for the anti-NMDAR1 C1 antibody being tested, this antibody with our human hippocampal tissue did not react. Correlation analysis showed

no significant association between panGluN1 protein expression and age or PMI.

Quantitative RT-PCR of the GluN1 Isoforms

The N1 cassette was expressed in all hippocampal tissue samples corresponding to NR1-1b, -2b, -3b or -4b splice variants, with the exception of three schizophrenia autopsies and one control. We focused on the C terminus domains to find any interaction of diagnosis (DG), sex, variants and laterality (four-way ANOVA). Because no significant effects (main or interaction) of sex were observed, the factor of sex was disregarded in further analyses. In the simplified model (three-way ANOVA), there was a trend for an interaction of laterality, splice variant (NR1-1, -2, -3, -4) and diagnosis (schizophrenia vs. control), but this failed to reach significance (F [3, 60] = 2.74, $P = 0.0511$) because of the different distributions of the four C-cassette variants when schizophrenia and controls were compared. Separate analyses of the data from the left and right hippocampi revealed significant left-sided interaction between the factors of splice variant and diagnosis (F [3, 81] = 3.23, $P = 0.0267$). Post-hoc analyses (*t*-tests) revealed a significant decrease of the splice NR1-4b variant (T [27] = 2.43, $P = 0.0281$) in the left hippocampi of schizophrenic patients in comparison with controls (Fig. 3a). Moreover, there was a trend towards an increase in the NR1-3b splice variant in schizophrenia (T [27] = 1.92, $P = 0.0659$). The interaction between variants and diagnosis in the right hippocampus was not reach significance (F [3, 66] = 2.41, $P = 0.0744$). The *t*-test of the NR1-2 variant pointed to a significant difference between schizophrenia and controls (T [22] = 2.99, $P = 0.0067$; Fig. 3b). To identify differences in the laterality of the C-terminal isoforms between schizophrenic and control persons, we analysed individual left/right differences by two-way repeated-measures ANOVA with the factors diagnosis and variants. There was a trend for the interaction of the diagnosis and variant factors (F [3.60] = 2.74, $P = 0.0511$)—a result equivalent to the ternary interaction of laterality, variant and DG cited above. The left/right differences of the NR1-2 variants were significantly different in the schizophrenia and control groups (T [20] = 2.25, $P = 0.0355$). In addition, differences in isoforms containing the C1 cassette (NR1-1; NR1-3; associated with the efficiency of NMDA-induced gene expression [27] and with the C terminus phosphorylation [9]) occurred more commonly in schizophrenic hippocampi than in control samples. *T* test analysis reached significance in the right hippocampus
(T [22] = 2.32, $P = 0.0297$) and showed a trend for the increase in the left hippocampus in schizophrenic patients (T [27] = 1.77, $P = 0.0876$). As in the previous analysis, the Spearman correlation was not significant.

Table 3 Summary of statistical results

Analysis	n	Data	Effect	$F(df_1, df_2)/t(df)$	P
panGluN1 RT-qPCR	8 CONT vs. 13 SCZ	Absolute left–right differences	Diagnosis (CONT vs. SCZ)	2.67 (19)	0.0152
GluN1 isoforms	10 CONT vs. 12 SCZ	All	Laterality × splice var. × diagnosis	2.74 (3, 60)	0.0511
RT-qPCR	13 CONT vs. 16 SCZ	Left side	Splice var. × diagnosis	3.23 (3, 81)	0.0267
		Left side, NR1-4b variant	Diagnosis (CONT vs. SCZ)	2.42 (27)	0.0281
		Left side, NR1-3b variant	Diagnosis (CONT vs. SCZ)	1.92 (27)	0.0659
	11 CONT vs. 13 SCZ	Right side	Splice var. × diagnosis	2.41 (3, 66)	0.0744
		Right side NR1-2b variant	Diagnosis (CONT vs. SCZ)	2.99 (22)	0.0067
		All, left–right differences	Splice var. × diagnosis	2.74 (3, 60)	0.0511
	10 CONT vs. 12 SCZ	Left–right differences, NR1-2 var.	Diagnosis (CONT vs. SCZ)	2.25 (20)	0.0355
		C1 cassette (sum of NR1-1, NR1-3)			
	11 CONT vs. 13 SCZ	Right side	CONT vs. SCZ	2.32 (22)	0.0297
	13 CONT vs. 16 SCZ	Left side	CONT vs. SCZ	1.77 (27)	0.0876
panGluN1 WB	7 M/6 F CONT vs. 8 M/9 F SCZ	All	Sex × laterality	11.74 (1, 26)	0.0020
			Sex × diagnosis	5.82 (1, 26)	0.0232
			Sex × laterality × diagnosis	4.63 (1, 26)	0.0409
	7 CONT vs. 8 SCZ	Males	Laterality	4.78 (1,13)	0.0477
			Diagnosis	8.91 (1, 13)	0.0105
		Males, left side	Diagnosis	2.94 (13)	0.0116

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WB, western blots; F, female; M, male; CONT, control group; SCZ, schizophrenia. F-test or, where only two groups are compared, Student t-test
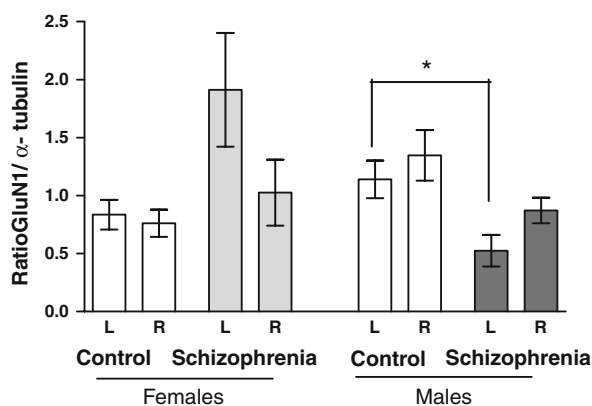

Fig. 2 Western blot quantification of the panGluN1 subunit in schizophrenic patients and controls. Designations: left hippocampus, L; right hippocampus, R. Values are given as mean ± SEM. Significance: * $P < 0.05$

Discussion

Hippocampal Levels of the panGluN1 Subunit Transcripts

In this study, we found no changes in the expression of the panGluN1 subunit in schizophrenic patients. Our results are in accordance with other studies [28]. There are also studies where the lower expression of GluN1 mRNA [15] was found specifically in the left hippocampus [17] of schizophrenic patients in comparison with non-psychotic patients. However, all mentioned studies used in situ hybridisation as the method for mRNA assessment, and the left-sided or area-limited decreases in the hippocampal GluN1 subunit transcripts were not accompanied by an analogous decrease in ligand binding to the glycine co-agonist sites [28].

We have seen bigger left/right differences in panGluN1 expression in the hippocampus of schizophrenic patients. Therefore, a laterality of panGluN1 expression in schizophrenia is highlighted in our sampling. Several researchers mention the differential involving of left and right temporal lobes in schizophrenia, and thereby asymmetrical alteration in hippocampal glutamate receptors might be one such manifestation [29]. In biochemistry, lateral changes in schizophrenic hippocampal activities of the nitric oxide mediator system have previously been reported [30]. Nevertheless, the elucidation of this phenomenon will be needed in future studies.
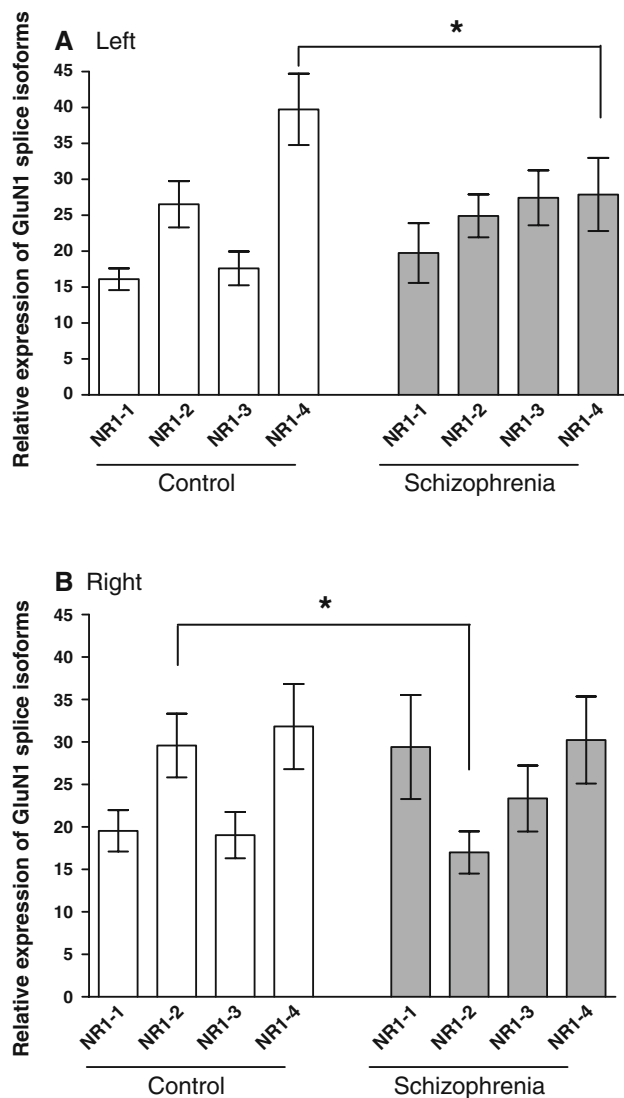
Fig. 3 **a**, **b** RT-PCR quantification of the C-terminal cassette variants in schizophrenic patients and non-psychiatric controls. Values of mRNA splice variants are given as mean ± SEM. Significance: * $P < 0.05$. NR1-1, -2, -3 and -4 splice variants in the left (**a**) and right hippocampus (**b**)

Amount of Subunit GluN1 Protein in the Schizophrenic Hippocampus

Protein expression revealed more pronounced laterality in the group of schizophrenic men and women than in controls, in accordance with our findings on the transcript of panGluN1. Additionally, the results displayed an important decrease in the panGluN1 protein in the left hippocampus of elderly male patients with schizophrenia that was comparable to the findings of left-sided reduction of GluN1 transcripts in some hippocampal areas [15, 17]. In another study, the left hippocampus of medication-free patients in vivo was shown to have a significant reduction in NMDA-R binding compared with antipsychotic-treated

patients and healthy subjects [24]. One explanation for the vulnerability in the brains of males might be delayed lateralisation and lack of oestrogen protection [31].

Our results introduce questions that will need to be answered in future studies. First, previously unreported strong sex differences in the expression of the panGluN1 protein exist. This might be an important factor in this type of assessment. Regarding the pathophysiology of schizophrenia, further research should be dedicated to the implications of RNA splice variants changing expression against a background of the unchanged expression of panGluN1 transcripts.

Hippocampal GluN1 Subunit and Its Alternative RNA Splicing

Through the alternative splicing of the *GRIN1* gene, which gave rise to eight isoforms (alternatively splice variants) of the GluN1 subunit, a multitude of NMDA-Rs were expressed in various regions of normal and schizophrenic brains [6, 32]. With the exception of three samples there was an expression of the N1 cassette in all autopsies. We only evaluated variants differing in the C terminus (cytoplasmic) domain.

The data on the quantitative determination of individual isoforms in schizophrenic tissue samples revealed lower amounts of NR1-4 and NR1-2 (subunits without the C1 cassette) in the left and right hippocampus, respectively. By contrast, there was a higher expression of NR1-1 and NR1-3 (subunits with the C1 cassette). When the C1 cassette is present (exon 20), serines 896 and 897 can be phosphorylated in parallel to NMDA-R trafficking [33]. However, serine 890 can also be phosphorylated, and this phosphorylation is connected with clustering modulation [34]. The phosphorylation of serines 896 and 890 by two different protein kinase Cs (PKCs) has been shown to cause different types of modulation of the NMDA-R [35]. We have seen an increased expression of C1 cassette variants in the schizophrenic group, and thereby there might be a higher presentation of serines 896, 897 and 890 and a higher opportunity for phosphorylation. It remains to be determined whether increased levels of the NR1-1 and NR1-3 isoform transcripts play a role in the dysregulation of NMDA-R phosphorylation.

The C1 cassette contains an ER retention signal (RRR) and presentation of this domain is connected with significantly less surface expression of this variant than those without C1 [36]. By contrast, the C2′ cassette, containing splice variants that include the terminal PDZ-interacting domain, showed significantly increased surface expression (compare NR1-3b to NR1-1b) [36]. Shorter C-terminal splice forms are expressed on the cell surface more than longer forms, so NR1-1 is almost completely retained inside the cell and NR1-4 is highly expressed [36]. Based

on our results, we speculated that there is a change in the trafficking of the NR1 subunit in schizophrenia and that it might play a crucial role in the availability of NR1 for assembly with NR2 subunits and thereby in creation of adequate receptors.

The different left/right distribution of the C-cassette in schizophrenia is an important finding and this phenomenon is in congruence with an older report [23]. There were no changes in expression of any variant in both hippocampi, which means all detected changes were unilateral. We speculated that in schizophrenia there is a different alteration in left and right hippocampi splicing of GluN1.

The C-terminal domains are also responsible for anchoring the GluN1/GluN2 heteromers into the postsynaptic density protein-95 (PSD-95) [37]. The heteromers containing different GluN1 isoforms associated with PSD-95 also exhibited a distinct affinity for the glycine co-agonist binding site as well as for the D-serine/D-alanine and the modification of the NMDA-R channel gating mechanism [37]. This is another mechanism of the NMDA-R hypofunction, which does not require a reduction in the number of receptor sites.

Limitation of the Study

Several limitations must be considered when interpreting data attained from postmortem studies. Firstly, we have not kept samples from all 31 persons at our disposal for all analyses because the tissue was used for another study.

For a prolonged period patients from this study were exposed to different antipsychotic drugs, whose effect on NMDA-R expression is still unclear. Previous reports have demonstrated no effect of either acute [38] or chronic antipsychotic administration [39] on the expression of GluN1 subunits in rats. However, other studies have reported the down-regulation of GluN1 in the dorsolateral prefrontal cortex in rats after chronic treatment [40]. A region-specific study showed no change in the hippocampus after chronic atypical antipsychotic administration [41].

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