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Research report

Expression of GABA_A receptor subunit mRNAs in hippocampal pyramidal and granular neurons in the kindling model of epileptogenesis: an in situ hybridization study

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Abstract

To investigate the molecular changes underlying kindling epileptogenesis in the rat hippocampus, the expression levels of the genes encoding for 13 different γ -aminobutyric acid type-A receptor (GABA_AR) subunits were measured in hippocampal principal neurons using in situ hybridization techniques and semi-quantitative analysis of the autoradiograms. Schaffer collateral-commissural pathway kindled rats were investigated at three different stages of kindling acquisition, at 24 h after the last seizure and at long-term (28 days) after termination of kindling stimulations. Changes were distinct for the different subunits in the three analyzed regions (CA1, CA3, fascia dentata) and also different for the various kindling stages. In all hippocampal areas at the early phases of kindling epileptogenesis, before the appearance of generalized seizures, an increase was found of those transcripts that constituted the majority of the expressed variants in control animals ($\alpha 1$, $\alpha 2$, $\alpha 4$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 2/\gamma 2L$ mRNA). In these stages, the increased levels of different variants in the granular neurons of the fascia dentata were more pronounced when compared to the pattern of changes in pyramidal cells of CA1 and CA3. In fully kindled animals, the expression levels of several subunits returned to control levels, whereas $\beta 3$ and $\gamma 2/\gamma 2L$ mRNA expression was still significantly enhanced in all areas. At long-term, few changes were encountered. The long-splice variant of $\gamma 2$ was decreased within pyramidal and granular neurons while the total level of $\gamma 2$ mRNA was not different from controls. The increased GABA_AR subunit expression in the fascia dentata may underly the reported increased GABA_AR ligand binding and the increased GABA mediated inhibition. However, the decreased GABA_AR binding and the attenuation of GABAergic inhibition in CA1, could not be explained by a decrement of receptor subunit expression.

Keywords: Epilepsy; In situ hybridization; Plasticity; Gene expression; GABA_A receptor subunit mRNA; Hippocampus

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1. Introduction

Kindling is an experimental model for the induction and expression of epilepsy with a focal onset. Kindling is realized by the repeated application of a short, high frequency, electrical stimulation that is capable of eliciting a focal electroencephalographic seizure called afterdischarge (AD) [12,17]. In the course of kindling a gradual prolongation of the AD takes place which is paralleled by the appearance of behavioural epileptiform seizure activity of increasing severity, culminating in generalized tonic-clonic convulsions [12,60]. Once

established, this enhanced susceptibility for the induction of seizures is persistent [12,22,40,47].

The hypothesis has been put forward that a gradual attenuation of the inhibitory GABAergic control over local neuronal circuits is one of the major factors contributing to the process of kindling epileptogenesis and may also contribute to the persistent state of hypersensitivity after cessation of the stimulations. In CA1 area of the hippocampus, experimental evidence has been found in support of this hypothesis; a reduction of inhibitory synaptic transmission takes place in the course of kindling as shown by a reduced paired-pulse inhibition of local evoked field potentials [22,24,26,77,78], a diminished sensitivity of the pyramidal neurons for iontophoretically applied GABA [20]

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and a reduced binding of the GABA_A receptor agonist [³H]muscimol [69] and of benzodiazepines [29,67]. In sharp contrast to the decreased inhibition in CA1, in the fascia dentata paired pulse inhibition is enhanced [7,21,26,70,79], together with an increase in the amplitude of miniature inhibitory postsynaptic currents [53], and a robust increase in both [³H]muscimol and [³H]flunitrazepam binding in this area [51,62,67,69]. The results of these studies imply local differences in the response of the GABAergic inhibition to kindling stimulations, specifically an impaired inhibition in CA1–3 area, leading to an increased propensity for seizures, along with a strengthened inhibition in fascia dentata, possibly limiting seizure activity. It may be hypothesized that these opposing changes of the GABAergic synaptic transmission are the consequence of dissimilar changes at the GABA_A receptor (GABA_AR) level.

The GABA_A/benzodiazepine receptor complex is a hetero-oligomeric complex composed of several distinct peptides. Molecular cloning studies revealed five different but homologous receptor subunits (α , β , γ , δ , and ρ), each of which exists in the brain in different variants. Additional diversity arises from RNA splicing [35,36,46,52,71]. In recombinant expression studies, the association of different cloned subunit variants leads to functional diversity of the GABA_A receptor complexes with respect to conductance and gating properties of the chloride channels, the allosteric modulation by benzodiazepines and the sensitivity for agonists. The type of α subunit, in combination with a β and a $\gamma 2$ subunit, is a major determinant of the type of benzodiazepine pharmacology displayed [13,35,39,52,57–59,72]. The GABA_AR affinity and efficacy for benzodiazepines is also influenced by the γ subunit present, with receptors containing a $\gamma 2$ subunit exhibiting the classical benzodiazepine modulation [59,63,76]. For the β class subunits, two domains were found that strongly influence the sensitivity for GABA. However, the naturally occurring sequence variations in these regions were not associated with clear differences in agonist sensitivity [1]. The type of β subunit does not appear to influence the pharmacological properties of the GABA_ARs [14,34,63].

In situ hybridization studies have shown that the distribution of subunit mRNAs displayed a marked regional heterogeneity in the brain suggesting the regionally specific assembly of subunits into various isoforms of the GABA_A-receptor [4,54,73]. Furthermore, regional differences in immunoprecipitation patterns, using subunit specific antibodies, supported the concept of structural heterogeneity [10]. Although a detailed insight into the precise subunit assembly and location of functional GABA_AR in the brain is at present lacking, biochemical, pharmacological and recombinant expression studies point to the preferential

existence of receptors formed by a combination of α , β and γ subunits [2,5,8,9,61].

The subunit diversity may sustain a functional diversity of GABA_AR mediated inhibition in different brain areas. By altering the expression pattern of GABA_AR subunit encoding genes, neurons may have the ability to fine tune their GABAergic signal transduction pathway in response to changing conditions. Changes in subunit expression profile during development [30] and in response to transient cerebral ischemia [33], chronic GABA_A/benzodiazepine receptor agonists/antagonists [15,25,42,56,63,80] or ethanol treatment [41,44,49] may underly functional changes of GABA_ARs. Modified GABA_AR subunit mRNA levels were also described in rats that were subjected to repeated electrical stimulations with short interstimulus intervals resulting in recurrent epileptiform seizures (rapid kindling procedure) [27].

To investigate the hypothesis that the kindling induced changes in GABA_A receptor binding and GABA-mediated synaptic inhibition in CA1 and fascia dentata are related to an altered GABA_AR subunit gene expression, the mRNA levels of GABA_AR subunits were determined by in situ hybridization. This technique allows a differential measurement of the levels in the hippocampal subregions. This report describes the results of the first comprehensive study on effects of conventional kindling epileptogenesis on gene expression of all relevant members of the GABA_AR subunit family [18].

2. Material and methods

2.1. Kindling

Male Wistar rats (200–225 g, Harlan Netherlands) were used. Stainless-steel electrodes were placed in the CA1 area of the left dorsal hippocampus of rats under pentobarbital anaesthesia (Nembutal, 1.05 ml/kg). The stimulation bundle was placed in the Schaffer-colateral/commissural fiber pathway and the recording bundle was positioned in stratum radiatum of CA1. The details of this procedure were described previously [24]. After two weeks of recovery, the rats were connected to a stimulation/recording device to enable the delivery of kindling stimulations consisting of a train of 50 Hz pulses of 1–2 s duration at an intensity of 200–300 μ A and to carry out local electroencephalographic recordings.

2.2. Experimental design and kindling

A group of 54 implanted animals was divided into a non-stimulated control group ($n = 25$) and a group ($n = 29$) that received, twice daily, kindling stimula-

Fig. 1. Mean afterdischarge duration in the course of Schaffer collateral/commissural fiber kindling. Notice the steady increase in afterdischarge length from session 1 to around session 16 and the stabilization thereafter.

tions at an intensity supra-threshold for the induction of an afterdischarge [12]. The animals of the control group were handled throughout the experimental period in a way comparable to the kindled rats but did not receive tetanic stimulations. The development of kindling epileptogenesis was followed by scoring the afterdischarge duration (Fig. 1).

The expression of GABA_AR mRNAs was studied in four groups of kindling stimulated animals: (i) a 6-AD group ($n = 7$) sacrificed after the 6th tetanic stimulation; (ii) a 14-AD group ($n = 6$) after the 14th afterdischarge; (iii) fully kindled group ($n = 8$) sacrificed after 22–33 afterdischarges when rats experienced generalized tonic-clonic convulsions (total number of class 5 seizures: 6 ± 1 [60]); (iv) a long-term group ($n = 8$), kindled to the same stage as the fully kindled animals (total number of 15–32 afterdischarges resulting in 6 ± 1 class 5 seizures, lasting 125 ± 18 s). Animals of the 6-AD, 14-AD and fully kindled groups were sacrificed 24 h after the last seizure and animals of the long-term group were killed 28 days after the last seizure. The rats of the control group were divided over the different stimulated groups and their brains were fixed at the same time.

2.3. *In situ* hybridization

Animals were deeply anaesthetised with ether and sacrificed by decapitation. The brain was rapidly removed and frozen in chilled isopentane at -35°C , further frozen on powdered dry-ice and, wrapped in aluminium foil, stored at -70°C . Coronal cryosections ($12\ \mu\text{m}$) were cut, thaw-mounted onto poly-L-lysine coated slides, and dried at room temperature. Sections were fixed for 5 min in 4% paraformaldehyde (RT), washed in phosphate buffered saline, dehydrated in

70% ethanol and stored in 95% ethanol at 4°C until use. Prior to hybridization, sections were removed from the ethanol storage boxes and air dried. For this study, sections from a confined region at a level of around -2.8 mm caudal to the bregma were selected, a region which is approximately 0.4 mm caudal with respect to the position of the stimulation electrode. In situ hybridization was carried out as described in detail previously [19,74]. Briefly; subunit specific 45-mer oligonucleotides were 3' end-labeled with [^{35}S]dATP (N.E.N., 1200–1500 Ci/mmol) using terminal deoxynucleotidyl transferase (Gibco BRL Life Technologies). The labeled probe was purified over a Sephadex G-25 spin column and diluted to $1\ \text{pg}/\mu\text{l}$ ($\pm 1500\ \text{cpm}/\text{pg}$) in a hybridization solution containing 50% formamide, 0.6 M NaCl, 0.06 M sodium citrate ($4 \times \text{SSC}$), 100 $\mu\text{g}/\text{ml}$ polyadenylic acid, 25 mM natriumphosphate, 1 mM pyrophosphate, $5 \times$ Denhardt's, 200 $\mu\text{g}/\text{ml}$ sheared salmon sperm DNA and 10% dextran sulphate. Sections were incubated with this hybridization solution overnight at 42°C . Sections were rinsed several times in $1 \times \text{SSC}$ at 20°C , and subsequently washed for 20 min at a stringency of $1 \times \text{SSC}$ at 60°C . Sections were dehydrated and opposed to Kodak XAR-5 film. The total exposure time was optimized, using test slides, for the different probes to obtain an approximately equal density of the autoradiograms allowing an equally accurate densitometric analysis.

The sequences and the specificity of oligonucleotides that were used here to examine the expression of GABA_AR subunit mRNAs in kindled rats are identical to those used by Wisden et al. [73] to describe the distribution of the subunit mRNAs in rat brain. In accordance with their findings, when a 50-fold excess of unlabeled probe was added to the hybridization mix no hybridization signal was detected. The long splice variant of GABA_A $\gamma 2$ mRNA (GABA_A $\gamma 2\text{L}$) was detected by a probe with the sequence 5'-AATGG-TAGGGGCCTTGAAGGAAAACATCCGAAGAAGAG-GGTTTTT-3'; the $\gamma 2\text{L}$ specific 24 nucleotide insert is bold [71]. Our probe is almost identical to the oligonucleotide used by Miralles et al. [46], who demonstrated the specificity of this probe on Northern blots.

2.4. Densitometric analysis

The densitometric analysis of the obtained autoradiograms for the different GABA_AR subunits was described previously in detail [19]. In short; the ipsilateral (left hemisphere) and contralateral hippocampal area within the autoradiogram was scanned and digitized. Of the three mounted sections only one was used for analysis since the variation between animals was found to be larger than the negligible variation between different sections of one animal (data not shown).

Table 1

Relative changes in mean extinction values of the in situ hybridization autoradiograms of GABA_A receptors in the different kindled groups (% ± S.E.M.). The value obtained in the control group was used as reference and set at 100%. Statistical comparisons with respect to controls were carried out on the determined extinction values using the Student's *t*-test.

	6-AD (n = 7)	14-AD (n = 6)	Fully kindled (n = 8)	Long-term (n = 8)
CA1				
α1	9.0 ± 6.6	−0.2 ± 5.6	3.4 ± 6.8	7.4 ± 6.9
α2	25.9 ± 5.4 ****	17.2 ± 6.5 ***	22.8 ± 5.6 ****	−2.6 ± 4.4
α3	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
α4	17.5 ± 3.2 ****	8.5 ± 3.3	0.6 ± 5.8	−3.1 ± 3.8
α5	4.3 ± 6.5	−3.3 ± 4.2	−1.7 ± 3.8	0.9 ± 2.2
α6	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
β1	4.3 ± 2.8	9.6 ± 4.5	9.4 ± 3.0	2.6 ± 2.6
β2	2.4 ± 3.0	−4.9 ± 3.5	2.5 ± 2.2	−2.6 ± 3.7
β3	6.5 ± 5.8	1.1 ± 4.1	7.2 ± 2.4	0.4 ± 4.8
γ1	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
γ2	19.0 ± 6.3 **	17.5 ± 6.0 **	19.0 ± 3.7 ***	0.0 ± 3.8
γ2L	23.2 ± 5.4 ***	18.5 ± 7.5 *	15.0 ± 4.7(*)	−20.5 ± 9.8(*)
γ3	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
δ1	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
CA3				
α1	1.2 ± 4.8	−2.6 ± 3.8	−10.5 ± 3.2 **	6.5 ± 6.8
α2	11.7 ± 2.8 **	2.8 ± 2.4	−0.9 ± 3.5	−8.9 ± 3.9
α3	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
α4	2.6 ± 4.7	0.3 ± 4.9	−4.8 ± 2.3	−5.5 ± 2.5
α5	6.8 ± 5.8	7.8 ± 5.0	6.2 ± 4.3	2.6 ± 2.3
α6	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
β1	3.4 ± 2.2	9.3 ± 1.8	4.3 ± 3.3	−2.3 ± 1.8
β2	−1.5 ± 3.3	−12.1 ± 3.8 ***	−0.5 ± 3.1	1.2 ± 4.5
β3	7.4 ± 3.5	3.7 ± 2.4	14.5 ± 2.9 ****	−2.2 ± 4.2
γ1	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
γ2	14.4 ± 4.5 *	9.1 ± 5.3	12.0 ± 2.9(*)	3.6 ± 4.2
γ2L	15.0 ± 4.5 *	9.2 ± 6.1	16.7 ± 4.2(*)	−21.0 ± 9.6(*)
γ3	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
δ1	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
Fascia dentata				
α1	14.6 ± 5.7 **	12.6 ± 6.1(*)	4.8 ± 5.0	1.5 ± 6.4
α2	21.8 ± 3.4 ****	11.7 ± 3.7 *	4.0 ± 3.0	2.0 ± 6.4
α3	0.6 ± 9.2	5.2 ± 8.1	13.9 ± 5.2	42.6 ± 13.5 *
α4	15.1 ± 3.4 ***	18.7 ± 2.0 ****	5.3 ± 4.5	6.4 ± 3.4
α5	1.5 ± 9.3	−3.9 ± 9.0	−5.3 ± 3.6	6.1 ± 3.3
α6	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
β1	13.8 ± 4.9 *	20.3 ± 6.6 ***	1.8 ± 5.3	9.8 ± 5.1
β2	15.0 ± 5.3 ***	14.0 ± 5.3 ***	17.0 ± 2.7 ****	−0.2 ± 5.0
β3	22.8 ± 5.9 ****	27.1 ± 5.5 ****	29.9 ± 4.2 ****	4.5 ± 6.0
g1	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
γ2	26.8 ± 9.3 ***	21.5 ± 8.9 **	20.5 ± 4.0 ***	15.3 ± 3.9 **
γ2L	41.6 ± 10.9 ****	27.0 ± 11.1 **	36.3 ± 5.2 ****	−26.7 ± 9.6 **
γ3	n.d.e.l.	n.d.e.l.	n.d.e.l.	n.d.e.l.
δ1	−19.6 ± 3.1 ****	−15.4 ± 4.1 ****	−5.1 ± 5.1	−5.6 ± 4.6

n.d.e.l.; non detectable expression level; (*) *P* < 0.05, one tailed Student's *t*-test; * *P* < 0.05; ** *P* < 0.025; *** *P* < 0.010; **** *P* < 0.003.

All stored images were assigned a code and quantification was carried out by an observer who had no knowledge of the treatment that the animals under study had

been subjected to. The codes were broken only after completion of the quantification. Using a gray level segmentation followed by a fixed sequence of standard

Fig. 2. GABA_AR α1–6, β1–3, γ1, γ2, γ2L, γ3 and δ mRNA distribution in coronal sections of control rats. The ipsilateral, electrode implanted, hemisphere is on the left side. Exposure times of the hybridized sections were chosen to allow accurate quantification of hippocampal regions. This resulted sometimes in a slight underexposure of the other brain regions (f.i. GABA_AR β3 and γ2). Note the different patterns of expression in hippocampus, thalamic and hypothalamic regions. Bar = 5.0 mm.

$\alpha 1$

$\alpha 2$

$\alpha 3$

$\alpha 4$

$\alpha 5$

$\alpha 6$

$\beta 1$

$\beta 2$

$\beta 3$

$\gamma 1$

$\gamma 2$

$\gamma 2L$

$\gamma 3$

$\delta 1$

image analysis steps, we constructed a mask leaving only the pyramidal and granular cell layer of the original image. Subsequently, the mean extinction value (absorbance) for the different selected regions was calculated. The linearity of the relation between the determined extinction value and the actual amount of probe specifically hybridized with the complementary mRNA sequence present in the section and also the reproducibility of the quantification procedure were described before [19].

2.5. Statistical analysis

Statistical comparison was carried out for each analyzed hippocampal region (CA1, CA3, fascia dentata) independently. The paired Student's *t*-test revealed no statistical differences between ipsi- and contralateral hemisphere showing that the implantation of the electrodes or a slight asymmetry of the sections does not affect the extinction value. Further analysis was, therefore, carried out on the mean extinction value of the two hemispheres. The variation in measured extinction values was comparable in control and kindled groups; S.E.M. represented typically 3–9% of the mean extinction value, dependent on the probe in question (Table 1).

For the statistical analysis of the kindling induced alterations of subunit mRNA expression, the following statistical analysis of the obtained extinction values was carried out. First, the control animals fixed along the 6-AD, 14-AD and fully kindled groups were compared and except for the GABA_A- β 1 expression, no significant differences were found between these control groups. Second, for further analysis these control animals were pooled into one group ($n = 17$) and the extinction values of the 6-AD, 14-AD and the fully kindled groups were compared using the Student's *t*-test. For some subunits (α 1, β 1, β 2, β 3) a small but significant decrease in expression, in one or all hippocampal areas, was observed in the long-term control group in comparison to the controls sacrificed 4–6 weeks earlier indicating age-dependent effects [45]. Therefore, the long-term kindled group was compared with the control group fixed at the same time.

To facilitate the presentation of the changes found in the different kindled groups, we determined the percentual change of the extinction values in the kindled group in comparison to the control groups by dividing the mean extinction of the kindled group by that of the control group and multiplying the outcome by 100. The difference from the 100% (= control) value and the outcome of the statistical analysis is presented in Table 1 for the pyramidal cell layer of CA1, CA3 and the granular cell layer of the fascia dentata.

3. Results

3.1. Expression pattern of GABA_AR subunits in control animals

The distribution of the mRNA in the coronal sections of control animals is illustrated in Fig. 2. The expression pattern of the GABA_AR mRNAs was also verified in horizontal brain sections (not shown). Of the α -type subunits, α 1, α 2, α 4 and α 5 were expressed by all principal neurons of the hippocampus. No detectable GABA_AR α 3 mRNA levels were obtained in the pyramidal neurons and, only after long exposure of the hybridized sections, low amounts were found in the granular neurons of fascia dentata, neocortex and some thalamic nuclei. GABA_AR α 6 could not be detected in the coronal sections and subsequent hybridization on horizontal brain sections revealed an exclusive expression of this subunit in the cerebellum. All three GABA_AR β subunits were expressed in significant amounts by the pyramidal and granular neurons of the hippocampus. With the GABA_AR γ 1 probe a weak expression was only found in the amygdala and hippocampal expression was barely detectable. The expression of GABA_AR γ 2 mRNA was studied using a pan probe that recognized both γ 2S and γ 2L forms and a second oligonucleotide probe specific for the 24 additional nucleotide stretch that typifies γ 2L mRNA. High expression of γ 2 was found in all brain regions. The γ 2L probe followed a similar widespread pattern of distribution in coronal and horizontal sections. The GABA_AR γ 3 specific probe revealed no significant amounts of expression in coronal sections at this plane. GABA_AR δ 1 transcript was low-abundant in the granular neurons of the fascia dentata, in some thalamic nuclei and high abundant in granular cells of the cerebellum.

The observed expression patterns in coronal and horizontal sections are in good agreement with the mRNA distributions described by Wisden et al. [73] and Persohn et al. [54]. Hippocampal GABAergic interneurons, located in stratum oriens, radiatum or moleculare did not express detectable hybridization signals using film detection, indicating that this type of cells did not significantly contribute to the extinction values measured in pyramidal and granular cell layers.

3.2. Kindling

In the course of kindling the afterdischarge duration increased linearly from a length of 18 s at the first session to 110 s at session 16: the acquisition phase (Fig. 1). In subsequent kindling sessions the afterdischarge duration, recorded from the dorsal hippocampus, stabilized around the latter length, while the afterdischarges were accompanied by the recurrent occur-

rence of class 5 behavioural seizures: the stabilization phase. The expression of GABA_AR mRNA was studied in four groups of animals that were sacrificed at different stages of kindling. Two groups were studied in the acquisition phase of kindling epileptogenesis, characterized by the steady increase of afterdischarge duration; (i) a 6-AD group ($n = 7$) sacrificed after the 6th tetanic stimulation which triggered afterdischarges lasting 39 ± 7 s (mean $\pm$ S.E.M.) and; (ii) a 14-AD group ($n = 6$) after the 14th afterdischarge, lasting 77 ± 11 s. The animals of these groups were fixed 24 h after the session because this interval allows short-last-

ing, seizure related, changes like post-seizure depression and the transient expression of immediate early genes to disappear [64]. The stabilization phase of kindling, characterized by the regular occurrence of generalized seizures, was investigated in the (iii) fully kindled group ($n = 8$) sacrificed 24 h after the 30th afterdischarge, lasting 107 ± 14 s, when rats exhibited generalized tonic-clonic convulsions (total number of class 5 seizures: 6 ± 1 [60]). The persistent changes in kindled tissue were studied in the (iv) long-term group ($n = 8$), these animals were kindled to the same degree as the fully kindled animals (total number of 30 after-

Fig. 3. GABA_AR $\alpha 2$, $\beta 3$ and $\gamma 2$ subunit mRNA distribution in the hippocampus of a control, a 6-AD, a 14-AD, a fully kindled and a long-term kindled animal to illustrate some of the quantitative changes. Photographs were obtained using identical processing conditions. The ipsilateral stimulated hemisphere is always on the left side. The significant increase of $\alpha 2$, $\beta 3$ and $\gamma 2$ expression in CA1 and CA3 in the 6-AD are difficult to visualize in these photographs. In the FD of the 6-AD, the 14-AD, and the fully kindled animal, bilateral enhanced levels of $\beta 3$ and $\gamma 2$ can be noted (*). Bar = 5.0 mm.

discharges resulting in 6 ± 1 class 5 seizures, lasting 125 ± 18 s) but they were killed 28 days after the last seizure.

3.3. GABA_AR subunit mRNA levels during kindling

The results of the quantitative analysis of the expression patterns is given in Table 1 and presented as percentual changes of the extinction values in the kindled groups in comparison to the extinction values of the control groups that were set at 100%. The following changes were found. The $\alpha 1$ expression in CA1 was not altered and in CA3 only in the fully kindled group a small reduction (-11%) was observed. In the fascia dentata, the levels were enhanced in the 6- and 14-AD groups, by 15 and 13%, respectively, returning to control values in fully kindled animals. GABA_AR $\alpha 2$ mRNA levels in CA1 were increased by approximately 22% during the whole kindling acquisition period (Fig. 3). In the CA3 and the fascia dentata an enhanced level was observed in the 6-AD animals but this increase disappeared in the 14-AD and fully kindled stages. GABA_AR $\alpha 3$ expression was only detectable at very low levels in the fascia dentata and was unchanged in the kindled groups fixed 24 h after the last stimulation. However, in the long-term group we encountered a significant increase of expression in the FD (Fig. 4). Repeated hybridization in the same series of animals revealed similar results ($85 \pm 10\%$; $P < 0.002$) and in another series of animals the increase was $68 \pm 18\%$ ($P < 0.01$), again restricted to the long-term group of kindled animals. GABA_AR $\alpha 4$ expression in CA1 was increased by 18% in the 6-AD group, returning to baseline expression thereafter. In the fascia dentata an increase of 15 and 19% was found for the 6-AD and 14-AD groups, respectively, but not for the fully kindled group. No significant changes were found in CA3. GABA_AR $\alpha 5$ mRNA levels were unaffected by kindling. Expression of $\alpha 6$ mRNA above background levels was not observed in the hippocampus in any of the kindled groups. The changes in the expression of the β -subunits have been published in abridged form [18]. For the densitometric data on the β -subunits presented here, a new series of hybridizations with the $\beta 2$ probe were carried out while the densitometric data of $\beta 1$ and $\beta 3$ were re-analyzed following the same statistical approach as for the other subunits. In the fascia dentata an enhanced expression of $\beta 1$ was detected in the 6-AD and 14-AD groups (14 and 20%) whereas in the fully kindled group no significant change was found. The $\beta 2$ mRNA levels in the fascia dentata were significantly increased in all kindled groups sacrificed 24 h after the last stimulation. In CA1 no significant changes were encountered, while in CA3 a significant decrease of $\beta 2$ was found after 14-ADs. GABA_AR $\beta 3$ mRNA levels in CA1 and CA3

Fig. 4. GABA_AR $\alpha 3$ expression levels remained unaltered during the development of kindling but in the LT-group an increased level was found in the fascia dentata although long exposure times were required to acquire densities that could be quantified indicating a relative low abundant expression in comparison to other subunits. Bar = 2.5 mm.

showed no clear trend in the pre-convulsive stages of kindling but expression was increased in fully kindled animals. Profound changes (23–30%) were found in the fascia dentata of the 6-AD, 14-AD and fully kindled groups (Fig. 3). However, 28 days after the last seizure the levels were not different from controls. The $\gamma 1$ and $\gamma 3$ subunits remained not detectable in all groups of kindled animals. The $\gamma 2$ subunit was increased in all hippocampal subregions and at all stages

Fig. 5. Normalized expression levels of the GABA_AR subunits in the three analyzed subfields of the hippocampus. Normalization was carried out by correcting the measured extinction value for variations in exposure time and in specific activity of the probe corrected for the isotopic decay over the exposure period.

of kindling, most noticeable in CA1 and in the fascia dentata. In the latter region, at long-term, expression levels were still significantly elevated by 15% (Fig. 3).

The conspicuous changes in $\gamma 2$ expression was reason for further investigation using a probe specific for the long splice variant of $\gamma 2$ which contains an 8-amino

Fig. 6. Schematic representation of the results presented in Table 1. Note the differences between CA1, CA3 and fascia dentata. Changes in the fascia dentata involve more subunits and increases are of larger magnitude in comparison to CA1 and CA3. Also note the tendency of changes to be more pronounced in the early stages. The percentages at the left represent the contribution of that subunit to the total mRNA population of the class it belongs to.

acid insert with a consensus substrate sequence for phosphorylation by PKC [28,50]. In the 6-AD, 14-AD and fully kindled groups the increased levels of $\gamma 2$ were paralleled by changes of comparable magnitude of $\gamma 2L$ expression. In the long-term group we observed a decreased mean expression of $\gamma 2L$ in all regions but the variability of the expression levels was much higher in the kindled group and, therefore, this decrease was not significant except in the fascia dentata. Finally, the expression of GABA_AR $\delta 1$ mRNA was present in detectable amounts only in granular neurons and showed a significant decrease in the 6-AD and 14-AD groups.

3.4. Normalized expression levels of GABA_AR subunits

The primary purpose of the experiments described, was to determine relative changes of expression levels in kindled animals in comparison to controls. In order to get autoradiograms with sufficient densities permitting accurate densitometric quantification, the film exposure length was prolonged for the less abundant subunits. Consequently, the obtained densitometric values do not provide precise information on the relative abundance of the different GABA_AR subunit mRNAs. This drawback may hamper the evaluation of the relevance of the observed relative changes for the studied subunits because it may be assumed that changes in low abundant subunits may have only a minor effect on the GABA_AR subunit population expressed in a particular region. Therefore, we calculated a normalized expression level taking into account differences in exposure time and in specific activity of the probe the latter corrected for the isotopic decay over the exposure period. Repeated measures with the same probe but with a different specific activity and different exposure periods resulted in comparable normalized expression values. The outcome of these calculations for CA1, CA3 and FD areas of control animals is presented in Fig. 5 and the contribution of the subunits to the total expression of the α , β , γ and δ class is given in Fig. 6. The expression levels revealed a similar profile in all hippocampal regions; the $\alpha 2$ subunit was the most abundant of the α class, followed by $\alpha 1$, $\alpha 4$

and $\alpha 5$. In the β class, the $\beta 3$ mRNA expression was by far the most abundant subunit followed by $\beta 1$ and $\beta 2$. These normalized expression values are in good agreement with the qualitative descriptions of signal intensity presented by Wisden et al. [73], Araki and Tohyama [4] and Persohn et al. [54]. This analysis showed that all quantified subunits, accounted for at least 10% of the total GABA_AR subunit expression within their class with one exception. The $\alpha 3$ mRNAs formed only 3% of the α type subunit mRNAs in the granular neurons and, therefore, the relevance of the long-term increase of this subunit may be questioned. Antibodies against $\alpha 3$ supported the absence of $\alpha 3$ subunits in bovine hippocampus [10]. As illustrated in Fig. 6, the most clear and consistent changes were found in $\alpha 2$, $\beta 3$ and $\gamma 2$ mRNAs which are also the most abundant variants within each class.

Using the normalized densitometric values, we estimated that the fraction of GABA_AR $\gamma 2L$ variant comprised approximately 39%, 36% and 27% of the total number of $\gamma 2$ subunit mRNAs in CA1, CA3 and fascia dentata, respectively (Table 2). This is in good agreement with the PCR based analysis of the relative abundance of the two isoforms revealing the dominance of the short isoform in the total hippocampus [71] and the reported 30–40% contribution of $\gamma 2L$ to the total $\gamma 2$ transcripts in the hippocampus [46]. In all kindled groups the ratio between $\gamma 2L$ and $\gamma 2$ remained at the same percentage found in controls, despite the increased expression levels. However, in the long-term group the contribution of the $\gamma 2L$ variant decreased significantly in all areas (Table 2). Since total $\gamma 2$ mRNA was either unchanged or increased at long-term it can be derived that the short $\gamma 2$ variant is enhanced at this stage.

4. Discussion

4.1. Kindling induced changes in GABA_AR subunit mRNA expression

In this report the changes that take place in the mRNA levels of the GABA_AR subunits in the hip-

Table 2

Percentage of GABA_AR $\gamma 2L$ mRNA of total GABA_AR $\gamma 2$ mRNA expression in CA1, CA3 and fascia dentata (FD) neurons using normalized expression levels. The percentage was determined for each individual animal and mean $\pm$ S.E.M. was calculated for the kindled groups and a pooled control group. Statistical comparisons with respect to controls were carried out using the Student's *t*-test. In the long-term group, the percentage $\gamma 2L$ was decreased in comparison to controls but the total expression of all $\gamma 2$ variants was either unchanged or increased (fascia dentata) suggesting a compensatory increase of $\gamma 2S$ mRNA levels.

	Controls (<i>n</i> = 17)	6-AD (<i>n</i> = 6)	14-AD (<i>n</i> = 6)	FK (<i>n</i> = 7)	LT (<i>n</i> = 6)
CA1	39 $\pm$ 1	41 $\pm$ 1	40 $\pm$ 2	38 $\pm$ 1	32 $\pm$ 5 *
CA3	36 $\pm$ 1	37 $\pm$ 1	37 $\pm$ 1	36 $\pm$ 1	29 $\pm$ 3 **
FD	28 $\pm$ 1	31 $\pm$ 2	29 $\pm$ 2	31 $\pm$ 1	18 $\pm$ 3 ****

* *P* < 0.05; ** *P* < 0.025; *** *P* < 0.010; **** *P* < 0.003.

pocampus during the acquisition, stabilization and the persistent stages of kindling are described. The kindled focus of epileptiform activity was obtained by stimulation of CA1 area of the hippocampal formation. The levels of all presently known GABA_AR subunit variants were investigated except the two retina specific members of the ρ class. The numerical changes in kindled animals in comparison to implanted age matched controls are presented in Table 1 and an overview is graphically illustrated in Fig. 6. The main results can be summarized as follows.

(i) The mRNA levels of most GABA_A subunits were increased in at least one of the analyzed areas and at one or more stages of kindling ($\alpha 1$, $\alpha 2$, $\alpha 4$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 2$ and $\gamma 2L$). There were marked differences in the level of increase between these subunits but, in general, the increases were most pronounced in the fascia dentata. In this area almost all expressed subunits were enhanced at the early stages of kindling acquisition ($\alpha 1$, $\alpha 2$, $\alpha 4$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 2$ and $\gamma 2L$), while in CA1 fewer subunits were increased ($\alpha 1$, $\alpha 2$, $\alpha 4$, $\gamma 2$ and $\gamma 2L$) and in CA3 even less ($\alpha 2$, $\gamma 2$ and $\gamma 2L$).

(ii) Reduced GABA_A subunit mRNA levels were observed for the $\delta 1$ subunit in the fascia dentata and for $\gamma 2L$ at long-term in all three investigated areas. The reduction of $\beta 2$ mRNA levels in CA3, as reported previously [18], was essentially confirmed in the present series although the decrease was less evident. A minor reduction (–8%) of $\beta 2$ in CA1 of the 6-AD and 14-AD groups, as reported before, was not significant in the present series [18].

(iii) The kindling protocol did not activate the expression of the subunits that were expressed in control animals at very low levels, i.e. the expression of $\alpha 6$, $\gamma 1$, $\gamma 3$ in all principal cells and $\alpha 3$ and $\delta 1$ in CA1 and CA3.

(iv) At long-term, 28 days after the last seizure, few significant changes in comparison to implanted age matched controls were revealed; a decreased $\gamma 2L$ expression in all areas and a marked relative increase for the low-abundant $\alpha 3$ subunit in the fascia dentata. Both changes were only found in this group. The decrease of $\gamma 2L$ expression is striking because during all kindling acquisition stages its levels closely followed the increase that took place in $\gamma 2$ levels. However, at long-term the changes in $\gamma 2L$ and $\gamma 2$ deviated, suggesting a persistent shift in mRNA splicing preferences in favour of the short variant (Table 2).

When analyzing the above mentioned trends in more detail, two general patterns of changes can be recognized: (a) enhanced levels already found after 6 kindling stimulations and maintained at a high level as long as afterdischarges are triggered; this is the case for $\alpha 1$ in CA1; $\beta 2$ and $\beta 3$ in the fascia dentata; $\gamma 2$ and $\gamma 2L$ in all three areas; (b) enhanced levels found in the 6-AD and the 14-AD groups but subsiding thereafter

to levels not significantly different from controls. For example the $\alpha 4$ levels were increased in CA1 and fascia dentata after 6 and 14 afterdischarges, but after fully kindled seizures no significant alteration was observed. A similar pattern was found for $\alpha 2$ in CA3 and fascia dentata; and $\alpha 1$ and $\beta 1$ in the fascia dentata. The latter type of changes, confined to the initial acquisition kindling stages, may be of particular interest since the 6-AD and 14-AD groups represent the kindling stages at which an ongoing process of epileptogenesis is taking place, as can be deduced from a progressive increase in afterdischarge duration and seizure complexity (Fig. 1).

The normalization of the expression levels revealed an expression profile that was in accordance with the relative hybridization signal intensity reported in literature [4,54,73]. All quantified subunits, contributed significantly to the GABA_AR subunit expression within their particular class, with the exception of $\alpha 3$ in fascia dentata that accounted for only 3% of the α type subunit mRNA expressed in that area. In all regions, the $\alpha 2$ transcript was the most abundantly expressed subunit of the α class (40–50%), $\beta 3$ dominated the β class expression (60%) and $\gamma 2$ accounted fully for the γ class due to the absence of both $\gamma 1$ and $\gamma 3$. Therefore, we conclude that the most conspicuous elevations provoked by kindling were manifested in the α , β and γ subunits that were also the most abundantly expressed variants within their class.

4.2. GABA_AR subunit mRNA expression in other models of synaptic plasticity

A variety of experimental conditions have been shown to either increase or decrease the expression levels of GABA_AR subunits. Rapidly declining levels of $\alpha 1$ and $\beta 2$ were observed in the hippocampal subfields after bilateral carotid occlusion, with a decrease of 43% developing within 4 hr after reperfusion indicating a rapid turn-over of these mRNAs [33]. While a single electroconvulsive shock did not lead to significant GABA_AR subunit mRNA changes in the neocortex or hippocampus [55], recurrent seizures were followed by changes in expression. Cortical levels of several subunits were long-lasting enhanced by seizure activity induced by β -carboline kindling [32]. Kokaia et al. [27] demonstrated a complex set of changes in the GABA_AR mRNA levels after rapid kindling in the hippocampus (i.e. tetanic stimulation every 5 min). Animals were fixed at different time intervals after 40 stimulations. Only $\alpha 1$, $\beta 3$ and $\gamma 2$ were investigated by *in situ* hybridization. During the first 4 hr after the last seizure all three subunits showed an initial decreased expression especially in the fascia dentata. Thereafter, up to 48 h, expression increased above control level and returned to control levels at 120 h. In CA1 and

CA3 no overt changes were found [27]. Friedman et al. investigated the effects of kainate induced seizures on $\alpha 1$ levels; 24 h after the seizures had started, a reduction of $\alpha 1$ was found in CA3 and no changes in CA1 or fascia dentata [11]. A Northern and slot blot analysis of mRNA from several brain regions failed to find any significant changes in $\alpha 1$, $\alpha 2$, $\alpha 4$ or $\beta 1$ transcripts, 30 days after hippocampal kindling or 24 h after rapid kindling [31]. Recently, an enhanced expression of $\alpha 4$, $\beta 1$ and $\beta 3$ subunits in the fascia dentata, 4 h after the last amygdala kindling induced seizure, was reported [6]. Based on the rather scarce number of publications addressing the subject of GABA_AR subunit expression in kindling and given the differences in the experimental models used, it is difficult to relate these data to the observations presented here. Nevertheless, the overall tendency suggests an enhanced expression, although seizure activity may, transiently, depress mRNA expression. In contrast, several studies on the effects of long-lasting application of GABA_AR agonists to cultured neurons, or the continuous treatment of animals with benzodiazepines, have quite consistently reported a decreased expression of the GABA_AR subunits $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$ and $\gamma 2$, which has been related to reduced GABA_AR mediated functions [16,42,48,65,80]. The administration of the inverse benzodiazepine agonist, FG 7142, for several days increased $\alpha 1$ and $\gamma 2$, while treatment with diazepam resulted in decreased levels [56]. Furthermore, the long term administration of ethanol, a positive modulator of GABA_AR mediated functions, also decreased the expression of several α class members in the neocortex [44,49]. These data suggest that a long-lasting activation of GABA_ARs is followed by a response at the mRNA level, presumably to compensate for the altered receptor efficacy.

4.3. Relation of the observed mRNA changes and alterations of GABAergic function during kindling

One important issue that must be considered is whether changes reported here at the mRNA level can be related to the kindling induced modifications in GABAergic inhibition in the hippocampus, i.e. changes in GABA_AR binding [29,51,62,67,69], in synaptic GABAergic inhibition [7,21,24,70,75,77–79], in sensitivity for iontophoretically applied GABA [19] and in GABA_AR mediated Cl^- uptake in synaptoneurosomes [66]. In this respect, it is of particular interest to compare the opposite changes in CA1–3 area, where a reduced GABAergic inhibition is manifested, with the changes in fascia dentata region where inhibition is strengthened [21,26,66,67,69,79]. Based on immunoprecipitation studies, it has been postulated that the muscimol and benzodiazepine binding GABA_ARs in the hippocampus are preferential assembled from associations of $\alpha(1,2,4)$, $\beta(1–3)$ and $\gamma(2)$ subunits [8–10,61].

Moreover, single-channel patch-clamp recordings from different combinations of co-expressed subunits in cultured cells confirmed the preferential assembly into $\alpha 1\beta 1\gamma 2$ S subunits over a combination of $\alpha 1\beta 1$ [2]. The observed enhanced expression of α , β and γ subunits in the fascia dentata in kindled rats, therefore, suggests an upregulation in the number of GABA_ARs, particularly of those assembled in $\alpha 1/2/4$, $\beta 1/3$ and $\gamma 2$ combinations. Since these subunits are dominant under normal conditions it can be concluded that there is no conspicuous change induced by kindling of the overall subunit composition of GABA_ARs. Moreover, the relative increases of the different α type subunits are comparable, suggesting no striking alteration in the pharmacological profile of benzodiazepine modulation in the fascia dentata [13,39,57–59,63,72]. This interpretation of our observations correlates well with the enhanced [^3H]muscimol, [^3H]flunitrazepam and [^{35}S]TBPS binding [51,62,67–69], the increased GABAR mediated Cl^- uptake by synaptoneurosomes prepared from fascia dentata [66] and the gradual strengthening of local recurrent inhibition in the course of kindling [7,21,26,53,70,79]. At long-term the changes in inhibition, receptor binding and in GABA-mediated flux had virtually subsided and the expression of GABA_AR subunits also returned to control levels. We concede that the extrapolation of changes detected at the mRNA level to changes at the receptor or even at the functional level of GABAergic inhibition should be made cautiously. However, parallel changes at mRNA and protein level have been reported in other models [41–43], and the relative expression of the subunit mRNAs is closely reflected at the receptor protein level [8,10,38,46].

For the CA1 subfield the interpretation of the changes in the GABAergic system induced by kindling is more complicated. A close monitoring of the temporal changes of GABAergic inhibition in the course of kindling acquisition, revealed an ongoing loss of paired pulse inhibition that started already after the first session and gradually became more evident during the acquisition phase to approximately the 16th kindling session [21,24]. In line with these observations, in fully kindled animals a decreased sensitivity of pyramidal neurons for GABA [20] and a reduced GABA_AR mediated Cl^- uptake by synaptoneurosomes prepared from CA1/3 region was reported [66]. Binding studies in the same region revealed decreased GABA_AR ligand binding although these changes were rather small [51,62,67–69]. In view of these observations, the increased expression of GABA_AR subunits is unexpected. We may speculate that the shift in equilibrium between number of binding sites and the number of mRNA copies indicates an aberrant translation or post-translational processing of the GABA_AR subunits. Another possibility is a gradual down-regulation

of the GABA_AR function caused by a persistently modified receptor phosphorylation or due to an increased receptor turn-over [3,28,37,50,81]. In this context, both the enhanced expression of subunits as well as the reported increased presynaptic GABA release [23] can be interpreted as a compensatory effort of the local neuronal circuit to nullify the GABA_AR down-regulation and to maintain the balance between excitation and inhibition as close as possible within normal limits.

At long-term, one month after cessation of kindling stimulations, when the enhanced sensitivity for seizures still persists, the expression of GABA_AR subunits had returned to control levels except for the selective decrease of γ 2L. Since the total expression of all γ 2 variants was either unchanged or increased (fascia dentata), a simultaneous increase of γ 2S mRNA levels is suggested, implicating a change in splicing route at the expense of the PKC/calmodulin-dependent protein kinase II phosphorylation sites containing variant [28,37,50].

4.4. Conclusions

Kindling epileptogenesis of the Schaffer collaterals in hippocampal CA1 area is associated with an upregulation of GABA_AR subunit mRNA levels in all subfields of the hippocampus but most prominent in the fascia dentata. The enhanced expression levels may be related to the increased receptor density and enhanced GABAergic inhibition in the dentate region. In contrast, the long-lasting reduced inhibition in CA1, underlying a local increased excitability, can not be accounted for by an impaired or altered pattern of subunit expression and must be explained by other, presently unknown, molecular alterations, such as a modified receptor phosphorylation.

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