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LONG-TERM AND REGIONAL SPECIFIC CHANGES IN ³H]FLUNITRAZEPAM BINDING IN KINDLED RAT HIPPOCAMPUS

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Abstract—The binding of the GABA_A/benzodiazepine receptor agonist [³H]flunitrazepam was studied in the hippocampus of rats kindled by daily stimulation of the Schaffer collaterals, using semi-quantitative autoradiography. Two kindled stages were investigated: (i) 24 h after the last generalized tonic-clonic seizure (fully kindled) and (ii) 28 days after the last generalized seizure (long-term). The binding of [³H]flunitrazepam was determined at two concentrations, 3 and 16 nM.

In the CA1 area, we found a small but significant decrease (*ca.* 10%), both in the 3 and 16 nM [³H]flunitrazepam binding at the fully kindled stage. In contrast, there was a significant increase in the 3 nM binding (*ca.* 15%) at the long-term stage. The 16 nM binding was not significantly different from control binding at this stage. In the granular and molecular layers of the fascia dentata, we found at both kindled stages a significantly increased 3 nM (*ca.* 9 and 19%, respectively) and 16 nM (*ca.* 19 and 14%, respectively) binding. Furthermore, we found that muscimol was still able to enhance the [³H]flunitrazepam binding in kindled animals, indicating that the GABA_A receptor agonist binding site and benzodiazepine agonist binding site are still functionally coupled.

The changes in [³H]flunitrazepam binding at the fully kindled stage are in agreement with the recently observed kindling-induced changes in [³H]muscimol binding in the hippocampal formation of the same animals [Titulaer M. N. G. *et al.* (1994) *Neuroscience* 59, 817–826] and extend these observations to the benzodiazepine modulatory site of the GABA_A receptor.

We conclude that the observed changes in GABA_A/benzodiazepine receptor complex binding may contribute to the induction and maintenance of a kindling-induced epileptogenic focus.

Kindling is widely used as a model for human temporal lobe epilepsy and complex partial seizures.¹⁵ The term “kindling” was first introduced by Goddard *et al.*¹⁰ in 1969, and refers to the process by means of which repeated periodic application of high-frequency electrical stimuli to certain brain areas results in the gradual appearance of increasingly complex behavioural and electrographic seizures, culminating in generalized tonic-clonic convulsions (class V seizures³⁷). Once this stage of kindling has been achieved, the susceptibility to seizures is long-lasting,¹⁰ indicating that the kindling process causes a permanent change in neuronal networks. Many neurotransmitter systems have been thought to be involved in kindling epileptogenesis, and both an enhancement of excitatory drive and/or a decrease in inhibitory control have been implicated.^{22,36} However, the exact underlying neurophysiological mechanisms are still unclear.

Several lines of evidence indicate the involvement of a disturbed GABAergic neurotransmission in kindling epileptogenesis. Pharmacological blockade of the GABAergic system accelerates kindling, whereas

pharmacological enhancement of GABAergic activity retards it.^{4,13,23} Benzodiazepines are also effective anticonvulsants, which can control a variety of partial and generalized seizures in experimental animal models.²⁶ They exert their effects via a positive allosteric modulation of the GABA_A/benzodiazepine receptor complex, resulting in facilitation of the inhibitory neurotransmission mediated by GABA.³⁸

In kindled animals, electrophysiological studies using paired-pulse stimulation protocols have revealed a decreased paired-pulse depression of local evoked field potentials in the CA1 area and an increased paired-pulse depression in the fascia dentata 24 h after the last seizure, and even at two to four weeks later.^{8,14,18,25,46,47} Paired-pulse depression is considered to be a sensitive measure of GABAergic inhibition in the hippocampus. The decrease in paired-pulse depression in the CA1 area may be caused by the long-lasting decrease of the responsiveness of pyramidal CA1 neurons to GABA observed by Kamphuis *et al.*¹⁶

To investigate whether the observed electrophysiological^{8,14,25,46,47} and iontophoretic¹⁶ changes can be related to changes in GABA_A/benzodiazepine receptor complex binding, we studied various binding sites of this receptor complex using the technique of semi-quantitative autoradiography.

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Abbreviations: FC, fully kindled control; FK, fully kindled; LC, long-term kindled control; LT, long-term kindled.

A transient increase in the [^3H]muscimol, [^3H]flunitrazepam and diazepam-insensitive [^3H]Ro 15-4513 binding restricted to the fascia dentata area at 24 h after the last class V seizure induced by entorhinal or amygdala stimulations has been reported.^{6,31,32,45,46} Nevertheless, clear changes in other hippocampal areas, namely in the CA1, were not reported. Recently, we reported long-lasting changes in [^3H]muscimol binding in opposite directions in the granular/molecular layers of the fascia dentata and in the CA1 area, induced by Schaffer collateral kindling,⁴¹ i.e. an increase in the former and a decrease in the latter. This prompted us to examine whether these two hippocampal subareas also reacted differently as regards the benzodiazepine agonist binding site.

Here, we report the results of a semi-quantitative autoradiographic study using [^3H]flunitrazepam on tissue sections obtained from the same animals as in the [^3H]muscimol study. The ligand binding was investigated in the CA1 area and the fascia dentata, at two ligand concentrations in the range of the estimated K_D (3 nM) and B_{max} (16 nM) values of the [^3H]flunitrazepam binding and at two kindled stages, namely at 24 h (fully kindled stage, FK) and at 28 days (long-term stage, LT) after the last class V seizure. In addition, in order to investigate the allosteric coupling between the GABA_A and benzodiazepine agonist binding site after kindling stimulations, we studied the enhancement of the [^3H]flunitrazepam binding by muscimol in the two hippocampal subareas at both stages.

EXPERIMENTAL PROCEDURES

Animals and implantation procedure

Thirty-one Male Wistar rats (250–300 g) were anaesthetized with sodium pentobarbital, and two bundles of isolated stainless-steel electrodes (80 μm diameter) were chronically implanted in the left dorsal hippocampus under electrophysiological control. The recording electrodes were placed in strata oriens and radiatum of the CA1 area (coordinates: 2.4 mm posterior to bregma and 1.4 mm lateral to the midline). Stimulation electrodes were placed in the Schaffer collateral fibre layer (coordinates: 2.8 mm posterior to bregma and 2.4 mm lateral to the midline). The electrodes were fixed to the skull with dental acrylic cement, when optimal field potentials to Schaffer collateral fibre stimulation were obtained. These procedures have been described in detail previously.¹⁴ After implantation, the rats were individually housed in plastic cages with free access to food and water in a temperature-controlled room (20–22°C) with a 12 h light: 12 h dark cycle (lights on 7.00 a.m.). After a recovery period of one week, the rats were divided into a control group and a group that was to be kindled.

Kindling procedure

The kindling stimulus (200–300 μA , 50 Hz, 0.1 ms duration biphasic pulses, 1 s) was applied twice daily. The animals in the control group were handled daily, in the same way as the kindled group. In the course of kindling, the animals were randomly assigned to two groups. A fully-kindled group (FK; $n = 8$), killed 24 h after the last class V seizure,³⁷ and a long-term group (LT; $n = 8$), killed 28 days after the last class V seizure. A “fully-kindled”

control group (FC; $n = 6$) and “long-term” control group (LC; $n = 6$) were killed along with the FK and LT groups. The FK group experienced a mean of 25 tetanic stimulations, resulting in a mean of five class V seizures. The LT group experienced a mean of 26 tetanic stimulations, resulting in a mean of four class V seizures.

Preparation of coronal brain sections

Rats were killed by decapitation and the brains were rapidly removed, dipped into ice-cold sucrose (0.32 M) and subsequently frozen by immersion in cooled (-30°C) isopentane. The brains were then stored at -60°C until sectioning. The frozen brains were brought into a cryostat microtome (-20°C). Consecutive coronal sections (12 μm) were cut from hippocampal brain area (plates 33–36³⁵) and thaw-mounted onto chrome-alum-gelatin-coated microscope slides. Four adjacent sections were mounted per slide. Mounted sections were air-dried for 10 min and subsequently stored at -20°C until incubation.

[^3H]Flunitrazepam binding assay “wipe off” studies

The [^3H]flunitrazepam binding assays were performed in the following way. Sections were placed at room temperature for at least 45 min before incubation. There was no difference between [^3H]flunitrazepam binding studies performed at $0-4^\circ\text{C}$ or at room temperature (data not shown). All procedures were therefore carried out at room temperature. The sections were preincubated three times for 10 min in a large volume of buffer (50 mM Tris-HCl, pH 7.4) to remove endogenous GABA. High-performance liquid chromatography analysis of the GABA content of the tissue sections revealed that after preincubation the GABA content in the sections was reduced by more than 99%, compared to the GABA content in the unwashed sections.⁴² Subsequently, the sections were dried under a stream of cold air. For the “wipe off” studies, the sections were incubated for 80 min in buffer containing various concentrations of [^3H]flunitrazepam (84 Ci/mmol; Amersham) in the range of 0.5–20 nM. After incubation, the sections were washed twice in buffer for 2 min each, followed by a dip in ice-cold distilled water to remove residual salts. Non-specific binding was determined in the presence of 2.5 μM diazepam and 200 nM clonazepam (diazepam was obtained from Sigma Chemical Co.; clonazepam was a gift from Dr M. Verhage, Rudolf Magnus Institute, University of Utrecht, The Netherlands). Specific binding accounted for more than 95% of the total binding. Sections were then quickly wiped off from the slides with filters (No. 6 Glasfaser Rundfilter, Schleicher and Schuell) and the radioactivity bound to the filter was determined by liquid scintillation counting (Packard, 1600 TR).

[^3H]Flunitrazepam autoradiography

The slide-mounted sections were incubated with two concentrations of [^3H]flunitrazepam in the range of the estimated K_D (3 nM) and B_{max} (16 nM) values determined previously in the “wipe off” study.

Allosteric modulation of the [^3H]flunitrazepam binding by muscimol

To investigate the coupling between the GABA_A and benzodiazepine agonist site, after Schaffer collateral kindling, incubation was carried out with 3 nM [^3H]flunitrazepam in the presence of 10 μM muscimol.

After incubation the sections were quickly dried under a stream of cold air. ^3H -sensitive film (^3H -Hyperfilm, Amersham), was apposed to the dried sections along with a ^3H -standard (Amersham) in light-tight cassettes for eight to 12 weeks. The levels of polymer activity on the ^3H -microscales are given in actual values, nominal values and tissue equivalent values in Bq/mg and nCi/mg wet weight. We used the tissue equivalent values in Bq/mg wet weight to convert the grey values measured on the autoradiographs into

radioactivity bound. In the CA1 area (strata oriens, pyramidale and radiatum), the absolute amounts of 16 nM [^3H]flunitrazepam binding (mean $\pm$ S.E.M.) were: in the FC group, 1137 ± 48 fmol/mg wet weight; in the fascia dentata (granular layer and upper and lower blades of the molecular layers), 1271 ± 139 fmol/mg wet weight; in the hilus, 783 ± 32 fmol/mg wet weight. However, due to the fact that the 3 and 16 nM incubations were not processed simultaneously in the same cassettes for the establishment of autoradiographs, the absolute amounts of the 3 and 16 nM binding cannot reliably be compared. All results are therefore presented graphically as relative changes in relation to binding in the control animals.

After exposure, the films were developed at room temperature (Kodak-D19, 4 min). Non-specific binding was not detectable on the autoradiographs. After exposition the sections were stained with Cresyl Violet to obtain Nissl images.

Data analysis

The autoradiographs were digitized and analysed using an image analysing system (IBAS). The image analyser was connected to a Bosch TYK9BTV camera equipped with a chalnicon tube mounted on a Zeiss microscope. The microscope was equipped with planapo objectives (magnification 1.25 times 1.6) and connected to a light source with stabilized power supply. Appropriate software, specifically designed for measuring ligand binding in the hippocampal formation by Dr C. Pool (Netherlands Institute of Brain Research), was used. The procedures for the analysis of ligand binding have been described in detail previously.⁴¹ The analysed hippocampal layers are indicated in Fig. 1a.

Quantification of the outlined areas was performed as follows: the image analyser scaled the mean grey values of the ^3H -standard into the known radioactivity in Bq/mg wet weight and a calibration curve was established. Subsequently, the mean grey values of the measured areas were determined and converted into specific ligand bound in Bq/mg wet weight by using the previously established ^3H -calibration curve.

Two of the four sections on a slide were used for analysis (in general the middle two sections), as the variation between animals was found to be larger than the variation between two different sections of one animal. The data from the ipsi- and contralateral sites were not significantly different (paired Student's *t*-test) and further analysis was therefore carried out on the mean specific ligand bound in Bq/mg wet weight of both hemispheres.

Statistics

All results are presented as relative changes in relation to binding in the control animals (mean percentage difference $\pm$ S.E.M.). Because strata oriens, pyramidale and radiatum of the CA1 area, on the one hand, and the granular and molecular layers of the fascia dentata, on the other, appeared to react in a similar way to kindling stimulation, we decided to test the null hypothesis (i.e. no difference between FK or LT groups and their controls) on the raw data, measured separately in all layers of the CA1 area and in the granular and molecular layers of the fascia dentata, using a two-way ANOVA, with repeated measurements, with treatments and the two hippocampal subareas as variables. Values of *P* less than 0.05 were considered significant. The data of the hilus were analysed using Student's *t*-test.

RESULTS

[^3H]Flunitrazepam binding in the rat hippocampus

The pattern of [^3H]flunitrazepam binding in the rat hippocampal formation showed a high binding in the granular and molecular layers of the fascia dentata,

an intermediate binding in the CA1 area and a low binding in the hilar formation (Fig. 1b). This is in agreement with the observations of Olsen *et al.*³³ and Unnerstall *et al.*^{43,44}

[^3H]Flunitrazepam binding kinetics

Figure 2a shows the saturation curve, in the concentration range of 0.5–20 nM, of the [^3H]flunitrazepam binding to coronal rat brain sections from three control Wistar rats. Non-specific binding was determined in the presence of 2.5 μM diazepam and 0.2 μM clonazepam. Computer-assisted non-linear least square curve fitting of the

Fig. 1. Outlined hippocampal areas for analysis of the [^3H]flunitrazepam binding with the aid of an image-analysing system (IBAS). 1, Stratum oriens; 2, stratum pyramidale; 3, stratum radiatum (CA1 area); 4, upper blade molecular layer; 5, granular layer; 6, hilus; 7, lower blade molecular layer (fascia dentata). See text (in Experimental Procedures) for explanation of the procedure of analysis and quantification. (b) Distribution of [^3H]flunitrazepam binding in the rat hippocampal formation. Highest binding is observed in the granular and molecular layers of the fascia dentata. Intermediate binding is present in the CA1–CA3 area, and the hilus shows low binding.

Fig. 2. Saturation plot of [^3H]flunitrazepam (0.5–20 nM) binding to cryostat-mounted coronal brain sections (12 μm thick) from three control Wistar rats. Specific binding is given in d.p.m./two sections $\pm$ S.E.M. Non-specific binding was determined in the presence of 2.5 μM diazepam and 200 nM clonazepam. The binding assay was carried out as described (see Experimental Procedures). Bound radioactivity was measured by liquid scintillation counting. (b) Scatchard analysis of the specific [^3H]flunitrazepam binding. Non-linear curve fitting indicated the presence of one binding affinity with an estimated K_D value of 3.2 nM.

Scatchard plot³⁰ of the specific [^3H]flunitrazepam binding (Fig. 2b) revealed the presence of one binding affinity of [^3H]flunitrazepam, with an estimated K_D of 3.2 nM (regression line correlation coefficient: $r^2 = 0.903$, $n = 3$). On the basis of these findings, the autoradiographic studies were carried out using two concentrations of [^3H]flunitrazepam, 3 and 16 nM, reflecting the observed K_D value and the corresponding B_{max} value, respectively.

[^3H]Flunitrazepam binding in kindled rat hippocampus: quantitative autoradiography

Table 1 presents the relative change in binding of the 3 and 16 nM [^3H]flunitrazepam concentrations to the GABA_A/benzodiazepine receptor agonist site, in relation to the corresponding control binding (mean percentage difference $\pm$ S.E.M.) within the two hippocampal subareas, CA1 (strata oriens, pyramidale, radiatum) and fascia dentata (upper and lower blades

of the granular and molecular layers and hilus), at the FK and LT stages. The main findings are as follows.

CA1 area. The CA1 area showed a significant decrease of approximately 10% ($P \leq 0.005$) in the 3 and 16 nM [^3H]flunitrazepam binding at the FK stage. The 3 nM binding was significantly increased ($P \leq 0.01$) by c. 15% at the LT stage, whereas at this stage no significant change in the 16 nM binding in comparison to control binding was observed.

Fascia dentata. In the upper and lower blades of the granular and molecular layers of the fascia dentata, we found a significant increase ($P \leq 0.01$) of 9% in the 3 nM and 19% in the 16 nM [^3H]flunitrazepam binding at the FK stage, and of 19% and 14% at the LT stage, respectively. The hilus did not show any significant change in the 3 and 16 nM [^3H]flunitrazepam binding in comparison to control binding.

Allosteric modulation of the [^3H]flunitrazepam binding by muscimol in kindled rat hippocampus

To investigate whether the allosteric coupling between the GABA_A receptor agonist site and the benzodiazepine binding site was changed as a result of kindling stimulations, we studied the enhancement of the 3 nM [^3H]flunitrazepam binding by 10 μM muscimol. This concentration of muscimol caused a

Table 1. Relative changes in [^3H]flunitrazepam binding in relation to binding in the control animals in the two hippocampal subareas, CA1 area and fascia dentata (upper and lower blades of the granular and molecular layers, and hilus)

		FK		LT	
CA1 area					
3 nM	str. or.	6.1 ± 1	**	20.0 ± 4	*
	str. pyr.	-9.1 ± 2.4		12.8 ± 5.7	
	str. rad.	-10.6 ± 0.6		14.6 ± 4.1	
16 nM	str. or.	-10.7 ± 3	*	5.0 ± 4.6	
	str. pyr.	-8.4 ± 4.5		1.3 ± 4.4	
	str. rad.	-10.2 ± 3.5		3.7 ± 4.1	
Fascia dentata					
3 nM	upp. bl.	11.6 ± 1.6	*	17.7 ± 3.1	*
	gran.	5.9 ± 3.2		20.8 ± 6.2	
	low. bl.	11.2 ± 2.1		17.7 ± 2.9	
	hilus	-6.9 ± 2.3		19.3 ± 9.2	
16 nM	upp. bl.	18.8 ± 5.6	*	17.5 ± 3.7	*
	gran.	18.4 ± 5.3		9.6 ± 3.1	
	low. bl.	19.1 ± 5.6		13.0 ± 2.7	
	hilus	-5.6 ± 3		-0.7 ± 2.2	

Statistical comparison in the CA1 area, and in the granular and molecular layers of the fascia dentata was done by two-way ANOVA with repeated measurements (see text). The data of the hilar region were analyzed using Student's *t*-test. Data are means $\pm$ S.E.M. for the following number of animals: three controls, six kindled (3 nM binding); six control, eight kindled (16 nM binding). * $P \leq 0.01$, ** $P \leq 0.005$. str. or., stratum oriens; str. pyr., stratum pyramidale; str. rad., stratum radiatum; gran., granular layer; upp. bl., upper blade; low. bl., lower blade.

Table 2. Relative increase (delta percentage) of the 10 μM muscimol-modulated 3 nM [^3H]flunitrazepam binding in comparison to the unmodulated 3 nM [^3H]flunitrazepam binding (set at 100%), in the CA1 area and fascia dentata, at the fully kindled and long-term stages

	Δ increase (%)	Experimental vs control
CA1		
FC	25.0 $\pm$ 2.0	
FK	36.2 $\pm$ 1.6	1.45**
LC	35.0 $\pm$ 3.7	
LT	29.2 $\pm$ 2.9	0.83 (N.S.)
Fascia dentata		
FC	28.4 $\pm$ 4.8	
FK	27.0 $\pm$ 3.8	0.95 (N.S.)
LC	19.9 $\pm$ 2.6	
LT	16.9 $\pm$ 3.3	0.85 (N.S.)

Comparison of the ratio muscimol modulation between the experimental groups (FK, LT) and their corresponding controls (FC, LC). Statistical analysis shows only a significant difference for the FK group in the CA1 area. Two-way ANOVA, with repeated measurements: ** $P \leq 0.01$.

maximal increase in the [^3H]flunitrazepam binding by approximately 50% in coronal rat brain sections from control rats, as determined in "wipe off" studies (not shown). Table 2 gives the relative increase (as a percentage) in the 10 μM muscimol-modulated 3 nM [^3H]flunitrazepam binding in comparison to the unmodulated 3 nM [^3H]flunitrazepam binding (set at 100%), in the CA1 area and fascia dentata, for the FC, FK, LC and LT stages. In all cases, the 3 nM [^3H]flunitrazepam binding was modulated by muscimol. However, the modulation observed in the kindled animals was in general somewhat smaller than the modulation seen in the corresponding control groups, although not significantly different. Only in the CA1 area, at the FK stage, was a significantly larger muscimol modulation of [^3H]flunitrazepam binding for the kindled animals in comparison to the corresponding controls found.

DISCUSSION

Main findings

Two main findings emerge from this study. First, kindling stimulations induce long-lasting and time-dependent changes in [^3H]flunitrazepam binding in the hippocampal formation, confirming previous reports regarding the [^3H]muscimol⁴¹ and [^{35}S]TBPS⁴² binding. Second, the changes in [^3H]flunitrazepam binding are regionally specific and can occur in opposite directions in the CA1 area and fascia dentata in FK rats. These findings are based upon the following observations: hippocampal kindling induces, 24 h after the last seizure, a significant decrease in both the 3 and 16 nM [^3H]flunitrazepam binding in the CA1 area, whereas at the LT stage, 28 days after the last seizure, there was a significant increase in the 3 nM [^3H]flunitrazepam binding and no significant change in the 16 nM binding in comparison to con-

trol binding in this hippocampal subarea. In the granular and molecular layers of the fascia dentata, kindling induces a significant increase in the 3 and 16 nM [^3H]flunitrazepam binding, both at the FK and LT stages.

Kindling-induced changes in [^3H]flunitrazepam binding

The presence of one [^3H]flunitrazepam binding site in coronal sections of rat brain, with an estimated K_D value of 3.2 nM estimated in our study, is in agreement with the reported values in the literature.^{3,24,28,46} In the present study, we investigated two concentrations of [^3H]flunitrazepam in the range of the estimated K_D value (3 nM) and corresponding to the value at which B_{max} is found (16 nM). Due to the fact that there is only one [^3H]flunitrazepam binding affinity in coronal rat brain sections (Fig. 2b), we made an effort to interpret the observed changes in [^3H]flunitrazepam binding in terms of specific changes in binding characteristics. At the FK stage, the CA1 area presented decreases both in the 3 and 16 nM binding, indicating a decrease in binding site density (B_{max} value); however, at the LT stage, the 3 nM binding was significantly increased, whereas the 16 nM binding was not different from control, suggesting an increase in binding affinity (decreased K_D value). In contrast, the upper and lower blades of the granular and molecular layers of the fascia dentata showed significant increases in the 3 and 16 nM [^3H]flunitrazepam binding at both kindled stages, indicating a long-lasting increase in binding site density. However, it has to be stressed that Scatchard analysis including more [^3H]flunitrazepam concentrations is needed to substantiate these interpretations.

Using quantitative autoradiography, an increase in the [^3H]flunitrazepam binding in the fascia dentata of rats kindled in the entorhinal cortex and amygdala, 24 h after the last seizure, has also been reported. However, neither long-term changes in the fascia dentata nor changes in the CA1 area were reported in these studies.^{6,31,39,45} The differences between our observations and the former studies may be related to the fact that amygdala or entorhinal kindling may cause less conspicuous changes in the different hippocampal subareas than kindling of the Schaffer collaterals.

Allosteric modulation of the [^3H]flunitrazepam binding by muscimol in kindled rat hippocampus

GABA, or GABA agonists, increase the affinity of [^3H]flunitrazepam for the GABA_A/benzodiazepine receptor complex.^{12,34,38} To investigate the allosterical coupling between the benzodiazepine agonist and GABA_A receptor agonist binding site after kindling stimulations, we studied the ability of 10 μM muscimol to increase the 3 nM [^3H]flunitrazepam binding in the two hippocampal subareas, at 24 h and 28 days

after the last class V seizure. In the CA1 area, muscimol was able to increase [^3H]flunitrazepam binding, 24 h after the last seizure, to a higher extent than in controls, thereby apparently compensating for the decrease in [^3H]flunitrazepam binding in the CA1 area at the FK stage. This is somewhat surprising, since we also observed a decrease in the high/intermediate (5–40 nM) and low-affinity (60–100 nM) [^3H]muscimol binding in the CA1 area at the FK stage.⁴¹ However, it should be noted that the [^3H]muscimol concentrations used in the autoradiographic study are very low and represent binding to relative high-affinity states of the GABA_A receptor, whereas the allosteric modulation of the [^3H]flunitrazepam binding by muscimol is tested at concentrations that are two orders of magnitude higher and are mediated by a “super-low-affinity” state (μM binding) of the GABA_A receptor.³⁸ The data indicate that under our experimental conditions the GABA_A and benzodiazepine sites are still functionally coupled. Muscimol was also able to increase the [^3H]flunitrazepam binding in comparison to the unmodulated binding, in the CA1 area at the LT stage, and in the granular and molecular layers of the fascia dentata at both experimental stages. However, in all these cases we observed a smaller, although not significantly different (see Table 2), increase in the muscimol-modulated binding in comparison to the unmodulated binding. Similar findings for the fascia dentata have also been reported by Fanelli and McNamara⁹ and Shin *et al.*³⁹ It might be speculated that the coupling mechanism between the GABA_A and benzodiazepine receptors is less effective in the granular and molecular layers of the fascia dentata at both kindled stages and in the CA1 area at the LT stage. However, the fact that muscimol is still able to modulate the [^3H]flunitrazepam binding appears to contradict this hypothesis. We cannot exclude that the super-low-affinity state of the GABA_A receptor, which mediates the effect of the 10 μM muscimol concentration, may be changed as a result of the kindling stimulations. To investigate this, the super-low-affinity binding site may be examined with specific ligands.

Kindling-induced changes in [^3H]flunitrazepam binding and GABAergic inhibition

The observed decrease in [^3H]flunitrazepam binding in the CA1 area and increase in the granular and molecular layers of the fascia dentata at the FK stage closely parallels the previously observed decreased high/intermediate and low-affinity [^3H]muscimol binding in the CA1 area and increased binding in the granular and molecular layers of the fascia dentata of the same animals, 24 h after the last class V seizure.⁴¹ These changes may underlie the electrophysiologically observed decreased paired-pulse depression in the CA1 area and increased paired-pulse depression in the fascia dentata at the FK stage.^{14,18,47,48} Kamphuis *et al.*¹⁷ reported a significant

increase of the GABA release in the CA1 area, already present after 14 tetanic stimuli of the Schaffer collaterals. The changes in GABA exocytosis and GABA_A/benzodiazepine receptor agonist binding in the CA1 area, reported here, may be related events. Chronic exposure of cultured neurons to GABA has been reported to induce down-regulation of GABA/benzodiazepine receptor binding, of GABA-gated chloride currents,^{11,27} and of GABA_A receptor subunit mRNA expression levels.²⁹ On the other hand, it may be speculated that the increase GABA release acts as a compensatory mechanism to overcome the observed decrease in [^3H]flunitrazepam and [^3H]muscimol⁴¹ binding sites in the CA1 area at the FK stage. However, a more detailed analysis of the time course of the reported changes is necessary, in order to obtain more insight into a possible causal relationship between these events.

There is evidence that the changes in paired-pulse depression are long-lasting. Zhao and Leung⁴⁷ observed a prolonged decrease in the paired-pulse depression in the CA1 area, *in vitro*, 23 days after the last class V seizure. Furthermore, the increased GABA release in the CA1 area was also still present at the LT stage.¹⁷ In agreement with these results, a long-lasting decrease of the responsiveness of the GABA_A receptors of pyramidal neurons, studied iontophoretically, in the CA1 area was reported¹⁶ after Schaffer collateral kindling. These results indicate that kindling stimulations cause a persistent change in the GABA_A receptor complex in the CA1 area. At the LT stage, the changes in GABA_A receptor binding in the CA1 area appear to be more complex. In the present study we found a significant increase in the 3 nM [^3H]flunitrazepam binding, whereas previously we observed a decrease in the low-affinity [^3H]muscimol binding.⁴¹ The increased 3 nM [^3H]flunitrazepam binding may represent an increased binding affinity and counteract the effects of the long-lasting decreased low-affinity [^3H]muscimol binding.⁴¹ Our results are in agreement with the observations of a regional increase in the ability of the benzodiazepine midazolam to potentiate GABA-mediated Cl^- flux one week after the last amygdala-kindled seizure in areas with reduced GABAergic function, particularly in hippocampus,⁴⁰ and with the report of an increase in the anticonvulsant efficacy of diazepam following amygdala kindling.¹

Using the *in situ* hybridization technique, Kamphuis *et al.*^{19,20} recently found that mRNA expression levels of various GABA_A receptor subunits in the pyramidal and granular neurons of the rat dorsal hippocampus were changed after Schaffer collateral/commissural kindling. In the early stages of the kindling process (after six and 14 afterdischarges) they observed in the CA1 area an increase in mRNA levels of the $\alpha 2$, $\alpha 4$, $\gamma 2$, $\gamma 2\text{L}$ subunits. In the fascia dentata, enhanced expression levels of the $\alpha 1$, $\alpha 2$, $\alpha 4$, $\beta 1$, $\beta 3$, $\gamma 2$ and $\gamma 2\text{L}$ subunits were found. At 24 h after

the last class V seizure, the increases were still present in some of the subunits in both areas: $\alpha 2$, $\beta 1$, $\beta 3$, $\gamma 2$ and $\gamma 2L$ in the CA1 area, and $\beta 2$, $\beta 3$, $\gamma 2$ and $\gamma 2L$ in the fascia dentata. At the LT stage, a few changes were encountered; the fascia dentata presented a significant increase in the low-abundant $\alpha 3$ and the $\gamma 2$ subunit mRNA expression levels, whereas the $\gamma 2L$ subunit now presented a significant decrease. In line with these results are the findings of Kokaia *et al.*,²¹ who observed a region-specific and differential regulation of the GABA_A receptor $\alpha 1$ -, $\beta 3$ - and $\gamma 2$ -subunit mRNA levels, following the induction of one, 10 and 40 hippocampal seizures. These kindling-induced changes in GABA_A receptor subunit mRNA expression levels are consistent with the binding studies for the fascia dentata at the fully kindled state,^{e.g. Refs 31,32,39,41} but are not easy to interpret with respect to the changes in the CA1 area. A possible explanation for the apparent contradiction between the increase in several subunits and the reduced GABA_A receptor binding in this area may be that kindling causes a modification in the translation or post-translational processing of the GABA_A receptor; for example, due to an increased receptor breakdown or internalization. Further investigations are necessary to elucidate this possibility.

CONCLUSION

The specific localization of the increase in [³H]flunitrazepam binding to the granular and molecular layers of the fascia dentata, at the FK and LT stages, and the process of mossy fibre axon sprouting and reactive synaptogenesis, which has been found to occur in several animal kindling models⁵ and in human temporal lobe epilepsy,² may be related events. Although mossy fibre sprouting is generally considered to be of excitatory nature, Davenport *et al.*⁷ found evidence of an increase of glutamate decarboxylase immunoreactivity in the dentate gyrus following intrahippocampal kainate. Therefore, it may be hypothesized that kindling-elicited sprouting in the dentate area may be accompanied by the formation of new GABAergic synapses, as revealed by the increased binding sites for [³H]muscimol⁴¹ and [³H]flunitrazepam reported here.

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