Plasticity of amino acid release and uptake during kindling epileptogenesis

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Chapter 5

Alterations in release and uptake of amino acids in rat hippocampus *in vivo* during kindling epileptogenesis
Amino acid release and uptake during kindling

Alterations in release and uptake of amino acids in rat hippocampus * in vivo during kindling epileptogenesis

Abstract

Changes in amino acid transmitter release and uptake during kindling epileptogenesis were investigated in rat hippocampus CA1 region by in vivo microdialysis. Elevations of extracellular GABA, glutamate and aspartate concentrations by the uptake blockers SK&F 89976-A and L-trans-PDC, indicated active regulation of these amino acids by their transporters. However, during kindling, the effects of both blockers disappeared, already after 14 afterdischarges for glutamate and aspartate, and after generalized seizures for GABA. Changes in vesicular and cytosolic, transporter-mediated, releasable pools during kindling were estimated by discriminating between K+-induced Ca²⁺-dependent and Ca²⁺-independent amino acid release, respectively. Shortly after generalized seizures, Ca²⁺-dependent GABA release increased faster, but the total releasable pool did not change. In contrast, 4-5 weeks after the last seizure, this GABA release component was decreased. Ca²⁺-independent GABA release was increased shortly after seizures, but was decreased 4-5 weeks thereafter. Both releasable pools of glutamate did not clearly change during kindling, whereas Ca²⁺-independent aspartate release was decreased 4-5 weeks after the last seizure. These results indicate complex changes in release and uptake of GABA during kindling. The reduced GABA release, at long-term, and the persistent decrease in glutamate/aspartate uptake, may contribute to enhanced seizure susceptibility.

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**Introduction**

Evidence is accumulating that temporal lobe epilepsy is the result of a disturbed balance between excitatory and inhibitory neurotransmission in limbic areas of the brain. Accordingly, the principal excitatory transmitter glutamate, and the main inhibitory transmitter GABA, have been the subjects of a variety of studies. Impairments in the synthesis and/or release of these amino acids as well as the function of their receptors have been observed in hippocampus tissue of epileptic patients and in animal models of epilepsy (Meldrum, 1994; Bradford, 1995; Glass & Dragunow, 1995). Several studies, using microdialysis, have related disturbed synaptic transmission to changes in extracellular levels of GABA and glutamate during epileptogenesis (Minamoto et al., 1992; During et al., 1992; Kaura et al., 1995). However, these amino acid levels are the net result of release and uptake by high-affinity transporters. Therefore, it is not known whether the observed changes reflect altered release and/or altered uptake. Changes in $\text{Ca}^{2+}$-dependent amino acid release from vesicular pools, evoked by high $\text{K}^+$, during epileptogenesis have been investigated mainly *in vitro*, and increases, decreases, or no changes were reported (Geula et al., 1988; Kapur et al., 1989; Jarvie et al., 1990; Kamphuis et al., 1990, 1991; see also Table 2 of Chapter 1). *In vivo*, increased $\text{K}^+$-stimulated GABA release both in amygdala-kindled rats and patients with temporal lobe epilepsy has been reported, but $\text{Ca}^{2+}$ dependency was determined only in the patients (During et al., 1992, 1995). The latter study was performed shortly after seizures (i.e. about 24 h), thus it did not provide information about persistent changes characteristic of the chronic epileptic state. Moreover, the time resolution of microdialysate collection was relatively poor (i.e. 30 min), which hampered the detection of dynamic changes during stimulation. During et al. (1995) proposed that a reduced GABA transporter activity would be the cause of the changes observed in extracellular GABA levels both in patients with temporal lobe epilepsy and amygdala-kindled rats. This conclusion was based on indirect observations of glutamate-induced increases in extracellular GABA. A reduced activity of glutamate transporters during epileptogenesis was also indicated by measuring aspartate uptake in cortical tissue *in vitro* of kindled rats (Leach et al., 1987). Although these studies yield fragmentary indications of impaired amino acid release and uptake during epileptogenesis, the relative importance of both processes regulating extracellular amino acid concentrations *in vivo* during development of epilepsy and thereafter was not studied before.
In the present study, we investigated changes in the regulation of extracellular amino acid levels comprehensively at different stages of epileptogenesis using local microdialysis in the CA1 region of freely moving rats. Epileptogenesis was generated by daily tetanic stimulation of the Schaffer-collateral fibres in the CA1 region accumulating to generalized seizures, the so-called kindling model of epilepsy (Goddard et al., 1969; Kamphuis & Lopes da Silva, 1990). Since extracellular amino acids monitored by in vivo microdialysis reflect the balance between neuronal release and uptake into surrounding neurons and glial cells (During, 1995; Herrera-Marschitz et al., 1996), we unravelled the effect of possible changes in amino acid transporter activity by estimating the effects of the GABA uptake inhibitor 1-(4,4-diphenyl-3-butenyl)-3-piperidine carboxylic acid hydrochloride (SK&F 89976-A) and the glutamate uptake inhibitor L-trans-pyrrolidine-2,4-dicarboxylate (L-trans-PDC) on basal extracellular amino acid levels. In addition, since changes in actual synaptic release of amino acid transmitters under baseline conditions will hardly be visible with microdialysis (During, 1995; Timmerman & Westerink, 1997a), we investigated changes in the capacity of releasable pools by depolarization with elevated K⁺. To determine the vesicular origin of these pools, the K⁺-stimulated Ca²⁺-dependent fraction of amino acid release was estimated as outlined previously (Zuiderwijk et al., 1996). Consequently, changes in Ca²⁺-independent (non-vesicular) pools, presumably representing reversal of GABA and glutamate transporters, were also determined. The collection of microdialysates every 2 min enabled us to monitor dynamic changes in extracellular amino acid levels during K⁺ stimulation. This study demonstrates that besides changes in vesicular GABA release, a long-term impairment in both in GABA and glutamate transporter activity is manifest in kindling-induced epilepsy.

Materials and methods

Materials

SK&F 89976-A was kindly provided by Dr. Skidmore (Smith, Kline & Beecham). L-trans-PDC was purchased from Tocris Cookson. Hypersil was from Shandon. Xylazine was from Bayer and ketamine from Aescoket. All other chemicals were of the purest grade available and were obtained from Sigma and
Merck. All solutions were prepared with HPLC-grade ultra-pure water generated by a "Milli-Q" purification system (Millipore).

**Kindling procedure**

All experimental procedures were controlled and approved by the Animal Experiments Committee of the University of Amsterdam (March, 1997).

The kindling procedure used in this study was described in detail by Kamphuis et al. (1988). Briefly, male Wistar rats (200-270 g body weight) were implanted with stainless-steel, trimel insulated, electrodes in the CA1 area of the right dorsal hippocampus under ketamine/xylazine (4 vol/3 vol) anaesthesia (0.1 ml/100 g body weight, i.m.). The stimulation electrodes were placed in the Schaffer-collateral/commissural fibre pathway and the recording electrodes in stratum radiatum. Concomitantly with the implantation of the electrodes, a guide cannula (CMA Microdialysis) was placed with its tip just above the CA1 area of the left dorsal hippocampus (A/P 2.4 mm, M/L 1.4 mm, D/V 1.9 mm, nose bar 5.0 mm above interaural line). After 1 week recovery, the rats were connected to a stimulation/recording device to enable kindling stimulations and local electroencephalographic recordings in freely moving conditions. The 42 implanted animals were divided into a non-stimulated control group (n = 21) and a kindling group (n = 21). The latter received, twice daily, kindling stimulations at an intensity suprathreshold for the induction of an afterdischarge (200-300 μA, 50 Hz, 1-2 s). The control group was handled in the same way as the kindling group throughout the experimental period, but did not receive tetanic stimulations. Microdialysis experiments were performed at different stages of kindling epileptogenesis: One group of animals (n = 7) was studied in the initial phase of kindling, after the induction of 14 afterdischarges (14AD). Microdialysis in this 14AD group was performed 24 h after the last afterdischarge. Along with this group, 8 animals of the control group were studied. Two groups of animals were studied after the occurrence of 7 generalized tonic-clonic class 5 convulsions (Racine, 1972): a fully kindled (FK) group (n = 7) in which microdialysis was performed 24 h after the 7th class 5 seizure (along with this group, 6 control animals were studied), and a so-called long-term kindled (LT) group (n = 7) in which microdialysis was performed 28-31 days after the 7th class 5 seizure (along with 7 control animals). The animals of this LT group were left unstimulated during this period, but it is known that a renewed kindling stimulation after
such a long period will result in a generalized convulsion, indicating that the excitability changes induced by kindling are long-lasting (Goddard et al., 1969).

**Microdialysis experiments**

To prevent gliosis around the microdialysis probe (Benveniste et al., 1987) and to obtain low and stable extracellular amino acid concentrations (Zuiderwijk & Ghijsen, 1997), the guide cannula obturators were replaced by CMA/11 microdialysis probes (membrane length 1.0 mm, CMA Microdialysis) filled with artificial cerebrospinal fluid (a-CSF) not until 20-24 h before the onset of microdialysis. The dialysis membrane covered all layers of the hippocampal CA1 region without penetrating into the dentate gyrus region. The next day, microdialysis probes were connected to a syringe-type infusion pump (Harvard Apparatus) via a liquid swivel, allowing the animals to move freely during the dialysis experiment. Probes were perfused with a-CSF containing (in mM) 150 NaCl, 3 KCl, 2 CaCl₂, 5 Hepes, pH 7.4, at a rate of 2.0 μl/min. Following a 2-h stabilization period, microdialysis samples were collected and perfusion media...

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**Fig. 1.** Time schedule of microdialysis procedure. 2 h after the beginning of microdialysis, 4-min baseline samples were collected, first in the absence (t = 120-136 min) and then in the presence (t = 170-186 min) of SK&F 89976-A (0.5 mM) and L-trans-PDC (1.0 mM). Subsequently, 2-min samples were collected around two stimuli of 153 mM K⁺ (16 min duration), applied sequentially at an interval of 90 min, first in the presence (t = 192-236 min) of Ca²⁺ and then in the absence (t = 282-326 min) of Ca²⁺, with SK&F 89976-A and L-trans-PDC present throughout. Sampling periods are indicated by the black bars.
were changed according to the time schedule outlined in Fig. 1. Firstly, four consecutive 4-min samples were collected under basal conditions. To study the regulation of extracellular amino acid levels by GABA and glutamate transporters, subsequently the uptake inhibitors SK&F 89976-A and L-trans-PDC were added to the perfusion medium at a concentration of 0.5 and 1.0 mM, respectively. These concentrations had previously shown clear transporter-mediated regulation of extracellular amino acid levels under basal and K+ depolarized conditions (Zuiderwijk et al., 1996). After 30 min, another set of four 4-min samples was collected. Thereafter, Ca2+-dependent and Ca2+-independent amino acid releasable pools were determined using a protocol of repetitive stimulation with elevated K+, while SK&F 89976-A and L-trans-PDC were present during the whole period, as slightly modified from previous experiments (Zuiderwijk et al., 1996). The K+ concentration in the perfusate was raised twice to 153 mM for 16 min (corrected for osmolarity by replacing NaCl by KCl), first in the presence of Ca2+ and 1.5 h later in the absence of Ca2+. Ca2+-free medium contained 10 mM Mg2+ (to block Ca2+ channel activity) and 2 mM EGTA (to chelate residual Ca2+) and was perfused 20 min after the end of the first high K+ period (Zuiderwijk et al., 1996). Before, during and after both high K+ periods, 2-min samples were collected. All collected samples were diluted to a total volume of 30 μl with a-CSF containing 1 % (wt/vol) trichloroacetic acid and 0.25 μM L-homoserine, as external standard, for amino acid content determination. To avoid amino acid metabolization, samples were kept cool (at 2°C) during the experiment by means of a recirculation cooler (B.Braun) and stored at -20°C until amino acid quantification. Rats were killed by decapitation immediately after the last sample had been collected.

Quantification of amino acids in dialysates

Amino acid content in the microdialysates was quantified by HPLC after precolumn derivatization of a 25-μl sample with 50 μl 10 mM o-phthalaldehyde/0.4 % (vol/vol) mercaptoethanol in 100 mM Na-tetraborate (pH 10.5), as described previously (Verhage et al., 1989). The 12.5-cm-long, 4.6-mm-diameter column packed with Hypersil C-18 (3 μm particle size), was eluted isocratically with a buffer (pH 7.0) containing 0.1 M Na2HPO4, 1 mM Na2-EDTA, 0.3 % (vol/vol) tetrahydrofuran and 35 % (vol/vol) HPLC-grade methanol. The o-phthalaldehyde derivates were detected by a Jasco (model FP-920) fluorimeter and evaluated by an on-line computerized data acquisition system (Gilson).
Amino acid release and uptake during kindling

Amino acid levels were calculated by comparison with a standard mixture of 0.25 \( \mu M \) of these amino acids.

**Statistical analysis**

Results were statistically evaluated using the paired or unpaired Student's *t*-test, or two-factor ANOVA with repeated measures, as indicated. Values were considered significantly different when \( P < 0.05 \).

**Results**

**Regulation of basal extracellular amino acid levels by GABA and glutamate transporters during kindling epileptogenesis**

To investigate possible changes in the activity of GABA and glutamate transporters during kindling epileptogenesis, extracellular amino acid concentrations in hippocampal CA1 region were measured under basal conditions successively in the absence and presence of the GABA uptake inhibitor SK&F 89976-A (0.5 mM) and the glutamate uptake inhibitor L-trans-PDC (1.0 mM). Since we had previously shown that SK&F 89976-A did not affect extracellular glutamate levels and L-trans-PDC did not affect extracellular GABA levels (Zuiderwijk et al., 1996), both compounds were applied simultaneously via the microdialysis probe. Because there were no significant differences between extracellular amino acid levels in the control groups corresponding to the different kindled groups, neither in the absence, nor in the presence of SK&F 89976-A and L-trans-PDC, all control experiments were pooled. In controls, the application of SK&F 89976-A and L-trans-PDC increased extracellular levels of GABA, glutamate, aspartate significantly (\( P < 0.01 \), paired *t*-test; Figs. 2A, B and C), indicating that both GABA and glutamate transporters are active under basal conditions. The increase of extracellular GABA in the presence of SK&F 89976-A and L-trans-PDC was still significant (\( P < 0.02 \)) in the initial phase of kindling, after the induction of 14 afterdischarges (14AD group) (Fig. 2A). However, the blocker sensitivity was absent in the fully kindled (FK) group, after 7 generalized seizures, and in the long-term kindled (LT) group, 28-31 days after the last seizure (Fig. 2A). Extracellular levels of aspartate and glutamate did not change significantly by the application of SK&F 89976-A and L-trans-PDC in any of the
kindled groups (Figs. 2B and C). In contrast, extracellular taurine levels were increased significantly by SK&F 89976-A and L-trans-PDC, both in control and kindled groups, regardless of the phase of kindling ($P < 0.05$, paired $t$-test; Fig. 2D), indicating that the impairment of amino acid uptake was selective for the transmitters GABA, glutamate and aspartate. Comparison of extracellular amino acid levels between control and kindled groups, both in the absence and in the

Fig. 2. Effects of 0.5 mM SK&F 89976-A (SK&F) and 1.0 mM L-trans-PDC (PDC) on basal extracellular levels of GABA, glutamate, aspartate and taurine in kindled and control animals. Kindled animals were divided into a 14 afterdischarge (14AD) ($n = 8$), a fully kindled (FK) ($n = 7$), and a long-term kindled (LT) ($n = 7$) group. Basal levels of non-kindled control animals from the 14AD, FK and LT group were pooled into one control group ($n = 21$). Each column represents the average of four 4-min samples. Within each kindled and control group, comparisons were made between basal levels in the absence and presence of SK&F 89976-A and L-trans-PDC (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, paired Student's $t$-test). Basal levels of each kindled group were compared with the control group, both in the absence and presence of SK&F 89976-A and L-trans-PDC (#$P < 0.05$, unpaired Student's $t$-test). Values are expressed as mean dialysate concentrations ± SEM.
Amino acid release and uptake during kindling presence of SK&F 89976-A and L-trans-PDC, did not show any significant differences, except for aspartate which was, in the presence of SK&F 89976-A and L-trans-PDC, decreased in the LT group (P < 0.04, unpaired t-test; Fig. 2C).

**Ca\(^{2+}\)**-dependent amino acid release during kindling epileptogenesis

Changes in the capacity of Ca\(^{2+}\)-dependent releasable pools of amino acids during kindling epileptogenesis were investigated by depolarization with high K\(^+\). It has been assumed generally that during K\(^+\) depolarization amino acid release originates not only from a Ca\(^{2+}\)-dependent (vesicular) pool, but also from a Ca\(^{2+}\)-independent (cytosolic) pool via reversal of the Na\(^+\)-dependent high-affinity transporters (Erecinska, 1987; Nicholls & Attwell, 1990; Attwell et al., 1993). To discriminate between these pools, two stimuli consisting of 153 mM K\(^+\) in the perfusate were applied sequentially at an interval of 1.5 h, first in the presence of Ca\(^{2+}\) and then in the absence of Ca\(^{2+}\) (see Fig. 1). The Ca\(^{2+}\)-dependent release fraction was obtained by subtracting the release levels evoked by both stimuli. Previous experiments in which high K\(^+\) was applied repeatedly without changing Ca\(^{2+}\) did not show significant differences in extracellular amino acid levels, indicating that time effects or depletion of intracellular amino acid stores due to repetitive K\(^+\) stimulation did not occur using this protocol (Zuiderwijk et al., 1996). We did not observe any behavioural seizure activity during or after

**Fig. 3.** (next page ⇒) Ca\(^{2+}\)-dependent GABA release in control and kindled animals. Kindled animals were divided into a 14AD (n = 7), a FK (n = 7), and a LT (n = 7) group, and compared with age-matched control groups of 8, 6 and 6 animals, respectively. All experiments were performed in the presence of SK&F 89976-A (0.5 mM) and L-trans-PDC (1.0 mM) (see Fig. 1). The Ca\(^{2+}\)-dependent release was obtained by subtracting the Ca\(^{2+}\)-independent release determined during the second high K\(^+\) stimulation from the total release determined during the first high K\(^+\) stimulation in the presence of Ca\(^{2+}\). K\(^+\) was raised to 153 mM in the perfusate for 16 min (indicated by the black bar). Comparisons were made between kindled and age-matched control groups before application of high K\(^+\), during application of high K\(^+\), and during the recovery-phase directly after removal of high K\(^+\) using repeated-measure ANOVA (\(*P < 0.05\)). In B, corresponding time-points were compared with an unpaired Student's t-test (\(\#P < 0.05\)). Values are expressed as mean dialysate concentrations ± SEM.
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Calcium-dependent GABA release

A

B

C

[Graphs showing calcium-dependent GABA release with control and treated conditions]
Fig. 4. Ca$^{2+}$-dependent glutamate release in control and kindled animals. See Fig. 3 for groups, statistics and experimental conditions.
stimulation with K⁺, neither in control, nor in kindled animals. Since we had previously shown that, even under K⁺-depolarized conditions, GABA and glutamate transporters actively regulate extracellular amino acid levels (Zuiderwijk et al., 1996), all experiments were performed in the presence of SK&F 89976-A and L-trans-PDC.

The total Ca²⁺-dependent release of GABA, measured during the application of high K⁺, did not change significantly both in the 14AD and FK group compared to their age-matched controls (repeated-measure ANOVA; Figs. 3A and B). However, the rise in release upon K⁺ was faster in the FK group (t = 14-16 min; P < 0.05, unpaired t-test; Fig. 3B). In the LT group, Ca²⁺-dependent GABA release, measured during application of high K⁺, was decreased significantly compared to the control group (P < 0.05, repeated-measure ANOVA; Fig. 3C).

The Ca²⁺-dependent release of glutamate did not change in any kindled group during application of high K⁺ (Figs. 4A, B and C), but its elevation was slightly prolonged in the LT group directly after removal of high K⁺ (t = 24-36 min; P < 0.04, repeated-measure ANOVA; Fig. 4C). The release of aspartate was totally Ca²⁺-independent and, therefore, Ca²⁺-dependent aspartate release is not shown (see also Zuiderwijk et al., 1996). Although taurine release showed a small Ca²⁺-dependent component, no significant changes were observed in any of the kindled groups (data not shown).

**Ca²⁺-independent amino acid release during kindling epileptogenesis**

Ca²⁺-independent amino acid release was measured during the second high K⁺ stimulation where Ca²⁺ was replaced by 10 mM Mg²⁺ and 2 mM EGTA (see Fig. 1). The K⁺-stimulated Ca²⁺-independent release of GABA did not change significantly in the 14AD group compared to the age-matched control group (Fig. 5A). In FK animals, a slow increase in Ca²⁺-independent GABA release was observed after 8 min of high K⁺ until shortly thereafter (t = 16-28 min; P < 0.05, repeated-measure ANOVA; Fig. 5B). Conversely, in the LT group, K⁺-stimulated Ca²⁺-independent GABA release was decreased compared to controls (P < 0.04, repeated-measure ANOVA; Fig. 5C).

The Ca²⁺-independent release of glutamate did not change significantly in any of the kindled groups when measured during application of high K⁺ (Figs. 6A, B and C), but was decreased in the LT group directly after removal of high K⁺ (t = 24-36 min; P < 0.05, repeated-measure ANOVA; Fig. 6C) In addition, K⁺-
Fig. 5. Ca\textsuperscript{2+}-independent GABA release in control and kindled animal. See Fig. 3 for groups, statistics and experimental conditions.
Chapter 5

Calcium-independent Glutamate release

A

\[ \text{Fig. 6.} \quad \text{Ca}^{2+}\text{-independent glutamate release in control and kindled animals. See Fig. 3 for groups, statistics and experimental conditions.} \]
Fig. 7. Ca²⁺-independent aspartate release in control and kindled animals. See Fig. 3 for groups, statistics and experimental conditions.
stimulated Ca$^{2+}$-independent aspartate release was not changed both in the 14AD and FK group (Figs. 7A and B), but was decreased in the LT group ($P < 0.03$, repeated-measure ANOVA; Fig. 7C). In this LT group, basal aspartate levels before K$^+$ stimulation were already significantly decreased compared to the control group ($P < 0.05$, repeated-measure ANOVA; see also Fig. 2C). Moreover, the significant increase of extracellular aspartate induced by high K$^+$ (compared to mean basal values before high K$^+$; $P < 0.05$, paired $t$-test) observed in the 14AD, FK and all control groups, was totally absent in the LT group. The K$^+$-stimulated Ca$^{2+}$-independent release of taurine did not change in any of the kindled groups (data not shown).

**Discussion**

The present study was performed to estimate whether mechanisms which regulate *in vivo* extracellular amino acid levels in rat hippocampus CA1 region change during kindling epileptogenesis by discriminating between amino acid release and uptake. Our main findings were: (1) Under basal conditions, the GABA transporter activity was lost both at fully kindled stage (24 h after the last generalized seizure) and long-term kindled stage (28-31 days after the last seizure). The glutamate/aspartate transporter activity was lost already during the early development of kindling (after 14 afterdischarges), and this loss persisted throughout the other kindled stages. (2) The early onset of K$^+$-stimulated Ca$^{2+}$-dependent (vesicular) release of GABA was increased at the fully kindled stage. However, at long-term kindled stage, this release component was decreased. The K$^+$-stimulated Ca$^{2+}$-dependent release of glutamate did not change during kindling, but was slightly prolonged at long-term kindled stage. (3) The K$^+$-stimulated Ca$^{2+}$-independent, presumably transporter-mediated, release of GABA was enhanced at fully kindled stage, whereas it was decreased at long-term kindled stage. This release component for aspartate was also decreased at long-term kindled stage.

These results demonstrate for the first time that kindling epileptogenesis is associated with a long-lasting dysregulation of both release and uptake of amino acid transmitters *in vivo*, being most prominent for GABA.
**Basal extracellular amino acid levels during kindling epileptogenesis**

Our results did not show significant differences in basal extracellular levels of GABA, glutamate, aspartate and taurine in the CA1 region during kindling epileptogenesis. However, it has to be emphasized that *in vivo* microdialysis is of limited use in monitoring changes in extracellular amino acid levels locally around synapses, since rapid diffusion and uptake into surrounding nerve terminals and glial cells will mask substantial changes at extrasynaptic sites which are accessible to the dialysis probe (During, 1995; Timmerman & Westerink, 1997a). Therefore, estimation of the effect of uptake inhibitors will unravel the importance of amino acid transporters in the clearance of possible local changes in extracellular amino acid levels. In accordance with our findings, During et al. (1995) also did not observe changes in basal GABA levels in the hippocampus of amygdala-kindled rats using microdialysis. Other microdialysis studies have reported changes in extracellular amino acid levels during amygdala kindling, although pointing into different directions (During et al., 1992; Minamoto et al., 1992; Kaura et al., 1995; Ueda & Tsuru, 1995). These deviating findings may be related to the different brain areas that were studied, i.e. dorsal raphe nucleus (During et al., 1992) and amygdala (Kaura et al., 1995), the use of chronically indwelling microdialysis probes (Minamoto et al., 1992), or multiple implantations in the same animal (Ueda & Tsuru, 1995). The latter procedures can induce gliosis around the probe, which may cause additional changes in the concentrations of extracellular amino acids (Benveniste et al., 1987).

**Vesicular amino acid release during kindling epileptogenesis**

Since changes in actual synaptic release of amino acid transmitters will hardly be visible with microdialysis (see above), we investigated changes in total releasable pools by massive depolarization with high K+. Using this approach we got a better insight in the capacities of releasable pools in the CA1 region of control and kindled rats. Although the K+ concentration perfused through the microdialysis probe was 153 mM, we estimated (according to the recovery for K+ given in the manufacturer's description) that the actual extracellular K+ concentration would not exceed 35 mM. Such a K+ concentration would mainly cause amino acid release from neuronal pools, since elevations to 40-50 mM, or higher, were necessary to trigger release from glial cells (Erecinska, 1987;
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Longuemare & Swanson, 1997; however, see Albrecht et al., 1998). Since presynaptic vesicular release should be exclusively Ca\(^{2+}\)-dependent, it is likely that the K\(^{+}\)-stimulated Ca\(^{2+}\)-dependent amino acid release represents the vesicular release pool from nerve terminals locally present in the CA1 region. The collection of 2-min samples allowed us to estimate dynamics of this release pool. Although the total releasable pool of GABA did not change during the development of kindling and at the fully kindled stage, the initial onset upon K\(^{+}\) stimulation appeared to be increased at the fully kindled stage, suggesting different presynaptic distribution of releasable vesicles, possibly due to seizure-related events. The reduction of K\(^{+}\)-stimulated Ca\(^{2+}\)-dependent GABA release at the long-term kindled stage, could be explained by a reduction of GABAergic neurons in the CA1 region at this kindled stage previously observed (Kamphuis et al., 1989). The K\(^{+}\)-stimulated Ca\(^{2+}\)-dependent release capacity of glutamate did not change during kindling. Remarkably, Ca\(^{2+}\)-dependent glutamate release was slightly prolonged at long-term kindled stage after removal of high K\(^{+}\). Interestingly, extracellular levels of glutamate also remained elevated postictally in the hippocampus of epileptic patients (During & Spencer, 1993). Although other microdialysis studies have also investigated K\(^{+}\)-stimulated amino acid release in different animal models of epilepsy (Lehmann et al., 1989; During et al., 1992, 1995), Ca\(^{2+}\) dependency was determined only in patients with temporal lobe epilepsy (During et al., 1995). This latter study reported increased GABA release, measured shortly (i.e. about 24 h) after seizures, which would be comparable to our results at fully kindled stage. In contrast to our study, no dynamic changes could be observed in the study of During et al. (1995) since the sample time was 30 min. Moreover, the possibility that seizures underlie the observed effect could not be excluded. Several in vitro studies have reported conflicting results with respect to K\(^{+}\)-stimulated Ca\(^{2+}\)-dependent GABA and glutamate release from hippocampal slices during kindling epileptogenesis (Geula et al., 1988; Kapur et al., 1989; Jarvie et al., 1990; Kamphuis et al., 1990, 1991b), but since most of these studies were performed without uptake inhibitors, it is possible that the observed changes in release were influenced by altered transporter activities. In agreement with our previous study (Zuiderwijk et al., 1996), we did not observe Ca\(^{2+}\)- dependent aspartate release, suggesting that aspartate is not released from vesicles. Although several studies have found Ca\(^{2+}\)-dependent aspartate release and a recent study has indicated that aspartate was enriched in the synaptic vesicle region of nerve terminals in the CA1 area (Gundersen et al., 1998), a direct coupling between Ca\(^{2+}\) influx and exocytosis of aspartate has not been shown yet.
Nicholls & Attwell (1990) have postulated that Ca\(^{2+}\)-dependent aspartate release is most likely the result of Ca\(^{2+}\)-dependently released glutamate, which subsequently activates glutamate/aspartate transporters causing release of aspartate from the cytosolic pool via heteroexchange.

**Amino acid transporter activity during kindling epileptogenesis**

**GABA transporter activity**

The increase in extracellular GABA levels by SK&F 89976-A observed under basal conditions in control animals, indicates an active regulation by GABA transporters. Since SK&F 89976-A is a non-transportable blocker of GABA uptake (Larsson et al., 1988) and highly selective for GABA transporter subtype GAT-1 (Borden et al., 1994), the loss of effect of this blocker at the fully kindled and long-term kindled stages indicates a reduction of GABA uptake activity by GAT-1 during kindling epileptogenesis. This reduced uptake activity, could work as a compensatory mechanism to diminish the spread of focal seizure activity. It should be emphasized that other GABA transporter subtypes such as GAT-3, which is expressed at low levels in the hippocampus (Borden, 1996) and is not blocked by SK&F 89976-A, may still regulate extracellular GABA concentrations. This could explain why extracellular GABA levels, in the absence of SK&F 89976-A, did not significantly change during kindling epileptogenesis.

The K\(^+\)-stimulated Ca\(^{2+}\)-independent release of GABA is assumed generally to originate from a cytosolic GABA pool and is mediated by GABA transporters acting in the reversed direction (Erecinska, 1987; Attwell et al., 1993; Richerson & Gaspary, 1997). Therefore, changes observed in Ca\(^{2+}\)-independent release may reflect altered transporter activity, but also changes in the cytosolic GABA pool. This latter possibility is, however, less likely, since total CA1 tissue levels of GABA, determined in the region where microdialysis experiments were carried out, did not change between control and kindled groups (Ghijsen, unpublished observation). Possibly, kindling is associated with changes in the number of GABA transporters, as suggested by During et al. (1995), who found a decrease in the glutamate-induced, assumably transporter-mediated, GABA release in the hippocampus of amygdala kindled rats, together with a reduction in the number of GABA transporters. Our results show relatively slow dynamics of Ca\(^{2+}\)-independent compared to Ca\(^{2+}\)-dependent GABA release during K\(^+\) stimulation. Such a delay could be explained by a gradually increasing extracellular K\(^+\)
concentration, which has to exceed a certain threshold before leading to reversal of GABA transporters. Since the actual elevation of extracellular K+ (i.e. ~ 30-35 mM, see above) was probably not sufficient to reverse glial transporters, it is likely that the K+-stimulated Ca2+-independent GABA release represents a different transporter population than under basal conditions, where glial transporters are involved. This would explain the apparent contradiction at the fully kindled stage between enhanced K+-stimulated Ca2+-independent GABA release (mainly mediated by neuronal transporters) and reduced uptake activity under basal conditions (involving glial transporters as well). Kamphuis et al. (1990) reported an increased K+-stimulated Ca2+-independent GABA release at the fully kindled stage as well, which persisted at long-term kindled stage. This latter discrepancy could be explained by the higher extracellular K+ concentration (i.e. 50 mM) applied in that in vitro study as compared to our study, which could activate a different transporter population, as mentioned above. It has been shown that seizure activity is accompanied by a rise in extracellular K+ to about 10 mM (Heinemann et al., 1977), which would be sufficient to reverse neuronal GABA transporters (Gaspary et al., 1998). In addition, it has been proposed that reversal of GABA transporters during seizures would play an important role in suppressing seizure activity (During et al., 1995). The enhancement of K+-stimulated Ca2+-independent GABA release at fully kindled stage, observed in our study, could work as a protective mechanism to compensate seizure activity, whereas the impaired GABA release at long-term kindled stage, would facilitate the propagation of seizures. Interestingly, although seizure activity was associated with slightly enhanced extracellular K+ (Heinemann et al., 1977), this is by itself probably not a causative factor for the induction of seizures, since we did not observe any behavioural seizure activity during or after K+ stimulation, neither in control, nor in kindled animals.

Glutamate/aspartate transporter activity

The increase in basal extracellular glutamate and aspartate levels induced by L-trans-PDC observed in control animals, was lost at all kindled stages, indicating impaired activity of glutamate/aspartate transporters during kindling epileptogenesis. Since L-trans-PDC can act as a substrate of glutamate/aspartate transporters (Madl & Burgesser, 1993; Griffith et al., 1994), thereby inducing additional glutamate and/or aspartate release from the cytosolic pool via heteroexchange, it is difficult to discriminate whether the absence of changes in extracellular levels of both amino acids, is the result of reduced uptake activity
and/or reduced reversal of the transporters. However, the reduction in extracellular aspartate levels in the presence of L-trans-PDC at the long-term kindled stage as compared to control, indicates a loss of L-trans-PDC-induced heteroexchange rather than a reduced uptake of aspartate. Such a decrease in heteroexchange was probably not due to changes in the cytosolic pool of aspartate, since no changes in total CA1 tissue levels of aspartate between kindled and control groups were observed (Ghijsen, unpublished observation).

The K⁺-stimulated Ca²⁺-independent release of glutamate and aspartate is assumed to represent reversal of glutamate/aspartate transporters (Nicholls and Attwell, 1990; Attwell et al., 1993; Fillenz, 1995). Our results show little changes in K⁺-stimulated Ca²⁺-independent glutamate release, being only decreased at long-term kindled stage directly after removal of high K⁺. However, the change in Ca²⁺-independent glutamate release during K⁺ stimulation was rather slow, implying that glutamate is released via indirect mechanisms. In contrast, K⁺-stimulated Ca²⁺-independent aspartate release, was more pronounced and showed a marked decrease at long-term kindled stage. This result could be explained by a lack of glutamate/aspartate transporter reversal, since depletion of the aspartate pool was not likely, as mentioned above. Consistent with our results, Leach et al. (1987) observed a loss of [³H]-aspartate uptake in cortical tissue of kindled rats 1 week after the last seizure, indicating impaired transporter activity. One possible explanation for reduced glutamate/aspartate transporter activity could be a down-regulation of transporters. Molecular studies have indicated, however, an upregulation in protein levels of the neuronal glutamate transporter EAAC1 and no changes of the glial transporters GLAST and GLT-1 in the hippocampus of amygdala-kindled rats (Prince Miller et al., 1997; Akbar et al., 1997). Interestingly, selective reduction of EAAC1 in rat brain resulted in epileptic seizures, but did not elevate extracellular glutamate levels in the striatum measured by microdialysis, whereas reduction of either GLT-1 or GLAST did not lead to seizures, but did elevate glutamate (Rothstein et al., 1996), indicating that by microdialysis primarily changes in glial transporters are observed.

**Implications of changes in the regulation of extracellular amino acid levels for the epileptogenic focus in the CA1 region**

The results from this study indicate changes both in GABA and glutamate releasable pools, as well as GABA and glutamate/aspartate transporter activity
during kindling epileptogenesis. The alterations found during the development of kindling and fully kindled stage may be seizure-related. On the one hand, the facilitated vesicular GABA release, together with the reduced GABA uptake activity, may work compensatory to oppose the reduced postsynaptic GABA<sub>A</sub> receptor function in the CA1 region at these kindled stages previously observed (Titulaer et al., 1994, 1995). On the other hand, the reduced activity of glutamate/aspartate transporters could result into glutamate and/or aspartate overflow in the extrasynaptic space thereby propagating the induction of seizures. At long-term kindled stage, the shift towards a decreased vesicular GABA release and a prolonged glutamate release, possibly underlies a permanent enhanced seizure susceptibility. The reduced transporter-mediated GABA release at this kindled stage, would facilitate the propagation of seizures.

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