Plasticity of amino acid release and uptake during kindling epileptogenesis
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Chapter 6

Compensatory change in EAAC1 glutamate transporter in rat hippocampus CA1 region during kindling epileptogenesis
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Abstract

Functional and molecular changes in glutamate transporters during kindling epileptogenesis were investigated in hippocampus CA1 region of rats. In control animals total glutamate transporter activity was indicated by the stimulatory effect of the high-affinity transporter blocker L-trans-pyrrolidine-2,4-dicarboxylate on extracellular glutamate and aspartate concentrations, as measured by in vivo microdialysis. This blocker-induced elevation was absent already early during epileptogenesis. CA1 levels of the glutamate transporter subtypes GLAST and GLT-1, analyzed by quantitative immunoblotting, did not change during kindling epileptogenesis. However, the 60 % decrease in EAAC1 level observed in age-matched controls was fully compensated for in kindled animals 4-5 weeks after the last generalized seizure. These results indicate a compensatory change of the neuronal EAAC1 glutamate transporter in CA1 region during kindling epileptogenesis, which may be the consequence of a decrease in total transporter activity.

Introduction

In epileptic patients and in kindled rats, increases in extracellular glutamate levels in hippocampus and/or amygdala just prior and during seizures have been observed (During & Spencer, 1993; Kaura et al., 1995; Ueda & Tsuru, 1995). Such increases could be the result of enhanced release, reduced uptake, or a combination of the two. With respect to the former, in vitro studies have indicated enhancement of stimulated glutamate and/or aspartate release in hippocampus slices in several animal models of chronic epilepsy (Geula et al., 1988; Kamphuis et al., 1991b; Flavin & Seyfried, 1994; Kaura et al., 1995). Regarding changes in glutamate uptake, contradictory observations have been reported. Down regulation of glutamate transporters was suggested in view of the reduced $[^3]$H glutamate and $[^3]$H D-aspartate uptake in brain tissue from kindled animals (Leach et al., 1987). In other studies, either no change (Slevin & Ferrara, 1985) or an increase in glutamate or aspartate uptake in epileptic tissue (Bruhn et al., 1997) were reported. The molecular characterization of diverse glutamate transporter subtypes, i.e. GLT-1, GLAST, EAAC1 (Kanai et al., 1993), permitted to test whether these molecules changed in epileptic tissue. These studies suggested that the mainly astroglial transporters GLT-1 and GLAST did not clearly change at long-term in hippocampus or amygdala tissue from kindled rats, or in biopsies of epileptic patients (Akbar et al., 1997; Prince Miller et al., 1997; Tessler et al., 1999). Interestingly, the level of the neuronal transporter EAAC1 increased transiently after seizures (Prince Miller et al., 1997), though this finding was not related to local changes in extracellular glutamate and aspartate concentrations during epileptogenesis.

In the present study, we investigated whether glutamate transport activity changed during kindling epileptogenesis in hippocampus CA1 region by measuring the effects of a high-affinity transporter blocker on in vivo extracellular glutamate and aspartate concentrations using microdialysis (Zuiderwijk et al., 1996). We related changes in transporter activity to levels of the glutamate transporter subtypes GLT-1, GLAST and EAAC1 by quantitative immunoblotting using subtype-specific antibodies.
Materials and methods

Kindling induced epilepsy was generated in hippocampus CA1 region of Wistar rats (200-270 g body weight) by twice daily tetanic stimulation of the Schaffer-collateral fibres, as described previously (Kamphuis et al., 1991b). Stainless steel electrodes were implanted in CA1 region of the right hippocampus, and simultaneously, a guide cannula for a microdialysis probe (CMA Microdialysis) was placed in the cortex just above the left hippocampus. Studies were performed at different stages of kindling, thereby discriminating between early seizure-dependent stages and a persistent, seizure-independent stage. Non-stimulated, age-matched animals with electrodes implanted were taken as controls. A CMA/11 microdialysis probe (1.0 mm membrane length) was placed in the left hippocampal CA1 region of kindled animals, either after induction of 14 afterdischarges (14AD group), or after reaching 7 generalized tonic-clonic seizures (fully kindled), or 4-5 weeks after the last seizure during which period the animals did not receive stimulations (long-term kindled), and in the corresponding controls. 20-24 h thereafter, local glutamate transport activity was estimated indirectly by measuring the effects of the high-affinity transporter blocker L-trans-pyrrolidine-2,4-dicarboxylate (L-trans-PDC, Tocris Cookson) on extracellular glutamate and aspartate concentrations (Zuiderwijk et al., 1996). Microdialysis was performed in freely moving animals with a perfusion buffer containing (in mM) 150 NaCl, 3 KCl, 2 CaCl₂, 5 Hepes (pH 7.4) at 2.0 µl/min. After 2 h perfusion four consecutive 4-min samples were collected. Subsequently, 1.0 mM L-trans-PDC was added to the perfusion buffer, and after 30 min another four 4-min samples were collected (Zuiderwijk et al., 1996). The samples were stored at -20°C until amino acid analysis. Amino acid concentrations were analyzed by reversed-phase HPLC after precolumn derivatization with o-phtalaldehyde (Zuiderwijk et al., 1996).

After microdialysis, the animals were decapitated, and CA1 tissue was rapidly dissected from 1 mm thick slices from the right hippocampus. CA1 tissue was homogenized using a Teflon-glass homogenizer in 500 µl of ice-cold buffer consisting of 0.32 M sucrose, 1.0 µl 0.5 M EDTA, 0.125 µl 1 M dithiothreitol, 50 µl complete protease inhibitor cocktail (Sigma) and 0.5 µl peptatin A. The homogenates were stored at -20°C until the Western blotting experiments.
Results

To prevent effects of local perfusion of L-trans-PDC on transporter subtype expression, the microdialysis probe was implanted contralaterally to the site in which the transporter levels were determined. Previous studies have shown that in vitro changes in glutamate and GABA release by kindling epileptogenesis occur bilaterally (Kamphuis et al., 1991b). As shown in Fig. 1, no changes in basal extracellular glutamate (Fig. 1A) or aspartate (Fig. 1B) concentrations in CA1 region could be observed at any epileptic stage as compared to the control animals. Perfusion of L-trans-PDC elevated the extracellular levels of both glutamate and aspartate in CA1 region in control animals, indicating active regulation of these levels by the transporters. However, already after 14 afterdischarges (14AD) this effect disappeared and remained absent at later stages, suggesting persistent impairment of glutamate transporter activity in epileptic animals. The extracellular aspartate levels were, in the presence of L-trans-PDC, significantly smaller in long-term kindled as compared to control animals (P < 0.04, unpaired t-test).

In order to determine the effect of kindling on the levels of the glutamate transporter subtypes GLAST, GLT-1 and EAAC1, 15 μg protein samples of CA1 homogenate from the ipsilateral hippocampus from kindled and control animals were run on 10 % sodium dodecyl sulfate-polyacrylamide gels in a Mini Protein II device (Bio-Rad), according to (Prince Miller et al., 1997). The separated proteins in the gel were transferred to Hybond nitrocellulose membrane by electroblotting, and incubated in Tris-buffered saline (pH 7.5) with 0.05 % Tween (TBS-T) and 5 % nonfat milk overnight at 4°C under shaking. Subsequently, the blots were incubated under shaking during 1 h, either with rabbit affinity-purified anti-GLT-1 (1:40.000), anti-GLAST (1:200) or anti-EAAC1 (1:200) in 10 ml TBS-T with 10 % (vol/vol) goat serum. After washing 5 times with TBS-T and 1 % nonfat milk, the blots were incubated with alkaline phosphatase-linked goat anti-rabbit IgG for 1 h (1:5000), washed again, and processed for quantitative immunoreactivity using enhanced chemifluorescence by 20 min incubation with Vistra ECF substrate (Amersham) and detection with a Storm FluorImager (Molecular Dynamics Inc. Gent, Belgium). The data were analyzed using ImageQuant software supplied by Molecular Dynamics. To correct for differences in blotting efficiency and antibody staining between the different experiments, fluorescence values were normalized by comparing to a standard whole forebrain homogenate of a control rat.
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**Fig. 1.** Extracellular concentrations of glutamate (A) and aspartate (B) in CA1 region during kindling in the presence and absence of L-trans-PDC. Microdialysis was performed 24 h after 14AD (n = 8), fully kindled (FK) (n = 7), and 4-5 weeks thereafter (LT) (n = 7). Since the glutamate and aspartate concentrations of the age-matched controls of the different stages did not differ, they were pooled (n = 21). Each bar represents the average of four 4-min samples without (basal, white) and with 1.0 mM L-trans-PDC (PDC-induced, shaded) ± SEM. Concentrations determined in the presence of L-trans-PDC were compared with values in its absence by the paired Student's t-test (**P < 0.01, ***P < 0.001). Comparisons between kindled and control groups were made by the unpaired Student's t-test (#P < 0.04). The data are replotted from Fig. 2 of Chapter 5.
containing all three proteins in each gel. In the Western blots clear bands of GLAST (MW = 65 kDa), GLT-1 (MW = 73 kDa), and EAAC1 (MW = 69 kDa) could be recognized (Rothstein et al., 1994). For all three antibodies at the dilution tested, the amount of 15 μg CA1 homogenate protein applied on the gel fell within a linear range.

In Fig. 2 the levels of the three glutamate transporters in CA1 homogenates prepared from rats at different kindling stages are shown, together with the corresponding age-matched controls. The astroglial GLAST protein did not change after 14AD and at the fully kindled stage, neither did the absolute levels (Fig. 2A). At long-term, GLAST levels tended to increase in both control and kindling tissue, but this increase did not reach statistical significance. The mainly astroglial GLT-1 protein did not change during kindling epileptogenesis (Fig. 2B). Surprisingly, the exclusively neuronal EAAC1 transporter showed a progressive decrease in the course of time both in controls and in kindled animals (Fig. 2C). This time (age)-dependent decrease was evident in control animals matched to the three kindled groups, being 64 % (P < 0.02, unpaired t-test) when going from 14AD towards long-term. However, in the corresponding kindled animals this decrease was only apparent between the 14AD and fully kindled stages. Between fully kindled and long-term kindled stages a stabilization occurred. At the latter stage the EAAC1 levels were significantly larger (P < 0.05) in kindled compared to control animals.

Discussion

This study shows for the first time persistent changes in levels of one of the glutamate transporter proteins, i.e. EAAC1, in hippocampal CA1 region of epileptic animals. The increase in EAAC1 level may compensate the impaired overall regulation of extracellular glutamate and aspartate concentrations by the transporters in kindled animals, as indicated by the microdialysis studies.

Fig. 2. ⇒ Effects of kindling on levels of the glutamate transporters GLAST (A), GLT-1 (B) and EAAC1 (C) in CA1 region. Data represent means of fluorescence values ± SEM after immunoblotting using different antibodies, from 8 (14AD), 6-7 (FK) and 7-8 (LT) independent experiments, together with the age-matched controls. Significance was evaluated by the unpaired Student's t-test (*P < 0.05; **P < 0.02).
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A

GLAST

B

GLT-1

C

EAAC1

(epileptic stage)
Dynamical changes of the EAAC1 transporter were also reported during CNS development; this transporter reaches its highest values around birth and decreases thereafter towards adulthood (Furuta et al., 1997). The progressive decrease in EAAC1 levels observed in CA1 region of control animals during young adulthood in our study, may indicate that this age-dependent decline continues during that period. The fact that such a decline in EAAC1 amount was not reflected in an increase in extracellular glutamate and aspartate levels monitored by microdialysis, could be explained by the negligible contribution of this transporter subtype in regulating resting amino acid concentrations as observed in rat striatum after its selective suppression by antisense oligonucleotides (Rothstein et al., 1996). More prominent roles in that respect have been proposed for the astroglial GLAST and GLT-1 subtypes (Rothstein et al., 1996), which did not change during kindling. Indeed, fast activation of glial transporters was exerted by stimulation of glutamatergic neuronal fibres, thereby rapidly clearing most of the elevated transmitter amount from the synaptic cleft (Bergles & Jahr, 1997). The relative importance of the EAAC1 glutamate transporter in glutamatergic transmission is not yet clear. Several studies have indicated localization of this protein in neuronal compartments outside the synaptic cleft of glutamatergic fibres, thereby making a role in local clearance of glutamate after its presynaptic secretion rather unlikely (Coco et al., 1997; Rothstein et al., 1994). However, since the transporter is located in the somatodendritic compartment in hippocampal neurons (Coco et al., 1997), it could suppress excessive elevations of glutamate and aspartate in the extrasynaptic space, that probably occur after excessive stimulation such as during epileptic seizures. Compensatory changes in EAAC1 levels during epileptogenesis would suppress such excessive glutamate elevations, while the glial transporters would be saturated. Normally, the astroglial transporters would sufficiently clear the extracellular amino acid rises in the synaptic space.

In amygdala-kindled rats a small significant increase in EAAC1 level was found in whole hippocampus tissue shortly after occurrence of generalized seizures (Prince Miller et al., 1997). In that study EAAC1 tended to increase similarly at long-term, but this increase was not statistically significant. Since the changes in EAAC1 levels were expressed relatively to normalized control values for each kindled stage, no discrimination between up-regulation and retarded down-regulation could be made. The absence of changes in GLT-1 and GLAST levels during kindling epileptogenesis confirms similar findings in earlier
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studies performed in brain tissue from kindled animals and epileptic patients (Akbar et al., 1997; Prince Miller et al., 1997; Tessler et al., 1999).

The disappearance of L-trans-PDC-induced elevations in extracellular concentrations of excitatory amino acids in kindled animals indicated reduced activity of the population of transporters sensitive to this blocker. Presumably, all three transporters subtypes investigated, will be blocked at high-affinity by L-trans-PDC (Vandenberg, 1998). Such an impairment in glutamate transporter activity during kindling is in apparent contradiction to the absence of changes of GLT-1 and GLAST levels. However, since the microdialysis probe primarily monitors extracellular amino acid concentrations at extrasynaptic sites which are subject to eventual changes in metabolism and turn-over, it is difficult to relate both observations directly (During, 1995). The reduction in extracellular aspartate levels in the presence of L-trans-PDC in long-term kindled as compared to control animals, probably indicates a loss of L-trans-PDC-induced heteroexchange (see Discussion of Chapter 5). This new in vivo observation confirms earlier findings in vitro, showing a reduced aspartate uptake in cortical tissue after kindling (Leach et al., 1987).

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