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Kainate-induced epilepsy alters protein expression of AMPA receptor subunits GluR1, GluR2 and AMPA receptor binding protein in the rat hippocampus

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Abstract Kainic acid induces seizures with consecutive degeneration of highly vulnerable hippocampal CA3 neurons in adult rats. An abnormal influx of calcium through newly synthesized α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) receptors lacking the GluR2 subunit, which normally renders AMPA receptors calcium impermeable, is thought to play a pivotal role for postictal neuronal death (GluR2 hypothesis). Using a specific GluR2 antiserum, postictal hippocampal GluR2 protein expression was investigated and compared to GluR1 between 6 and 96 h after seizure induction. In addition, postictal protein expression of a recently cloned AMPA receptor binding protein (ABP), which anchors AMPA receptors in the plasma membrane was also analyzed, to address the question of whether its protein expression is associated with neuronal death or survival. At 6 h after seizure induction, GluR2 immunoreactivity (IR) in CA3 was more markedly reduced compared to GluR1, but at 24 h GluR2 IR reattained control levels. More importantly, GluR2 IR was also markedly, but transiently decreased between 6 and 48 h in hippocampal CA1 neurons, but no significant cell loss was observed. These findings modify the GluR2 hypothesis in so far as only a subset of, but not all, hippocampal CA1 and CA3 pyramidal neurons may die due to reduced GluR2 levels with consecutive calcium overload through calcium-permeable AMPA receptors. ABP was induced postictally in presumed CA2 and a subpopulation of CA3 neurons and seems not to be involved in mechanisms of delayed neuronal death.

Keywords α-Amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) receptors · AMPA receptor binding protein (ABP) · Kainic acid · Immunohistochemistry · Rat

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Introduction

Limbic or temporal lobe epilepsy (TLE) is the most common and often intractable type of human epilepsy [18, 24]. Morphological alterations consist of neuronal cell loss in the hippocampal subfields CA1 and CA3 and less pronounced loss in the dentate gyrus (DG) [2, 60]. Both behavioral and histopathological changes closely resembling human TLE can be induced in adult rats by systemic application of kainic acid (KA), a cyclic analogue of the major excitatory neurotransmitter glutamate and a potent neurotoxin [5, 41, 42, 47, 55, 61]. The exact molecular basis of KA-induced epileptogenesis and neurotoxicity, however, is poorly understood. Antagonists of *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA)/kainate receptors possess anticonvulsant properties; hence, glutamate receptors are thought to participate in seizure induction and postictal neuronal degeneration (for review see [45]). Of particular interest are calcium-permeable AMPA receptors. AMPA receptors are thought to be pentamers assembled from four different subunits, GluR1 to GluR4 (GluR-A to GluR-D) [31, 43]. Additional structural diversity is generated by alternative splicing of the flip/flop module [59]. Calcium permeability crucially depends on the presence of at least one GluR2 subunit in its edited form [19, 26, 66]. Previous work demonstrated that GluR2 editing is not altered in the hippocampal kindling model of epilepsy and thus is unlikely to contribute to postictal neuronal degeneration [29]. The observation that the GluR2 subunit determines the calcium permeability of AMPA receptors in conjunction with a preferential postictal down-regulation of GluR2 mRNA levels led to the so-called 'GluR2 hypothesis', suggesting a contribution of Ca^{2+}-permeable AMPA receptors to neuronal death in epilepsy [15, 50]. Two recent studies using the KA model in rats also demonstrated a selective reduction of GluR2 protein in CA3 neurons prior to cell death, a finding supporting the GluR2 hypothesis [14, 22].

Up to now little is known about whether the expression of anchoring proteins of glutamate receptors is also in-

volved in mechanisms responsible for neuronal death or survival of vulnerable nerve cell populations. Recently, a novel component of the postsynaptic density, AMPA receptor binding protein (ABP) was cloned, which binds to the C termini of AMPA receptors via PDZ domains (named after three proteins containing the motif: PSD-95, Dig-A and ZO-1 [12, 32, 34]) of GluR2/3 subunits [62]. ABP is expressed in the hippocampus in pyramidal cells as well as in some GABAergic interneurons [62]. ABP is thought to be involved in the regulation of AMPA receptor function by regulating the synaptic localization of AMPA receptors during neosynaptogenesis or functional and structural synaptic modification [36, 62].

The present study was designed to test, at the protein level, whether postictal changes of the hippocampal AMPA receptor subunit GluR1, GluR2 and/or ABP protein expression are associated with neuronal degeneration or survival. In the present study a specific antiserum against the C terminus of GluR2 [52], which contains the binding site for ABP, was used.

Materials and methods

Animal experiments

The experimental model has been given in detail by others in previous publications [5, 16, 61]. In brief, adult male Sprague-Dawley rats (260–310 g) were injected i.p. with 10 mg/kg KA (Sigma, Deisenhofen, Germany), dissolved in 0.9% saline at pH 7.0 (n=31). Sham-treated control animals received an equivalent volume of 0.9% physiological saline instead of KA (n=5). Both experimental and control animals were pretreated with atropine (1.25 mg/kg body weight; Sigma) 15 min before injection of KA or saline, respectively, to reduce mortality during status epilepticus in KA-treated rats. All animals were monitored for 4–6 h after KA injection to determine the severity of seizures. Two animals died during status epilepticus, four animals who developed no or only very mild seizures were excluded from the study. Postictal protein expression of AMPA receptor subunits GluR1, GluR2 and ABP was investigated in sham-treated control rats at 96 h and in experimental animals at 6, 12, 24, 48 and 96 h after KA application (n=5 each). All procedures were carried out according to the guidelines of the German animal protection law.

Neuropathological evaluation

Neuronal cell densities within the hippocampal pyramidal layers and the granule cell layer of the DG were quantitatively assessed after labeling neurons with an antibody against the neuronal marker protein NeuN. Sham-treated rats (n=5) were compared to KA-treated rats (n=5) 96 h after saline injection or seizure induction, respectively. Hippocampal subfields CA1 and CA3 as well as the DG of both hemispheres were scanned at a magnification of ×300. Two sets of three adjacent regions per area per hemisphere were recorded, averaged and expressed as mean cell number/mm^2 (neuronal density). Since the CA2 subfield was not clearly identifiable in the NeuN immunostaining, cell counts of this area were not included. All data were analyzed statistically using the General statistics module of Analyse-it for Microsoft Excel (Analyse-it Software, Leeds, UK). Values were expressed as mean ± standard deviation (SD). Significant group effects were confirmed by analysis of variance (ANOVA) and Bonferroni error protection with a significance level at P<0.05.

Immunohistochemistry

At the determined endpoints of the experiment, the animals were deeply anesthetized and perfused through the ascending aorta with saline for 2 min followed by 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer for 10 min. Brains were removed, post-fixed overnight in the same fixative and then transferred to 0.5% PFA/0.1 M phosphate buffer until further processing. Immunohistochemistry was performed on coronal free-floating 50-µm vibratome sections. Antisera against GluR1 (obtained from Chemicon International, Temecula, Calif.), GluR2 (kindly provided by R. J. Wenthold, Laboratory of Neurochemistry, NIDCD, NIH, Bethesda, Mass.) and ABP (kindly provided by E. B. Ziff, Howard Hughes Medical Institute, Department of Biochemistry, NYU Medical Center, N.Y.) were generated in rabbits immunized against C-terminal peptides of rat origin [51, 52, 62]. All antibodies used are well characterized and the specificity of antisera has been previously demonstrated [52, 62, 67]. After pretreatment with methanol 70%/1% H_2O_2 for 20 min, free aldehyde moieties were blocked by a 10-min incubation in PBS/0.4% borhydride for detection of GluR1 and GluR2. Sections were then incubated in normal swine serum (10% in phosphate-buffered saline) for 30 min, followed by the primary antisera for 24 h (ABP) or 72 h (GluR1, GluR2) at 4°C. The primary antibodies were diluted 1:100 (GluR1), 1:200 (GluR2) and 1:250 (ABP), respectively. To assess neuronal densities, sections were immunostained with the neuron-specific NeuN monoclonal antibody (Chemicon International) at a dilution of 1:2,000. Immunoreactivity (IR) was visualized by the avidin-biotin-complex method (Vectastain, Vector Laboratories, USA). Sections were developed in 0.02% diaminobenzidine with 0.02% hydrogen peroxide. The reaction product was intensified by addition of 0.02% cobalt chloride and nickel ammonium sulfate. Omission of the primary antisera resulted in no immunostaining. For semiquantitative analysis of the receptor distribution, hippocampi were investigated by optical densitometry. All sections were examined at a final magnification of ×40 with a Zeiss Axiophot microscope (Jena, Germany). Images were scanned under equal light conditions with a DMC video camera (Polaroid, Offenbach, Germany). From hippocampal subfields CA1, CA3 and DG, four regions of interest (ROI) both in dendritic layers and the pyramidal layer were selected on the monitor. Optical density was automatically determined using the AIS imaging research software (Imaging Research, St. Catharines, Ontario, Canada). For ABP, the presumed CA2 subfield was additionally scanned as described above. The optical density of the corpus callosum was used as reference value for background staining (OD_{USP}) and subtracted from total optical density in the ROI (OD_{TOT}). Specific immunolabeling was calculated as ratio OD_{SP}/OD_{TOT}. Values (in %) are expressed as mean ± SEM. Statistical analysis was performed using the General statistics module of Analyse-it for Microsoft Excel (Analyse-it Software). Significant group effects were confirmed by ANOVA and Bonferroni error protection with a significance level at P< 0.05.

Results

Animal experiments

As in published data [5, 6, 16, 61], administration of KA caused characteristic sequential behavioral changes. During the first minutes after KA injection, rats demonstrated strong immobility. After 30–45 min, this behavior was replaced by ‘staring spells’, followed by repetitive head nodding and ‘wet dog shakes’. During the following 2 h, progressive motor seizures developed. Finally, animals suffered from limbic status epilepticus with continuous convulsions, lasting for several hours.

Table 1 Neuronal cell densities in sham and kainic acid-treated rats 96 h after seizure induction. Values represent neuronal densities/mm^2 ± standard deviation (*DG* dentate gyrus)

Group	*n*	CA1	CA3	DG
Sham	5	4633±287	2770± 88	7758±544
Kainate	5	4450±546	2010±526*	8125± 98

*Significant (ANOVA and Bonferroni error protection with significance level at $P<0.05$)

Neuropathological evaluation

Focal neuronal cell loss in CA3 was first detected at 12 h after KA injection (not shown). Almost complete degeneration of CA3 neurons in individual animals had occurred at 24 h. In contrast, loss of pyramidal CA1 neurons was first detectable at 48 h (not shown). In the piriform cortex, four of five rats exhibited shrinkage of neuronal perikarya at 6 h. From 24 h on, the piriform cortex showed necrosis in all animals investigated (not shown). After 48 h, the lateral nuclei of the amygdala were also necrotic (not shown). Immunohistochemical analysis of NeuN-stained neurons in the hippocampus revealed significant cell loss only in the CA3 subfield at 96 h after KA application (Table 1).

Immunohistochemistry (GluR1, GluR2 and ABP)

CA3

In sham-treated control rats, prominent immunostaining for GluR1 and GluR2 was present in the somata of pyramidal cells and hilar interneurons, followed by dendritic layers (Figs. 1, 2, 3). At 6 h after KA injection, both GluR1 and GluR2 IR were significantly reduced in pyramidal layer and dendritic layers of CA3 (Figs. 1, 2, 3). In contrast to GluR1, which largely showed a rather uniform and moderate decrease of immunostaining, GluR2 IR in somata of CA3 pyramidal neurons was strongly, but not uniformly diminished (Figs. 1, 2). However, in CA3 a few small foci with loss of GluR1 IR were also detectable (Figs. 1, 2). At 12 h after epilepsy induction, GluR1 IR reattained control levels (Fig. 3). GluR2 IR was also only transiently reduced and returned to control levels at 24 h (Fig. 3).

ABP IR was most prominent in dendritic fields, but only moderately in the somata of neurons in the pyramidal

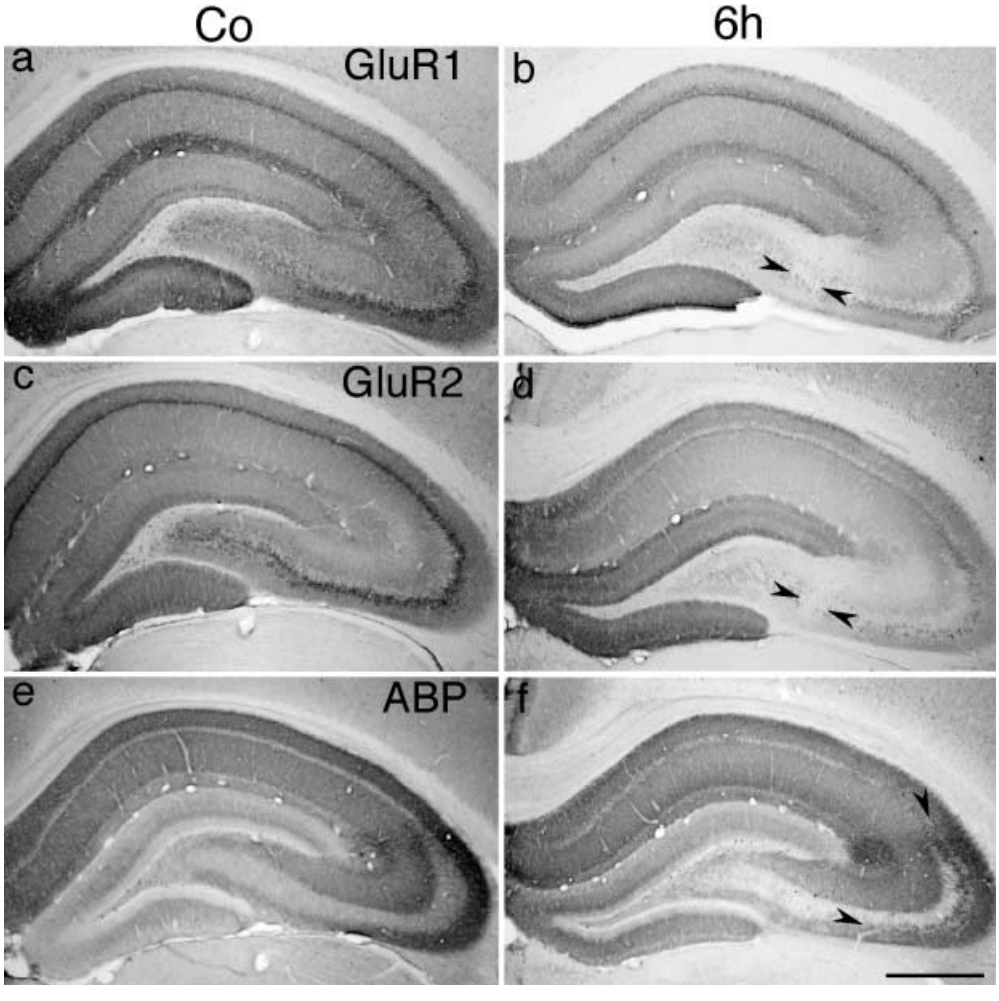

Fig. 1 Hippocampal immunostaining in the same animal, respectively, for GluR1 (**a,b**), GluR2 (**c,d**) and ABP (**e,f**) in a sham control (*Co*) rat (**a,c,e**) and 6 h after limbic epilepsy induction (**b,d,f**). Compared to the Co rat (**a**), reduction of GluR1 IR in CA3 6 h after KA treatment is largely uniform (**b**), except for one focus with complete abolishment of IR (**b**, *arrowheads*). In contrast, GluR2 IR in CA3 is markedly, but non-uniformly reduced (**d** vs **b**). The focus colocalizes with that of GluR1 IR (**d**, *arrowheads*). In comparison to the sham Co (**e**), ABP is strongly induced 6 h after KA injection in CA2 and CA3a (**f**, *arrowheads*) (*ABP* AMPA receptor binding protein, *KA* kainic acid, *IR* immunoreactivity). *Bar* 800 μm

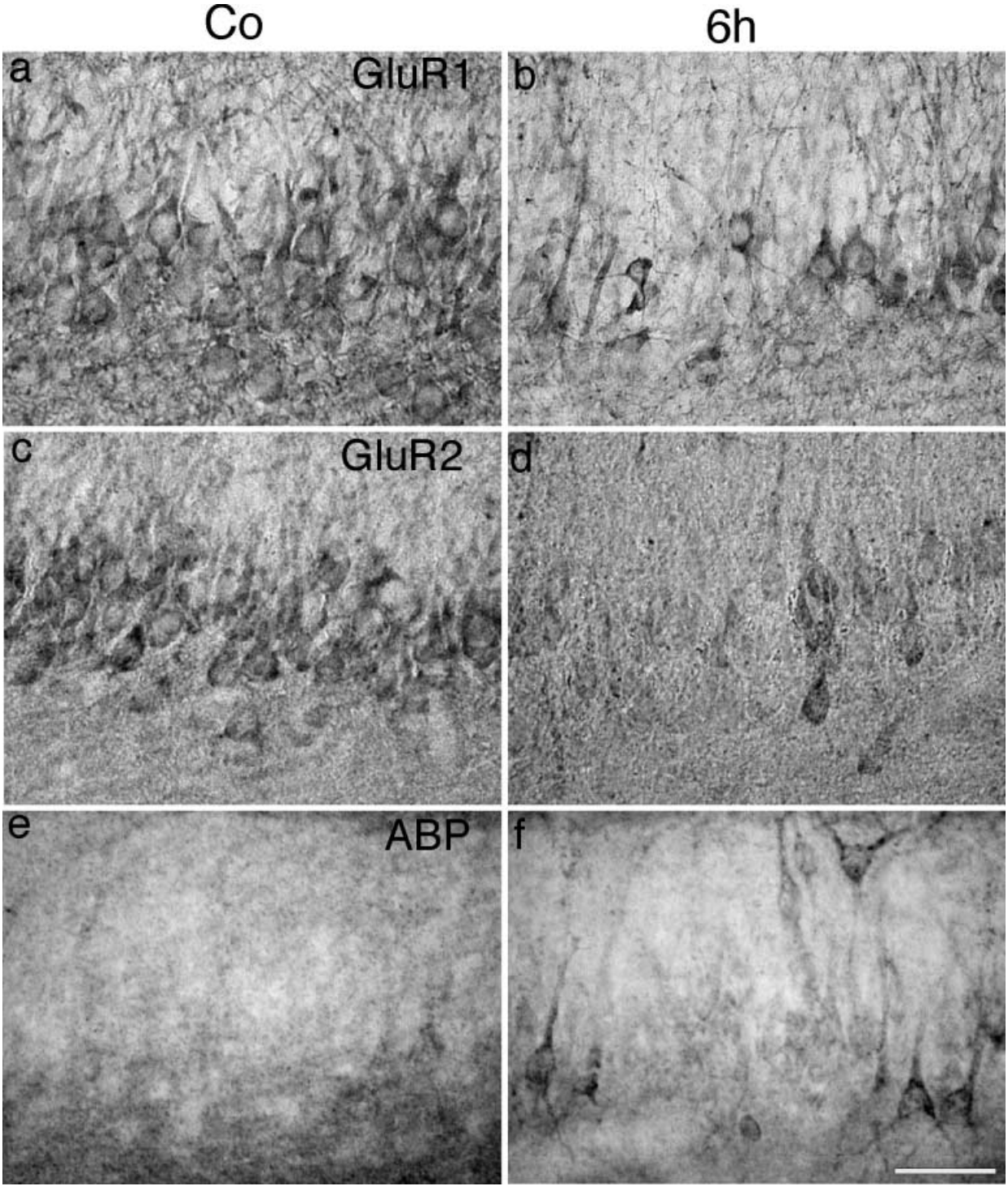

Fig. 2 CA3a immunostaining for GluR1 (**a,b**), GluR2 (**c,d**) and ABP (**e,f**) in a sham-treated control (Co) rat (**a,c,e**) and 6 h after seizure induction (**b,d,f**) in the same animal, respectively. At higher magnification, patchy reduction of GluR1 immunostaining is seen at 6 h (**b** vs **a**). GluR2 IR is also focally decreased, but exceeds reduction of GluR1 IR (**d** vs **c**). In contrast, ABP is strongly induced in scattered neurons of CA3 (**f** vs **e**). *Bar* 50 μm

cell layer (Figs. 1, 2, 4). At 6 h after seizure induction, somal ABP IR was strongly induced in scattered pyramidal neurons in the lateral areas of CA3 (CA3a) (Figs. 1, 2), but otherwise unchanged (Fig. 4). In contrast, ABP IR in dendritic fields was significantly reduced at 12 and 96 h (Fig. 4).

CA1

In sham-treated control animals, strong GluR1 and GluR2 IR was present in somata of pyramidal neurons (Figs. 1, 3). In dendritic layers, distinct GluR1 and GluR2 im-

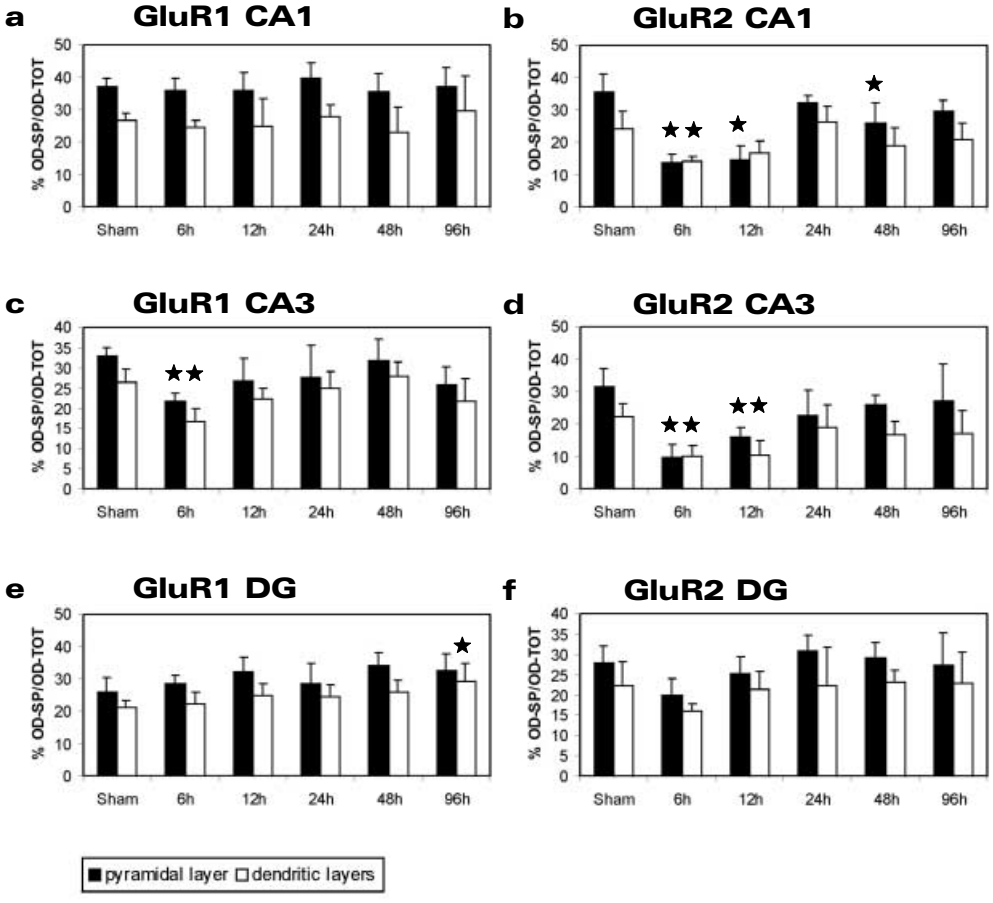

Fig. 3a–f Semiquantitative assessment of postictal GluR1 and GluR2 IR in hippocampal subfields CA1, CA3, and DG. GluR1 IR is unaltered in CA1 (**a**), whereas in CA3 a transient reduction occurs (**c**). In the DG, a slight increase is detectable 96 h after seizure induction in dentritic layers (**e**). GluR2 IR is transiently lowered both in relatively resistant CA1 and vulnerable CA3 (**b,d**), suggesting that temporary GluR2 reduction alone may not trigger neuronal death. No significant alteration of GluR2 is present in the DG (**f**) (*OD-SP* specific optical density, *OD-TOT* total optical density, *DG* dentate gyrus; *: significant; ANOVA and Bonferroni error protection with significance level at $P<0.05$)

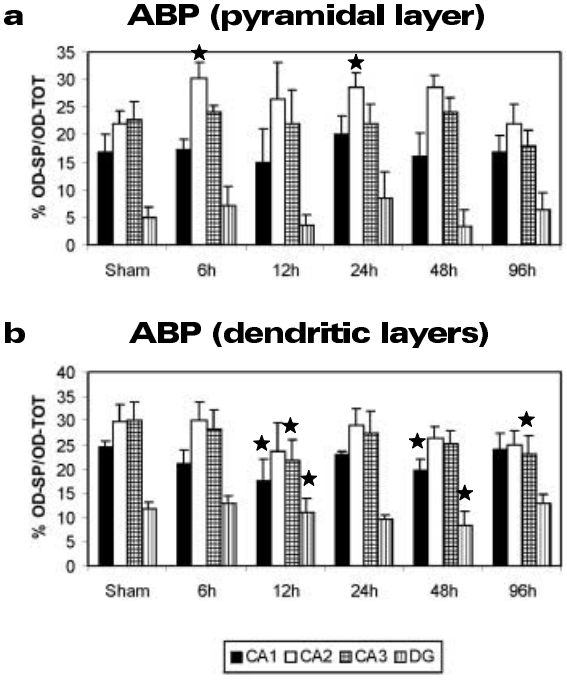

Fig. 4 Semiquantitative assessment of postictal hippocampal ABP IR inCA1, CA2, CA3, and DG. ABP is significantly induced in the pyramidal layer of presumed CA2 at 6 and 24 h after epilepsy induction (**a**). No induction is measurable in dendritic layers. In contrast, in conjunction with ongoing neuronal death dendritic ABP IR decreases (**b**); *: significant (ANOVA and Bonferroni error protection with significance level at $P<0.05$)

munostaining was also detectable (Figs. 1, 3). GluR1 IR was not significantly altered at any time point investigated (Fig. 3). In contrast, GluR2 IR in CA1 was transiently decreased at 6 h both in the pyramidal layer and in dendritic layers. Dendritic GluR2 IR returned to control levels at 12 h, whereas immunostaining in the pyramidal layer did not reattain control levels until 24 h with a second slight but significant decrease at 48 h (Fig. 3).

In sham-treated rats, ABP IR was only weak in the pyramidal layer, but prominent in dendritic layers (Figs. 1, 4). ABP immunostaining was first induced in somata of a few single CA1 pyramidal neurons at 6 h after KA treatment. Postictal ABP IR in dendritic layers was reduced at all time points investigated, but reached significance only at 12 and 48 h (Fig. 4).

CA2

Immunostaining for ABP in sham-treated animals was most prominent in a small area at the border zone between CA1 and CA3 marking the presumed CA2 subfield (Figs. 1, 4, 5). The most obvious change of ABP levels was also detected in this area. At 6 and 24 h, a significant induction of ABP IR in the pyramidal layer was seen with a secondary moderate decline at 48 and 96 h (Figs. 4, 5).

GluR1 and GluR2 IR in this area showed no distinct staining pattern compared to CA1 and CA3.

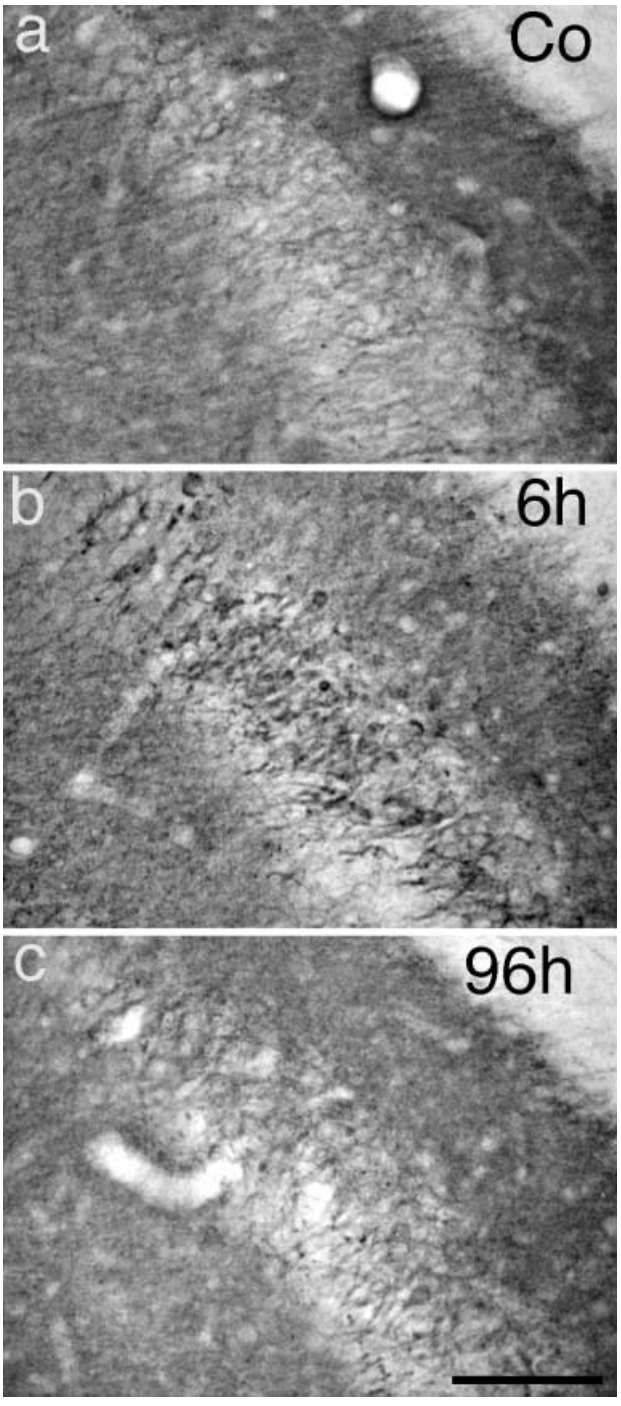

Fig. 5a–c ABP immunostaining in presumed CA2 after limbic epilepsy. In control rats, distinct ABP IR is detectable in somata of CA2 neurons (**a**). Note the strong induction of ABP immunostaining in the pyramidal layer at 6 h (**b**). At 96 h after seizure induction, intensity of ABP immunoreactivity has largely returned to control levels (**c**). *Bar* 100 μm

Dentate gyrus

Both GluR1 and GluR2 were detectable by immunohistochemistry in granule cells and dendritic layers of the DG in sham-treated rats (Figs. 1, 3). GluR1 IR in dendritic layers showed a slight, but continuous increase, which became significant at 96 h after seizure induction (Fig. 3). No significant changes were detectable for GluR2 at any time point investigated (Fig. 3). ABP IR in the DG was also unchanged throughout the investigation period (Fig. 4).

Discussion

In the present study, experimental limbic epilepsy induced changes in the protein expression of AMPA receptor subunits GluR1 and GluR2 as well as AMPA receptor binding protein (ABP), were found between 6 and 96 h after systemic injection of KA. Particular interest focused on the question of whether GluR2 protein is preferentially down-regulated in vulnerable neurons as predicted by the GluR2 hypothesis, and whether corresponding changes are observed for ABP.

A number of studies in experimental animals and humans have investigated GluR2 expression after limbic epilepsy. However, data are controversial and either restricted to the mRNA level [11, 15, 21, 53] or – when AMPA receptor subunit protein was analyzed – the respective antibody was nonspecific and did not differentiate between GluR2 and GluR3 [3, 7, 39] or GluR2 and GluR4 [8], respectively. To our knowledge, only two studies investigated postictal GluR2 protein expression using a specific antibody directed against the N terminus of GluR2 [14, 22]. Compared to GluR1, the authors demonstrated a selective decrease of GluR2 protein in CA3 neurons prior to neuronal degeneration. In conjunction with previous mRNA data after KA epilepsy [15, 22] and data indicating that GluR2 mRNA is also preferentially reduced in vulnerable neurons after global ischemia [20, 49], it was concluded that neuronal death in various neurological diseases can be attributed to de novo synthesis of AMPA receptors, lacking the GluR2 subunit. Such AMPA receptors would no longer be calcium impermeable and are thought to kill neurons by enhanced calcium influx followed by a deleterious increase of cytosolic free calcium (GluR2 hypothesis [50]).

Using a specific GluR2 antiserum against the C terminus of GluR2 [52], our study corroborates that GluR2 IR is significantly reduced in CA3 neurons of adult rats at 6 h after KA application, i.e., prior to neuronal cell loss (Figs. 1, 2, 3). However, GluR2 IR is only transiently lowered and reattains control levels at 24 h. Since only about 30% of CA3 neurons have died 96 h after epilepsy induction (Table 1), these findings strongly suggest that at least a subpopulation of CA3 neurons survive, despite a transient but severe decrease of GluR2 protein. Similarly, postictal GluR2 IR was also significantly but transiently reduced between 6 and 48 h in all hippocampal CA1 neurons (Figs. 1, 3), but no significant cell loss was detectable at 96 h (Table 1). These data modify the GluR2 hypothesis in so far as some, but not all, vulnerable neurons with lack of GluR2 subunit protein and consecutive calcium overload after status epilepticus may undergo cell death. Interestingly, DG granule cells known to be relatively re-

sistant to epileptic seizures, exhibited only a slight transient, but not significant, down-regulation 6 h after KA treatment (Fig. 3). According to the GluR 2 hypothesis, this finding would suggest that these neurons survive due to maintenance of GluR2 protein. However, the validity of this hypothesis has recently been restricted to CA1 and CA3 hippocampal neurons by their original proponents. Knockdown experiments with GluR2 antisense treatment led to a reduction of GluR2 expression in DG granule cells, but resulted in no significant cell loss in this area, whereas CA1 and CA3 neurons underwent cell death [46].

Evidence against a close association between lack of GluR2 and neuronal death came from studies with transgenic animals. GluR2 knockout mice were viable and showed no neuronal degeneration in the entire CNS, despite a ninefold increase of AMPA receptors in calcium permeability [28]. Mice with different levels of unedited GluR2 exhibited neurological deficits depending on the expression levels of the allele and consecutive calcium permeability, but this was not directly accompanied by cytotoxicity [10, 13, 30]. The more severe phenotype of editing deficient mice compared to GluR2 knockouts may be due to an important role of correctly edited GluR2 in AMPA receptor assembly and targeting [55, 68]. In addition, it was shown in mice deficient for the RNA-editing enzyme ADAR2 that editing is a prerequisite for efficient splicing and processing of the pre-mRNA [25].

The authors of the GluR2 hypothesis argue that neurons under these circumstances survive, since they permanently express calcium permeable AMPA receptors, whereas only neurons that have suddenly to cope with a high calcium influx, through newly formed AMPA receptors lacking the GluR2 subunit, degenerate. However, a recent study by Tóth and McBain [65] demonstrated that interneurons of the stratum lucidum constitutively have calcium permeable AMPA receptors, but die after transient ischemia or kainate-induced seizures [27, 38]. In general, the notion that calcium influx and an increase of cytosolic free calcium alone result in irreversible cell damage needs to be re-examined. A growing number of *in vitro* studies indicate that depletion of endoplasmic reticulum calcium stores and/or elevated calcium uptake in mitochondria cause neuronal death irrespective of free cytosolic calcium concentration [48, 63].

There has been substantial controversy in the literature as to whether a separate CA2 subfield in the rat hippocampus exists or not. It is well known that presumed CA2 pyramidal cells are morphologically different from CA3 neurons with regard to their lack of thorny excrescences [37, 64]. More recently, a growing number of immunohistochemical studies have suggested that an individual CA2 region can be identified. In particular, CA2 neurons demonstrate a denser immunostaining for calcium-binding proteins [4, 35, 58] compared to the adjacent CA1 and CA3 subfield. In the human hippocampus, chromogranin A IR was found to be particularly intense in CA2 [40]. It has been speculated that the high abundance of these calcium-buffering proteins contributes to the known relative resistance of the CA2 sector against epileptic damage [1, 42, 56, 61]. The present finding of a more intense basal ABP IR in presumed CA2 pyramidal cells at the border between CA1 and CA3 supports the idea of a separate CA2 subfield in the rat (Fig. 1). Markedly enhanced ABP IR after kainate-induced seizures was restricted to resistant CA2 and to scattered CA3 neurons, suggesting that ABP may not be causally related to neuronal survival. Interestingly, heat shock protein 72, a marker protein for reversible neuronal injury and thought to be involved in protective mechanisms [9, 44, 57] is not induced after KA administration in CA2, but in CA1 and CA3 [17]. These findings suggests that the relative resistance of CA2 does not exclusively depend on gene expression, but also on different innervation effects. In fact, the CA2 subfield in general shares the majority of neurophysiological connections to CA3, but receives a particularly prominent transsynaptic input from the posterior hypothalamus [23] and from the tuberomammillary nucleus [33], which themselves are not targets of KA-induced excitation [54].

Conclusions

The present data modify the GluR2 hypothesis as a molecular switch for the development or absence of KA-induced hippocampal neuronal death: (i) only 30% of hippocampal CA3 neurons degenerate after a pronounced transient reduction of GluR2 IR; (ii) despite a marked and similar postictal reduction of GluR2 protein expression in all hippocampal CA1 neurons, no significant cell loss occurs.

ABP, a novel GluR2/3 anchoring protein, is apparently not related to neuronal death in CA3, but its strongest induction at the border between CA1 and CA3 argues for a separate CA2 subsector in the rat brain.

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