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ORIGINAL RESEARCH

3-Mercaptopropionic Acid-Induced Repetitive Seizures Increase GluN2A Expression in Rat Hippocampus: A Potential Neuroprotective Role of Cyclopentyladenosine

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Abstract The *N*-methyl-D-aspartate receptor (NMDAR) is involved in synaptic plasticity, learning, memory, and neurological diseases like epilepsy and it is the major mediator of excitotoxicity. Functional NMDARs in the mature brain are heteromeric complexes composed of different subunits: GluN1 and GluN2. There are four different GluN2 subunits (A–D) and each of them critically determines the pharmacological and electrophysiological properties of NMDARs. GluN1 is ubiquitously expressed in the central nervous system while the highest GluN2A expression is in the hippocampus. Adenosine, an endogenous anticonvulsant, is a neuromodulator with a critical role in the regulation of neuronal activity, mediating its effect on specific receptors, among which adenosine A_1 receptor is highly expressed in the hippocampus. In the present work hippocampal GluN2A expression after the convulsant drug 3-mercaptopropionic acid (MP) induced seizures and the effect of cyclopentyladenosine (CPA) given alone or prior to MP (CPA + MP) in an acute or repetitive experimental model was studied. CPA administered to rats for one or 4 days increases seizure threshold induced by MP. After one administration of MP, no significant difference in GluN2A expression was observed in CPA and CPA + MP by Western blot, although immunohistochemistry revealed an increase in CA2/3 area. However, repetitive MP administration during 4 days showed a significant increase of GluN2A expression, and the repetitive administration of CPA 30 min prior to MP caused a significant decrease of GluN2A expression with respect to MP treatment, returning to control levels. These results show that GluN2A subunit is involved in repetitive MP-induced seizures, while CPA administration displays a protective effect against it.

Keywords NMDAR · GluN2A · Hippocampus · Epilepsy · Cyclopentyladenosine · 3-Mercaptopropionic acid

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Introduction

Glutamate mediates most excitatory synaptic transmission in the mammalian central nervous system (CNS) by activation of ionotropic receptors. One of them, *N*-methyl-D-aspartate receptor (NMDAR) is involved in synaptic plasticity, learning, memory, and neurological diseases like epilepsy, and it is the major mediator of excitotoxicity (Dingledine et al. 1999; Cull-Candy et al. 2001). Most NMDARs in the mature brain are heteromeric complexes composed of different subunits: GluN1, GluN2, and GluN3 (nomenclature Collingridge et al. 2009). Expression of functional recombinant NMDARs requires the co-expression of at least one NR1 and one NR2 subunit. The GluN1 subunit is essential and binds glycine, whereas GluN2 subunits bind glutamate (Yoneda and Ogita 1991; Doble 1999; Köhr 2006). Although the exact subunit composition has not yet been entirely established, there is consensus that NMDAR is a tetramer of two GluN1 and two GluN2 subunits of the same or different subtypes. There are four different NR2 subunits (A, B, C, and D) and each of them critically determines the pharmacological and electrophysiological properties of NMDARs (Cull-Candy et al. 2001; Mayer 2005; Köhr 2006; Paoletti and Neyton 2007; Kopp

et al. 2007). GluN1 is ubiquitously expressed in the CNS while the highest GluN2A expression is in the hippocampus (Jang et al. 1998; Köhr 2006). Different results about seizure effects on GluN2A expression were reported. In the model of seizure induced by pentylenetetrazol (PTZ), no significant alteration of GluN2A expression appeared in the hippocampus (Zhu et al. 2004), and kindling method does not produce long-lasting changes in NMDAR subunits' expressions in the same area (Kraus and McNamara 1998); while in chronic kainic hippocampal seizures an increase in GluN2A/B in the inner molecular layer of the dentate gyrus and a decrease in CA3-4 pyramidal cell layer and hilus were reported (Mikuni et al. 1999).

On the other hand, current treatments for epilepsy are based on the suppression of symptoms (i.e., seizures) by antiepileptic drugs (AEDs). Adenosine—a neuromodulator with endogenous anticonvulsant properties—inhibits neuronal firing and synaptic transmission, acting mainly via A_1 receptors, the most abundant of the four kinds of adenosine receptors described (Deckert and Gleiter 1994; Dunwiddie and Masino 2001; Ribeiro et al. 2002; Sebastiao and Ribeiro 2009). Adenosine is formed almost exclusively by the enzyme 5′-nucleotidase during seizure activity (Girardi et al. 1989a, b; Latini and Pedata 2001) and acts through specific receptors: A_1, A_{2A}, A_{2B}, and A_3, being A1 the most abundant in the CNS and highly expressed in the hippocampus (Jarvis 1991; Giraldez et al. 1998; Sebastiao and Ribeiro 2009). A_1Rs and A_{2A}Rs are primarily responsible of the central effects of adenosine, especially in the modulation of excitatory synaptic transmission. A_1R activation inhibits glutamatergic synaptic transmission mainly by presynaptic inhibition of glutamate release resulting in a potent anticonvulsant effect; these receptors also act as excitotoxic protectors hyperpolarizing the membrane when they are activated postsynaptically, thus raising the threshold for NMDARs opening (de Mendonça et al. 1995; Li and Henry 2000; Giraldez et al. 1998; Giraldez and Girardi 2000; Sichardt and Nieber 2007; Gomes et al. 2011). A_1Rs have the highest affinity for adenosine and are suggested as mediators of antiexcitotoxic effects, and suppressors of seizures and neuroprotectors (Sebastião et al. 2001; Boison 2005, 2008; Stone et al. 2009).

Adenosine—exogenously added—can achieve a greater anticonvulsant effect, suggesting that the neuromodulator adenosine system has a potential therapeutic benefit in seizure control. The administration of the adenosine analogue cyclopentyladenosine (CPA) increases 3-mercaptopropionic acid (MP)-induced seizure threshold (Giraldez and Girardi 2000; Girardi et al. 2007). Previous studies from our laboratory have shown that administration of CPA alone or prior to MP-induced changes the NMDA receptor–ligand ^{3}H-MK-801 (open-channel blocker) binding in different regions of the hippocampus in a stratum and area-dependent manner (Giraldez and Girardi 1998) and the administration of the convulsant MP modifies the [3H]-2-chloro-N6-cyclopentyl-adenosine (^{3}H-CCPA) ligand binding to A_1R receptor in different regions of the CNS (Giraldez et al. 1998; Vanore et al. 2001).

In the present study, we have investigated whether hippocampal GluN2A expression is altered after MP-induced seizure and the effect of adenosine analogue CPA given alone or prior to MP (CPA + MP).

Methods

Materials

MP, CPA, propidium iodide (PI), streptavidin complex, secondary antibodies for immunohistochemistry, trizma, and protein determination reagents were obtained from Sigma-Aldrich. Polyclonal rabbit antiserum against GluN2A, and actin were purchased from Santa cruz biotechnology (Santa cruz biotechnology Inc., Santa cruz, CA, USA). All materials used in immunoblot assays, including ECL chemiluminescence detection system were from Amersham Biosciences (GE Healthcare, Buckinghamshire, UK). Protease inhibitor cocktail and peroxide hydrogen 30 % were purchased from Merck and unless indicated otherwise the chemical substances of analytical grade were from Merck.

Animals and Treatments

Male Wistar rats (250–300 g) were maintained on a 12/12 h diurnal cycle. They were divided into four experimental groups. Each experimental group was divided in two subgroups: one single dose in 1 day (1D) and one single dose daily during 4 days (4D). Animal care was in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and the principles presented in the guidelines for the use of animals in neuroscience research by the Society for Neuroscience.

In the 1D subgroup, rats were injected intraperitoneally (i.p.) only once with 45 mg of MP/kg body weight (MP_1 group), CPA (CPA_1 group) 2 mg of CPA/kg body weight, CPA 30 min before MP injection ($CPA_1 + MP_1$ group), and saline as vehicle ($Control_1$). The MP and CPA + MP animal groups were sacrificed after 30 min of seizure onset and control and CPA groups 30 min after the respective drug administration.

In the 4D subgroup, MP (MP_4), CPA (CPA_4), CPA 30 min before MP injection ($CPA_4 + MP_4$), and saline as vehicle ($Control_4$) were administered once daily during 4 days. All animals of this treatment were sacrificed on the fifth day. All drugs were freshly prepared immediately

before administration: MP dissolved in saline, neutralized with tris base and CPA with saline as vehicle.

Isolation of Membranes and Immunoblot Procedure

The hippocampi of the treated and control animals ($n = 5$ rats/group) were dissected in cold and pooled in the homogenizing solution containing 0.32 M sucrose neutralized with Tris base solution (0.2 M) pH 7.2 and 0.05 ml/g proteinase inhibitor cocktail. Tissues were rapidly homogenized at 10 % (w/v) in this solution, in a Teflon glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at $900\times g$ for 10 min at 4 °C. The supernatants were spun at $100{,}000\times g$ for 30 min. The resulting pellets were resuspended in 20 mM Tris–HCl, 0.25 M sucrose, and 0.5 mM EDTA. Protein concentration was determined using Folin–Ciocaulteu reagent with bovine serum albumin as the standard (Lowry et al. 1951). For gel electrophoresis, 23 µg of protein were denatured at 95–99 °C and loaded on each lane. After separation by SDS-PAGE 8 %, the resolved proteins were electro-transferred onto a nitrocellulose membrane. The membranes were placed in a blocking buffer (5 % non-fat milk in TBS-T (20 mM Tris–HCl; pH 7.4, 140 mM NaCl, 0.1 % Tween-20)) for 1 h at room temperature and then incubated overnight with rabbit anti-GluN2A primary antibody (1:500) at 4 °C (or without antibody for no antibody controls). Anti-actin primary antibody (1:4000) was used on the same blots as loading control. After three washes (2 min each) with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody in buffer (1:5,000) for 1 h and then again washed (three times) with TBS-T. Protein detection was made with the ECL chemiluminescence detection system (Amersham ECL Western blotting detection reagents and analysis system from GE Healthcare, Buckinghamshire, UK). Densitometry of immunoreactive bands was performed using ImageJ 1.43u software.

Immunohistochemical Experiments

This technique was applied as previously described (Girardi et al. 2007). Briefly, 24 h after the last injection, animals were deeply anesthetized with 300 mg/kg of chloral hydrate, perfused and fixed with paraformaldehyde 4 %. Each hippocampus was removed and 50-µm-thick sections were cut using an Oxford vibratome. Sections were cryoprotected with 25 % sucrose in 0.1 M phosphate buffer pH 7.4 and stored at −20 °C. Free-floating tissue sections of all groups were simultaneously processed for immunostaining. Endogenous peroxidase activity was inhibited on tissue sections using 0.5 % v/v H_2O_2 in methanol for 30 min at room temperature. Tissue sections were blocked for 1 h with 3 % (v/v) normal goat serum in phosphate buffer saline (PBS) and then were incubated for 48 h at 4 °C with rabbit anti-GluN2A primary antibodies (1:200). After washing with PBS-X (PBS + 0.02 % Triton X-100) tissue sections were incubated with biotinylated secondary antibody (1:200), and then with streptavidin-peroxidase complex (1:200). Development of peroxidase activity was performed with 3,3′-diaminobenzidine plus nickel ammonium sulfate and H_2O_2 in acetate buffer. Sections were mounted on gelatin-coated slides, dehydrated and coverslipped using DPX mountant (Fluka) for light microscopic observation. Negative controls were processed simultaneously by omitting the primary antibodies (data not shown).

PI Staining in the Hippocampus After MP-Induced Seizures

PI staining was performed according to Hezel et al. (2012). Hippocampal slices (50 µm thick) were incubated in 2.5 µg/ml of PI solution in PBS for one hour at room temperature with gentle mixing and washed twice with PBS for 10 min. Slices were mounted on gelatin-coated slides and coverslipped using glicerine–PBS (3:1).

Image Analysis

Optical density (OD) of immunostained cells were measured using an Axiophot Zeiss light microscope, equipped with a digital camera micro publisher with a CCD Bayer pattem. Images obtained from the light microscope were analyzed with ImageJ 1.43u software (National Institute of Health, USA). The resolution of each pixel was 256 gray levels (8 bits). The projected surface of cells was measured using mean gray values. Relative optical density (ROD) was obtained after a transformation of mean gray values into ROD by using the formula ROD: log (256/mean gray). A background parameter from each section out of the labeled structures was obtained and subtracted to each cell ROD before statistically processing values. Seven to ten fields were digitalized for each stained section.

PI filter was used to detect the PI staining (Red color). PI staining images were observed in an Axiophot Zeiss fluorescent microscope and confocal microscope Olympus, FV1000 espectral. Photographs were taken with a soft imaging systems video camera.

Statistics

All statistics were performed by GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). In both 1D and 4D treatments, immunoblot values represent the

means of five separated rats per each experimental condition, while immunohistochemistry results were obtained from 3 to 4 animals per experimental condition. Individual immunohistochemistry experiments were composed of 5–9 hippocampal tissue sections from each experimental condition. Ten to twelve readings in each field were measured from each hippocampal section. Results are expressed as mean ± SEM. Statistical comparisons were performed using two-tailed *t* test when two groups were compared and one-way ANOVA followed by Student–Newman–Keuls post test when multiple comparisons were made. Statistical significance was set to $p < 0.05$.

Results

Upon the administration of the convulsant drug MP, either alone or with CPA, GluN2A expression was evaluated in the rat hippocampus.

MP and CPA Effect

MP administration induced seizures at 10.3 (± 1.382; $n = 9$) min after injection in animals treated with a single dose and at 12.0 (± 0.936; $n = 9$) min after 4D treatment (Fig. 1), without significant changes among each day of the 4D treatment (data not shown). Seizures were characterized by excitation with sudden running fits and convulsions, which lasted 1–3 min.

CPA administration alone resulted in a relaxed and inactive state in CPA group animals.

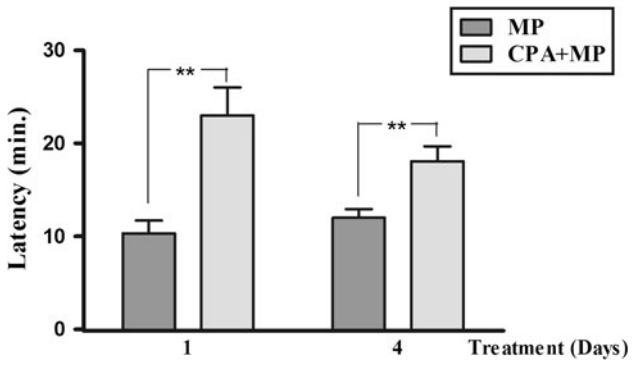

Fig. 1 Effect of CPA on latency time in seizure onset. Rats were injected i.p. with MP (*dark gray bars*) or CPA 30 min before MP (*light gray bars*), once for 1 day (1D treatment) or once daily during 4 days (4D treatment). Graph represents mean ± SEM (min), $n = 9$ in each condition. Data from 4D treatment represent the mean of 36 events corresponding to 4 days for each 9 rats. Statistics was performed by Student *t* test. $^{**}p < 0.01$. MP, CPA 30 min prior to MP (CPA + MP)

Pre-treatment with CPA Delays the Seizures Onset

CPA administration 30 min prior to MP delayed seizures onset in both 1D treatment 23 (±3.0 min; $n = 9$) and 4D treatment 18 (±1.6 min; $n = 36$) showing a significant increase in seizure latency with respect to MP group (Fig. 1), according to the previous studies (Girardi et al. 2007). No significant difference on seizure duration of both CPA + MP or MP treatments were observed (data not shown).

GluN2A Expression After MP and CPA Administered for Only 1 Day

Western blot analysis was performed on the whole hippocampal tissue showing a 177-kDa band, which stained positive for GluN2A. MP administration only for 1 day did not show any significant change of GluN2A expression with respect to control group (109 ± 11.5 %; $p > 0.05$; $n = 5$). CPA administration 30 min before MP increased seizure latency with respect to MP group, but similar bands in CPA + MP and CPA alone were detected after 1D treatment (105 ± 5.03 %; $p > 0.05$; $n = 5$). The Western blot assays in 1D treatment showed that there was no significant difference in the GluN2A expression between all the analyzed groups (Fig. 2a).

Repeated Pre-treatment with CPA Prevents MP-Induced GluN2A Expression in the Hippocampus

Western blot assays of hippocampal homogenates after 4D treatment showed a significant increase in GluN2A expression after MP administration (138 ± 8.91 %; $p < 0.05$ $n = 5$) and a significant decrease in GluN2A expression after CPA administration (47 ± 8.07 %; $p < 0.01$; $n = 5$) with respect to control. CPA administration 30 min before MP showed no significant difference in GluN2A expression compared to control (84 ± 9.75; $p > 0.05$; $n = 5$), but there was a significant difference with respect to MP ($p < 0.01$), showing a reversion of MP effect to control values (Fig. 2b).

After 1 Day of Treatment No Difference in GluN2A Expression was Observed in Most Analyzed Areas by Immunohystochemistry

To determine if there are differential changes of GluN2A expression depending on area, an immunohistochemistry technique was performed. Hippocampal GluN2A expression has a heterogeneous pattern of distribution and density in the different areas, showing positive staining in different strata in control slices, with higher expression in pyramidal layer, subiculum, and dentate gyrus. Immunohistochemistry assays of hippocampi treated with a single dose of

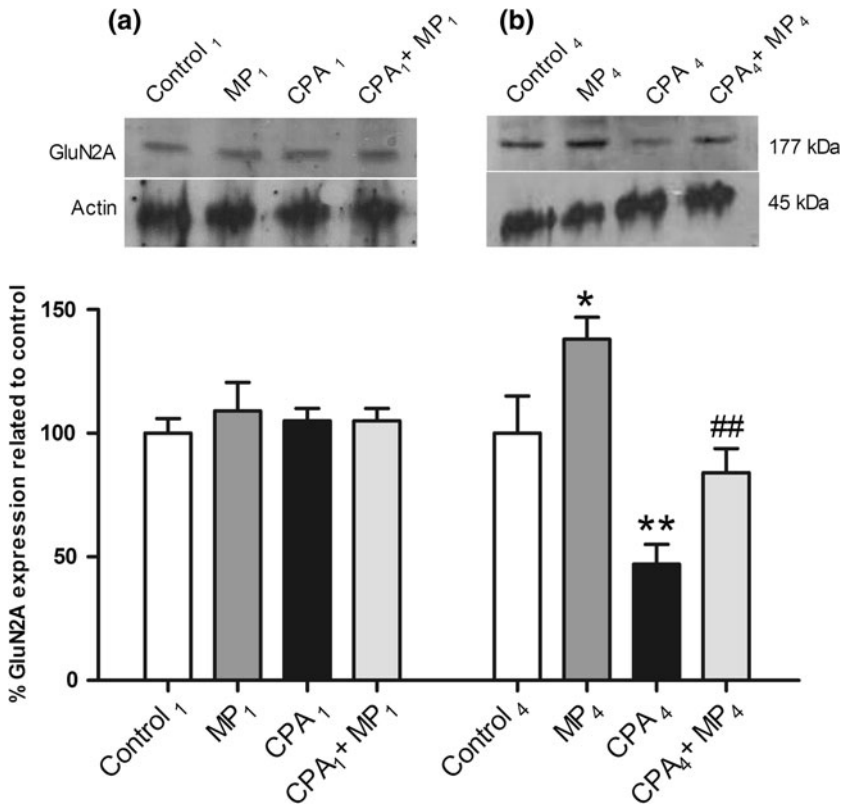

Fig. 2 Immunoblots of GluN2A expression from hippocampi of both 1D (**a**) and 4D (**b**) treatments. *Graphs* represent percentage of GluN2A immunoreactivity in relation to control. *Subscripts* indicate days of treatment ($Control_1$, MP_1, CPA_1, and $CPA_1 + MP_1$: 1D treatment, $n = 5$ in each condition; $Control_4$, MP_4, CPA_4, and $CPA_4 + MP_4$: 4D treatment, $n = 5$ in each condition). Actin was used as loading control. All values are mean ± SEM. Significance was evaluated by one-way ANOVA followed by Student–Newman–Keuls post test. $^{*}p < 0.05$, $^{**}p < 0.01$ significant versus $control_4$; $^{\#\#}p < 0.01$ significant versus MP_4

convulsant drug showed no significant difference in GluN2A expression in MP_1 group except in CA2–3 area where an increase of 53 % ($p < 0.001$) in pyramidal cell layer was observed (Figs. 3h; 4). After one single administration of CPA, no difference in GluN2A expression was observed in the analyzed area and strata with respect to control group, except an increase of 27 % ($p < 0.05$) in CA2/3 area (Figs. 3m; 4). CPA administered prior to MP ($CPA_1 + MP_1$) showed no significant differences with respect to control. Yet, a significant reduction of GluN2A expression (30 %; $p < 0.001$) in CA2–3 area and (38 %; $p < 0.05$) in hilus versus MP_1 was observed, showing a reversion of MP effect (Figs. 3r, s; 4).

Pre-treatment with CPA Prevents MP-Induced Increase in Hippocampal GluN2A Expression Levels After 4 Days of Treatment

Four daily injections of MP resulted in a significant increase in GluN2A expression in the subiculum (38 %; $p < 0.01$; Fig. 5f), CA1 area (73 %; $p < 0.05$; Fig. 5g), and hilus (26 %; $p < 0.05$; Fig. 5i); and a tendency to rise in CA2–3 (48 %; $p > 0.05$; Fig. 5h) and a slight reduction (12 %; $p > 0.05$; Fig. 5j) in the granular cell layer with respect to control group was observed (Fig. 6). CPA administration did not show significant differences in GluN2A expression compared to control, except in granular cells of dentate gyrus, where a decrease of GluN2A expression (37 %; $p < 0.05$; Fig. 5o) was observed. As in Western blot assay, there was no significant difference in $CPA_4 + MP_4$ group with respect to control. However, CPA administration prior to MP decreased GluN2A expression in all analyzed areas compared to MP group, showing a significant reversion of MP effect in the subiculum (21 %; $p < 0.01$; Fig. 5p) and hilus (14 %; $p < 0.05$; Fig. 5s) (Fig. 6).

PI Staining in the Hippocampus After MP-Induced Seizures

PI is a fluorescent marker of cell death. To investigate whether MP-induced seizure during 4 days cause excitotoxic effect on hippocampal cells, we analyzed PI staining in brain slices from MP-treated rats. PI staining reveals the entire cell cytoarchitecture. Intact and bright cells with dark nuclei and in some cells bright nucleoli were observed. Images did not reveal either markedly condensed nuclei or apoptotic cells. No differences between control and treated rats were observed with a fluorescent and a confocal microscope analysis (Fig. 7).

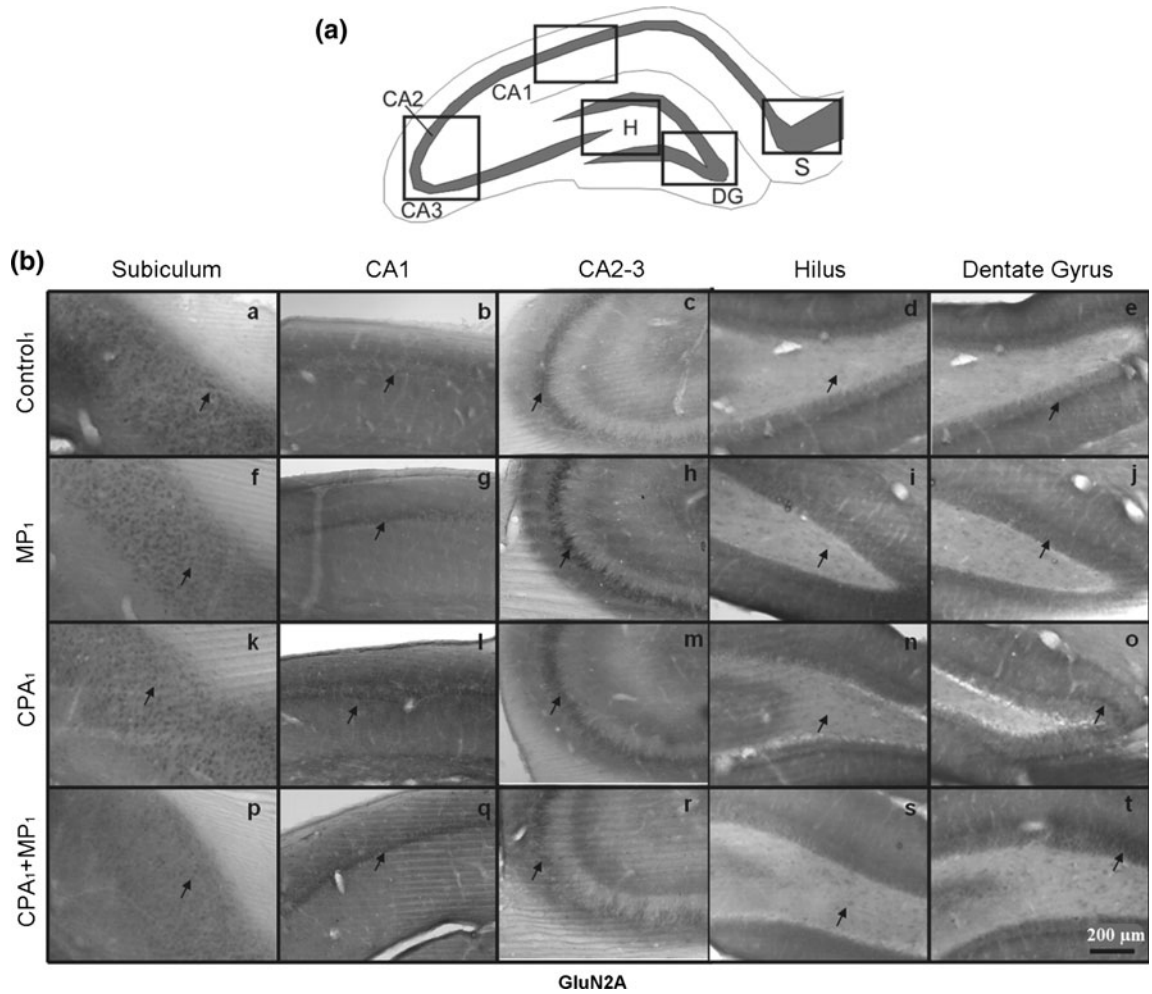

Fig. 3 GluN2A immunostaining on hippocampal areas at 1 day (1D) treatment. **a** Schematic representation of the hippocampus (adapted from Paxinos and Watson 2005). *Black squares* indicate the analyzed areas. Subiculum (*S*); Cornus Ammonis 1 and 2–3 (CA1 and CA2–3); Hilus (*H*); Dentate Gyrus (*DG*). **b** Each picture shows the different drug treatments (*rows*) and studied areas (*columns*). *Black arrows* indicate staining layers. $Control_1$: *a*, *b*, *c*, *d*, *e*; MP_1: *f*, *g*, *h*, *i*, *j*; CPA_1: *k*, *l*, *m*, *n*, *o*; and $CPA_1 + MP_1$: *p*, *q*, *r*, *s*, *t* correspond to the subiculum, CA1, CA2–3, hilus, and dentate gyrus, respectively, in each group. ($Control_1$: $n = 4$; MP_1: $n = 4$; CPA_1: $n = 3$; $CPA_1 + MP_1$: $n = 4$). Ten to twelve readings per field were averaged from 5 to 9 hippocampal tissue sections of each animal. *Scale bar* 200 µm

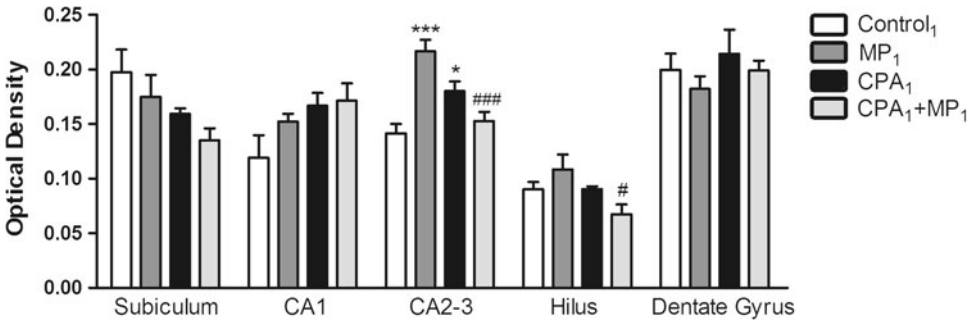

Fig. 4 Effect of MP, CPA, and CPA + MP administration during 1D treatment on GluN2A expression in different hippocampal areas. Data are expressed as mean ± SEM from averaging 10–12 readings per field from 5 to 9 hippocampal tissue sections of each animal ($Control_1$, MP_1 and $CPA_1 + MP_1$: $n = 4$; CPA_1: $n = 3$). One-way ANOVA analysis followed by Student–Newman–Keuls comparison test. $^{*}p < 0.05$, $^{***}p < 0.001$ significant versus $control_1$; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ significant versus MP_1

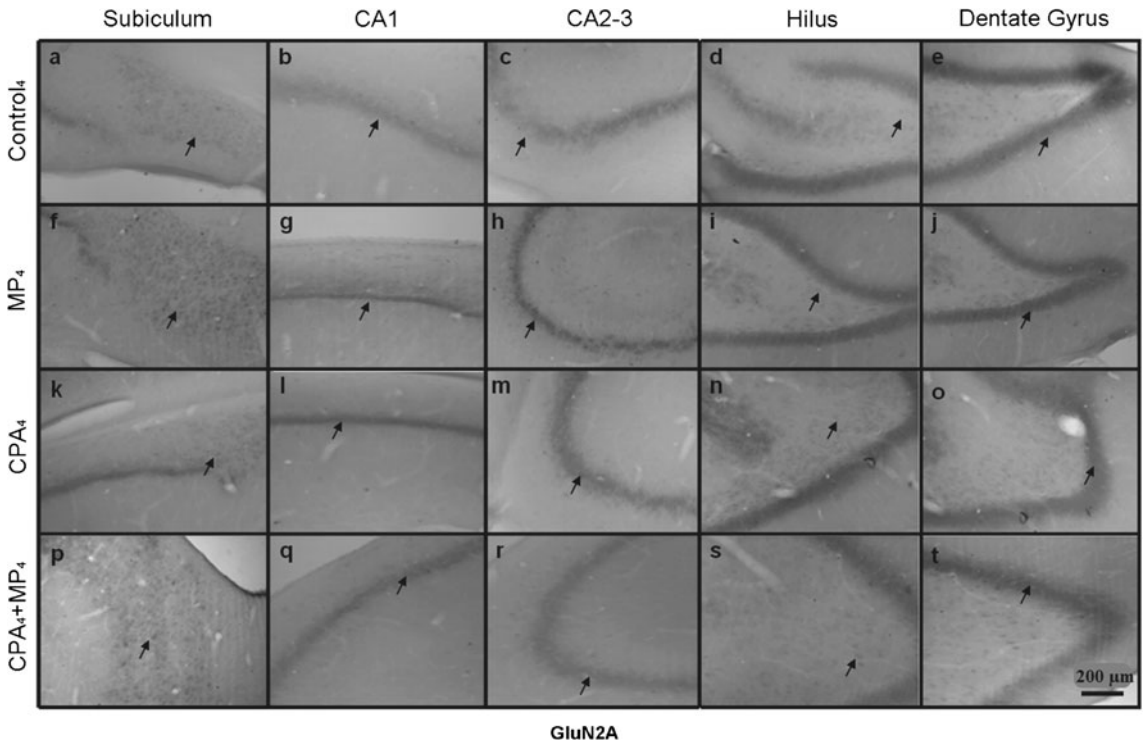

Fig. 5 GluN2A immunostaining on hippocampal areas at 4 days (4D) treatment. Each picture shows the different drug treatments (rows) and studied areas (columns). *Black arrows* indicate staining layers. $Control_4$: *a*, *b*, *c*, *d*, *e*; MP_4: *f*, *g*, *h*, *i*, *j*; CPA_4: *k*, *l*, *m*, *n*, *o;* and $CPA_4 + MP_4$: *p*, *q*, *r*, *s*, t correspond to subiculum, CA1, CA2–3, hilus, and dentate gyrus in each group. $Control_4$: $n = 4$; MP_4: $n = 4$; CPA_4: $n = 4$; $CPA_4 + MP_4$: $n = 4$. Ten to twelve readings per field were averaged from 5–9 hippocampal tissue sections of each animal. *Scale bar* 200 µm

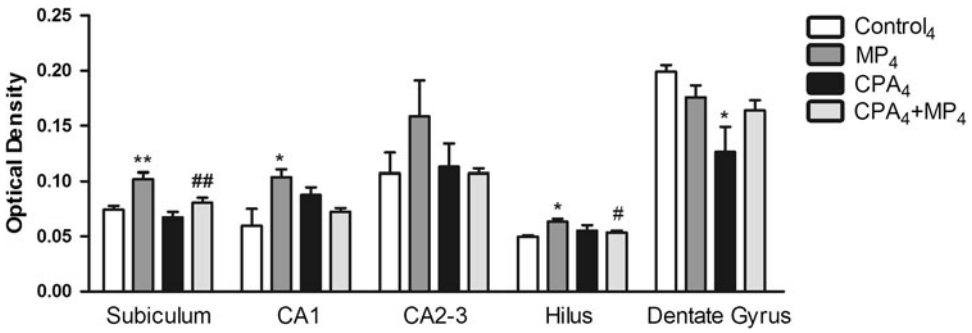

Fig. 6 Effect of MP, CPA, and CPA + MP administration during 4D treatment on GluN2A expression in different hippocampal areas. Data are expressed as mean ± SEM from averaging 10–12 readings per field from 5 to 9 hippocampal tissue sections of each animal ($Control_4$, MP_4; CPA_4 and $CPA_4 + MP_1$: $n = 4$). One-way ANOVA analysis followed by Student–Newman–Keuls comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$ significant versus $control_4$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significant versus MP_4

Discussion

The administration of MP is widely used by a variety of authors, as a classic inducer of tonic-clonic seizures (Sprince et al. 1969; Giraldez et al. 1998; Crick et al. 2007). In order to elucidate the involvement of NMDAR which is extremely important in seizure activity induced by the convulsant MP and the CPA anticonvulsant effect, we have analyzed the GluN2A subunit expression using molecular and immunohistochemistry assays in the two models: 1 day (1D) and 4 days (4D) treatments.

Single administration of MP led to generalized tonic-clonic seizures in treated animals. Despite this, no changes in GluN2A expression was observed. These results agree with those obtained using a single seizure model induced by PTZ, another inducer of tonic-clonic seizures (Zhu et al. 2004) and this would suggest that a single seizure after a single MP applied dose is not enough to cause significant

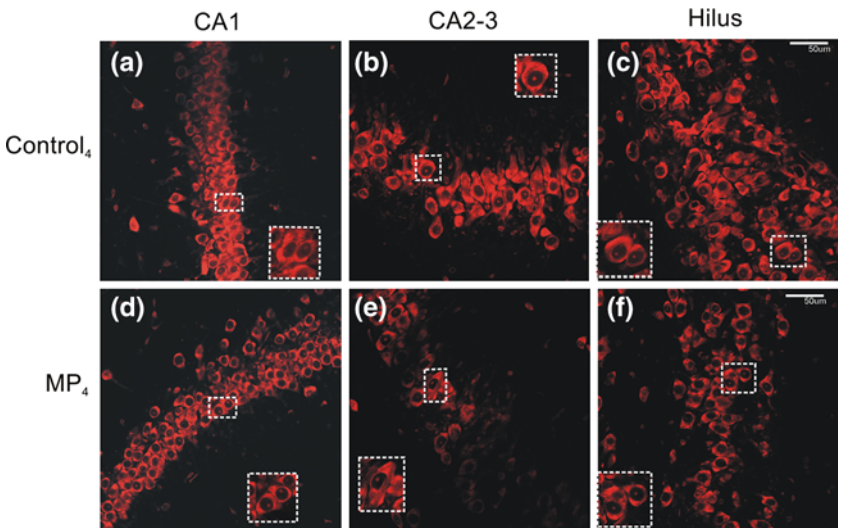

Fig. 7 PI staining in the hippocampus after MP-induced seizures. PI analysis of neurodegeneration in the hippocampus from control (**a**–**c**) and MP-induced seizures rats (**d**–**f**) during 4 days (4D treatment). Images revealed neither markedly condensed nuclei nor apoptotic cells. *Insets* show neurons at higher magnification. *Scale bar* 50 μm

changes in GluN2A subunit expression. Although in CA2/3 area an increase in GluN2A expression after MP and CPA was observed (Fig. 4), these results could indicate that CA2/3 could be a more vulnerable area and would be altered with a single MP induction.

On the other hand, repeated administration of MP showed a significant increase of GluN2A expression in molecular and immunohistochemical analysis, according to those results obtained by kainic acid intrahippocampal administration (Mikuni et al. 1999) and after repeated seizures induced by i.p. 4-aminopyridine (Borbély et al. 2009).

It is considered that synaptic activity influences the transcription of NMDAR subunits and that the overactivity would destabilize the GluN1/GluN2B complexes' connection at the synapse causing a replacement by the GluN1/GluN2A complex (Ehlers 2003; Pérez-Otaño and Ehlers 2005). This is consistent with the increase of GluN2A expression after MP-induced synaptic activity and this increase could be correlated with the previous observation on MP repeated seizure activity, made in our laboratory, which caused a significant decrease in GluN2B subunit expression in the hippocampus (Auzmendi et al. 2009). These could support the idea that repeated seizure activity causes an increase of GluN2A at synapses due to a reduction in GluN2B subunit expression, or GluN2B decreases as a consequence of GluN2A increase as opposite possibilities. This fact could be related to the segregated subcellular localizations of these subunits, GluN2A being predominantly expressed at synaptic sites (Pérez-Otaño and Ehlers 2005; Petralia 2012), although it was also suggested that it could be also at extrasynaptic sites, (Gladding and Raymond 2011). According to this, it was reported that activation of either synaptic or extrasynaptic GluN2A receptors promotes neuronal survival, triggers intracellular cascades promoting cell survival, and exerts a neuroprotective action against neuronal damage (Liu et al. 2007; Lujan et al. 2012), Zhang et al. (2011) reported that synapse-to-nucleus signaling triggered by synaptic NMDA receptors can lead to the buildup of a neuroprotective shield. These results could be related to the increased GluN2A expression observed after repetitive MP administration.

Considering that no altered cells were determined in PI-stained MP hippocampal slices, GluN2A increase could be related to reduce toxic effect. Using the fluorophore PI, which is a well-accepted marker for degenerative cells, we have observed similar images from the control and MP hippocampus. Entire neuronal cells, no shrinkage cells, and no apoptotic degeneration was observed according to Hezel et al. (2012). These results could indicate that with MP_4 no relevant damage was caused although some authophagic images could not be discarded, hence these will be further analyzed. These results are according to Borbély et al. (2009) who did not find hippocampal neurodegenerative symptoms after 4-aminopyridine-induced seizure during 12 days.

It is noteworthy that NMDARs are associated with a network of proteins that mediate their clustering, activity, and signaling events, the most important being the post-synaptic density (PSD-95), a member associated with guanylate kinase family of scafolding proteins (Forder and Tymianski 2009; Cousins et al. 2009; Sanz-Clemente et al. 2013). The effect of MP-induced seizures and this protein expression could give more information about NMDAR subunits, considering that PSD-95 binding domains differed between GluN2A and GluN2B subunits (Sanz-Clemente et al. 2013).

It has been shown that seizure activity increases extracellular adenosine levels (Deckert and Gleiter 1994; Sebastiao and Ribeiro 2009). Although adenosine levels in MP seizures have not been reported so far, it has been found that MP administration enhances the activity of 5′-nucleotidase, a key enzyme in adenosine formation in

different areas (Girardi et al. 1989a, b). The fact that extracellular adenosine levels are increased in situations which also lead to the release of excitatory aminoacids (EAAs) and the distribution of A1 receptors resembles the distribution of NMDA binding sites and confers to adenosine the ability to regulate NMDAR function, participating in the processes of seizures as an endogenous anticonvulsant agent (Giraldez et al. 1998; Giraldez and Girardi 2000; Girardi et al. 2007).

The administration of CPA 30 min before MP caused an increase in seizure latency without significant changes between both 1D and 4D treatments. In addition, the molecular changes in GluN2A expression observed in MP repetitive seizures and the reversal effect due to pre-treatment with CPA showed that the adenosine analogue avoids the MP-induced increase on GluN2A expression trying to protect the brain, even with a single administration of CPA (1D treatment).

The significant rise of GluN2A expression in subiculum and hilus observed after the administration of CPA prior to MP during 4 days could be related to the afferents and efferents of these areas. Nevertheless, the increase of GluN2A expression under MP-induced seizure activity was decreased when CPA was administered as MP pre-treatment toward control levels.

It is known that CPA decreases the synaptic activity by presynaptic inhibition of glutamate release and by hyperpolarization of the postsynaptic membrane (Boison 2005; 2008; Gomes et al. 2011). The decreased GluN2A expression observed in the CPA group could be due to the lower synaptic activity induced by the adenosine analogue. The return to control levels in CPA + MP group suggests a neuroprotective effect of CPA against the molecular changes induced by MP. These GluN2A expression changes could be observed only in 4D treatment and it could be explained as a consequence of repeated treatment. While, in a single-day treatment, a unique dose and the short time between injection and sacrifice might not be enough to observe changes in expression levels, although with a higher dose of MP we have previously determined changes in the hippocampal binding of radioactive ligand of adenosine A1 receptors ^{3}H-CCPA (Giraldez et al. 1998; Girardi et al. 2007) and ^{3}H-MK-801 (Giraldez and Girardi 1998, 2000) at seizure and post-seizure period. We cannot discard that awaiting for a time longer than 30 min between the single dose and the sacrifice might show some difference in GluN2A expression, taking into account that an adenosine antagonist and agonist effect with longer incubation times has been observed (Deng et al. 2011). On the other hand, in relation to GluN2A increased expression during repeated treatment, a reversion to control values when CPA was administered prior to MP was observed. A possible effect of astrocytes in neuronal excitability on NMDAR subunits and an adenosine A_1R involvement cannot be disregarded considering that reactive astrocytes after repetitive MP injection were previously found (Girardi et al. 2004). According to this, Deng et al. (2011) have reported that astrocytes can affect neuronal excitability by regulating the NMDA receptors expression through the activation of specific intracellular signaling pathways by agonist or antagonist of A_1 receptors. Therefore, the possibility that the GluN2A expression changes observed during repeated treatment (4D) are due to regulation by CPA through the signaling pathways that could be carried out after the complete treatment, cannot be disregarded.

In our work, the studied area has been the hippocampus, rich in A_1R, and its activation by CPA inhibits the release of EAAs and hyperpolarizes the postsynaptic membrane. Girardi et al. (2010) have also observed the protective effect of CPA on cerebellar GluN2B expression, an area with high expression of A_1Rs. These results suggest that CPA would act as a protective agent to reverse the effects of MP toward control conditions acting on A_1Rs signaling pathways (Milton et al. 2008; Nayak et al. 2011).

It has become clear that NMDAR's activation can drive to promote cell survival or lead to neuronal death, depending on their subcellular location and a variety of pathways that would be involved (Xiao et al. 2010; Nayak et al. 2011; Sanz-Clemente et al. 2013). Further studies related to signaling pathways would determine the specific role of GluN2A subunit during MP seizure activity.

Conclusion

A single seizure induced by MP is not enough to alter the hippocampal GluN2A expression pattern. Immunohistochemical studies only detected an increase in CA2/3 area, which indicates that it could be a more susceptible area.

Repeated seizures increased GluN2A subunit expression, which could be related to a neuroprotective role of this subunit in MP-induced seizure activity. However, further studies about signaling pathways must be performed. CPA administration before MP delays seizure onset and reduces GluN2A expression to control levels, showing a protective effect.

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