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SHORT COMMUNICATION

3-Mercaptopropionic Acid-Induced Seizures Decrease NR2B Expression in Purkinje Cells: Cyclopentyladenosine Effect

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Abstract Inhibitory mechanism of cerebellum epileptic activity can be involved depending on the intensity and frequency of seizure convulsions. *N*-methyl-D-aspartate receptors (NMDARs) play key roles in excitatory synaptic transmission and have been implicated in neurological disorders: in cerebellum, they have specific characteristics. NMDARs are heteromeric complexes, and the expression of functional receptors in mammalian cells requires the subunit NR1 (essential) and one NR2 subtype of the four isoforms: NR2A–NR2D. In mature Purkinje cells, the combination of NR1 with NR2B subunits forms functional NMDARs; NR2B subunit may be altered in exocitotoxic events. Cyclopentyladenosine (CPA), an adenosine analogue, administered to rats, for one or more days, increases seizure threshold induced by the convulsant drug 3-mercaptopropionic acid (MP). In this study, we focused on the expression of NR2B in cerebellum after repetitive seizures induced by MP and the effect of adenosine analogue CPA administered alone or previous to MP (CPA + MP). A significant decrease in NR2B in the whole cerebellum was observed after MP and CPA administration with a tendency to recover to normal values in the combined treatment of CPA administered 30 min before MP by Western blot assay. In immunohistochemical studies, NR2B expression was observed and analysed in Purkinje cells. NR2B expression was decreased after MP (55%) and CPA (12%) administration, and CPA injected 30 min before MP led to 28% reduction in Purkinje cells. These results could be related to Purkinje cell damage or alternatively to avoid the excitotoxic effect. Results recorded after CPA + MP treatment seemed involved in decreasing the convulsant MP effect.

Keywords NR2B · Cerebellum · Epilepsy · Adenosine · Purkinje cells · 3-Mercaptopropionic acid · Seizures

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Introduction

N-methyl-D-aspartate receptor (NMDAR), a subclass of ionotropic glutamate receptor, plays a crucial role in synapse formation, plasticity and diseases like epilepsy (Dingledine et al. 1999; Cull-Candy et al. 2001). NMDARs are heteromeric complexes composed of different subunits within three subtypes: NR1, NR2 and NR3. Expression of functional recombinant NMDARs requires the co-expression of at least one NR1 and one NR2 subtype. Although the exact subunit composition has not yet been entirely established, there is consensus in that NMDAR is a tetramer of two NR1 and two NR2 subunits of the same or different subtypes (Cull-Candy et al. 2001; Köhr 2006; Paoletti and Neyton 2007). There are four types of NR2 subunits, NR2A–D. In cerebellum, NR1 subunit is ready to combine with the NR2B subunit to form functional NMDAR in mature Purkinje cell (Kakegawa et al. 2003). Multiple NMDAR subtypes are indeed expressed in Purkinje cells of young and adult mice; both NR2A- and NR2B-containing subtypes are present in the adult (Renzi et al. 2007).

Cerebellar NMDARs behave very differently to those present in other brain areas. This may be due to the different subunit compositions of the receptor and also to

differences in mRNA alternative splicing, phosphorylation, trafficking, etc. (Llansola et al. 2005). Although an inhibitory effect on seizure is proposed to cerebellum, cerebellar dysplastic lesions can be epileptogenic (Mesiwala et al. 2002). Besides focal seizure discharges of cerebellar origin has been reported (Harvey et al. 1996), and a cerebellar arachnoid cyst is associated with epilepsy (Gan et al. 2007).

The repetitive administration of the convulsant drug 3-mercaptopropionic acid (MP) induces tonic–clonic seizures and reactive astrogliosis in CNS (Girardi et al. 2004), and the administration of the adenosine analogue cyclopentyladenosine (CPA) increases seizure threshold (Girardi et al. 2007). Adenosine—a neuromodulator with endogenous anticonvulsant properties—inhibits neuronal firing and synaptic transmission acting mainly via A1 receptors, the most abundant of the four kinds of adenosine receptors described (Dunwiddie and Masino 2001, Ribeiro et al. 2003), being cerebellum, a rich region in A1 receptors (Giraldez et al. 1998). The convulsant MP increases [^{3}H]-2-chloro-N6-cyclopentyl-adenosine (CCPA) binding to A1 receptors in cerebellum, and this increase was maintained when CPA was administered before MP (Giraldez et al. 1998).

The objective of this study was to study cerebellar NR2B expression after repetitive convulsant drug MP, the adenosine analogue CPA administration and CPA + MP.

Methods

Animals and Treatments

Male Wistar rats (250–300 g) were maintained on a 12/12 h diurnal cycle. They were divided in four experimental groups (n = 4/group). Rats were daily injected intraperitoneally (i.p.) during 4 days with a single dose of: (a) 45 mg of 3-mercaptopropionic acid/kg body weight (MP group), (b) 2 mg of cyclopentyladenosine/kg body weigh (CPA group), or (c) the same dose of CPA 30 min before MP injection (CPA + MP group) and (d) control animals (C) were injected with saline as vehicle. 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. Daily MP injection (freshly prepared pH neutral MP solution) resulted in the onset of seizures episodes which occurred 5–8 min later, characterized by excitation with sudden running fits and convulsions which lasted 3–5 min. Rats injected i.p. with CPA (freshly dissolved in saline) resulted in a relaxed and inactive state at 30 min. The third group of rats injected with CPA 30 min before MP showed an increase in MP seizure latency. Control group was injected with vehicle for 4 days.

Homogenate Preparation and Western Immunoblot Procedure

Each experiment included lots of three rats in each condition. Treated and control cerebellum was separated in the cold and pooled. Tissues were rapidly homogenized at 10%(w/v) in 0.32 M sucrose neutralized with Tris base solution (0.2 M) pH 7.2 and 0.05 ml/g proteinase inhibitor cocktail, in a Teflon glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 900g for 10 min at 4°C. The supernatants were spun down at 100,000g 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 determinated using Folin–Cicaultau reagent with bovine serum albumin as standard (Lowry et al. 1951).

For gel electrophoresis, each lane was loaded with 23 µg of protein. After separation by SDS–PAGE 8%, the resolved proteins were electro-transferred onto a nitrocellulose filter. The filters 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 NR2B primary antibody (1:500) at 4°C. After three, 5-min washes with TBS-T, the filters were incubated with horseradish peroxidase-conjugated secondary anti-body in buffer (1:5000) for 1 h, washed (three times) with TBS-T and the blots were revealed by ECL-chemiluminescence. β-tubuline (1:1500) was revealed on the same blots. The resulting film was scanned, and the quantification of the gel bands was analysed by computer-assisted software (Image J). Values represent the means of 4–6 independent experiments. Percentages from the control values were statistically analysed by one sample t-test using Graph Pad prism, and were compared to control.

Immunohistochemistry

This technique was applied as previously described (Girardi et al. 2007). In brief, 24 h after last injection, animals were deeply anaesthetized with 300 mg/kg of chloral hydrate, perfused and fixated. Each cerebellum 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 the 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 (RT). Tissue sections were blocked for 1 h with 3% v/v normal goat

serum in phosphate buffer saline (PBS) and then incubated for 48 h at 4°C with rabbit anti-NR2B primary antibodies, incubated with biotinylated secondary antibody (1:100), followed by incubation with streptavidin–peroxidase complex diluted 1:200. Development of peroxidase activity was performed with 3,3′ diaminobenzidine plus nickel ammonium sulphate 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.

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 analysed with an image analysis software. The resolution of each pixel was 256 grey levels (8 bits). The projected surface of cells was measured using mean grey values. Relative optical density (ROD) was obtained after a transformation of mean grey values into ROD by using the formula ROD: log (256/mean grey). A background parameter was obtained from each section out of the labelled structures and subtracted to each cell ROD before statistically processing values.

Statistics

Values represent the means of 3–4 independent experiments. Individual experiments were composed of 4–7 tissue sections of each animal from each group. Differences among the means were analysed using one-way ANOVA and Student–Newman–Keuls post test. Statistical significance was set to $P < 0.05$.

Materials

MP, CPA, streptavidin complex, and secondary antibodies were obtained from Sigma–Aldrich. Polyclonal rabbit antiserum against NR2B was purchased from Santa Cruz Biotechnology. All other chemical substances were of analytical grade.

Results

The NR2B expression after the administration of the convulsant drug MP, the adenosine analogue CPA and both compounds together, CPA 30 min before MP, were studied in cerebellum.

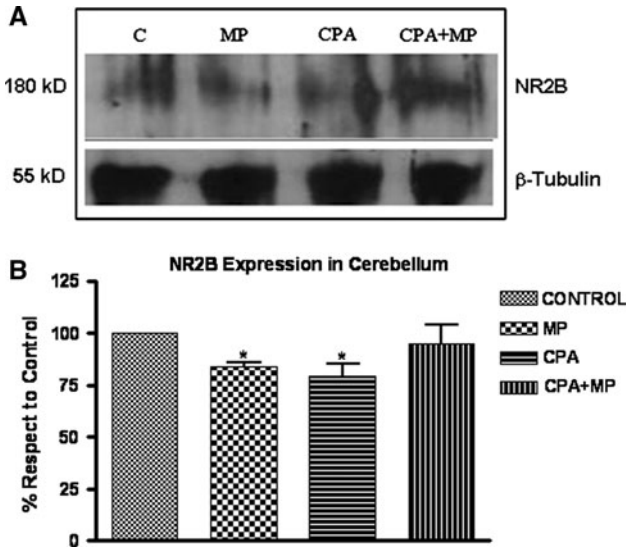

Fig. 1 NR2B cerebellar expression. **a** Western blot for NR2B. Control(*C*). 3-mercaptopropionic acid (*MP*), Cyclopentyladenosine (*CPA*) and CPA 30 min before (CPA + MP). Each *lane* represents 23 μg of pooled membrane proteins. **b** The *bar graphs* illustrate the percent change with respect to control. $P < 0.05$

Western blot analysis was performed on the whole cerebellum tissue showing a 180-kDa band which stained positive for NR2B. There was a decrease in NR2B expression of 17% and 21% after MP and CPA treatments, respectively, while in CPA + MP group, no significant difference was observed (Fig. 1a, b).

NR2B immunoassays showed an intense staining in Purkinje cell layer, in cell bodies and dendritic prolongations of Purkinje cells (Fig. 2). A light immunostaining in granular layer was observed but could not be measured. Repetitive MP administration decreased Purkinje cell NR2B expression; when CPA was injected, NR2B had a light difference with the control group, while CPA before MP decreased the MP effect alone (Fig. 3).

Densitometric analysis quantification showed that the convulsant drug administration decreased NR2B expression in Purkinje cells (55%) with respect to control. The adenosine analogue CPA decreased by 12%, while the combined treatment CPA + MP led to 28% decrease in NR2B subunit expression (Fig. 4).

Discussion

The administration of the convulsant drug MP and the adenosine analogue CPA decrease NR2B expression in Purkinje cells; this decrease can be reduced when CPA is administered before MP.

Cerebellar NMDARs behave very differently from NMDARs in other brain areas. In most cases, this is due to

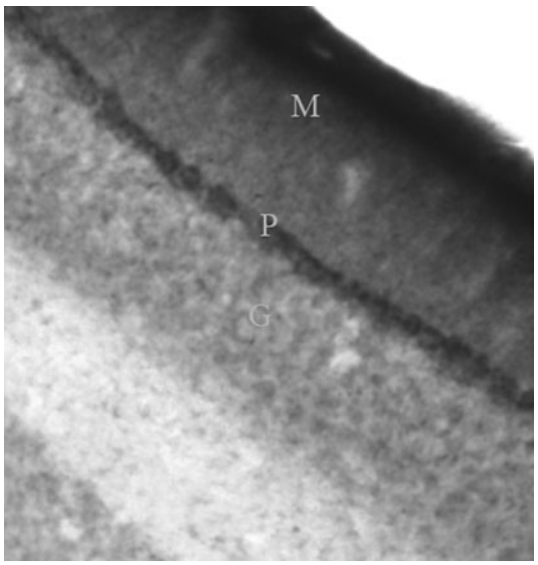

Fig. 2 Photograph of NR2B immunostaining in cerebellum. Different layers are indicated: Molecular (*M*), Purkinje cell (*P*), and Granular (*G*). Original magnification ×40

the different subunit compositions of the receptor, but there are also studies showing differences in mRNA alternative splicing, phosphorylation, trafficking, etc., and all these differences may have important consequences on the receptor function and regulation (Llansola et al. 2005). The affinity of the antagonist at the glutamate site was higher in NMDAR from forebrain than from the cerebellum (Reynolds and Palmer 1991). Accordingly, the study of NMDA antagonist ^{3}H-MK-801 binding to CNS areas disclosed that cerebellum failed to show detectable ligand binding (Giraldez and Girardi 1998). However, other authors have reported an important NR2B expression in Purkinje cells (Thompson et al. 2000, Kakegawa et al. 2003), and in support it was reported that functional NMDARs find expression in mature Purkinje cells that become detectable at 21 days after birth and control the complex spike waveform (Piochon et al. 2007).

Adenosine, which is concentrated in the soma and dendrites of Purkinje cells, plays an important role in the processing of information by cerebellar circuits (Jarvis and Williams 1990). The control of the extracellular adenosine concentration in the cerebellum appears comparable to that in other brain regions with a non-uniform distribution of the transporters relative to synapse (Wall et al. 2007). Adenosine acts through specific receptors being AR1 widely expressed in cerebellum (Giraldez et al. 1998). Adenosine agonist ^{3}H-CCPA binding to A1R in this area is increased after the administration of either MP alone or together with CPA (Giraldez et al. 1998).

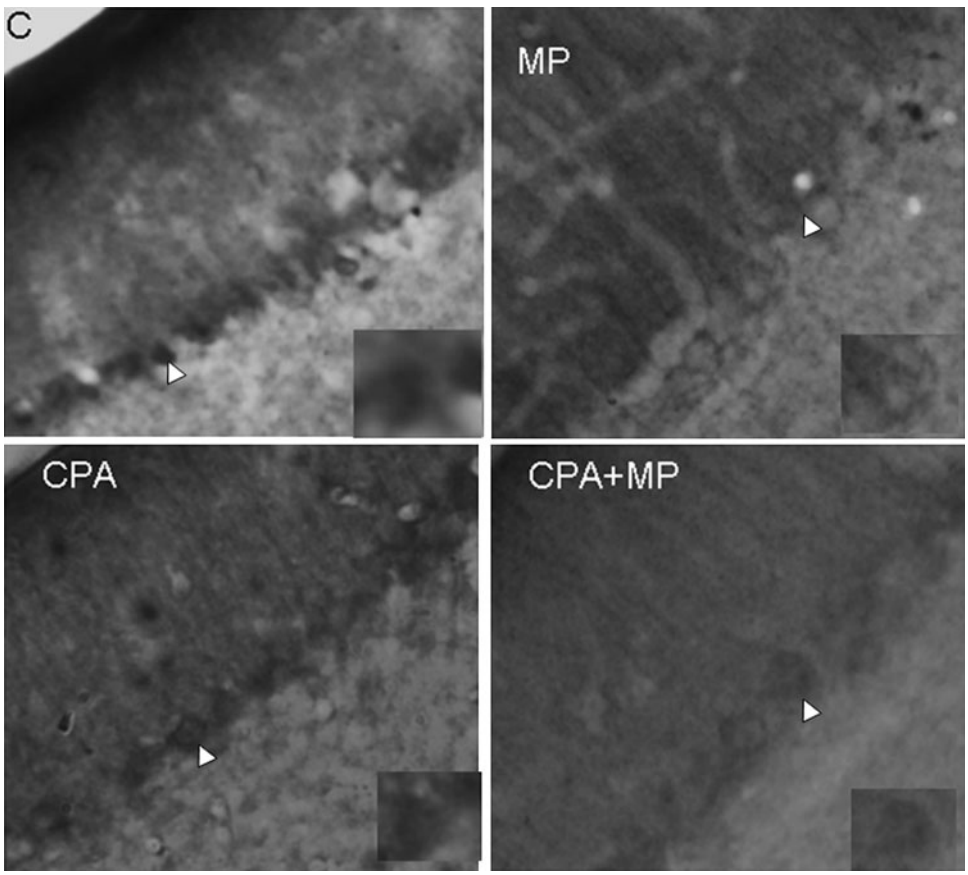

Fig. 3 Photograph of cerebellum in each treatment. *C* Control, *MP* convulsant treatment, *CPA* cyclopentyladenosine, an adenosine analogue; CPA + MP CPA administered 30 min before MP. Original magnification ×100. *White arrows* indicate Purkinje cells

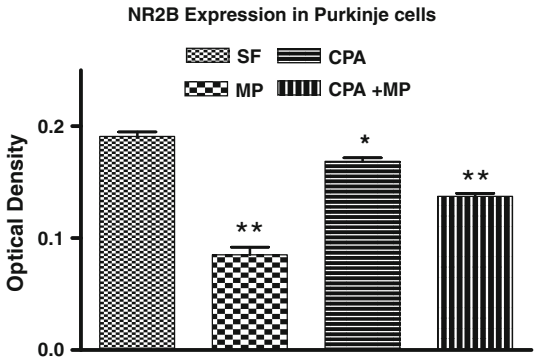

Fig. 4 Relative optical density of NR2B immunostained Purkinje cells after Control, MP, CPA and CPA + MP treatment. Values are expressed as the mean ± SEM. ** $P \leq 0.001$ and * $P \leq 0.01$ related to control. Statistical difference was observed among different treatments ($P \leq 0.01$)

We have also reported an increase in 5′ nucleotidase activity, the key enzyme in adenosine formation during seizure, in cerebellar subcellular fractions (Girardi et al. 1989), thus indicating a participation of cerebellar adenosine metabolism at seizures. This finding may indicate the involvement of cerebellar adenosine metabolism at seizure states. CPA and MP administration effects on NR2B expression give rise to a similar NR2B expression pattern, which is in accordance with a decrease in 3H–MK801 binding to NMDAR after MP and CPA administration in different brain areas (Giraldez and Girardi 1998). The reduction of the decreased NR2B expression in CPA + MP may be attributable to a compensatory mechanism to diminish the MP effect.

During CPA + MP treatment, where was observed a tendency to reduce MP effect which was greater in the whole homogenates. This could be due to an increase in NR2B expression on the entire Purkinje cells, and to a light NR2B expression in Golgi interneurons in granular layer (Thompson et al. 2000), reducing the difference from the control in the whole homogenates, considering that a light immunostaining in granular layer was observed but could not be measured.

Purkinje cells present a unique cellular profile; they are the only output of the cerebellar cortex, and play a vital role in the normal function of the cerebellum. The Purkinje cells are located in a monolayer between the molecular and granular layers, and the Purkinje cell dendritic branches orientate towards the pial surface (Goldowitz and Hamre 1998). The inhibitory Purkinje cells—the sole output of the cerebellum—project to deep cerebellar nuclei in brainstem and subsequently to widespread frontal lobe and subcortical structures. The reduced expression of NR2B could be associated to an internalization of the receptor or to an apoptotic effect on Purkinje cells, considering that a single MP administration induces morphological changes in these cells and reduces GABA levels and the glutamate decarboxilase cerebellar activity (Rodríguez de Lores Arnaiz et al. 1972). In this way, the interneuron gabaergic signals may be altered in their regulatory role of glutamate excitability in the Purkinje cells. Besides, the inhibitory Purkinje cell outputs may be changed.

Cerebellum serves as an integrator of sensory information and regulator of motor coordination and training, which is considered a broad learning machine for neuronal control (Ito 2006), it has inhibitory effects on seizures (Cooke and Snider 1955; Velasco et al. 2005), and it can be also epileptogenic in cerebellar dysplastic lesions (Mesiwala et al. 2002). In this regard, it has been reported that focal seizure discharges of cerebellar origin (Harvey et al. 1996) and the Purkinje cells of tottering mice with altered functional expression of calcium channel results in the absence epilepsy and motor dystonia linked to cerebellar dysfunction (Erickson et al. 2007). Increased activity in cerebellum observed in human secondary generalizes tonic–clonic seizures may be crucial for motor manifestation and may be related with cortical and subcortical networks (Blumenfeld et al. 2009).

The repetitive MP administration induces a reduction in cerebellum NR2B expression that could indicate a tendency to avoid the excitotoxicity due to an increased convulsive hyperactivity. CPA alone decreased this subunit expression and CPA administered before MP reduces MP effect trying to diminish seizure changes.

It is worthwhile to mention that the decreased NR2B expression observed after repetitive MP administration could be related to a protective effect from released glutamate excitotoxicity. In relation to this, it was reported that there is a tightly controlled NMDAR activity by endogenous substances, and during an intense neuronal activity, increases NMDAR inhibition to limit an overstimulation and to prevent neuronal injury (Gielen et al. 2008). These effects may occur in our experimental epileptic model; in the presence of adenosine analogue, the lower inhibitory effect on NR2B expression may be due to the concentration of the endogenous substances.

The decreased NR2B expression in the cerebellum (present finding) and in certain hippocampal areas and strata (Auzmendi et al. 2009) may indicate an injury or cognitive inhibition considering that NR2B subunit overexpression in mice have been observed with cognitive enhancement (Cull-Candy et al. 2001).

We have observed Purkinje cell NR2B expression in coincidence with other authors (Thompson et al. 2000; Kakegawa et al. 2003), and this NMDAR subunit expression was decreased after MP repetitive convulsive seizure, thus indicating the Purkinje cell NR2B participation in this hyperactivity. A decreased NR2B cerebellar expression could be related to a tendency to reduce the epileptogenesis

though an alteration and reduction of Purkinje cells could not be disregarded. Results recorded after CPA + MP treatment seemed involved in decreasing the convulsant MP effect.

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