Neurochem Res (2007) 32:1120–1128
DOI 10.1007/s11064-006-9275-8

ORIGINAL PAPER

Differential Expression of Cerebellar Metabotropic Glutamate Receptors mGLUR2/3 and mGLUR4a after the Administration of a Convulsant Drug and the Adenosine Analogue Cyclopentyladenosine

Elena Silvia Girardi · Juan Canitrot · Marta Antonelli · Nélida N. González · Héctor Coirini

Accepted: 28 December 2006 / Published online: 31 March 2007

Abstract Metabotropic glutamate receptors (mGluR) play a role in synaptic transmission, neuronal modulation and plasticity but their action in epileptic activity is still controversial. On the other hand adenosine acts as a neuromodulator with endogenous anticonvulsive properties. Since cerebellum from epileptic patients has shown neuronal damage, sometimes associated with Purkinje cells loss, we have explored the effect of repetitive seizures on two types of mGluR in the cerebellum. Seizures were induced by the convulsant drug 3-mercaptopropionic acid (MP) and the effect of the adenosine analogue cyclopentyladenosine (CPA) alone or before MP administration (CPA+MP) were also evaluated. The expression of the receptors subtypes 2/3 (mGluR2/3) and 4a (mGluR4a) was assessed by immunocitochemistry. Granular cell layer was labeled with mGluR2/3 antibody and increased immunoreactivity was observed after MP (60%), CPA (53%) and CPA + MP (85%) treatments. Control cerebellum slices showed mGluR4a reactivity around Purkinje cells, while MP, CPA and CPA+MP treatment decreased this immunostaining. Repetitive administration of MP and CPA induces an increased cerebellar mGluR2/3 and a decreased mGluR4a immunostaining, suggesting a distinct participation of both receptors that may be related to the type of cell involved. A protective action and /or an apoptotic effect may not be discarded. CPA repetitive administration although increase seizure latency, cannot prevent seizure activity.

Keywords Metabotropic receptors · mGluR2/3 · mGluR4a · Cerebellum · Epilepsy · Adenosine · Seizure · Convulsant drug · Cyclopentyladenosine · 3-Mercaptopropionic acid

E. S. Girardi (✉) · J. Canitrot · N. N. González
Instituto de Biología Celular y Neurociencia ''Prof. Eduardo De Robertis'' Facultad de Medicina, Universidad de Buenos Aires. Buenos Aires. Argentina y Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Paraguay 2155, 3°piso, ZIP C1121ABG Buenos Aires, Argentina
e-mail: egirardi@mail.retina

M. Antonelli
IQUIFIB, UBA-CONICET, Buenos Aires, Argentina

H. Coirini
Department of Human Biochemistry, IBYME-CONICET, Facultad de Medicina. UBA, Buenos Aires, Argentina

Introduction

Glutamate, the major excitatory neurotransmitter in the central nervous system (CNS), plays a crucial role in seizure generation and maintenance as well as in excitotoxic epileptic brain damage [1, 2]. Glutamate acts through two principal classes of receptors, ionotropic and metabotropic (mGLuR) receptors. The latter have been involved in synaptic transmission, synaptic modulation and plasticity. These receptors present seven transmembrane domains that modulate intracellular second messengers via GTP-binding proteins [3, 4]. It has been described at least eight types of mGluRs that have been subdivided into three main groups based on sequence homology, signal transduction mechanism and response to receptor agonists. Group I (mGluR1; mGluR5) is linked to stimulation through inositol triphosphate (IP3) formation. Group II (mGluR2; mGluR3) and group III (mGluR4,

mGluR6, mGluR7 and mGluR8) are coupled to adenylcyclase inhibition [3, 5]. The cellular distribution of the metabotropic receptors in the rat brain has been achieved by immunohistochemitry and electron microscopy studies in the rat brain, exhibiting a complex regional pattern of differential localization [6–9]. Although members of each of the mGluRs groups can be located at both pre and postsynaptically, group II and III typically act as presynaptic receptors involved in neurotransmitters release regulation [10, 11].

Adenosine, considered an endogenous anticonvulsant, inhibits neuronal firing, synaptic transmission and modifies cyclic AMP levels [12, 13].These effects are due principally to the activation of the inhibitory A1 receptors, the most abundant of the four kind of adenosine receptors described [12, 13]. In relation to this protective effect, we have found that the administration of cyclopentyladenosine (CPA), an adenosine analogue, and the convulsant 3-mercaptopropionic acid (MP) increase [^{3}H]-2-chloro-N6-cyclopentyl-adenosine (CCPA) binding to A1 receptors in different regions of the central nervous system [14]. In addition the administration of one unique dose of CPA before the convulsant MP produces a delay in seizure appearance [15], and it has been associated to changes on the specific [^{3}H]-MK-801 binding. It was observed a decrease in this ligand binding to NMDA receptor in different brain areas after MP and CPA treatment [16].

The cerebellar cortex has a well characterized ultrastructure and cellular organization. [17, 18]. This brain area is rich in adenosine A1 receptors and expresses the different groups of mGluR [8, 14]. The cerebellum is involved in the control of movements, particularly those where timing is crucial or is linked to the voluntary actions. Changes in excitatory synaptic strength in the cerebellar cortex have been associated to motor learning [19]. In addition it is an essential node in the central integration of somatic and visceral activities adapting the changes of internal and external environments [20]. Cerebellar stimulation is known to have inhibitory effects on seizures [21, 22] but cerebellar dysplastic lesions can be epileptogenic. In this regard it has been reported that a hemorrhagic cerebellar lesion produced epilepsia partialis continua in a patient [23].

The aim of this study was to evaluate cerebellar mGluR2/3 and mGluR4a alterations in an MP-induced seizure model and assess the role of the adenosine analogue as a neuromodulator, using immunocytochemical techniques.

Experimental procedures

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 intra peritoneal (ip) during 4 days with a single dose of: a) 45 mg of 3-mercaptopropionic acid/kg body weight (MP group), b) 2 mg of cyclopentyl-adenosine/kg body weigh (CPA group) or c) the same dose of CPA 30 min before MP injection (CPA+MP group), control animals (CTL) were injected with saline as vehicle. Animal care was in accordance with the National Institutes of Health guideless 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 after MP injection, 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 saline for 4 days.

Immunohistochemistry Twenty four hours after the last injection, animals were deeply anaesthetized with 300 mg/kg of chloral hydrate and perfused through the left ventricle, initially with a cold solution containing 0.05% w/v $NaNO_2$ plus 50 IU of heparin in 0.1 M phosphate buffer (pH 7.4) followed by a fixative solution (4% paraformaldehyde plus 0.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). Each cerebellum was removed and kept in the same cold fixative solution for 4 h. After that, tissue was washed three times in cold 0.1 M phosphate buffer pH 7.4 containing 5% w/v sucrose, and left in this washing solution for 18 h at 4°C. Saggital 40 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 (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-mGluR2/3 (1:400) or anti-mGluR4 (1:800) primary antibodies.

After five rinses in PBS, sections were incubated for 1 h at RT with biotinylated secondary antibody (1:100). After further washing in PBS, sections were incubated for 1 h with streptavidin-peroxidase complex diluted 1:200. Section were washed 5 times in PBS and 2 times in 0.1 M acetate buffer pH 6 (AcB), development of peroxidase activity was performed with 0.035% w/v 3,3'-diaminobenzidine plus 2.5% w/v nickel ammonium sulphate and 0.1% v/v H_2O_2 in AcB. Sections were then washed in AcB 3 times and once in distilled water. Sections were mounted on gelatin-coated slides, dehydrated and cover-slipped using Permount (Sigma) for light microscopic observation. All antibodies, as well as streptavidin complex, were dissolved in PBS containing 1% v/v normal goat serum and 0.3% v/v Triton X-100 (pH 7.4). PBS buffer used in washing steps included 0.025% Triton X-100. Negative controls were processed simultaneously by omiting the primary antibodies. Four to six assays for each primary antibody using 6–10 tissue sections for animal/group were made.

Image analysis

Optical density (OD) and morphometric parameters of immunostained cells were measured using an Axiophot Zeiss light microscope, equipped with a video camera on line with a Zeiss-Kontron Vidas image analyzer. Images obtained from the light microscope were transferred to an interactive image analysis system on line using a video camera. The images were digitized into an array of 512×512 pixels corresponding to 140×140 μm ($40 \times$ primary magnification). The resolution of each pixel was 256 gray levels (8 bits). After automatic normalization of gray scale, an interactive delineation and contrast enhancement of the cell images were performed, following the removal of interfering nonspecific images. 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 labeled structures and subtracted to each cell ROD before statistically processing values. Seven to ten fields were digitalized for each stained section. All cells from every field were measured.

Statistics

Values represent the means of 3–4 independent experiments. Inter-animal differences inside the groups were not significant. Individual experiments were composed of 6–10 tissue sections of each animal from each group. Seven to ten fields were measured for each cerebellum. Differences among the means were analyzed using one-way ANOVA and Student-Newman-Keuls post test. Statistical significance was set to $P < 0.05$.

Materials

MP, CPA, streptavidin complex, secondary antibodies were obtained from Sigma–Aldrich. Polyclonal rabbit antiserum against mGluR2/3 and mGluR4a were purchased from Upstate Biotechnology. All other chemical substance were of analytical grade.

Results

Immunocitochemical analysis of the expression of mGluR2/3 and mGluR4a in cerebellar cortex showed differential localization and concentration (Fig. 1).

mGluR2/3 immunostaining was intense on the granular layer closely apposed to the Purkinje cell layer (Fig. 2A). Treatments with MP, CPA and CPA+MP increased mGluR2/3 immunoreactivity (Fig. 2). Densitometric analysis showed that mGluR2/3 staining intensity was significantly increased in both independent drug treatments: MP (61%), and CPA (53%). When CPA was administered 30 min before MP the staining intensity showed a greater increase (85%) that was not statistically significant among treatments (Fig. 3).

On the other hand, mGluR4a reactivity was mainly observed around Purkinje cells. The immunoreactivity in the molecular layer appeared as a shadow, avoiding a precise quantitative analysis (Fig. 4). Treatment with MP, CPA and CPA+MP decreased the immunostaining. Densitometric analysis showed that mGluR4a staining intensity was significantly decreased after MP (35%) and CPA (54%) treatment when compared to control. However when CPA was administered before MP the staining intensity was lower than the observed for each individual treatment (89%) (Fig. 5).

CPA administration previous MP during four days, shows a delay on the onset of seizure (Fig. 6).

Discussion

In the present study, the effect of MP-induce seizures and adenosine analogue CPA on group II and III mGluR expression was evaluated on rat cerebellum.

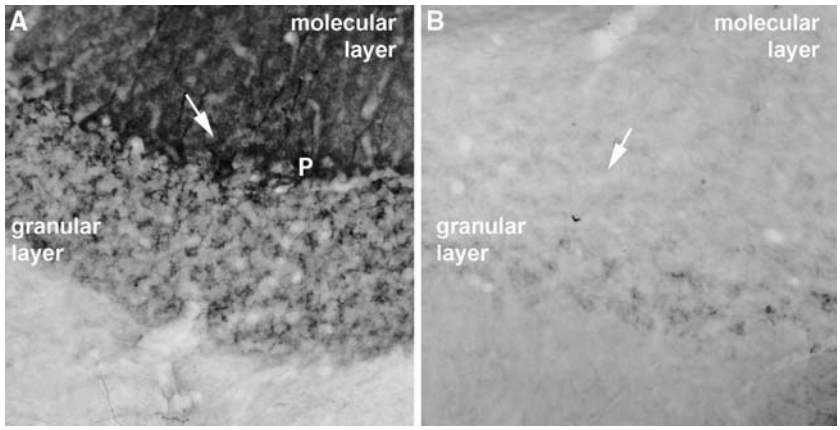

Fig. 1 Photograph of mGluR immunostaining in cerebellum. (A) mGluR 4a. The three layers are named. Molecular, Purkinje and Granular layers. Arrow shows Purkinje cells. (B) mGluR2/3 molecular and Purkinje cell layers are not stained (arrow). Original magnification 100×

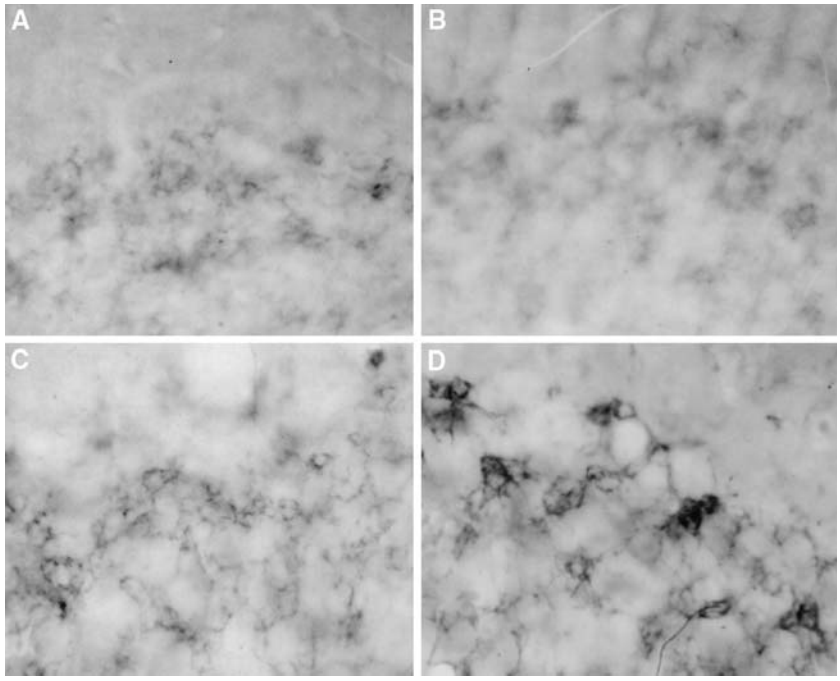

Fig. 2 Photograph of mGluR 2/3 immunostaining. (A) Control, (B) CPA, (C) MP, (D) CPA+MP. Original magnification 400×. Note the increased immunostaining in CPA + MP (D)

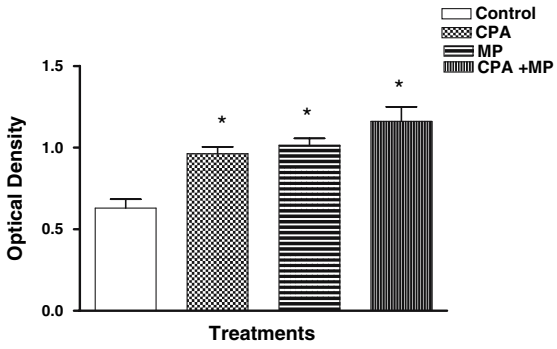

Fig. 3 Relative optical density of mGluR2/3 immunostained granular cells after control, CPA, MP and CPA+MP treatments. Values are expressed as the mean ± SEM. Data were analyzed by one way ANOVA followed by Student-Neumann-Keuls test. *$P < 0.05$ related to control. No statistically difference was observed among different treatments

In a previous work we demonstrated that MP administration induced an increase in [^{3}H]-CCPA binding to adenosine receptor in cerebellum slices [14] as well as an increase in 5′nucleotidase activity, the key enzyme in adenosine production during seizure, in cerebellar subcelullar fractions [24]. In addition, stimulation of this enzyme by neurotoxic concentrations of glutamate on culture granulare cells has been reported [25].

The role of the cerebellum in epilepsy is controversial, some reports indicate inhibitory effects on seizures after cerebellar stimulation [21, 22] and there are reports of Epilepsia partialis continua possibly caused by cerebellar lesion [23] and an epilepsy syndrome of infant with seizures of cerebellar origin [26]. Moreover Purkinje cell loss/injury was observed in the cerebellum of epileptic patients [27].

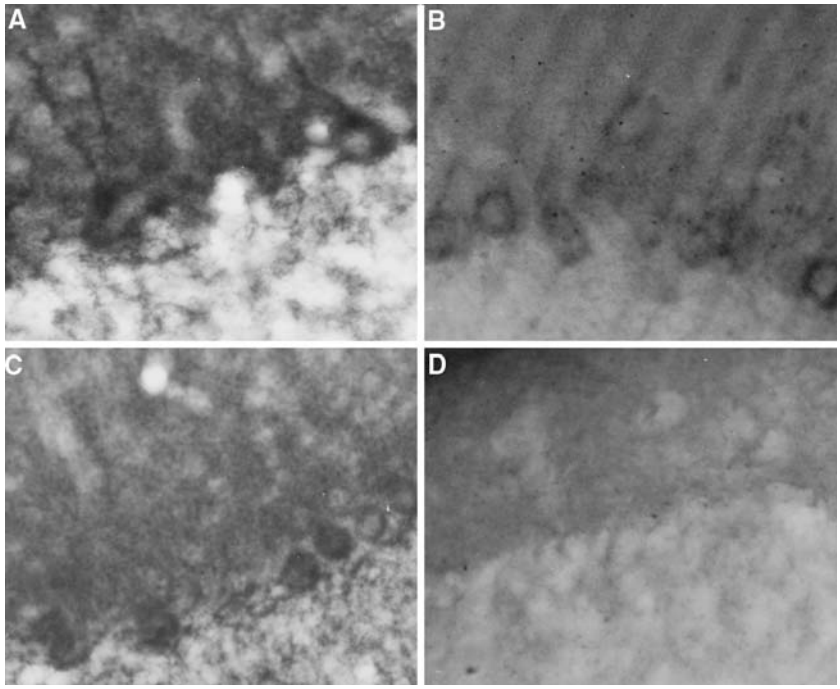

Fig. 4 Photograph of mGluR 4a immunostaining. (A) Control, (B) CPA, (C) MP, (D) CPA+MP. Original magnification 400×. Note the reduced immunostaining in treatments respect to control

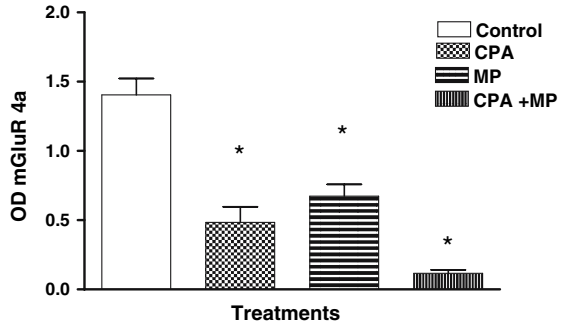

Fig. 5 Relative optical density of mGluR4a immunostained Purkinje cells after control, CPA, MP and CPA+MP treatment. Values are expressed as the mean ± SEM. Data was analyzed by one way ANOVA followed by Student-Neumann-Keuls test. *$P < 0.001$ related to control. Statistically difference was observed among different treatments ($P < 0.01$) except CPA versus MP

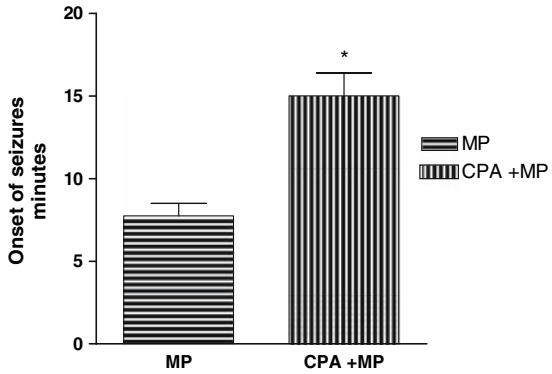

Fig. 6 Effect of CPA (2 mg/kg) administration 30 min previous to MP (45 mg/kg) during 4 days, CPA delays seizure appearance. Statistically difference $P < 0.001$

The cerebellar cortical structure consists in three layers: the molecular, the Purkinje cell and the granular cell layers. We observed that mGluR2/3 is preferentially expressed in the granular layer. Our results are in agreement with those reported by other authors [9, 28] that described mGluR2/3 expression in the cell body of the granular layer, dendrites and axon terminals of Golgi cells. Daily and repetitive MP-induced seizures increased mGluR2/3 expression, in granular layer. In addition CPA treatment alone and CPA injected before MP tends to enhance the MP alone treatment effect. In this regard activation of the mGluR2/3 receptors provides a negative feedback mechanism to prevent excessive presynaptic glutamate release in brain regions that have been implicated in pathologic disorders and it was reported that mGluR2/3 agonists prevent or inhibit different induced seizure models through activation of its presynaptic group II mGluRs receptors. In addition DBA/2 mice were protected against audiogenic seizures with (S)-4-carboxy-3-hydroxyphenyl glycine, an antagonist of mGluR1a that also acts as an agonist of mGluR2 [29].

In the model of seizures induced in immature rats by bilateral intracerebroventricular infusion of DL-homocysteic acid, the group II mGluR agonist (2R, 4R)-4-aminopyrrolidine-2,4-dicarboxylate (2R,4R-APDC)

has an anticonvulsant and potent neuroprotective effect against seizures [30]. The authors concluded that seizures induced in their model indicate a complex role of mGluR in epilepsy suggesting that the response will depend not only of the levels of mGluR subtype modified but also of the concentration of agonists and/or antagonists employed and perhaps the seizure model used [31]. According with this Tang et al. [32] were unable to find a protective effect using different intravenous injection doses of 2R,4R-APDC in a model of pilocarpine-induced status epilepticus in adult rats. On the other hand the selective group II mGluR agonist (+)-2-aminobicyclo-[3.1.0]hexane-2,6-dicarboxylic acid (LY 354740) administered prior to the injection of pentetrazole or picrotoxin produced a dose-dependent decrease in the number of mice exhibiting clonic convulsions [33]. Moreover it is interesting to mention that the group II receptor agonist failed to suppress the hypersusceptibility to pentylenetetrazol- induced seizure in diazepam-withdrawn mice, but significantly increased the threshold for pentylenetetrazol-induced seizure in control mice [34].

As far as the agonists for group III mGluRs are concerned, the application of classical agonists L-AP4 (L-2-amino-4-phosphonobutyric acid) or L-SOP (L-serine-*O*-phosphate) attenuated the seizures induced by 3,5-DHPG ((R,S)-3,5-dihydroxyphenylglycine) [35].

Adenosine, a protective neuromodulator, is involved along with the mGluRII receptors in presynaptic inhibition. The increase in mGluR2/3 immunostained cells after CPA administration could be associated to this inhibitory effect. Our experiments show an increase in mGluR2/3 immunostaining that was enhanced when CPA has been administered previously to MP. Although this difference was not statistically significant, it could be indicating a probable protective effect.

In agreement, Glitsch et al. [36] suggested that depolarization -induced Ca^{2+} entry leads to the release of glutamate to the extracellular space. This release results sequentially in an activation of presynaptic mGluR2/3 receptors, (located in interneurons), inhibition of adenylate cyclase, decrease of the presynaptic cAMP concentration and decrease of GABA release. It is noteworthy that MP administration reduces GABA level and glutamate decarboxilase cerebellar activity [37]. Then the GABA levels or similar induced responses could be the link between CPA and MP effect on both mGluR

The effect of repetitive CPA and MP administration on the mGluR2/3 and mGluR4a immunoreativity showed an analogous behavior that our previous observations on [^{3}H]-CCPA and [^{3}H]-MK 801 binding to different brain areas, after a single injection of these compounds [15, 22]. Similar observations were reported respect to oxygen consumption which is increased after MP administration, or after the administration of phenobarbital alone, the later having anticonvulsant properties, that avoids MP seizures [38].

About the mechanism involved on the CPA administration on mGluR 2/3 and 4a on MP-induced seizures in cerebellum, we might suggest that the effect of CPA administration on mGluR 2/3 could be associated to a protective mechanism. CPA effect administered alone or before MP, on Purkinje cells containing mGluR4a might induce either structural changes on these cells or an internalization of membrane receptors, although an apoptotic effect could be also considered.

In agreement with this it was reported that chronic treatment with CPA results in a pronounced increase in seizure intensity and seizure-associated mortality, which is an observation opposite to the protective effect observed in acute treatment [39]. Boeck et al. [40] have described that A1 receptors antagonists protect cells, but CPA alone had no effect on glutamate-induced damage, glutamate neurotoxicity, and cellular death in cerebellar granulare cells.

Although adenosine is considered to act as a neuroprotector having anticonvulsant properties, it was reported that CPA administration activate adenosine A1R and induces white matter and neuronal loss in 3-to 14 postnatal day rats [41]. Moreover the activation of adenosine receptor A1R with CPA enhances neurotoxicity caused by kainate in cortical cultures neurons [42]. Besides adenosine did not promote Purkinje cell survival in rat cerebellar culture [43] and repeated treatment with the analogue 2-chloro-N6-cyclopentyladenosine, a selective adenosine A1 receptor agonist, decreases the anticonvulsant properties of 3-(2 carboxypiperazine-4y) propenyl-1-phosphonic acid (CPPene) against audiogenic seizures [44].

In our experiments we have observed that CPA administration during 4 days induce mGluR2/3 and 4a changes in a similar way to MP administration. We have previously described an increased expression of the mdr-gene product, the P-gp protein after 4 days of MP treatment [45]. Increased P-gp indicates an augmented extrusion of unspecific drug administration and a possible effect on refractory epilepsy. This might explain in part the synergistic effect of both compounds. The effect of CPA after repetitive injection suggests that the modification of mGluR may be due to a long term exposure of these animals to the adenosine analogue, showing sedation and depressive symptoms. Although we have not studied the mdr-gene expression

in this particular case, changes on its expression could not be disregarded.

It is important to note that the cerebellum presents a unique organization with regards the distribution of afferent information. It receives impulses via mossy and climbing fibers from several brain regions such as the spinal cord, the vestibular nucleus and the cerebral cortex. Purkinje axons are the only output from the cerebellar cortex. Certain neurochemical characteristic of the cerebellum may contribute to the sensitivity and vulnerability of this tissue to different compounds [17]. For example in a pentylenetetrazole seizure-induced model [46], cerebellum showed to be the most susceptible: a decrease in the amount of glutamate, glutamine, GABA, aspartate and taurine levels in cerebellum was described. However none of these amino acids change in cortex and subcortical regions [46]. Cerebellar GABAergic neurons showed a pronounced decrease in GABA synthesis as well as a decrease in glucose consumption [47]. Pratt et al. [48] reported that a single electroconvulsive shock seizure caused an increase in mRNA levels for some of the GABAA receptors subunits in the cerebellum without effect in cerebral cortex or hippocampus. In addition Purkinje cell reduced capability to recapture glutamate and to generate energy during anoxia makes them susceptible to ischemic death [49].

All treatments decreased mGluR4a immunoreactivity. MP administration can result either in a toxic effect on Purkinje cells or in a severe alteration in certain groups of cells that leads to a decrease in cerebellar immunostaining considering that it was reported that an unique dose of MP induce Purkinje morphological alteration, condensation, darkening and retraction of some of the neuronal perikaryon [37]. About this possible effect Snead et al. [50] reported a resistance to low dose pentylenetetrazol-induced absence seizures in mGluR4 knock out mice. This effect was mimicked by administration of the mGlur4 antagonist alpha-cyclopropyl-4-phosphono phenyl glycine (CPPG) in wild type mice [50].

We observed an intense immunostaining in the neuropil of the molecular layer that is in agreemente with Kinoshita et al. [51]. In addition we found mGluR4a expression around Purkinje cells. It was reported that the clustering of the group III metabotropic glutamate receptor in the cerebellar molecular layer, along the presynaptic membrane of parallel fiber synaptic terminals, might help to a rapid and effective activation of the mGluR4a by glutamate [52]. It is interesting to note that a decreased expression of the mGluR4 gene has been associated with neuronal apoptosis meanwhile an elevated mGluR4a expression or the activation of this receptor promotes survival [53]. Considering our experimental results an apoptotic event could not be disregarded.

It is worthwhile to mention that the existence of neuron interaction between mGluRs and adenosine receptors seems to involve protein kinase C that plays a wide spread role in the regulation of the three mGluRs' groups inhibiting neurotransmitter release at glutamate synapses [54, 55]. Recently it was reported the mGluR4 is internalized and desensitized upon protein kinase C activation [56]. A similar situation could not be rule out in our model. According with the different expression of mGluR in MP-induce seizure it was reported that group II and III are segregated at the presynaptic terminal and there are distinct differences between the properties of the presynaptic action mediated by these two groups of receptors [57].

The present study shows that repetitive administration of MP and CPA induces an increased cerebellar mGluR2/3 and a decreased mGluR4a immunostaining, suggesting a distinct participation of both receptors that may be related to the differential cell distribution. A protective action and /or an apoptotic effect may not be discarded. CPA repetitive administration although increase seizure latency, cannot prevent seizure activity.

Acknowledgment This work was supported by grants of UBACYT M033 and M020 from Universidad de Buenos Aires, and CONICET (PIP N° 02267, 5798) from the Consejo Nacional de Investigaciones Científicas y Técnicas. We are grateful to Mrs Susana Buglione for her collaboration in the bibliographic management of the manuscript.

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