Neurotox Res (2009) 15:127–132
DOI 10.1007/s12640-009-9013-5

Chronic Dietary Administration of Valproic Acid Protects Neurons of the Rat Nucleus Basalis Magnocellularis from Ibotenic Acid Neurotoxicity

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Received: 4 July 2008 / Revised: 18 November 2008 / Accepted: 1 December 2008 / Published online: 4 March 2009

Abstract Valproic acid (VPA) has been used for many years as a drug of choice for epilepsy and mood disorders. Recently, evidence has been proposed for a wide spectrum of actions of this drug, including antitumoral and neuroprotective properties. Valproic acid-mediated neuroprotection in vivo has been so far demonstrated in a limited number of experimental models. In this study, we have tested the neuroprotective potential of chronic (4 + 1 weeks) dietary administration of VPA on degeneration of cholinergic and GABAergic neurons of the rat nucleus basalis magnocellularis (NBM), injected with the excitotoxin, ibotenic acid (IBO), an animal models that is relevant for Alzheimer's disease-like neurodegeneration. We show that VPA treatment significantly protects both cholinergic and GABAergic neurons present in the injected area from the excitotoxic insult. A significant level of neuroprotection, in particular, is exerted towards the cholinergic neurons of the NBM projecting to the cortex, as demonstrated by the substantially higher levels of cholinergic markers maintained in the target cortical area of VPA-treated rats after IBO injection in the NBM. We further show that chronic VPA administration results in increased acetylation of histone H3 in brain, consistent with the histone deacetylase inhibitory action of VPA and putatively linked to a neuroprotective action of the drug mediated at the epigenetic level.

Keywords Valproic acid · Neurotoxicity · Neuroprotection · Basal forebrain cholinergic neurons · Cholinergic cortical innervations · GABAergic neurons

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Introduction

Valproic acid (VPA—2-propylpentanoic acid), a medium branched chain fatty acid, has been used for decades as an antiepileptic and mood stabilizing drug (Johannessen and Johannessen 2003). Recent evidence for its action on cell growth, differentiation, and apoptosis, through the control of various cellular mechanisms, has promoted research on its role as an antitumoral and neuroprotective agent (reviewed in Monti et al. 2009). In in vitro, VPA protects neurons against several types of neurotoxic insults (Chuang 2005; Monti et al. 2009). In animal models, VPA was found to be neuroprotective towards ischemic brain damage, malonate-induced neurotoxicity in the striatum, a model for Huntington's disease (Morland et al. 2004; Kim et al. 2007) and rotenone-induced degeneration of dopaminergic nigro-striatal neurons (Monti et al., personal observations). Based on these evidences, VPA is going to be tested in clinical trials for Alzheimer's disease, Parkinson's disease, Huntington's disease, and spino-muscular atrophy (reviewed in Chuang 2005; Monti et al. 2009). It becomes, therefore, very important to further expand the knowledge on the potential neuroprotective role of VPA to other well established models of neurodegeneration that may be relevant for better understanding and treating human diseases. One such model is represented by lesions of the basal forebrain cholinergic neurons (basal nucleus of Meynert of humans), which provide most of the cholinergic innervation to the cortex and whose degeneration results in the loss of cortical cholinergic activity during the progression of Alzheimer's disease (Winkler et al. 1998). Among the various ways of lesioning these neurons, constituting the so called nucleus basalis magnocellularis (NBM) in rodents, are stereotaxic injections of the excitotoxin, ibotenic acid (IBO), which result in large and

highly reproducible depletion of cortical cholinergic activity (Winkler et al. 1998; Contestabile et al. 2004). As the corticopetal cholinergic projection from the NBM is strictly ipsilateral, this model offers the advantage of allowing the possibility to use the contralateral hemisphere as control. While the lesion is not specific for the corticopetal cholinergic neurons, as amygdalopetal cholinergic projection is also affected, and local GABAergic neurons are killed as well (Winkler et al. 1998), the model appears to be useful to test VPA potential neuroprotection in conditions of a rather large and widespread brain insult. We report here that long-term (4 + 1 weeks) dietary administration of VPA strongly counteracts the extent of the neurotoxic insult caused by IBO injection in the NBM of rats.

Materials and Methods

Male Wistar rats from Harlan Italy were used in this study. Starting from 2 months of age, when the animals weighed from 220 to 240 g, part of them were switched for 4 weeks to a diet containing 2% VPA, obtained by mixing VPA to the powdery chow and reconstituting chow pellets, while the others remained on standard diet. Based on mean daily food consumption (71 g/Kg body weight), VPA intake was in the range of 1.4 g/Kg body weight/day. In rats of the same strain, this dietary regimen has been shown to result in blood levels of VPA of 42 ± 6 mg/l which is close to the human therapeutic window (Hao et al. 2004). After 4 weeks, animals were anesthetized with ether, fixed to a David Kopf stereotaxic apparatus and injected slowly with 6 μg/0.75 μl IBO (Sigma, St. Louis, MO), dissolved in phosphate-buffered saline, into the left NBM at the following coordinates: anterior/posterior, 1 mm behind bregma; medial/lateral 2.7 mm from the midline; dorsal/ventral, 7.8 mm below dura. Sham operated rats, either fed with the normal diet or VPA supplemented, were injected with 0.75 μl PBS. After surgery, animals remained on their previous diet. No animal died as a consequence of the lesion. Seven days after surgery, rats were killed by decapitation, the brains were immediately removed from the skull and sliced with a Sorvall tissue chopper. Samples of the left or right frontoparietal cortex (three consecutive slices of approximately 0.5 mm thickness each) and of the left or right NBM area were separately microdissected under the stereomicroscope, immediately frozen in dry ice and kept at −80°C until assayed. For neurochemical analysis, tissue was homogenized in 50 mM Tris buffer at pH 7.4 and added with Triton X-100 to a 0.5% final concentration. The activity of choline acetyltransferase (ChAT) a specific marker for cholinergic neurons, glutamate decarboxylase (GAD) a specific marker for GABAergic neurons, acetylcholinesterase (AChE) a mixed marker for cholinergic/cholinoceptive neurons and protein content were measured in the homogenates according to standard radiochemical (Fonnum 1975; Fonnum et al. 1977) or colorimetric (Lowry et al. 1951; Ellman et al. 1961) methods. Samples from the left or right sides of each brain were always assayed separately as the projection from NBM to the cortex is strictly ipsilateral in the rat.

For immunohistochemistry, animals were deeply anesthetized with ether and perfused through the heart with saline followed by 4% paraformaldehyde in 100 mM phosphate buffer at pH 7.4. Brains dissected from the skull were further fixed by immersion in the same fixative at 4°C overnight, washed in buffer, and immersed in 18% sucrose in phosphate buffer overnight. Brains were transversely sectioned at 40 μm thickness with a freezing microtome and alternate sections including the NBM were incubated overnight at 4°C with an anti-ChAT antibody (goat polyclonal 1:200, Chemicon), followed by 1.5 h incubation with an HRP-conjugated secondary anti-goat antibody (1:400, Invitrogen). Detection was performed with DAB kit (Vector laboratories Burlingame, CA).

To extract nuclear proteins (Caruccio and Banerjee 1999), samples from cortex and NBM were homogenized in low salt extraction buffer (20 mM HEPES, pH 7.9, 10 mM NaCl, 3 mM MgCl2, 0.1% Nonidet P-40, 10% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol [DTT], protease inhibitor cocktail, all from Sigma) and left on ice for 10–15 min with occasional tapping. Nuclei were pelleted by centrifugation at 700×*g* for 5 min at 4°C and washed with 200 μl of a washing buffer (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, protease inhibitor cocktails) to remove NP-40 and pelleted again. Pellets were then resuspended into 60 μl of an extraction buffer with salt (20 mM HEPES, pH 7.9, 400 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, protease inhibitor cocktail) on ice for 45 min, mixing by tapping at intervals to extract nuclear proteins. Following centrifugation at 14,500×*g* for 15 min at 4°C, the supernatants were removed, aliquoted, frozen in dry ice, and stored at −80°C. After determination of protein content (Lowry et al. 1951), equal amounts of protein (20 μg) from each sample were resolved on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis before electroblotting. Membranes were incubated with antibodies against acetylated (lys 9/14) histone H3 or histone H3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:1,000 in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% defatted dry milk (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were then incubated with horse radish peroxidase-linked secondary antibodies (anti-goat 1:4,000 or anti-rabbit 1:2,000, Santa Cruz Biotechnology) and visualized by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK). The films were scanned and

densitometry was performed using the software 'NIH Image' (Scion Image, Frederick, MD, USA).

Data from neurochemical assays were subjected to statistical analysis through ANOVA followed by post hoc analysis through Bonferroni's test to evaluate specific differences among groups, while data from western blot analysis were analyzed with the Student's *t*-test. Experiments were approved by a local bioethical committee and conformed to Italian and EU regulations for the experimental use of laboratory animals. Animal health and surgical procedures were supervised by veterinary control.

Results

Valproate treatment did not overtly affect rat behavior. While food intake was only slightly reduced in VPA-fed rats (−7% compared to controls), the body weight gain during the 4 weeks of treatment was approximately 50% less than normally-fed animals. Main convulsive symptom observed after IBO injection consisted in animals turning around themselves on the bottom of the cage for 2–3 times at intervals of few minutes during 6–10 h after surgery. This was followed by a couple of days of apathy and reduced motor behavior. No apparent differences in these behaviors were observed among VPA-fed and control rats. Unilateral IBO injections in the NBM resulted, as expected, in large depletion (−65%) of ChAT activity in the ipsilateral cortex while leaving unaffected the levels measured in the contralateral one (Fig. 1a). Chronic VPA treatment significantly reduced the depletion of the cholinergic marker in the cortex ipsilateral to toxin injection in the NBM (−30% with respect to the contralateral cortex of the same animals, Fig. 1a). Essentially, similar results were obtained by assaying the same cortical homogenates activity of the cholinergic/cholinoceptive marker, AChE (Fig. 1b).

As most of the cholinergic innervation to the cortex comes from the cholinergic neurons of NBM (Winkler et al. 1998), these results suggested that these neurons were significantly protected from IBO-dependent degeneration by the chronic treatment with VPA. To obtain quantitative evidence for that, we assayed ChAT activity in homogenates from samples obtained by microdissection of the NBM area in animals of the various experimental groups. The results showed that the only significant decrease of ChAT activity was measured in the injected NBM of normally-fed animals and that VPA treatment significantly counteracted this decrease (Fig. 2a). To better characterize this datum with respect to the cholinergic neuronal population of the NBM, we performed immunocytochemical staining of ChAT-containing neurons in brain sections including this area in both the injected and the contralateral

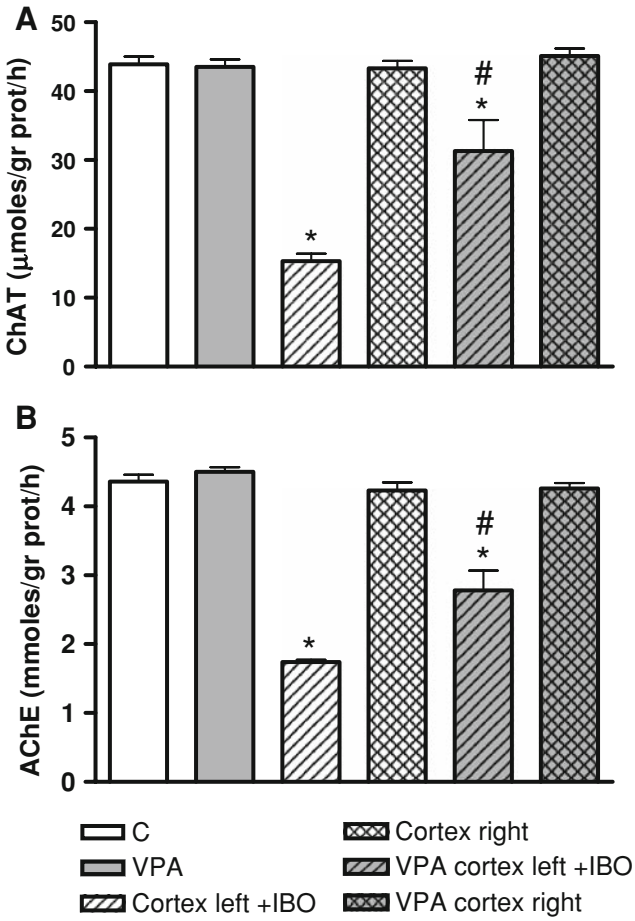

Fig. 1 **a** ChAT activity in the fronto-parietal cortex of sham operated rats fed with normal diet (Control) or VPA (VPA) and rats injected with IBO into the left NBM either fed with normal diet (cortex left + IBO and cortex right) or VPA (VPA cortex left + IBO and VPA cortex right). **b** Measurement of AChE in the same samples. Data are expressed as the mean ± S.E. of 6–8 experiments. $^{*}P < 0.001$ vs. Control; $^{\#}P < 0.001$ vs. cortex left + IBO

sides. As shown in Fig. 2, ChAT-immunoreactive neurons present in control conditions (Fig. 2b) were dramatically decreased in their number in IBO-injected NBM of animals fed with the standard diet (Fig. 2c), while they were remarkably preserved in IBO-injected NBM of animals supplemented with VPA in their diet (Fig. 2d). A rough quantitative estimate of the effect of IBO injection, in the presence or absence of VPA treatment, on the number of cholinergic neurons of the NBM was made by counting the number of ChAT-positive neurons in the left and right side of eight corresponding sections from two animals injected with IBO and fed with the normal diet and two animals injected with IBO and treated with VPA added to the diet. This count indicated that approximately 60% of cholinergic neurons of NBM were disappeared after IBO injection and that this value was reduced to less than 30% of cholinergic neuron loss in VPA-treated rats.

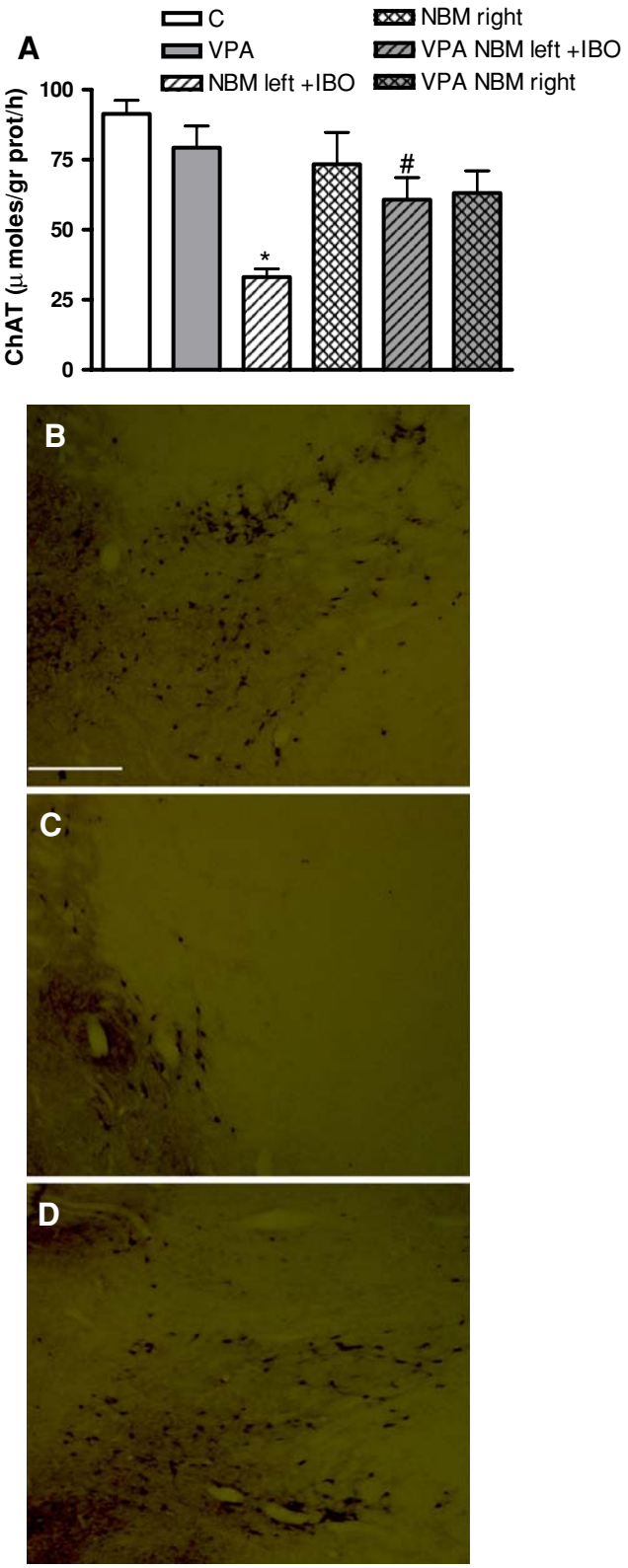

Fig. 2 **a** ChAT activity in the NBM of the various experimental groups. Same indications of Fig. 1. Data are expressed as the mean ± S.E of 6–8 experiments. $*P < 0.001$ vs. Control; $^{\#}p < 0.05$ vs. NBM left + IBO. **b**–**d**. Cholinergic neurons of the NBM revealed through ChAT immunohistochemistry in the side contralateral to IBO injection (**b**), and in the side ipsilateral to IBO injection of a rat fed with normal diet (**c**) or a rat fed with VPA. Calibration bar: 400 μm

To further confirm the neuroprotective effect of the long-term VPA treatment against IBO neurotoxicity, we assayed in NBM homogenates the activity of GAD, the marker enzyme for GABAergic neurons that are abundant in this brain region and are sensible to the neurotoxic action of IBO (Winkler et al. 1998). Indeed, IBO dramatically decreased GAD activity in the injected NBM in animals fed with the normal diet and VPA treatment significantly counteracted this decrease (Fig. 3).

Among the various molecular targets of VPA action on nerve cells, interest has been recently focused on the inhibition of histone deacetylases (Phiel et al. 2001; Monti et al. 2009). To gain novel insight into possible mechanistic links between VPA neuroprotection and altered state of acetylation of histones in the brain tissue we examined, by western blot through a specific antibody, the acetylation state of lysines 9/14 of histone H3, a specific substrate for histone deacetylase 1 (HDAC1) in nuclear extracts from the cortex and the NBM of control rats or of rats subjected to the chronic treatment with VPA. As shown in Fig. 4a, an increased level of acetylated histone H3 was found in samples from VPA-fed rats while the levels of total histone H3 were unchanged. Densitometric quantification demonstrated a statistically significant increase of the acetylation state of histone H3 in both brain regions (Fig. 4b).

Discussion

The present report demonstrates for the first time a remarkable neuroprotective effect of VPA, chronically administered with the diet, in an animal model of neurodegeneration that holds relevance for Alzheimer's-type neurodegeneration (Winkler et al. 1998). Considering together our neurochemical data obtained both at the injection site in the NBM and at the level of the cholinergic projection area in the cortex, it can be estimated that the massive degeneration of cholinergic neurons, and of GABAergic neurons as well, caused by IBO is rescued to a more than 50% extent by long-term VPA administration. Quantitatively, similar results have been reported by studies based on the effects of VPA administration on lesion/infarct size in rats subjected to malonate injection in the striatum, a model for Huntington's disease and in a model of middle cerebral artery occlusion (Morland et al. 2004; Kim et al. 2007). In addition, we have recently found evidence for VPA neuroprotection of nigro-striatal dopaminergic neurons in a rat model of Parkinson's disease (Monti et al., personal observations). Considered together, these results support the importance of research aimed at expanding our

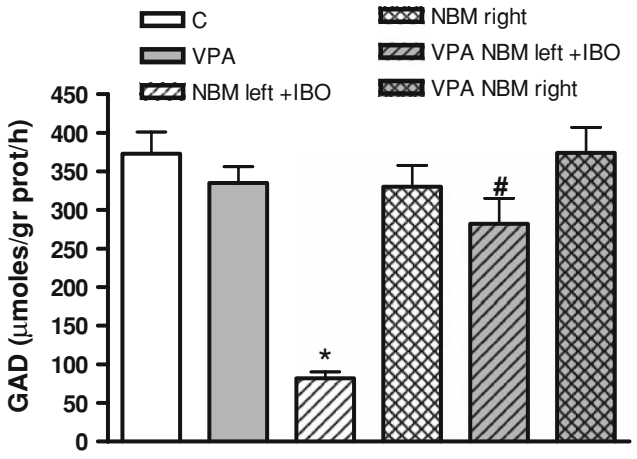

Fig. 3 GAD activity in the NBM of the various experimental groups. Same samples and indications of Fig. 2a. Data are expressed as the mean ± S.E of 6–8 experiments. $^{*}P < 0.001$ vs. Control; $^{\#}P < 0.01$ vs. NBM left + IBO

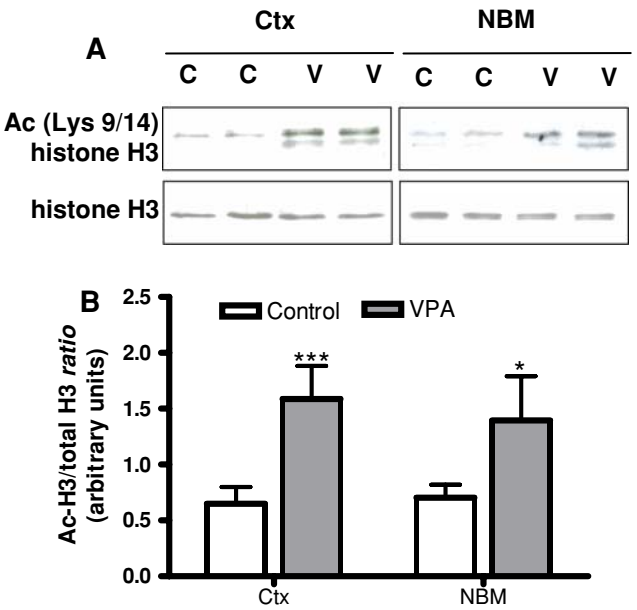

Fig. 4 Histone H3 acetylation in brain samples from control and VPA-treated rats. **a** Western blot analysis of histone H3 acetylation on lysine 9/14 and of histone H3 performed on nuclear extracts from cortex and NBM (C, control; V, VPA-fed). **b** Densitometry relative to the western blot analysis: data are expressed as acetylated H3/total H3 ratio in arbitrary units. Each data is the mean ± S.E. of 6 samples. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to its control

knowledge on the neuroprotective potential of VPA, in view of further extension of the studies to genetic models for human neurodegenerative diseases and possible novel trials on human patients. Previous trials conducted on Parkinson's and Huntington's disease patients were inconclusive (reviewed in Monti et al. 2009). Interestingly, a meta-analysis on clinical trials based on VPA administration to Alzheimer's disease patients to relieve psychopathological symptoms, has revealed the possible occurrence of neuroprotection, thus stimulating the proposal of performing specific trials to confirm this possible effect (Tariot et al. 2002). Future trials should take advantage of second generation VPA-based drugs with improved potency, better pharmacokinetics, and lower side effects, such as liver toxicity (Johannessen and Johannessen 2003).

An additional point of interest related to the action of VPA, is to ascertain whether neuroprotection against the insult may be accompanied by subsequent beneficial effects on the recovery from the lesion consequences by continuing the treatment for longer periods. Indeed, in the case of neuroprotection observed in the experiments on middle cerebral artery occlusion, neuroprotection was obtained by starting treatment shortly after the insult and by continuing it for some time (Kim et al. 2007). Preliminary data obtained by us in the course of the present study and based on a 6-week post-operative period during which the dietary administration of VPA was continued after the 4-pre-operative weeks, apparently failed to further improve the neuroprotective efficacy (data not shown).

Several actions of VPA, most notably against depressive disorders, are very similar to those exerted by lithium. However, significant differences exist in some of the cellular pathways on which the two drugs impinge (reviewed by Chuang 2005; Monti et al. 2009). It is not, therefore, surprising that lithium and VPA have different neuroprotective effects on the same or on very similar types of neurodegeneration. Regarding the experimental model used here, it is interesting to recall that chronic lithium treatment resulted ineffective for neuroprotection on the same model of IBO injection in NBM (Sparapani et al. 1997).

The multiplicity of cellular targets of VPA action, from channels and receptors to pathways involved in gene expression regulation (reviewed in Monti et al. 2009) makes the identification of neuroprotective mechanisms a particularly difficult task. Our present observation of increased histone H3 acetylation state in NBM and cortex as a consequence of chronic VPA treatment, point at VPA-mediated transcriptional upregulation, a main effect of increased histone acetylation (Kazantsev and Thompson 2008), as likely involved in the observed neuroprotective effect. This is consistent with previous reports that have linked neuroprotection and increased brain plasticity with upregulation of histone acetylation state and consequent promotion of gene transcription (Fischer et al. 2007; Kazantsev and Thompson 2008; Nott et al. 2008). It is unlikely that VPA may directly interfere with IBO excitotoxic action mediated by glutamate receptors. The transitory convulsive state induced by IBO infusion was not apparently different in VPA-fed and control animals.

Furthermore, IBO toxicity in primary neuronal cultures is not prevented by concomitant administration of VPA (personal unpublished results). Future development of present observations should be aimed at identifying putative genes whose transcription, epigenetically promoted by increased histone acetylation, may be involved in VPA-mediated neuroprotection.

Acknowledgments The present research was supported by grants from the Italian Ministry for Universities and Research and from the Institute for Advanced Studies of the University of Bologna. The skillful technical assistance of Miss Monia Bentivogli is gratefully acknowledged.

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