NMDA receptor-dependent CREB activation in survival of cerebellar granule cells during in vivo and in vitro development

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Abstract

During both in vivo and in vitro development, cerebellar granule cells depend on the activity of the NMDA glutamate receptor subtype for survival and full differentiation. With the present results, we demonstrate that CREB activation, downstream of the NMDA receptor, is a necessary step to ensure survival of these neurons. The levels of CREB expression and activity increase progressively during the second week of postnatal cerebellar development and the phosphorylated form of CREB is localized selectively to cerebellar granule cells during the critical developmental stages examined. Chronically blocking the NMDA receptor through systemic administration of the competitive antagonist, CGP 39551, during the in vivo critical developmental period, between 7–11 postnatal days, results in increased apoptotic elimination of differentiating granule neurons in the cerebellum [Monti & Contestabile, Eur. J. Neurosci., 12, 3117–3123 (2000)]. We report here that this event is accompanied by a significant decrease of CREB phosphorylation in the cerebellum of treated rat pups. When cerebellar granule neurons are explanted and maintained in dissociated cultures, the levels of CREB phosphorylation increase with differentiation, similar to that which happens during in vivo development. When granule cells are kept in non-trophic conditions, their viability is affected and both CREB phosphorylation and transcriptional activity are decreased significantly. The neuronal viability and the deficiency of CREB activity, are both rescued by the pharmacological activation of the NMDA receptor. These results provide good circumstantial evidence for a functional link between the NMDA receptor and CREB activity in promoting neuronal survival during development.

Introduction

The cAMP response element binding protein, CREB, belongs to a family of transcription factors possessing the cAMP response element (CRE) in their promoter (Shaywitz & Greenberg, 1999). Other transcription factors (Sheng et al., 1988; Sakamoto et al., 1994; Robertson et al., 1995; Yukawa et al., 1998), growth factors (Tao et al., 1998), enzymes (van Steeg et al., 1990; Sasaki et al., 2000) and antiapoptotic proteins (Wilson et al., 1996; Riccio et al., 1999) are among CREB-regulated genes. CREB is activated by stimuli, such as synaptic activity and neurotrophins (Ginty et al., 1994; Moore et al., 1996; Finkbeiner et al., 1997), that induce its phosphorylation at the ser 133 residue (Gonzalez & Montminy, 1989). CREB is phosphorylated by several kinases: protein kinase A (PKA), Ca2+-calmodulin-dependent kinases (CaMK), protein kinase B (Akt/ PKB) and mitogen-associated protein kinase (MAPK) (Gonzalez et al., 1989; Dash et al., 1991; Moore et al., 1996; Xing et al., 1996; Finkbeiner et al., 1997; Deisseroth et al., 1998; Du & Montminy, 1998) and its activity is involved in several brain functions, such as synaptic plasticity, learning and memory (Dash et al., 1990; Tully, 1997; Shieh & Ghosh, 1999; Taubenfeld et al., 1999). Recently, it has been proposed that CREB is also involved in neuronal survival and brain development (Riccio et al., 1999; Sala et al., 2000; Walton & Dragunow, 2000). The relationship between CREB phosphorylation and survival has been demonstrated in focal ischemia (Tanaka et al., 1999) and in PC12 cell degeneration (Beiter-Johnson & Millhorn, 1998; Walton et al., 1999). It has also been demonstrated recently that mice lacking CREB function undergo neurodegeneration both in the peripheral (Lonze et al., 2002) and in central nervous system (Mantamadiotis et al., 2002). Several factors promoting neuronal survival and/or differentiation can activate CREB: among them are oestrogens (Goodman et al., 1996; Green et al., 1997), pituitary adenylate cyclase activating peptide (PACAP) (Villalba et al., 1997), insulin-like growth factor-1 (IGF-1) (Kulik et al., 1997) and neurotrophins (Walton et al., 1999). Neurotransmitter-mediated neuronal survival may also involve CREB activation, as the antiapoptotic effect of NMDA receptor activity on differentiated cerebellar granule neurons is stimulated by brain-derived neurotrophic factor (BDNF), whose expression is under the control of CREB (Bhave et al., 1999). Cerebellar granule cells are a good model to study the functional links between synaptic activity mediated by glutamate receptors and survival, as the role of NMDA receptor for their survival has been firmly established (Contestabile, 2002). In vivo, these neurons depend on the trophic effect of glutamate acting at the NMDA receptor for their survival and in vitro, this requirement can be replaced by maintaining granule neurons at relatively depolarized membrane potentials (Balazs et al., 1988; Burgoyne et al., 1993; Contestabile, 2002). We have demonstrated previously that, during a specific developmental window [between postnatal day 7 (P7) and P11] the chronic blockade of the NMDA receptor through the competitive antagonist CGP 39551 increases the rate of apoptotic elimination of granule cells (Monti & Contestabile, 2000).
This model should, therefore, be exploited in order to obtain evidence for the involvement of CREB activity in developmental NMDA-mediated survival of cerebellar granule cells. We report here that the same pharmacological blockade of the NMDA receptor that increases developmental apoptosis of cerebellar granule cells (Monti & Contestabile, 2000), also results in decreased CREB activity. Moreover, to study the relationship among NMDA receptor activity, CREB expression, its phosphorylation state and CRE-driven gene expression, we have used primary cultures of cerebellar granule cells which allow one to replicate some of the developmental conditions in a controlled and easily modifiable system.

Materials and methods

In vivo experiments

Wistar rat pups were injected subcutaneously with CGP 39551 (kindly provided by Novartis International AG, Basel, Switzerland) dissolved in saline according to one of the following schedules of treatment (day of birth being considered as P0): Five mg/kg on P9 or 6 mg/kg on P11 or 7 mg/kg on P13 (acute treatments); 5 mg/kg per day from P7–P9 (chronic P7–P9 treatment); 5 mg/kg per day from P7–P9 and 6 mg/kg per day from P10–P11 (chronic P7–P11 treatment); 5 mg/kg per day from P7–P9, 6 mg/kg per day from P10–P11 and 7 mg/kg per day from P12–P13 (chronic P7–P13 treatment). The total daily administration was divided into two doses delivered at 08.00 h and 20.00 h. These schedules of treatment were derived from those adopted for previous studies (Virgili et al., 1998; Monti & Contestabile, 2000) and, while all animals survived, the doses were close to the maximum tolerated for these stages of development. Control pups received subcutaneous injections of equivalent volumes of the vehicle. All animals were used in the evening of the day of the last treatment, approximately 12 h after it, or after single P9 or P11 or P13 administration. Animals were killed by decapitation and the cerebellum was dissected immediately and frozen until used. The experiments were performed in accordance with the requirements of the Italian law on the use of animals for experimental purposes. The experimental protocols were approved by a local bioethical committee and the experiments were performed under the supervision of a veterinary commission for control of animal health and comfort.

Immunoblot detection

For immunoblot detection of P-Akt, Akt, P-CREB and CREB, samples were homogenized in 100 mM HEPES containing 200 mM NaCl, 10% glycerol, 2 mM NaF, 2 mM Na2PO4, 2 mM DTT, 1 mM EDTA, 1 mM benzamidine, 0.1 mM Na3VO4, 1 mM pestatine, 10 μg/mL trypsin inhibitor, 10 μg/mL aprotinin, 10 μM leupeptin and 10 μM phenylmethylsulfonyl fluoride (PMSF) at pH 7.4 (all chemicals were from Sigma Chemical, St. Louis, MO, USA). After lysis for 15 min in ice, samples were centrifuged at 20 500 g for 15 min. After determination of protein content using the Lowry method (Lowry et al., 1951), 30 μg of each sample were separated by electrophoresis on 10% acrylamide gel and blotted onto nitrocellulose membranes (Amersham International, Buckinghamshire, UK). After blocking the aspecific sites with PBS containing 0.1% Tween 20 (Sigma) and 15% defatted Milk (Bio-Rad Laboratories, Hercules, CA, USA) for 1 h at room temperature, the membranes were exposed to the primary antibody overnight at 4 °C. For Akt or CREB detection, polyclonal antibodies against Akt, independent from its phosphorylation state, or phosphorylated (ser 473) Akt only (New England Biolabs, Beverly, MA, USA), or total CREB or CREB phosphorylated on its ser 133 (Upstate Biotechnology, Lake Placid, NY, USA) or β-actin (Sigma) were used. The membrane was then incubated with an anti-rabbit, HRP-linked secondary antibody (Amersham) and visualized by ECL (Amersham). The films were scanned and densitometry was performed using the software ‘NIH Image’ (Version BETA 4.0.2).

Immunohistochemistry

For the immunohistochemical study, cerebella were dissected, fixed (by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 24 h) and immersed overnight in 15% sucrose in the same buffer. Parasagittal sections were sliced with a freezing microtome at 40 μm thickness and stained overnight at 4 °C with the polyclonal antibody against total CREB or against ser 133-phosphorylated CREB (Upstate) in PBS containing 0.1% Triton X-100 and 4% BSA. After incubation with a secondary biotinylated anti-rabbit antibody (Amersham), the reaction was visualized by the avidin–biotin–peroxidase reaction with diaminobenzidine (Vectastain ABC and DAB kit, Vector Laboratories, Burlingame, CA, USA).

In vitro experiments

Primary cultures of cerebellar granule cells were prepared from 7 day-old Wistar rat pups (Gallo et al., 1987). Briefly, cerebella were removed and dissected from their meninges in Krebs’ buffer (132 mM NaCl, 5 mM KCl, 8.5 mM Na2HPO4, 1 mM NaHPO4 and 10 mM Glucose) containing 0.3% BSA (Sigma). The cerebella were dissociated with trypsin (Sigma) at 37 °C for 15 min and triturated in a DNaase I (Sigma)/soybean trypsin inhibitor (Sigma) solution. Dissociated cells were collected by centrifugation, resuspended in Basal Medium Eagle (BME, Invitrogen, DH Breda, NL), supplemented with 2 mM glutamine, 10% heat-inactivated fetal calf serum (Invitrogen) and 25 mM KCl, and plated at 2.2 × 106/dish in 35 mm plastic dish precoated with poly-L-lysine (Sigma). After incubating for 16 h at 37 °C in an 95% air–5% CO2 atmosphere, 10 μM cytosine arabinono-furanoside (Sigma) was added to block the proliferation of non-neuronal cells.

Treatments were performed after 1 day in vitro (DIV). Cells were switched to fresh medium supplemented with both 10% fetal calf serum and 10 μM cytosine arabinono-furanoside, containing either 25 mM or 15 mM KCl. Control cultures were grown with 25 mM KCl (K25), while other cultures were exposed to 15 mM KCl, in the absence (K15) or presence (K15 + NMDA) of 140 μM NMDA: these KCl and NMDA concentrations were selected as they had been shown previously to be effective on cerebellar granule cell survival at equivalent stages of culture (Balazs et al., 1988). Moreover, some of the K15 + NMDA cultures were treated with a competitive (CGP 39551, Novartis; 50 μM) or a noncompetitive (MK801, RBI/Sigma; 5 μM) NMDA antagonist or with 10 μM Forskolin (Calbiochem Corporation, La Jolla, CA, USA).

Cell survival assay

Cell survival was assayed after 5 DIV with the MTT assay (Lobner, 2000). Briefly, cells were incubated with MTT [3-(4, 5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (Sigma)], for 15 min at 37 °C in an 95% air–5% CO2 atmosphere and subsequently lysed in a buffer containing 50 mM Tris and 5% Triton X-100. Reading of the coloured formazan product was performed at 570 nm in a spectrophotometer and neuronal death was evaluated by the decrease in MTT reduction by mitochondrial dehydrogenases.
CREB/p-CREB visualization

To visualize CREB expression and phosphorylation, in preliminary experiments cells were lysed in 100 mM HEPES, 200 mM NaCl, 10% glycerol, 2 mM NaF, 2 mM Na₃P₂O₇, 2 mM DTT, 1 mM EDTA, 1 mM benzamidine, 0.1 mM Na₃VO₄, 1 µM pepstatine, 10 µg/mL trypsin inhibitor, 10 µg/mL aprotinin, 10 µg/mL leupeptin and 10 µM PMSF at pH 7.4 (all chemicals were from Sigma). After lysis for 15 min in ice, cell suspension was centrifuged at 20,500 g for 15 min and protein content was determined (Lowry et al., 1951). Then, considering that there were no significant differences in total protein content depending on treatments, cultures plated at the same initial cell density were resuspended directly in loading sample buffer and frozen immediately at −80 °C. To confirm that the loading was equal, the same membrane was first incubated with the antibody against the phosphorylated protein, then stripped and exposed to the antibody against the total protein. Western blot analysis and quantification of total and phosphorylated CREB of these samples were then performed as above (see, *In vivo* experiments).

Transcriptional CREB activity

In order to assay transcriptional CREB activity, 1 DIV cerebellar granule cell cultures were transfected in 24-well plates for 60 min with 1 µg/mL plasmid containing the luciferase reporter gene (Pardy, 1994) under the control of a promoter with eight CREB Response Elements (CRE), by using PEI 25K as the DNA carrier as described previously (Boussif et al., 1995). Plates were spun for 5 min at 200 g, then cells were shifted to fresh complete medium, at different KCl concentrations, in the presence or absence of NMDA, MK801, CGP 39551 and forskolin (K25, K15, K15 + 140 µM NMDA, K15 + 140 µM NMDA + 5 µM MK801, K15 + 140 µM NMDA + 50 µM CGP 39551 and K15 + 10 µM Forskolin). After 5 DIV, luciferase activity of cultures grown under the different conditions was assayed by using luciferin (Roche L.T.D., Basel,

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**Fig. 1.** Western Blot analysis of CREB expression and phosphorylation (A) and densitometry (B) of CREB, P-CREB, Akt and P-Akt in control, chronically and acutely treated rats with CGP 39551 (A) C, control; CGP, chronically treated from P7–P9 or from P7–P11; CGPsi, acute treatment with single injection at P11. (B) Each bar represents the mean ± SEM of 4–6 animals. *P < 0.05 compared to control after Student’s t-test.
Switzerland) as the substrate in the cell lysates. The luciferase activity measured with a TD-20/20 luminometer was expressed as arbitrary units/h per mg of total protein in the samples. Parallel cultures were transfected with a plasmid containing the luciferase reporter gene under the control of the CMV promoter and other parallel cultures were tested with the MTT assay to control the effectiveness of the treatments in each experiment.

Results

In order to study the effect of blockade of the NMDA receptor (during the developmental phase of NMDA-dependency for cerebellar cell survival) on the phosphorylation and the expression of CREB the competitive antagonist, CGP 39551 was administered either acutely or chronically to rat pups: it had been shown previously that drug administration from P7–P11 resulted in excess apoptotic elimination of developing granule cells (Monti & Contestabile, 2000). As shown by Fig. 1A, both total CREB and phosphorylated CREB levels increased with age from P7–P11. This was not true for other phosphorylated proteins functionally linked to CREB; for instance, Akt/PKB total level increased with age but this was not paralleled by increased levels of its phosphorylated form (Fig. 1B). CREB phosphorylation decreased significantly after chronic administration of CGP 39551 from P7–P11, but not from P7–P9 or after acute administration at P7, P9 or P11 (Fig. 1B).

Immunohistochemical analysis of cerebella from control rats at P11 (Fig. 2A and B) showed that phosphorylated CREB was localized to granule neurons of the internal granular layer as well as to some scattered neurons of the molecular layer, but not to Purkinje cells. Immunohistochemistry of cerebellar sections from rats treated with CGP 39551 from P7–P11 showed a similar topo-

![Image](https://example.com/image.png)

**Fig. 2.** Immunohistochemistry of phosphorylated CREB localization in control pups at P11 (A and B) and in pups treated with CGP 39551 from P7–P11 (C and D). Each section comes from a different animal. Activity is mainly localized to the internal granular layer and to cells scattered in the molecular layer. The reaction intensity appears somewhat decreased in treated animals at both localizations. M, molecular layer; Gr, granular layer. Calibration bar in A, 20 μm (A–D).
phosphorylation in cerebellar granule cell cultures at 2, 4, 7, 9 and 11 DIV. 25 mM) to less trophic (KCl 15 mM) potassium levels at 1 DIV, in the ment, neuronal cultures were shifted from highly trophic (KCl con®rm that CREB phosphorylation is regulated by NMDA receptor by reproducing, in the environment, developmental conditions that temporally and experimentally overlapped those that had been studied in vivo, i.e., preparing cerebellar granule cell suspensions from 7-day-old-rat pups and culturing them for various periods in standard or modi®ed media. As shown by Fig. 3, the level of phosphorylation of CREB and of the protein kinase, Akt, have been studied at different DIV and, while phosphorylated CREB increased with culture maturation, phosphorylated Akt did not change appreciably, thus replicating the results obtained in vivo. In order to con®rm that CREB phosphorylation is regulated by NMDA receptor activity during the critical phase of cerebellar granule cell development, neuronal cultures were shifted from highly trophic (KCl 25 mM) to less trophic (KCl 15 mM) potassium levels at 1 DIV, in the absence or presence of NMDA at a concentration (140 µM) that had been shown previously to sustain granule cell survival at K15 (Balazs et al., 1988). In parallel experiments, either CGP 39551 or MK801 were added to cells cultured with K15 in the presence of NMDA, to keep the receptor blocked pharmacologically. As shown by Fig. 4A, and consistent with that reported by the literature (Balazs et al., 1988), the shift at K15 decreased viable granule cells to about 60% of the K25 condition at the 5th DIV, while the presence of NMDA rescued neuronal survival to a normal level. The rescuing effect of NMDA was abolished when the NMDA receptor block was maintained by the antagonists, CGP 39551 or MK801 (Fig. 4A). Forskolin, an activator of CREB phosphorylation through the induction of cAMP synthesis, when added to the K15 medium at 10 µM, also appeared effective as survival was not signi®cantly affected (Fig. 4A). Neither CGP 39551 or MK801 signi®cantly affected survival of granule neurons kept in trophic (K25) conditions (Fig. 4A). In parallel cultures, the levels of CREB phosphorylation and total CREB were analysed by Western blotting. Phosphorylated CREB was almost completely undetectable in cultures grown at K15, compared to the control ones (Fig. 4B). Normal levels of CREB phosphorylation were, instead, maintained in K15 cultures with added NMDA (Fig. 4B). A similar effect on CREB phosphorylation was obtained by including forskolin in the K15 medium (Fig. 4B). We also checked the effect of low potassium medium on Akt expression and phosphorylation, only noticing a moderate decrease of Akt phosphorylation (data not shown).

To assess whether CREB phosphorylation correlated, under the same culture conditions, to its transcriptional activity, cultures of cerebellar granule cells were transfected with either a plasmid containing the luciferase reporter gene under the control of a promoter with eight CRE replicas or, as a control, with a plasmid containing the same reporter gene under the control of the viral promoter, CMV. By transfecting cerebellar granule cells after 1 DIV, and maintaining them up to the 5th DIV in the various conditions, measurement of luciferase activity constituted a quantitative marker for CREB transcriptional activity. The luciferase activity driven by the promoter containing the CRE replicas, significantly decreased in cerebellar granule cells grown at K15, compared to the K25 condition, but the concomitant presence of 140 µM NMDA restored transcriptional levels to that of the controls (Fig. 5A). The concomitant presence of NMDA receptor antagonists, however, completely blocked the rescuing effect of NMDA on CRE driven luciferase activity (Fig. 5A). Cultures transfected with the plasmid containing the luciferase reporter gene under the control of the CMV promoter did not show any signi®cant difference in their transcriptional activity under the various growth conditions (Fig. 5B), thus excluding aspecifc effects due to a generalized alteration of transcription efficiency.

Discussion

It is well known that, during development, cerebellar granule cells are produced in a larger number than that needed; therefore apoptotic elimination of part of these cells takes place naturally (Wood et al., 1993; Contestabile, 2002). It has been proposed that granule cell survival depends on NMDA receptor activation sustained by the glutamate input that they receive from the mossy ®bers (Burgaione et al., 1993; Contestabile, 2002). In vivo pharmacological blockade of this receptor, at the developmental stages in which granule neuron survival depends on the NMDA trophic effect, induces an increase in their spontaneous rate of apoptotic elimination (Monti & Contestabile, 2000). To investigate whether CREB activation accompanies this developmental phase, critical for NMDA-receptor mediated survival of cerebellar granule cells, we studied the expression and the phosphorylation of CREB in the cerebellum of control rat pups and of pups treated either chronically or acutely with the NMDA receptor competitive antagonist, CGP 39551. Then, we used primary cultures of cerebellar granule cells to replicate, in the controlled in vitro environment, conditions similar to those created experimentally in vivo.

The main results of our study are: (i) phosphorylated CREB is selectively localized to granule cells at the targeted developmental stage of the cerebellum; (ii) both in vivo and in vitro, the levels of total and phosphorylated CREB increase with the maturation of these neurons; (iii) in vivo, a schedule of blockade of the NMDA receptor that has been previously shown to increase apoptosis, signi®cantly decreases the level of phosphorylated CREB; (iv) in vitro, growing cerebellar granule cells from 1–5 DIV in non trophic (K15)

**Fig. 3.** Western blot analysis of CREB and Akt expression and phosphorylation in cerebellar granule cell cultures at 2, 4, 7, 9 and 11 DIV.
conditions causes a decrease in CREB phosphorylation and transcriptional activity, which is paralleled by decreased survival. Both these effects are rescued by NMDA exposure and this trophic action is reversed by the simultaneous application of NMDA receptor antagonists. These results provide good circumstantial evidence for the involvement of CREB activity in the NMDA-mediated survival of cerebellar granule cell development, both in vivo and in vitro. Theoretically, blocking the NMDA receptor in culture could have resulted in decreased viability of granule neurons even when kept in trophic, high potassium medium, thus miming what happens in vivo. This was not, however, the case, thus confirming the original hypothesis that the depolarization state promoted by high potassium overcomes the unfavourable condition caused by insufficient NMDA receptor activation in culture (Balazs et al., 1988; Burgoyne et al., 1993).

That CREB expression and function are regulated during brain development has been observed in organotypic cultures of developing striatum (Liu & Graybiel, 1996), as well as in the hippocampal dentate gyrus, where phosphorylated CREB is expressed by granule cells at early stages of their differentiation (Bender et al., 2001). More recently, it has been shown that, in the thalamic circuit, both CREB protein and phosphorylation are highly regulated during development and that CRE-mediated gene expression is enhanced transiently in the dorsal thalamus during the developmental phase — critical for the refinement of thalamic connections — (Pham et al., 2001). We report here that in the cerebellum, the levels of total and phosphorylated CREB increase selectively during the most critical phase of survival and subsequent differentiation of granule neurons. Furthermore, our immunohistochemical analysis shows that phosphorylated CREB, at least in these developmental phase, is restricted to cerebellar granule cells, while it is completely absent from Purkinje cells, as has been observed previously in adult rats (Yang et al., 1998). This could be related to the fact that Purkinje cells express functional NMDA receptors only during the first postnatal

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**Fig. 4.** MTT assay analysis of neuronal survival (A) and Western blot analysis (B) of CREB phosphorylation and expression in cerebellar granule cells kept in different growth conditions from 1–5 DIV. Each bar corresponds to the mean ± SEM of at least six experiments run in quadruplicate from different culture preparations. ***P < 0.001 compared to K25; *P < 0.05 compared to K15; **P < 0.05 and ***P < 0.01 compared to K15 + 140 μM NMDA, after Student’s t-test.
week (Akazawa et al., 1994) and we have, indeed, observed that early postnatal Purkinje cells exhibit a detectable level of CREB phosphorylation (B. Monti and A. Contestabile, personal observations).

By studying CREB expression and phosphorylation after blockade of NMDA receptor activity in the critical phase for cerebellar granule cell survival, we show here that the chronic treatment with CGP 39551 from P7–P11 results in a statistically significant decrease in CREB phosphorylation, but not in CREB expression. Noticeably, this decrease in CREB phosphorylation exactly matches the period during which the same treatment increases the apoptotic elimination of cerebellar granule cells (Monti & Contestabile, 2000). The link between decreased CREB phosphorylation and increased apoptosis is further strengthened by the fact that both events fail to occur as a consequence of single acute treatments or of treatments extending to later periods of development (Monti & Contestabile, 2000; also present results).

A very similar picture, concerning the relationship among NMDA receptor, CREB and survival can be derived from our in vitro results. In fact, we showed that in cerebellar granule cells kept in non-trophic conditions (K15) from 1–5 DIV, a period that corresponds to the time window between P7–P11 in vivo, both phosphorylated CREB and CRE-mediated gene expression were significantly decreased compared to control cultures. The link between NMDA receptor and CREB activity was confirmed by the prevention of this decrease, obtained with exposure to the agonist, and by the abolition of this rescuing effect, by coapplying receptor antagonists. It has been shown previously that CREB, activated through the Ca²⁺/calmodulin protein kinases, plays a role in differentiated cerebellar granule cells survival (Bonni et al., 1999; See & Loeffler, 2001; See et al., 2001), but this is the first report of CREB involvement in survival of these neurons while undifferentiated.

CREB activity has been demonstrated to be regulated by glutamate, especially through the NMDA receptor activation, in several neuronal types. Calcium entry through either calcium channels or NMDA receptor channels induces the phosphorylation of CREB at Ser 133 and the transcription of CREB-regulated genes (Ghosh et al., 1994). This has been shown in several neural systems, from the suprachiasmatic nucleus upon light stimulation (Ghosh et al., 1994; Ding et al., 1997), to the demonstration that NMDA receptor regulates, through CREB modulation, dopamine-induced gene expression in striatal neurons (Konradi et al., 1996; Das et al., 1997; Macias et al., 2001). In hippocampal neurons, CREB phosphorylation is thought to play a key role in synaptic plasticity and long-term memory. In fact, it has been observed that CREB is phosphorylated in response to high frequency LTP stimulation in vivo and that this is completely prevented by pretreatment with NMDA receptor antagonists (Schulz et al., 1999). Moreover, NMDA receptors seem to be a starting point for the activation of CREB during learning in the hippocampus (Cammarota et al., 2000). Calcium influx through postsynaptic NMDA receptors also regulates neuronal gene expression via the transcription factor CREB, during the development of hippocampal neurons in culture, suggesting a parallel with neuronal plasticity occurring during brain development (Sala et al., 2000).

As well as CREB, other members of the CREB transcription factor family seem to be regulated through NMDA receptors. In fact, it has been demonstrated that in vivo, acute administration of the noncompetitive NMDA receptor antagonist, MK-801, increases the mRNA and protein expression of the cAMP response element modulator (CREM) and of the inducible cAMP early repressor (ICER), that negatively regulate CREB activity (Shaywitz & Greenberg, 1999), especially in hippocampus and cortex (Storvik et al., 2000). Moreover, in hippocampal neurons it has been shown that the calcium influx through both NMDA receptors and voltage-sensitive calcium channels induces the activation of the transcriptional coactivator CREB-binding protein (CBP), which positively influences the coupling of activated CREB to the basal transcriptional machinery, and CBP-mediated transactivation of CREB (Hardingham et al., 1999; Hu et al., 1999).

Under the experimental conditions of the present study, Akt activity did not appear as crucial as CREB activity in promoting NMDA mediated survival of cerebellar granule neurons in vivo as well as in vitro. An important role for Akt, together with CREB activation, has been demonstrated previously as a consequence of NMDA receptor stimulation in striatal neurons (Perkinton et al., 2002) or as a consequence of nitric oxide activity in cerebellar granule neurons (Ciani et al., 2002a, b). Concerning NMDA receptor mediated survival of these latter neurons, it appears from the present results that the role of CREB is more relevant than the one played by Akt.

The present results provide evidence for a functional link between NMDA receptor and CREB activation in promoting neuronal survival during development. Such a functional relationship is suggested by the comparative examination of the literature. By studying the CRE/CREB pathway in thalamic circuit development, it was suggested for the first time that CREB could be involved in coordinating the remodeling of developing circuits, when apoptotic elimination of neurons occurs in the forebrain after the establishment of the first connections (Pham et al., 2001). Considering that during development only neurons that have established appropriate connections survive, CREB is a good candidate for this action on neuronal
survival (Riccio et al., 1999; Walton & Dragunow, 2000). In addition, it has been demonstrated that CREB is involved in neuronal differentiation by regulating neurite extension (Shмуeli et al., 2001). Moreover, in cerebellar granule cells, CREB phosphorylation seems to be the critical event that terminates proliferation and allows the differentiation program to proceed (Pons et al., 2001). All of these developmental effects of CREB activation are exactly matched by similar effects attributed to NMDA receptor activity during brain development (Contestabile, 2000). This concordance clearly puts CREB activation as one of the main cellular signalling pathways activated during brain development not only in response to classical neurotrophic agents (Ginty et al., 1994; Finkbeiner et al., 1997; Kulik et al., 1997; Villalba et al., 1997; Riccio et al., 1999; Walton et al., 1999; Walton & Dragunow, 2000), but also in response to neurotransmitter-related, and in particular NMDA-related, neurotrophic signals (Balazs et al., 1988; Lipton & Kater, 1989; Burgoyne et al., 1993; Leviit et al., 1996; Cameron et al., 1998; Komuro & Rakic, 1998; Contestabile, 2000).

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