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Creb is modulated in the mouse superior colliculus in developmental and experimentally-induced models of plasticity

Gabriela Vierci[a], Camila Salum De Oliveira[b,1], Lucia Ruiz Perera[a], Natalia Bornia[a], Rodrigo Bainy Leal[b], Francesco Mattia Rossi[a,c,*]

[a] Laboratorio de Neurociencias "Neuroplasticity Unit", Facultad de Ciencias, UdelaR, Iguá 4225, esq. Mataojo, 11400 Montevideo, Uruguay
[b] Laboratório de Neuroquímica-3, Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil
[c] Neurodegeneration Laboratory, Institut Pasteur de Montevideo, Mataojo 2020, 11400 Montevideo, Uruguay

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ABSTRACT

In the central nervous system long-term plastic processes need the activation of specific gene expression programs and the synthesis of new protein in order to occur. A transcription factor fundamental for several plasticity mechanisms in various CNS areas is the cAMP response element-binding protein, CREB. This factor is activated through phosphorylation at its Serine 133 residue by multiple signaling pathways. Little is known about CREB role in the superior colliculus, a midbrain area considered an experimentally useful model for the study of neuronal plasticity processes. In the present work we studied by Western blot analysis the modulation of CREB expression and activation in the mouse superior colliculus in three models of neuronal plasticity: (1) developmental plasticity; (2) lesion-induced plasticity; (3) and fluoxetine-induced restored plasticity. We used an antibody that detects endogenous level of the total CREB protein (anti-TCREB) to identify possible modulations at CREB expression level, and a second antibody (anti-PCREB) that detects endogenous level of CREB only when it is phosphorylated at Ser133, to identify modifications of CREB activation state. The results showed that: (1) the expression and activation of CREB increase during the development of the superior colliculus in temporal correlation with the plastic process of refinement of retino-collicular projections; (2) the activation of CREB is induced by a monocular lesion performed during the critical period for plasticity in young animals but not when performed in less plastic juvenile mice; (3) the expression and activation of CREB increase in adult animals treated with fluoxetine, known to restore high levels of plasticity in adult animals. These results suggest that CREB transcription factor plays a fundamental role in plasticity processes also at the level of the mouse superior colliculus.

1. Introduction

The development and maturation of retino-fugal projections is a widely used model for the study of plasticity processes in the CNS. In the visual system retinal ganglion cells (RGCs) send projections mainly to the thalamic dorso-lateral geniculate nucleus (dLGN) and the midbrain superior colliculus (SC). The majority of RGC axons cross at the level of the optic chiasm so that each eye provides input mainly to the targets on the contralateral side of the brain. In these nuclei, retinal afferents from the two eyes are initially intermixed within each other. Later on, a process of activity-dependent segregation and refinement of connections will give rise to the extremely precise and adult-like pattern of afferent distribution. In rodents, this process occurs during the first two weeks of postnatal life, the so called critical period of plasticity. The emergence of eye-specific territories and retinotopic maps in the dLGN and SC and the corresponding critical periods are the subjects of intense studies on physiological plastic processes during CNS development

Abbreviations: CREB, cAMP response element-binding protein; RGCs, retinal ganglion cells; dLGN, dorso-lateral geniculate nucleus; SC, superior colliculus; CRE, cAMP response element; P, postnatal day; EDTA, ethylenediaminetetraacetic acid; NaF, sodium fluoride; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, tris buffer saline solution; HRP, horse radish peroxidase; OD, optical density; INTOD, integrated optical density; ERK1/2, extracellular signal-regulated kinase 1/2.

* Corresponding author at: Laboratorio de Neurociencias "Neuroplasticity Unit", Facultad de Ciencias, UdelaR, Iguá 4225 Esq. Mataojo, 11400 Montevideo, Uruguay. Tel.: +598 2525 8618x7138; fax: +598 2525 8617.
E-mail addresses: gaby_vierci@hotmail.com (G. Vierci), CAMILA.DEOLIVEIRA@NEWCASTLE.EDU.AU (C.S.D. Oliveira), lularuiz@gmail.com (L.R. Perera), natibornia@gmail.com (N. Bornia), rbleal@gmail.com (R.B. Leal), fmrossi@pasteur.edu.uy (F.M. Rossi).
[1] Present address: Cancer Research Unit, School of Biomedical Sciences and Pharmacy, University of Newcastle, Callaghan, New South Wales 2308, Australia.

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(Land and Lund, 1979; Fawcett et al., 1984; Godement et al., 1984; Shatz and Stryker, 1988; Sretavan et al., 1988; Hahm et al., 1991; Katz and Shatz, 1996). The possibility of remodeling the distribution pattern of retino-fugal terminals in the targets is scarce, even though present, in adult animals, as the system is already mature and characterized by a low degree of plasticity. As an example, a strong reorganization of the ipsilateral projections to the SC can be induced by lesioning the visual input in young still plastic animals (during the critical period), but little if the lesion is induced in adult animals once the critical period is closed (Chalupa and Henderson, 1980; Godement et al., 1980). Recently, a few experimental strategies for potentiating the residual plasticity in adult animals have been identified in the visual system (Baroncelli et al., 2010; Sale et al., 2009; Castrén and Rantamäki, 2010). In the SC it has been shown that treatment with the serotonin reuptake transporter inhibitor fluoxetine, and also treatment with the Brain-Derived Neurotrophic Factor and the protease Chondroitinase ABC, amplify the lesion-induced plastic reorganization of ispilateral projections (Bastos et al., 1999; Tropea et al., 2003). The cellular and molecular mechanisms regulating the physiological and experimentally-induced plasticity of retino-collicular projections are not well characterized. A central role may be played by the transcription factor cAMP response element-binding protein (CREB) which has a well-documented role in neuronal plasticity and long-term memory formation in the brain (Carlezon et al., 2005; Benito and Barco, 2010; Sakamoto et al., 2011). CREB is a member of a large family (CREB/ATF) of structurally related transcription factors that regulate transcription *via* their specific DNA target, the cAMP response element, CRE, with the obligatory co-activation by the CREB-binding protein, CBP (Brindle and Montminy, 1992; Sassone-Corsi, 1995). CREB transcriptional activity is induced by multiple signaling cascades, in turn activated by different stimuli that phosphorylate its Ser133 residue. CREB activation may be induced also by phosphorylation at other residues, however these mechanisms are poorly characterized (Parker et al., 1998; Wu and McMurray, 2001; Gau et al., 2002; Johannessen et al., 2004; Tardito et al., 2006; McClung and Nestler, 2008). In the present work we studied the expression and activation level (phosphorylation at Ser133) of the CREB transcription factor in three different models of plasticity of the mouse SC by Western blot analysis with specific antibodies.

2. Experimental procedures

2.1. Experimental animals and treatments

For the present study C57B6/J mice were obtained from the animal house of the Transgenic and Experimental Animals Unit of the Institute Pasteur of Montevideo, Uruguay. Experiments were performed following approval of the Institutional Ethics Committee ("Comisión Honoraria de Experimentación Animal" of Uruguay). A total of 90 animals were used for the complete study. Mice were group-housed under standard conditions with food and water *ad libitum* in Plexiglas cages (42 cm × 26 cm × 18 cm) and kept in a 12:12 light/dark cycle. For the developmental study, animals at postnatal day (P)0, 4, 9, 15, 30 and 45 were used. For the lesion study, young (P2) and juvenile (P21) mice were anesthetized by hypothermia or with ketamina/xylazine (5/1 mg/100 g of body weight, i.p). The right eye was treated with a local anesthetic (lidocaine in gel), proptosed by gentle lid retraction and enucleated with scissors by cutting the optic nerve. The eyelids were sutured and the animals re-warmed and returned to the animal facility. Mice were sacrificed one week after the enucleation (young mice at P9, and juvenile ones at P28), and left and right SC were separated following dissection. For the fluoxetine study, adult mice (P70) were treated with fluoxetine hydrochloride at approx. 15–20 mg/kg mouse weight/day delivered in the drinking water during four weeks (P70 to P98). This dose of fluoxetine was chosen because similar doses have been found to be efficacious on cellular, molecular or functional readouts in previous studies (Dulawa et al., 2004; Karpova et al., 2009; Rantamäki et al., 2007; Maya-Vetencourt et al., 2008; Chen et al., 2011). Adult mice (of approx. the same body weight, ~28 g) were housed in their home cages at 3 animals per cage. Fluoxetine was administered as 0.1 mg/ml solution in the drinking water in light protected bottles during four weeks and changed each 3–4 days with fresh one. Control mice were treated in the same way but with no addition of fluoxetine in the drinking water. The average fluid volume consumed per animal per day was recorded before starting and throughout the experiment to verify that each animal consumed approximately the same amount of water. No differences were detected in the total fluid intake among the treatment groups. At the end of the treatment the recorded volume intake and body weight values were used to calculate the effective drug intake and to verify that this corresponded to the selected one (~15–20 mg/kg mouse weight/day). Animals were killed by cervical dislocation at the same time of the day (between 8:00 and 10:00 a.m.) to avoid misinterpretations of results due to possible CREB circadian fluctuations (Obrietan et al., 1999; Katoh-Semba et al., 2008). Brain areas of interest (the whole superior colliculi) were rapidly dissected under a stereoscopic microscope on ice in saline solution (NaCl 0.9%) and samples stored at −70 °C for later analysis.

2.2. Western blotting

Proteins were extracted from brain areas according to Rossi et al. (2002). Briefly, samples were mechanically homogenized in 100–150 μl of lysis buffer (Tris 50 mM, EDTA 1 mM, NaF 100 mM, PMSF 0.1 mM, Na_3VO_4 2 mM, 1% Triton X-100, 10% Glycerol) and then incubated for 30 min on ice. Lysates were centrifuged (13,000 *g* for 1 h, at 4 °C) to eliminate cellular debris. Finally the supernatant was recovered and a small aliquot was removed for estimation of protein concentration. The remaining was added with 4× loading buffer (2% SDS, Tris 0.375 M, 10% Glycerol, 5% beta-mercaptoethanol, 0.2% Bromophenol Blue) for subsequent separation of proteins by electrophoresis. Protein concentration was estimated by the microtiter plate Bradford method in a 96-well microplate at λ = 595 nm. Concentration of samples was estimated through extrapolation from a standard curve using bovine serum albumin. To compare signals obtained in different samples, the same amount of protein per sample (70 μg) was electrophoresed in a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) at 160 V for one hour. Following electrophoresis, the proteins in the gel were transferred onto a nitrocellulose membrane (GE Healthcare) at 100 V for two hours. To verify transfer efficiency process, gels were stained with Coomassie blue (0.1% Coomassie blue R-250, 50% ethanol, 7% acetic acid) and membranes with 0.5% Ponceau in 1% acetic acid. In each experiment two gels were loaded with the same samples: one gel was treated for analysis with a rabbit monoclonal antibody that detects endogenous level of the total CREB protein (anti-TCREB, Cell Signaling) and the second with a rabbit monoclonal antibody which detects endogenous levels of CREB only when phosphorylated at Ser133 (anti-PCREB, Cell Signaling). In detail, blots were incubated in a blocking solution (5% non-fat dry milk in Tris Buffer Saline solution, TBS) for 1 h at RT and then probed o/n at 4 °C with anti-TCREB (1:1000) or anti-PCREB (1:750) primary antibodies in TBS containing 0.05% Tween 20 (TBST). Following several washes in TBST, blots were incubated with horse radish peroxidase (HRP)-linked anti-rabbit (Cell Signaling, 1:2000) secondary antibody for 1 h at RT. Immunoreactive bands were visualized using enhanced chemioluminescence system (Amersham). The antibody against TCREB detected a single band of approximately 43 kDa. The antibody against PCREB detected a band of approximately 43 kDa corresponding to the CREB protein phosphorylated at Ser133. In some experiment this antibody detected also a second band of approximately 30 kDa corresponding to the phosphorylated form of the CREB-related protein, ATF-1 (activating transcription factor 1), as specified in the Cell Signaling data sheet (#9198). The specificity of the signals was confirmed by using other monoclonal and polyclonal antibodies anti-TCREB (Cell Signaling #m4820) and anti P-CREB (Cell Signaling #p9191, Upstate NY-Millipore #p06-519) which gave similar results (not shown).

2.3. Densitometry analysis, quantification and presentation of data

To assess semi-quantitatively the intensity of the different signals obtained in the Western blot analysis, several sheets of X-ray film were exposed to each blot for varying lengths of time (10–30 min). The bands of the developed films were quantified using the NIH Image J 1.46r free analysis system. A window size was chosen to include one band for each measurement. Only the band at 43 kDa (corresponding to CREB protein) was quantified. For each band, an index of the precipitated silver in the emulsion of the film was calculated by multiplying the mean optical density (OD) by the total area of the band (area), resulting in the integrated optical density (INTOD). Quantification of β-actin signal (obtained using a rabbit anti- β-actin antibody, Cell Signaling, 1:1000) in each membrane was exclusively used as an additional control that equal loading of protein was used in the gel (not shown). For each study 4–16 tissue samples were used. To control inter-experimental variability each Western blot experiment was repeated 2–3 times. Data in the graphs are presented as mean ± S.E.M.

2.4. Statistical analysis

To identify statistical significance in the modulation of the Western blot results obtained in the three studies, all data were analyzed by using one-way analysis of variance (ANOVA) followed by Levene's *post hoc* test, with $p < 0.05$ as threshold for significant difference. Statistical analysis of data was carried out using Past 2.14 free analysis system (Hammer et al., 2001).

3. Results

CREB is a transcription factor which is activated by phosphorylation at its Ser133 residue by various signaling pathways. In order to