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The activation of ERK1/2 and p38 mitogen-activated protein kinases is dynamically regulated in the developing rat visual system

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Abstract

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that play an instrumental role in signal transduction from the cell surface to the nucleus. These enzymes are major intracellular mediators of developmental events and recently have been shown to control also synaptic plasticity processes [Sweatt, J.D., 2004. Mitogen-activated protein kinases in synaptic plasticity and memory. Curr. Opin. Neurobiol. 14, 311–317; Thomas, G.M., Huganir, R.L., 2004. MAPK cascade signalling and synaptic plasticity. Nat. Rev. Neurosci. 5, 173–183]. Mammalian members of this family are extracellular signal-regulated kinases 1/2 (ERK 1/2), c-Jun amino-terminal kinases or stress-activated protein kinases (JNK/SAPKs) and p38 kinases ($p38^{MAPK}$). At the level of the visual system, it has been demonstrated that the ERK pathway regulates developmental plastic processes at both retino-thalamic and thalamo-cortical level and that $p38^{MAPK}$ controls a peculiar form of long-term depression in the visual cortex [Di Cristo, G., Berardi, N., Cancedda, L., Pizzorusso, T., Putignano, E., Ratto, G.M., Maffei, L., 2001. Requirement of ERK activation for visual cortical plasticity. Science 292, 2337–2340; Naska, S., Cenni, M.C., Menna, E., Maffei, L., 2004. ERK signaling is required for eye-specific retino-geniculate segregation. Development 131, 3559–3570; Xiong, W., Kojic, L.Z., Zhang, L., Prasad, S.S., Douglas, R., Wang, Y., Cynader, M.S., 2006. Anisomycin activates p38 MAP kinase to induce LTD in mouse primary visual cortex. Brain Res. 1085, 68–76]. Here, as a first approach to gain more insight on the role of two MAPKs – ERK1/2 and $p38^{MAPK}$ – in visual system maturation, we characterized by western blot the regulation of their phosphorylation/activation in rat retina, superior colliculus and visual cortex, during postnatal development from birth to adult age. Our main results show that: (i) in the retina $p38^{MAPK}$ activation peaks at P4, and then, from P15 to P45, both ERK1/2 and $p38^{MAPK}$ phosphorylation increases; (ii) in the superior colliculus phosphorylation of both MAPKs increases between P4 and P15; (iii) in the visual cortex ERK1/2 phosphorylation increases from P15 to P45, while phosphorylation of $p38^{MAPK}$ increases starting from P4. The present data demonstrate a distinct regulation of the activation of ERK1/2 and $p38^{MAPK}$ in the three visual areas analyzed which occurs in temporal correlation with critical events for visual system maturation. These results suggest an important role for ERK1/2 and $p38^{MAPK}$ in the postnatal development of the rat visual system.

Keywords: Development; Retina; Superior colliculus; Visual cortex; Phosphorylation

The mammalian visual system is characterized by a high level of spatial specificity in that axons emerging from neighboring retinal ganglion cells specifically innervate neighboring neurons in the dorso-lateral geniculate nucleus (dLGN) of the thalamus and in the superior colliculus. Retinal projections from the two eyes are initially mixed in these targets during early phases of development, but then, through a process of selection and retraction, they will form eye-specific territories. In turn, projections from the dLGN to the primary visual cortex will segregate into ocular dominance

Abbreviations: dLGN, dorso-lateral geniculate nucleus; ERK1/2, extracellular signal-regulated kinases 1/2; INTOD, integrated optical density; JNK/SAPK, c-Jun amino-terminal kinases or stress-activated protein kinases; MAPKs, mitogen-activated protein kinases; p38 kinase, $p38^{MAPK}$; P, postnatal day.

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columns. This remarkable specificity of connections is, in part, obtained through an initial overproduction of neurons and synapses that are then selectively eliminated during critical periods of development (Cowan et al., 1984; Oppenheim, 1991).

It is well known that electrical activity and neurotrophic factors of the Nerve Growth Factor family play a fundamental role in visual system development (Katz and Shatz, 1996; McAllister et al., 1999; Berardi et al., 2003). However, how neurons sense these converging inputs and react to modify their function and structure is still relatively unknown. In recent years many studies have tried to identify the intracellular signaling cascades mediating these processes. Among others, the pathway of mitogen-activated protein kinases (MAPKs) resulted to be a predominant player in many physiological aspects. Mammalian MAPKs include extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino-terminal kinases or stress-activated protein kinases (JNK/SAPKs) and p38 kinases ($p38^{MAPK}$). These enzymes are serine/threonine kinases that become functional upon phosphorylation at both a threonine and a tyrosine residue by an upstream MAPK (English et al., 1999; Chang and Karin, 2001; Chen et al., 2001). MAPKs have been initially identified as enzymes controlling both proliferation and differentiation. These enzymes also control cell survival, with, by and large, ERK1/2 activation playing a protective role, while activation of JNK/SAPK and $p38^{MAPK}$ is linked to induction of apoptosis (Xia et al., 1995; Takeda and Ichijo, 2002). More recently, it has been shown that ERK1/2 and $p38^{MAPK}$ play a fundamental role also in synaptic plasticity processes in the hippocampus and in the visual system (Sweatt, 2004; Thomas and Huganir, 2004). Here, the ERK pathway is required for structural and functional plasticity during the development of the visual retino-thalamo-cortical pathway and $p38^{MAPK}$ activation has been linked to long-term depression at cortical level (Di Cristo et al., 2001; Naska et al., 2004; Xiong et al., 2006). Notwithstanding this information, very little is known on the role played by ERK1/2 in other visual structures and almost no information is available on $p38^{MAPK}$. In the present study, we contribute to deciphering the role of ERK1/2 and $p38^{MAPK}$ in rat visual system development by analyzing the regulation of their phosphorylation/activation. Our results demonstrate that the activation of ERK1/2 and $p38^{MAPK}$ is dynamically regulated during the development of the visual system. Moreover, the temporal correlation of MAPKs activation with critical periods for programmed cell death and axonal remodeling suggests an important role for these enzymes in specific events of visual system development.

1. Experimental procedures

1.1. Animals

For the present study $n = 54$ Long–Evans hooded rats from the animal house of the Federal University of Santa Catarina, Brazil, of different ages (postnatal day P0, 4, 9, 15, 30, 45) were used. Experiments were performed following approval of the Institutional Ethics Committee.

1.2. Western blot

Animals were killed at the same time of the day (between 8:00 and 10:00 a.m.) by decapitation and brains rapidly removed in saline solution (NaCl 0.9%) on ice. Brain areas of interest (retina, superior colliculus and visual cortex) were rapidly dissected under a stereoscopic microscope and proteins extracted according to Rossi et al. (2002). Briefly, samples were mechanically homogenized in 100–150 μl of Tris–base 50 mM pH 7.0, EDTA 1 mM, NaF 100 mM, PMSF 0.1 mM, Na_3VO_4 2 mM, Triton X-100 1%, glycerol 10%, and then incubated for 30 min in ice. Lysates were centrifuged ($10000 \times g$ for 1 h, at 4 °C) to eliminate cellular debris, and supernatants diluted 1/1 (v/v) in Tris–base 100 mM pH 6.8, EDTA 4 mM, SDS 8%, glycerol 16%. Protein content was estimated with the method described in Peterson (1977) at 750 nm wavelength and concentration calculated by a pattern curve with bovine serum albumin. To compare signals obtained at different postnatal ages, the same amount of protein (70 μg per lane) for each sample was electrophoresed in 10% SDS–PAGE minigels (after addition of bromophenol blue 0.2% and β-mercaptoethanol 8%) and transferred to nitro-cellulose membranes (Amersham). To verify transfer efficiency process, gels were stained with Coomassie blue (Coomassie blue R-250 0.1%, methanol 50%, acetic acid 7%) and membranes with Ponceau 0.5% in acetic acid 1%.

The same membrane was processed in three sequential steps, accordingly to Cordova et al. (2004): (i) incubation with antibody against the phosphorylated form of the enzyme (phospho-$p38^{MAPK}$ or phospho-ERK1/2); (ii) antibody against the total form of the enzyme ($p38^{MAPK}$ or ERK1/2); (iii) antibody against a housekeeping protein (β-actin). In between each step membranes were stripped to eliminate residues of the previous process and then re-probed.

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-phospho-ERK1/2 (Sigma, 1:10000) or anti-phospho-$p38^{MAPK}$ (Cell Signaling or Calbiochem 1:1000) primary antibodies in TBS containing 0.05% Tween 20 (TBST). Following several washes in TBST, blots were incubated with horse radish peroxidase (HRP)-conjugated anti-mouse or -rabbit (Amersham 1:4000, for detection of anti-phospho-ERK1/2 or anti-phospho-$p38^{MAPK}$, respectively) secondary antibodies for 1 h at RT. Immunoreactive bands were visualized using enhanced chemioluminescence system (Amersham). Blots were then stripped with NaOH 0.2 M for 5 min, abundantly washed in TBST and re-probed with anti-total-ERK1/2 (Sigma, 1:40000) or anti-total-$p38^{MAPK}$ (Sigma, 1:10000) antibody. Following several washes, blots were incubated with HRP-conjugated anti-rabbit (Amersham, 1:4000) secondary antibodies for 1 h at RT and bands visualized as described above. Finally, following a second stripping step, membranes were incubated with rabbit anti-β-actin antibody (Cell Signaling, 1:1000) to verify that equal amounts of proteins were loaded on the gel, and the signal revealed as above.

The antibody against $p38^{MAPK}$ detected a single band of approximately 38 kDa, while the antibody against ERK1/2 detected two bands, one at approximately 44 kDa and the second at approximately 42 kDa, corresponding respectively to the two ERK isoforms, ERK1 and ERK2. The anti-β-actin antibody detected a single band of approximately 45 kDa (data not shown).

1.3. Densitometric analysis, quantification and presentation of data

To assess semi-quantitatively the different signals obtained in Western blot analysis, several sheets of X-ray film were exposed to each blot for varying lengths of time (1 h for detection of phospho-$p38^{MAPK}$ protein and 5–15 min for detection of the others). The bands of the developed films were quantified using the Scion Image® analysis system. A window size was chosen to include one band for each measurement. 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 (giving the integrated optical density, INTOD).

MAPKs are enzymes that become activated exclusively following double phosphorylation (English et al., 1999; Chang and Karin, 2001; Chen et al., 2001). Thus, the comparison between the amount of the enzyme in the phosphorylated state and the total amount of the enzyme is a representative index of MAPK activation. The ratio between the INTOD value obtained with the antibody against the phosphorylated form of each enzyme and the INTOD value obtained with the antibody against the corresponding total form was