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Mitochondrial extracellular signal-regulated kinases 1/2 (ERK1/2) are modulated during brain development

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Abstract

Intracellular activation and trafficking of extracellular signal-regulated protein kinases (ERK) play a significant role in cell cycle progression, contributing to developmental brain activities. Additionally, mitochondria participate in cell signalling through energy-linked functions, redox metabolism and activation of pro- or anti-apoptotic proteins. The purpose of the present study was to analyze the presence of ERK1/2 in mitochondria during rat brain development. Immunoblotting, immune electron microscopy and activity assays demonstrated that ERK1/2 are present in fully active brain mitochondria at the outer membrane/intermembrane space fraction. Besides, it was observed that ERK1/2 translocation to brain mitochondria follows a developmental pattern which is maximal between E19-P2 stages and afterwards declines at P3, just before maximal translocation to nucleus, and up to adulthood. Most of mitochondrial ERK1/2 were active; upstream phospho-MAPK/ERK kinases (MEK1/2) were also detected in the brain organelles. Mitochondrial phospho-ERK1/2 increased at 1 μM hydrogen peroxide (H_2O_2) concentration, but it decreased at higher 50–100 μM H_2O_2, almost disappearing after the organelles were maximally stimulated to produce H_2O_2 with antimycin. Our results suggest that developmental mitochondrial activation of ERK1/2 cascade contributes to its nuclear translocation effects, providing information about mitochondrial energetic and redox status to the proliferating/differentiating nuclear pathways.

Keywords: development, extracellular signal-regulated kinases 1/2, hydrogen peroxide, MAPK/ ERK kinases 1/2, mitochondria, rat brain.
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The extracellular signal-regulated kinases (ERK) are evolutionary conserved serine/threonine protein kinases implicated in signal transduction from cell-surface to regulatory targets in cell-nucleus (Chang and Karin 2001). Originally discovered as critical regulators of cell division and differentiation, the ERK1/2 (p44/p42) mitogen-activated protein kinases (MAPK) subgroup of enzymes is specifically and differentially activated in response to growth factors and other intercellular messengers (Derkinderen *et al*. 1999; Sweatt 2001). The classic ERK1/2 cascade involves a first kinase so-called MAPK3 (Raf) which activates downstream MAPK/ERK kinases 1/2 (MEK1/2) by serine/threonine phosphorylation; MEK1/2 in turn, activate ERK1/2 by dual phosphorylation of a threonine and a tyrosine residue (Derkinderen *et al*. 1999).

ERK1/2 have been involved in a wide variety of functions in the mammalian brain. In neuronal cells, activation of ERK1/2 is an important mediator of neurotrophin signalling, resulting in brain growth and differentiation. Accordingly, during brain development, ERK1/2 mRNA and protein levels tend to increase from the embrionary stage up to adulthood, suggesting a role in the establishment of the primary neuronal network (Boulton *et al*. 1991). In addition, recent

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Abbreviations used: ERK, extracellular signal-regulated kinases; MAPK, mitogen-activated protein kinases; MEK, MAPK/ERK kinases; H_2O_2, hydrogen peroxide; PBS, phosphate-buffered saline; BMP, basic myelin protein; SDS–PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; mGluR1, metabotropic glutamate receptor type 1; OM+IMS, outer membrane and intermembrane space.

studies demonstrate that ERK1/2 cascade subserves the cognition process and contributes to hippocampal synaptic plasticity, learning and memory formation (Cammarota *et al*. 2000; Sweatt 2001; Alonso *et al*. 2002; Selcher *et al*. 2003).

From early embryogenesis to young adulthood, brain mitochondria evolve with growing and differentiation (Riobó *et al*. 2002). It has been extensively shown that mitochondrial integrity and function directly participate in the signalling pathway that culminates in apoptosis. In the CNS, due to its high capacity to sequester calcium, mitochondria have also been involved in important synaptic plasticity events as post-tetanic potentiation (Tang and Zucker 1997) and recovery from synaptic depression after periods of moderate activity (Billups and Forsythe 2002); moreover, new data, suggest a role for the mitochondrial permeability transition pore and voltage-dependent anion channels in mitochondrial synaptic calcium buffering and in hippocampal synaptic plasticity (Levy *et al*. 2003).

Although a significant connection between ERK and mitochondria has not been reported in the past, phospho-ERK1/2 may regulate mitochondrial activities related to cell life and death. ERK1/2 has been associated with neuronal survival by inhibiting cytochrome *c*-mediated apoptosis, through Bad phosphorylation (Jin *et al*. 2002). Furthermore, in a pioneer work, Baines and co-workers have shown the presence of mitochondrial ERK in murine heart; PKCε-ERK modules appears to play a role in PKCε-mediated cardioprotection, in part through the phosphorylation and inactivation of Bad (Baines *et al*. 2002).

Therefore, the purpose of the present study was to analyze whether ERK1/2 are present in brain mitochondria and whether they are modulated throughout the different developmental stages.

Materials and methods

Subcellular fractionation

Male Wistar rats were killed by decapitation at embryonic days 17 and 19 (E17-E19), post-natal days 2 and 3 (P2 and P3) and 60 day-old adults. The brain was rapidly removed and homogenized in 320 mM sucrose and 20 mM HEPES homogenization buffer, pH 7.2, containing 1 mM EDTA, 1 mM dithiothreitol, 10 μg/mL leupeptin, 2 μg/mL aprotinin, 10 μg/mL phenylmethylsulfonylfluoride, 50 mM sodium fluoride, 1 mM sodium orthovanadate and 0.1 mM ammonium molybdate. The homogenate was centrifuged for 10 min at 630 *g* to obtain the nuclear fraction. The low-speed supernatant was centrifuged for 10 min at 7600 *g* to provide the mitochondrial pellet; the resulting supernatant was stored at −70°C as the crude cytosolic fraction. The mitochondrial pellet was re-suspended in 1 mL of homogenization buffer per gram of the original tissue and diluted with four volumes of Percoll buffer (225 mM D-mannitol, 1 mM EGTA, 270 mM potassium HEPES, 1% bovine serum albumin, 37% Percoll). This suspension (10 mL) was ultracentrifuged at 30 000 *g* for 30 min in a Beckman Ti 90 rotor. To detach unspecific bound proteins, synaptosomes (white upper band) and mitochondria (brownish lower band) were subjected to repeated washing with 150 mM potassium chloride and homogenization buffer, respectively. The pellets of pure mitochondrias and synaptosomes were stored at − 70°C. Experiments were performed in duplicate using pools of two to 14 animals for each experiment, depending on the developmental stage; to obtain representative samples from embryos and neonates, brains from one litter were pooled.

Submitochondrial fractionation

Percoll-purified mitochondria were osmotically broken by diluting the mitochondrial pellet in four volumes of distilled water and centrifuged for 10 min at 12 000 *g* to give a supernatant containing the mitochondrial outer membrane and the intermembrane space and a mitoplast pellet, inner membrane enclosing the matrix. Then, the mitoplast fraction was sonicated twice at 40 W for 10 s with a Cole-Parmer sonicator (WPI, Sarasota, FL, USA). Subsequently, samples were centrifuged for 10 min at 8000 *g* to precipitate unbroken mitochondria, and the supernatant was centrifuged for 30 min at 100 000 *g*. The supernatant (mitochondrial matrix fraction) was separated from the pellet (inner membrane), and the latter was re-suspended in the homogenization buffer. Experiments were performed by duplicate using pools of 18 animals for each experiment.

Brain enzyme activities

Lactate dehydrogenase activity was assayed spectrophotometrically by following NADH oxidation at 340 nm. Twenty-five micrograms of protein were added to 100 mM phosphate buffer, pH 7.0 in the presence of 1 mM piruvic acid, 0.1% triton X-100 and 0.22 mM NADH; $\varepsilon_{340} = 6.22$ mM/cm. NADH-cytochrome-*c* reductase (Complexes I-III) activity was assayed spectrophotometrically by determining cytochrome *c* reduction at 550 nm in the presence of 30 μM cytochrome *c*, 1 mM potassium cyanide and 150 μM NADH, as electron donor; $\varepsilon_{550} = 21$ mM/cm.

Immunoblotting analysis

Subcellular and submitochondrial fraction samples (25–30 μg of protein) were subjected to sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE, 7.5–10% gels), and immunoblots were performed as described previously (Cammarota *et al*. 2000). Membranes were incubated with the following antibodies: anti-mGluR1 (1 : 1000, Upstate Biotechnology, Lake Placid, NY, USA), anti-GluR2 (1 : 1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ERK1/2 (1 : 3000), anti-phospho ERK1/2 (1 : 4000), anti-phospho-MEK1/2 (1 : 1000) and anti-phospho-RSK90 (1 : 1000); antibodies for ERK, MEK and RSK90 were from New England Biolabs, Beverly, MA, USA.

Immunoelectron microscopy

Purified mitochondria were suspended in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 2 h at 4°C, then dehydrated in 70, 96 and 100% ethanol (30 min each step) and embedded in LR White. Slides were obtained with glass knifes and placed on nickel grids over Formvar membranes. Immunocytochemistry was performed using a primary rabbit anti-ERK1/2 or anti-phospho ERK1/2 (New England Biolabs) at a dilution of 1 : 100 in PBS, pH 7.4. After washing with PBS, a goat anti-rabbit

serum conjugated with colloidal gold (10 nm-diameter particles; Jackson Immunoresearch, West Grove, PA, USA) was used at 1 : 100 in PBS, pH 7.4. Grids were slightly counterstained with 1% uranyl acetate in water for 5 min. Non-specific background was blocked by incubating the grids with 5% normal goat serum in PBS, at the beginning of the procedure. Specimens were observed in a Zeiss EM-109-T transmission electron microscope at 80 kV.

ERK activity of mitochondria and immunoprecipitation

ERK activity in mitochondrial fraction was evaluated using basic myelin protein (BMP) as specific substrate. Briefly, purified brain mitochondria (300 μg) isolated from adult and P2 rats were re-suspended in 400 μL of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% triton X-100, 5 mM sodium orthovanadate, 0.1 mM ammonium molybdate, 0.1 mM phenylmethylsulfonylfluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin and 50 mM sodium fluoride) and incubated 30 min at 4°C. Samples were centrifuged at 12 000 *g* and the supernatant was re-collected. The ERK activity assay was carried out incubating lysis supernatant samples (20 μg of total protein) with 50 μL of activity buffer (20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 200 μM sodium orthovanadate, 20 mM glycerol phosphate), 5 μCi ATP (Amersham, Buckinghamshire, UK), 50 mM ATP (Sigma-Aldrich, St Louis, MI, USA) and 0.25 mg/mL of BMP (Sigma-Aldrich) during 30 min at 30°C. The reaction was stopped with the addition of Laemmli sample buffer 4 ×, heated at 85°C for 5 min and then submitted to SDS–PAGE and autoradiography.

To immunoprecipitate ERK1/2, lysis supernatant samples (300 μg) were incubated overnight with 4 μg of anti-ERK1/2 total (New England Biolabs) and 40 μL of protein A Sepharosa. Samples were centrifugated at 2000 *g* and washed three times in lysis buffer. The pellet was solubilized with sample buffer and submitted to SDS–PAGE (gel 12%). Immunoblotting analysis was performed as previously described using an anti-phospho ERK1/2 antibody (New England Biolabs).

Mitochondrial production of hydrogen peroxide and ERK activity

Mitochondrial H_2O_2 production was continuously monitored in a Hitachi F-2000 spectrofluorometer (Hitachi Ltd., Tokyo, Japan) with excitation and emission wavelengths at 315 and 425 nm, respectively (Poderoso *et al*. 1999). The assay medium consisted of 50 mM buffer phosphate, 50 mM L-valine, pH 7.4, supplemented with 12.5 U/mL horseradish peroxidase, 250 μM *p*-hydroxyphenylacetic acid and 0.15 mg mitochondrial protein/mL, with 10 mM succinate as substrate. In order to explore effects of H_2O_2 on ERK activity, the organelles were re-suspended in the described homogenization buffer and supplemented with 1–100 μM H_2O_2, 2 μM antimycin or 3 μM catalase, in the presence of 10 mM sodium succinate. At the end of incubations (20 min), aliquots (25 μg protein) were taken for immunoblotting analysis.

Results

The mitochondrial fraction

To confirm the purity of the mitochondrial fraction, immunoblots were carried out in the rat brain subcellular fractions obtained at E17, E19, P2, P3 and adult with specific antibodies against synaptic membrane proteins, such as the metabotropic glutamate receptor type 1 (mGluR1). mGluR1 was early detected in cytosol, synaptosomes and nucleus but it was absent in the mitochondrial fraction at any developmental stage (Fig. 1a); another synaptic protein, the GluR2 AMPA receptor subunit, was not detected in the mitochondrial fraction (data not shown). Although nuclear factors are not expressed homogeneously throughout development, neither Fra-1 nor NF-κB (nuclear transcription factors) were detected at any stage in mitochondria fraction (data not shown). In addition, the activity of cytosolic lactate dehydrogenase was less than 5% in the mitochondrial fraction (Fig. 1b). Finally, NADH-cytochrome *c* reductase activity further confirmed the identity and functional aptitude of the mitochondrial preparation (Fig. 1c).

Ultrastructural and functional ERK1/2 detection in mitochondria

Immune electron-microscopy was utilized to detect mitochondrial localization of total ERK1/2 and dually phosphorylated, active phospho-ERK1/2 in neonate and adult brain. In these conditions, colloidal gold particles were clearly detected in mitochondria from animals at P2 (Figs 2a and b). Instead, particles were scarce in the adult organelles (Fig. 2c), thus indicating the developmental modulation of these kinases. The background levels were low and samples, processed with normal rabbit serum as a primary antibody, did not show any labelling, confirming the specificity of the reaction (Fig. 2d). In addition, ERK activity was found in the mitochondrial fraction, using BMP as specific substrate. In accordance with ultrastructural changes, ERK activity was higher in P2 neonates than in adults rats supporting the developmental modulation of these kinases in brain mitochondria (Fig. 3a). Moreover, active ERK1/2 dually phosphorylated forms were specifically detected in immunoprecipitates obtained from the pure mitochondrial fraction with antibodies anti-total ERK1/2 (Fig. 3b).

The localization of ERK 1/2 and MEK 1/2 in brain mitochondria

Matrix, internal membrane, mitoplasts, and a fourth fraction composed of both outer membrane and intermembrane space (OM+IMS) from brain mitochondria were analyzed for the presence of phospho and total ERK1/2. A marked enrichment of both forms was observed in the OM+IMS fraction and a slight enrichment in the matrix fraction (Figs 4a and b). In addition, immunoblot showed specific reactivity for phospho-MEK1/2; in this case, reactivity was enriched in the matrix fraction (Fig. 4c).

The RSK90 serine/threonine kinase is a downstream target of ERK1/2 that regulates gene expression by phosphorylating a number of transcription factors (Chen *et al*. 1992). To search for common brain ERK1/2 targets in the mitochondrial

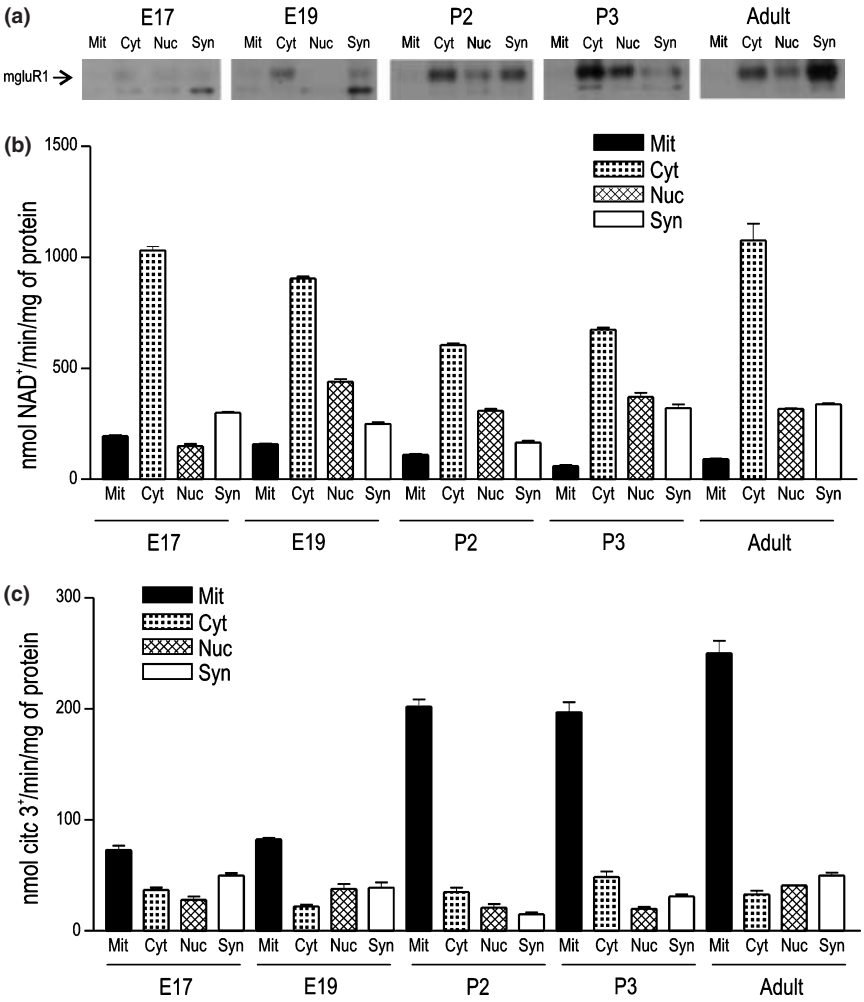

Fig. 1 Assessment of brain mitochondrial fraction identity and purity. (a) Western blot using an antibody against receptor mGluR1 in the brain subcellular fractions obtained at different developmental stages (embryonic days 17 and 19 (E17 and E19), post-natal days 2 and 3 (P2 and P3) and adults. (b) Lactate dehydrogenase activity was monitored spectrophotometrically in brain subcellular fractions at the same ages through oxidation of NADH at 340 nm. (c) NADH-cytochrome *c* reductase activity was followed through reduction of cytochrome *c* at 550 nm. (b, c) Data are expressed as mean ± SEM of three independent experiments. Mit, mitochondria; Cyt, cytosol; Nuc, nuclei; Syn, synaptosomes.

preparations, the level of activated RSK90 was analyzed; the results showed that, although phospho-RKS90 was detected in cytosol, nuclear and synaposomal fraction, this kinase was not present in brain mitochondria (Fig. 4d).

The subcellular distribution of ERK 1/2 during development

In addition to immune electron microscopy and activity assays, the distribution, dual phosphorylation and activation of ERK1/2 in rat brain was monitored during development by immunoblotting analysis. Phospho-ERK1/2 immunoreactivity at 42–44 kDa were differentially detected in cytosolic, nuclear and synaptosomal fractions; in addition, active ERK1/2 could be clearly detected in the highly purified mitochondrial fraction as well (Fig. 5a). Considering the expression level of total and activated protein in the different fractions, an evident spatio-temporal modulation of ERK and phospho-ERK1/2 was observed throughout brain development. At E17, ERK1/2 and phospho-ERK1/2 were observed in cytosol and synaptosomes, but hardly detected in mitochondria and nucleus. From E19 to P2, phospho-ERK1/2 markedly increased in mitochondria with respect to E17 levels. However, at P3, mitochondrial ERK1/2 and phospho-ERK1/2 content decayed sharply with respect to the precedent stages, and up to the levels found in the adult animals (Figs 5a and b). In addition, we preliminarily observed that mitochondrial phospho-ERK1/2 remains relatively unchanged in the transition from P3 to adulthood (data not shown). Instead, phospho-ERK1/2 increased in cytosol and nucleus from P2 up to the adult stage (Figs 5a, c and e). Consequently, the mitochondria/nucleus phospho-ERK1 and ERK2 ratios gave positive results at the P2 stage and negative in the adult brain (Figs 5d and f; $p < 0.05$); a similar trend was observed at the same ages in median mitochondria/cytosol ratios [phospho ERK1: 5.63 (0.63–56) vs. 0.12 (0.014–0.136); phospho-ERK2: 4.48 (1.2–12.79) vs. 0.37 (0.066–0.342)]. As a marker of neurite growth and plasticity, synaptosomal phospho-ERK1/2 were stably expressed in the brain from late fetal life to adulthood (Figs 5a and b).

In mitochondria, the described developmental pattern was due to a regulation of the specific protein localization because, as shown in Fig. 5(b), a similar profile was obtained

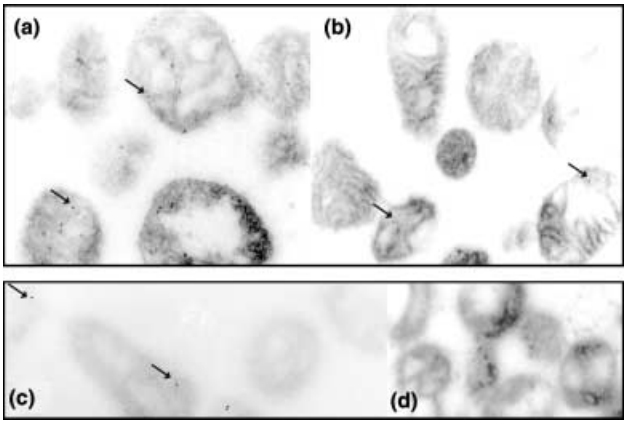

Fig. 2 Immune electron microscopy detects phospho-ERK1/2 and ERK1/2 in purified rat brain mitochondria. Mitochondria isolated at P2 were fixed, sliced and labelled with primary rabbit total ERK1/2 antibody (a) and phospho-ERK1/2 antibody (b) at 1 : 100 dilution in PBS. (c) Adult brain mitochondria were labelled with anti-ERK1/2 antibody in the same conditions. All samples were treated with a goat anti-rabbit serum conjugated with colloidal gold (10 nm-diameter particles), as indicated by arrows. To test unspecific effects, a set of control samples was solely incubated with the secondary antibody (d). Magnification = × 50 000.

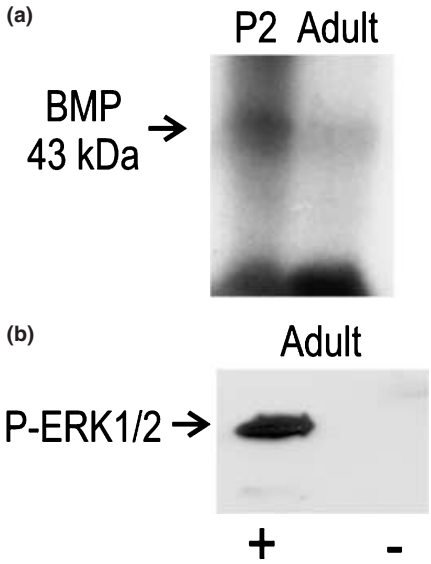

Fig. 3 Mitochondrial ERK activity. (a) Representative autoradiograph showing differential ERK activity of brain mitochondrial homogenates from P2 and adult rats. Activity was measured after 30-min incubation at 30°C with 5 μCi $P^{32}\gamma$ATP and 0.25 mg/mL of basic myelin protein (BMP) as substrate. (b) Representative immunoblot using anti-phospho-ERK1/2 shows the presence of ERK1/2 activated in adult animals after immunoprecipitation of mitochondrial proteins with anti-ERK total antibody (+) or in absence of the antibody (–).

with an antibody against total ERK1/2. Different modulation of phospho- and total ERK1/2 in the subcellular fractions is consistent with the purity of preparations. In addition, phospho-MEK1/2 was clearly detected in mitochondria at all stages, although a specific modulatory pattern could not be defined.

Effects of hydrogen peroxide on mitochondrial ERK1/2 activity

ERK1/2 activity is under redox influence in different systems (Kuruganti *et al*. 2002; Samavati *et al*. 2002). Mitochondria is the main cell producer of H_2O_2 at complexes I and III (Boveris and Cadenas 1997). On these bases, effects of exogenous or endogenously formed H_2O_2 on mitochondrial ERK were explored. The results show that, at low 1 μM H_2O_2, mitochondrial phospho-ERK1/2 levels are retained; although non-significantly, mitochondrial phospho-ERK/total ERK ratios resulted 20–25% increased in respect to controls (Figs 6a–c). In contrast, supplementation of purified mitochondria with 50–100 μM H_2O_2 decreased ERK1/2 activity by about 40–50%, below the control values ($p < 0.05$; Figs 6a–c). In addition, catalase that turns endogenous H_2O_2 up to a lower steady-state concentration increased mitochondrial phospho-ERK1/2 by 50% ($p < 0.05$). In contrast, effects of increasing endogenously formed H_2O_2 by inhibiting electron transfer at complex III with antimycin, resulted in a marked inhibition of mitochondrial ERK1/2 activation; in this case, phospho-ERK/total ERK ratios significantly decreased to 9–18% of the control values (Figs 6a–c). It is noticeable that, under the experimental conditions, 2 μM antimycin increased the basal mitochondrial H_2O_2 production rate by about fourfold (from 0.05 ± 0.001 to 0.18 ± 0.01 nanomoles H_2O_2/min.mg. mitochondrial protein, $p < 0.001$).

It is important to stress here that, in different redox conditions, no significant changes were found in total ERK1/2 content between groups (Fig. 6a).

Discussion

Our results show for the first time that: (i) ERK1/2 subfamily of MAPK is localized in rat brain mitochondria; this finding confirms and extends a recent finding demonstrating the presence of mitochondrial ERK localization in murine heart (Baines *et al*. 2002); (ii) ERK1/2 are confined to the fraction containing the mitochondrial outer membrane and the intermembrane space; (iii) ERK1/2 protein expression, traffic to mitochondria and activity are under developmental modulation.

Targeting of kinases close to their substrates should be crucial to ensure tight regulation of the phosphorylation events in a molecular framework (Pearson *et al*. 2001). In resting cells, ERK1/2 are anchored to cytosolic MEK and upon mitogenic stimulation are rapidly translocated to the nucleus that represents a site for ERK1/2 action, sequestration and signal termination (Volmat *et al*. 2001). It is accepted that nuclear migration requires ERK1/2

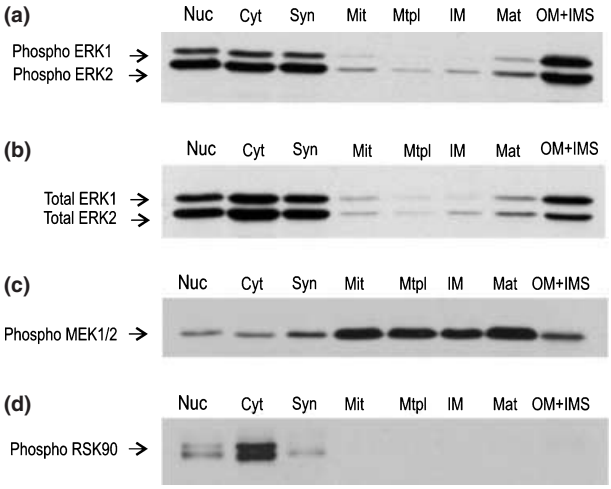

Fig. 4 Localization of ERK cascade in the subcellular and submitochondrial fractions from adult rat brain. (a) Representive western blots using phospho-ERK1/2 antibody in brain subcellular fractions. (b) The same blot as in (a) stripped out and incubated with ERK1/2 total antibody. (c) Same as in (b) using an antibody against phospho-MEK1/2. (d) Same in (b) using an antibody against phospho-RSK90. In all cases, 30 μg of proteins was loaded in each lane. Nuc, nuclei; Cyt, cytosol; Syn, synaptosomes; Mit, mitochondria; Mtpl, submitochondrial fractions mitoplasts; IM, internal membrane; Mat, matrix; OM+IMS, a fourth fraction composed of both outer membrane and intermembrane space.

phosphorylation to promote dissociation of ERK-MEK complex and further dimerization (Kyriakis and Avruch 2001). The present results propose a similar process involving the mitochondrial compartment considering that: (i) in the mitochondrial transit, ERK1/2 were predominantly found as phospho-ERK; (ii) mitochondria are targeted by upstream members of the ERK1/2 cascade like MEK1/2 and Raf-1, supporting the existence of a complete sequence for ERK1/2 activation in mitochondria (Nantel *et al*. 1999; Jin *et al*. 2002); (iii) in accordance, we found activated phospho-MEK1/2 in the brain organelles. In adult organelles, phospho-MEK1/2 was predominantly localized in the mitochondrial matrix (Fig. 4c) while ERK1/2 were confined to the OM+IMS fraction (Figs 4a and b). A different submitochondrial localization could modulate the MEK/ERK interaction and, therefore, ERK1/2 phosphorylation and the overall ERK activity in brain mitochondria. However, ERK-binding domain of MEK only represents the NH_2-terminal 32 amino acids (Kyriakis *et al*. 2001) and transient or stable transmembrane localization of this specific motif is also possible. In these conditions, a reported co-operation with other kinases like PKCε, which favours ERK activation in mitochondria (Baines *et al*. 2002) should probably rely on steric changes that facilitate phosphorylation by the specific kinases. In addition, some adapters or scaffolding proteins which favour MAPK interactions like Grb 10 are localized in mitochondria (Nantel *et al*. 1999), while others like 14.3.3

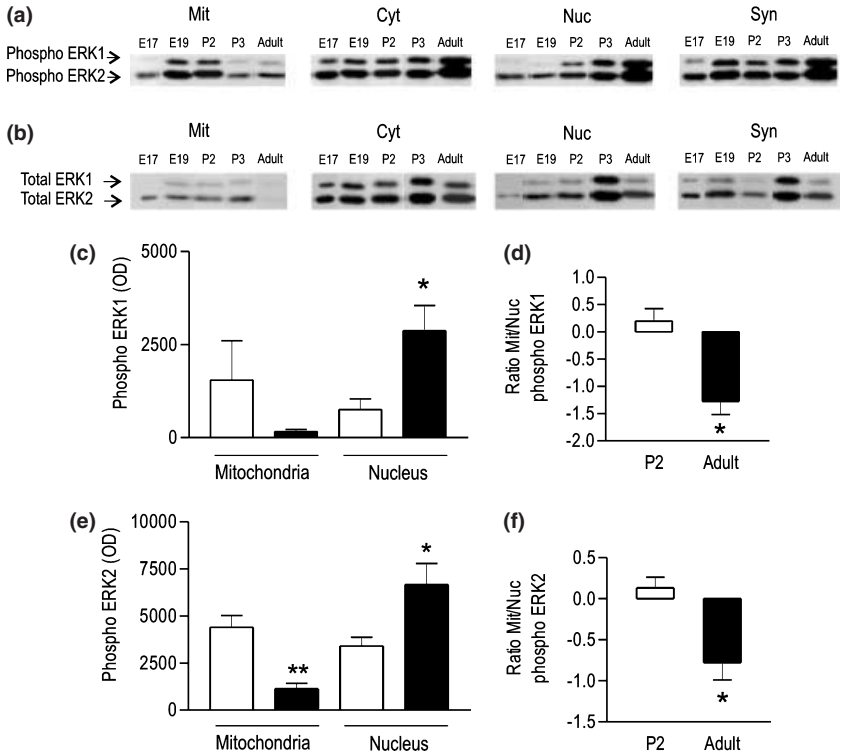

Fig. 5 Brain mitochondrial ERK1/2 are modulated during rat development. (a) Representive western blot reveals the comparative variations of phospho-ERK1/2 expression in subcellular fractions isolated during rat brain development (abbreviations as in Fig. 4), as detected with a specific antibody. (b) The same blot as in (a) was stripped out and incubated with total ERK1/2 antibody. In all cases, 25 μg of proteins was loaded in each lane. (c, e) The respective densitometric analysis of phospho-ERK1 and phospho-ERK2 expression in purified brain mitochondria and nuclei from P2 (open bars) and adult rats (closed bars) ($n = 3$). On the right, the mitochondria/nucleus ratio of phospho-ERK1 (d) and phospho-ERK2 (f) expression is compared at the different ages. To normalize the distribution of ratios, data are expressed as mean ± SEM of logarithm of calculated ratios ($n = 3$). *$p < 0.05$ and **$p < 0.01$, by Student *t*-test.

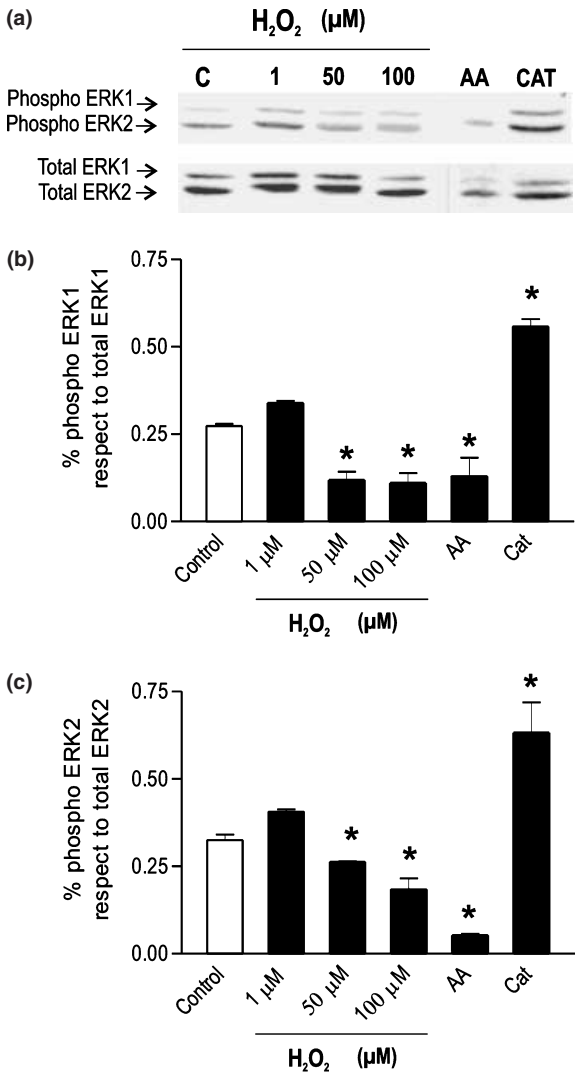

Fig. 6 Modulatory effects of H_2O_2 on mitochondrial ERK1/2. Freshly isolated rat brain mitochondria (4 mg protein/mL) were incubated by 20 min at 37°C with 1–100 μM H_2O_2 and compared with controls without H_2O_2. To test effects of mitochondrial endogenous H_2O_2 yield, the organelles were supplemented with 2 μM antimycin (AA) or 3 μM catalase (Cat) in the same conditions and in the presence of 10 mM sodium succinate. At the end of incubations, aliquots (25 μg protein) were taken for immune blotting, as described in Materials and methods. (a) Representative western blots of mitochondrial phospho-ERK1/2 and total ERK1/2 are shown in different conditions. (b, c) Respective phospho-ERK1 and 2/total ERK1 and 2 ratios are expressed. Data represent mean ± SEM from three independent experiments. *$p < 0.05$ denotes significantly different versus control group by Dunnet's test after ANOVA; logarithm of percentages were used to normalize the distribution of the data in the calculations.

proteins are probably not, though they may contribute to mitochondrial protein import (Pierrat *et al*. 2000).

In the present study, developmental trafficking of cytosolic ERK to mitochondria followed a different temporal course than trafficking to nucleus. ERK1/2 protein expression and specially activated phospho-ERK1/2 were evidenced in mitochondria from fetal E19 to P2 and decayed sharply at post-natal P3 while, conversely, they predominantly increased in nucleus and cytosol from P2-P3 up to the adult condition. Although a role for temporal ERK1/2 shuttling to mitochondria is not yet defined, our results suggest that this developmental pattern is sequentially co-ordinated.

Inhibition of ERK nuclear translocation, by overexpression of a cytoplasmic ERK-docking molecule (inactive MKP3) prevented growth factor-stimulated DNA replication (Brunet *et al*. 1999). In this way, it may be surmised that translocation to mitochondria in late fetal development could prevent early ERK nuclear migration. In addition, mitochondria is the main ATP producer; it has been reported after ischemia–reperfusion that ERK activation and phosphorylation requires ATP and full mitochondrial activity (Abas *et al*. 2000). Considering that ATP concentration is maximal in the outer membrane at the ADP/ATP translocase site and that migration to nucleus requires ERK1/2 phosphorylation, it may be alternatively hypothesized that a previous mitochondrial passage of ERK1/2 is required to be maximally phosphorylated and, finally, to simply reach the nuclear structures.

It is known that redox condition modifies the activation of ERK1/2 (Kuruganti *et al*. 2002; Samavati *et al*. 2002). In the presence of H_2O_2, increased amounts of ERK are activated to phospho-ERK by mitogens or growth factors in different cell lines, while inhibition of MEK1/2 decrease H_2O_2-mediated ERK1/2 activation (Bhat and Zhang 1999). Similarly, in this study, mitochondrial ERK1/2 are subjected to modulatory effects of H_2O_2; preliminary data of our group, indicating that both phospho-ERK1/2 and phospho-MEK1/2 levels could be under redox influence in brain mitochondria (data not shown). It is apparent that a low oxidative stress level is compatible with mitochondrial ERK full activity while, at high H_2O_2 concentration, phospho-ERK1/2 and probably phospho-MEK1/2 could decline in parallel. Effects of H_2O_2 may rely on the effects on cysteine residues of mitochondrial upstream kinase MEK1/2 (Kim *et al*. 2001). Moreover, MEK activity may considerably increase in the presence of anti-oxidants like N-acetylcysteine (Kim *et al*. 2001); redox effects on the activity of ERK phosphatases (MKP1-3) which, in turn, are phosphorylated and activated by ERK itself (Lu *et al*. 2002) deserve a further analysis.

Alternatively, a developmental increase of mitochondrial H_2O_2 yield observed in the rat brain after delivery (Riobó *et al*. 2002) may attenuate ERK activation and nuclear translocation. Likewise, H_2O_2 decreases mitochondrial membrane potential and phosphorylation efficiency, as required for ERK activation (Abas *et al*. 2000; Eckert *et al*. 2003). High H_2O_2 may also contribute to regulate the mitochondrial ERK availability; stress-activated ERK undergo to ubiquin-

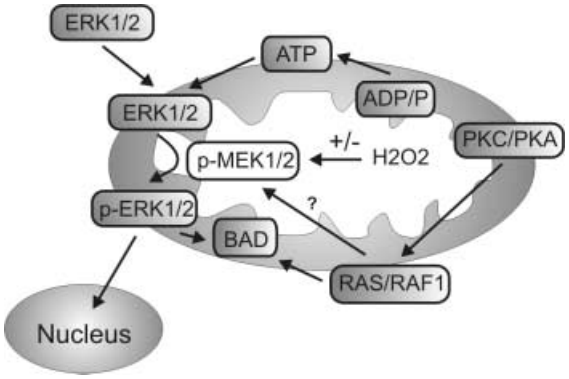

Fig. 7 The scheme summarizes putative ERK1/2 interactions at mitochondria in rat brain developmental cell trafficking. Based on data from literature and from this work, we illustrate that mitochondrial ERK1/2 transit and activation should depend on mitochondrial ATP production (Abas *et al.* 2000) and endogenous H_2O_2 yield (Fig. 6), thus favouring interactions with other mitochondrial signalling cascades, like Bad (Bonni *et al.* 1999; Jin *et al.* 2002), the redox-sensitive upstream ERK1/2 activators MEK1/2 (Kim *et al.* 2001), Raf/Ras (Wang *et al.* 1996) and PKC/PKA kinases (Schonwasser *et al.* 1998; Harada *et al.* 1999; Baines *et al.* 2002; Dumaz and Marais 2003).

itation and degradation (Lu *et al*. 2002), a process that may take place in mitochondria (Cogswell *et al*. 2003).

On the basis of the present results, it is believed that mitochondrial ERK1/2 may play different specific roles in neuron maturation and brain development. For instance, mitochondrial ERK1/2 contribute to phosphorylation and inactivation of Bad, a mitochondrial pro-apoptotic protein (Bonni *et al*. 1999; Jin *et al*. 2002). In the general context of mitochondria-nucleus energetic communication, ERK cascade could, however, be not only an amplifier of extracellular stimuli, but also an informational molecule which provides the status of the two most important mitochondrial functions – ATP synthesis and the formation of O_2 active species – to the proliferating/differentiating nuclear pathways (Fig. 7).

Our results provide clear evidence about the existence of constitutive ERK cascade in the whole brain pure mitochondrial fraction, and its developmental trafficking. In addition, further experiments will be required to differentiate neurons and glial cells contribution to mitochondrial ERK and also to elucidate its physiological relevance within discrete regions of the brain throughout the different developmental stages.

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