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# Estradiol facilitates neurite maintenance by a Src/Ras/ERK signalling pathway

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ABSTRACT

Different reports suggest the estrogens are involved in neuritic outgrowth, maintenance of dendritic morphology and spine formation in the CNS. However, the molecular mechanisms regulated by estrogens on neuronal integrity are not fully understood. We have addressed the relationship between 17β-estradiol-dependent ERK pathway stimulation and the maintenance of neuritic morphology in cerebellar granule cell cultures (CGC). We report that 17β-estradiol clearly activates ERK phosphorylation in CGC cultured in low potassium via ERα localized in the plasma membrane and without the activation of the insulin-like growth factor-I receptor. 17β-estradiol activates the ERK pathway through Ras-dependent Src kinase activity. A concomitant activation of the cAMP-response element-binding protein (CREB) is observed. Moreover, we demonstrate that 17β-estradiol-mediated ERK activation is involved in the maintenance of neuritic arborisation and neuronal morphology in proapoptotic conditions.



## Introduction

17β-estradiol has been associated with a variety of CNS effects including cell survival, differentiation and morphology. Thus, 17β-estradiol regulates the density of spines and dendrites in the hippocampus (Gould et al., 1990; Woolley and McEwen 1994; Murphy and Segal 1997) and in the cerebellum (Sakamoto et al., 2003). It has been also described that 17β-estradiol induces neurite outgrowth and branching in cholinergic neurons (Dominguez et al., 2004). The morphological effects of 17β-estradiol in the CNS are observed in normal and damaged tissue. The effect of 17β-estradiol seems to be partly mediated by changes in the expression pattern of several cytoskeletal proteins such as MAP2 isoforms or tau (Reyna-Neyra et al., 2002; Shah et al., 2003).

The molecular mechanisms regulated by 17β-estradiol that elicit the effects described above are not fully understood. The mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinases (ERK) pathway is considered a major contributor of neuronal plasticity by affecting activity-dependent formation of dendrites (Vaillant et al., 2002; Goldin and Segal 2003). Several studies have reported that 17β-estradiol activates the MEK/ERK pathway in various tumour cell lines, adipocytes and neurons (Singer et al., 1999), (Watters et al., 1997; Wong et al., 2003), likely by activating 17β-estradiol receptors (ER) located in the plasma membrane (Stevis et al., 1999; Toran-Allerand et al., 2002). Although still controversial, several studies supported that activation of ERα induces MEK/ERK pathway stimulation (Watters et al., 1997; Singh et al., 2000; Dos Santos et al., 2002). In some cases the transactivation of IGF-IR is needed for ERα-mediated activation of MEK/ERK pathway (Kahlert et al., 2000; Cardona-Gomez et al., 2002). Besides ERα, it has been recently described that 17β-estradiol-mediated MEK/ERK activation could be also mediated by GPR30, a seven-transmembrane G-protein-coupled receptor located in the endoplasmic reticulum (Revankar et al., 2005). A role of MEK/ERK pathway in the 17β-estradiol-mediated effect on structural plasticity in the CNS has been reported in the hippocampus (Bi et al., 2000) and in basal forebrain cholinergic neurons (Dominguez et al., 2004). However, the mechanisms upstream and downstream of ERK activation that mediates the effects of 17β-estradiol on neuronal morphology are largely unknown. Some reports suggest that activation of the cAMP-response element-binding protein (CREB) could be involved. CREB has been implicated in synaptic plasticity (Finkbeiner et al., 1997) and phosphorylation of CREB by 17β-estradiol is dependent on ERK activity in hippocampal cultures (Lee et al., 2004) and in the basal forebrain (Szego et al., 2006).

During the last years it has become apparent that 17β-estradiol has an important role in the development of the cerebellum. It has been suggested that estrogens could be involved in the regulation of dendritic growth, spine formation and neuron number in the cerebellum (Wong et al., 2003) (Sakamoto et al., 2003). Interestingly, expression levels of ERβ correlate with the differentiation of Purkinje

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cells, whereas the expression of ERα is maximal during the postnatal differentiation and migration of the cerebellar granule cells (CGCs) towards the internal granular layer (Altman 1972; Belcher 1999). It is well known that CGCs migration and differentiation is dependent on synaptic activity and the presence of trophic factors (Burgoyne et al., 1993; D'Mello et al., 1997). CGCs that do not receive trophic support lose their neuritic morphology and die afterwards by apoptosis (Gallo et al., 1987). This situation could be mimicked in CGCs cultures, as they undergo spontaneous apoptosis when grown in physiological KCl concentrations (Gallo et al., 1987; Xifro et al., 2005).

In a previous work (Miñano et al., 2007) we have described that 17β-estradiol is able to activate the MEK/ERK pathway in CGCs cultures grown in the absence of depolarizing or trophic factors support. In the present study we have examined the upstream and downstream mechanisms involved on ERK activation and, whether the activation of this pathway reverses the altered neuritic morphology observed in CGS.

## Results

### *17β-estradiol activates ERK1/2 in cerebellar granule neurons*

Previous results have clearly shown that 17β-estradiol activates ERK1/2 in a number of different cell types including neurons. Whereas in cortical neurons ERK1/2 activation by 17β-estradiol has been related to estrogen-mediated neuroprotection from excitotoxicity (Bi et al., 2000; Singer et al., 1999), the relationship between estrogen neuroprotection and ERK1/2 activation is less clear in other neuronal

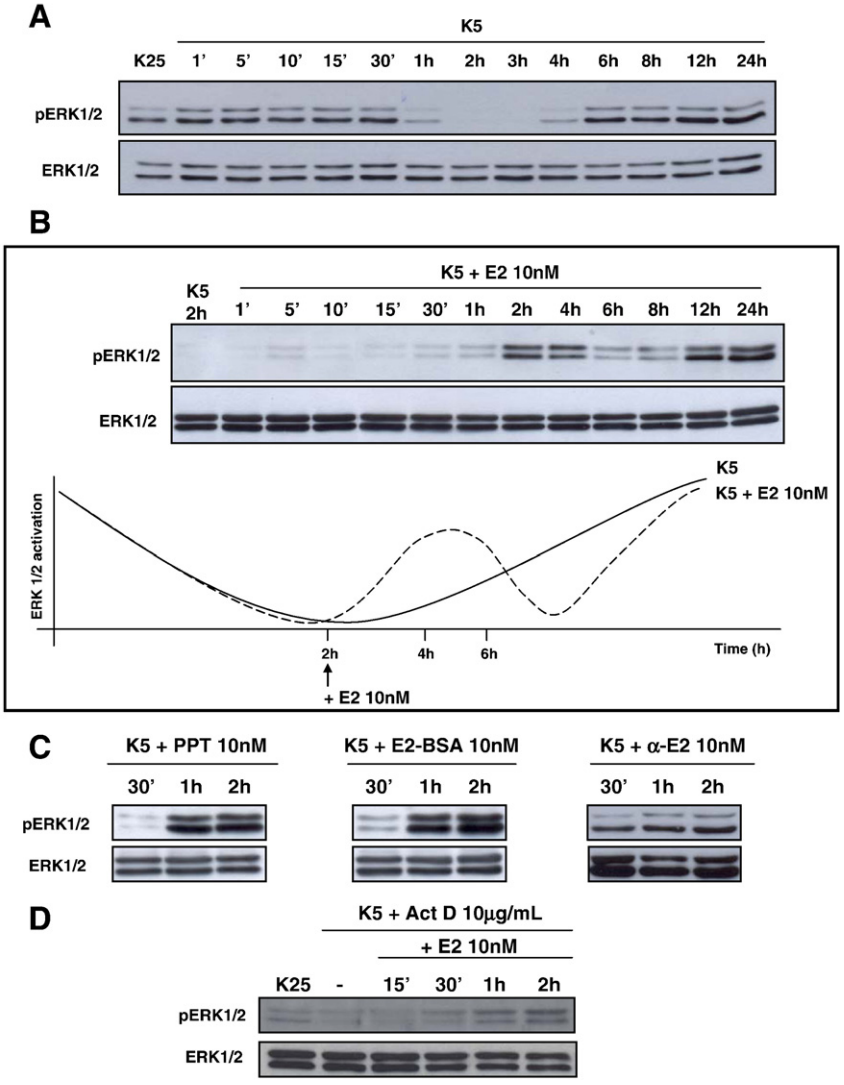


**Fig. 1.** 17β-estradiol activates ERK pathway via ERα. (A) Rat cerebellar granule cells (CGC) maintained in 25 mM KCl and B27 (K25) were placed in B27-free media containing 5 mM KCl (K5) for the indicated times (upper panel). The phosphorylation status of MAPK (Thr 202 and Tyr 204) from whole-cell lysates was assessed using an antibody recognising phosphorylated MAPK (top). Levels of total MAPK protein were determined on the same blot to control for loading variations (bottom). (B) ERK1/2 activation is shown in CGCs that were B27-starved and incubated in low KCl during 2 h (K5 2 h), then stimulated with 17β-estradiol (E2; 10 nM) for the indicated times. Diagram shows differential activation of ERK1/2 in CGCs in K5 compared to K5+E2 (lower panel). (C) ERK1/2 activation is also shown in CGCs that were B27-starved and incubated in low KCl during 2 h, then stimulated with 10 nM of the ERα agonist 4,4′,4″-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT) or the membrane impermeable estrogen analog E2-BSA for indicated times. ERK1/2 phosphorylation is not affected by the biological inactive isomer 17α-estradiol (α-E2). The membrane impermeable estrogen analog E2-BSA mimicked the effects of 17β-estradiol on ERK1/2 phosphorylation. The maximum activation of ERK1/2 phosphorylation was between 1 and 2 hours. The effect of PPT also paralleled the effect of 17β-estradiol. By contrast, the structural isomer 17α-estradiol was not able to stimulate ERK1/2 at any time. (D) ERK1/2 activation is also shown in the absence of transcriptional activity. CGC were B27-starved, incubated in low KCl, and pre-treated with actinomycin D 10 μg/mL (Act D), then stimulated with E2 (10 nM) for the indicated times. These results are representative of at least three independent experiments.