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G protein-coupled estrogen receptor is required for the neuritogenic mechanism of 17β-estradiol in developing hippocampal neurons

Isabel Ruiz-Palmero, Maria Hernando, Luis M. Garcia-Segura *, Maria-Angeles Arevalo

Instituto Cajal, CSIC, E-28002 Madrid, Spain

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ABSTRACT

Estradiol promotes neuritogenesis in developing hippocampal neurons by a mechanism involving the upregulation of neurogenin 3, a Notch-regulated transcription factor. In this study we have explored whether G-protein coupled estrogen receptor 1 (GPER) participates in this hormonal action. GPER agonists (17β-estradiol, G1, ICI 182,780) increased neurogenin 3 expression and neuritogenesis in mouse primary hippocampal neurons and this effect was blocked by the GPER antagonist G15 and by a siRNA for GPER. In addition, GPER agonists increased Akt phosphorylation in ser473, which is indicative of the activation of phosphoinositide-3-kinase (PI3K). G15 or GPER silencing prevented the estrogenic induction of Akt phosphorylation. Furthermore, the PI3K inhibitor wortmannin prevented the effect of G1 and estradiol on neurogenin 3 expression and the effect of estradiol on neuritogenesis. These findings suggest that GPER participates in the control of hippocampal neuritogenesis by a mechanism involving the activation of PI3K signaling.

1. Introduction

G-protein coupled estrogen receptor (ER) 1 (GPER), also known as G protein-coupled receptor 30 (GPR30), is a seven-transmembrane receptor that was first cloned by Owman et al. (1996), and was independently identified in ER-positive breast carcinoma cell lines using the technique of differential cDNA library screening (Carmeci et al., 1997). Then, Filardo et al. (2000) reported that GPER was required for estradiol induced activation of Erk-1 and Erk-2 in breast cancer cells. Further studies showed the involvement of GPER on estradiol actions in other cell types. These included macrophages, where GPER mediates the production of nerve growth factor in response of estradiol (Kanda and Watanabe, 2003a) and keratinocytes, where GPER mediates estradiol-induced expression of Bcl-2 and estradiol-induced cell proliferation (Kanda and Watanabe, 2003b, 2004). In 2005, two laboratories proposed that GPER is a membrane ER involved in rapid estrogen signaling (Thomas et al., 2005; Revankar et al., 2005; Filardo and Thomas, 2005). After this identification of GPER as a membrane ER, numerous studies have reported that GPER mediates different biological actions of estradiol; although the precise role of GPER as an ER is still controversial (Levin 2009; Langer et al., 2010; Maggiolini and Picard, 2010; Barton, 2012).

Early Northern blot and in situ hybridization analyses identified GPER mRNA in different brain regions of human and rat brains, including the hippocampus (O'Dowd et al., 1998). The abundant expression of GPER in the central nervous system has been confirmed by other studies and using different techniques, including in situ hybridization, Western immunoblotting and immunohistochemistry (Brailoiu et al., 2007; Canonaco et al., 2008; Matsuda et al., 2008; Dun et al., 2009; Hazell et al., 2009; Isensee et al., 2009; Takanami et al., 2010; Hammond et al., 2011). The function of GPER in the brain has also been studied. GPER has been shown to regulate intracellular calcium oscillations in LHRH neurons (Terasawa et al., 2009), to depress exogenous NMDA-elicited currents (Liu et al., 2012), to modulate cholinergic and serotoninergic function (Xu et al., 2009; Hammond et al., 2011; McAllister et al., 2012) and to influence anxiety behavior (Kastenberger et al., 2012) and spatial learning (Hammond et al., 2009). In addition, GPER is involved in the neuroprotective mechanisms of estrogens in different experimental neurodegenerative models (Blasko et al., 2009; Lebesgue et al., 2009; Gingerich et al., 2010; Yates et al., 2010; Zhang et al., 2010; Bodhankar and Offner, 2011; Liu et al., 2011, 2012; Al Sweidi et al., 2012; Bourque et al., 2012). However, the possible role of GPER during neuronal development has not been explored.

Estradiol regulates neuritogenesis in different neuronal populations of the central and peripheral nervous system by a variety of mechanisms (Arevalo et al., 2012). We have recently identified an interaction of estradiol with Notch signaling in the regulation of neuritogenesis in primary hippocampal neurons (Ruiz-Palmero

* Corresponding author. Address: Instituto Cajal, CSIC, Avenida Doctor Arce 37, E-28002 Madrid, Spain. Tel.: +34 915854729; fax: +34 915854750.
E-mail address: lmgs@cajal.csic.es (L.M. Garcia-Segura).

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et al., 2011). The hormone decreases the expression of Hairy and Enhancer of Split-1 (HES-1), a Notch regulated gene that represses the transcription of neurogenin 3 (Ngn3), a proneural gene from the basic helix-loop-helix superfamily that promotes neuritogenesis (Salama-Cohen et al., 2006). This mechanism was not impaired by ICI 182,780, an antagonist of classical nuclear ERs and was not imitated by ERα and ERβ selective agonists (Ruiz-Palmero et al., 2011). In contrast, G1, an agonist of GPER (Bologa et al., 2006), imitated the effect of estradiol on Ngn3 expression and neuritogenesis (Ruiz-Palmero et al., 2011). This finding suggested a possible involvement of GPER in the control of neuritogenesis in hippocampal neurons. However, G1 may target other molecules, in addition to GPER (Kang et al., 2010). Therefore, in the present study we have directly assessed the role of GPER on Ngn3 expression and neuritogenesis in primary hippocampal neurons using the GPER antagonist G15 (Dennis et al., 2009) and blocking GPER expression with a small interfering RNA. In addition, we have also analyzed the role of phosphoinositide-3-kinase (PI3K)/Akt in the action of GPER on neuritogenesis, because estradiol activates PI3K/Akt signaling in the nervous system (Cardona-Gomez et al., 2004; Zhang et al., 2008; Varea et al., 2009; Garcia-Segura et al., 2010) and because GPER is known to regulate PI3K signaling in cancer cells (Revankar et al., 2005; Wei et al., 2012). Finally, we have studied whether PI3K/Akt signaling is involved in the actions of estradiol and GPER on Ngn3 expression, since PI3K and Notch interact in other cell types (Wong et al., 2012; Shepherd et al., 2013).

2. Materials and methods

2.1. Animals

CD1 mice were raised in the Cajal Institute and used to generate embryos for this study. The day of vaginal plug was defined as E0. All procedures for handling and killing the animals used in this study were in accordance with the European Commission guidelines (86/609/CEE and 2010/63/UE) and were approved by our institutional animal care and use committee.

2.2. Hippocampal neuronal cultures and incubation conditions

The hippocampus was dissected out from embryonic day 17 mouse embryos and dissociated to single cells after digestion with trypsin (Worthington Biochemicals, Freehold, NJ) and DNase I (Sigma–Aldrich) (Goslin and Banker, 1989). Neurons were plated on 6-wells plates or glass coverslips coated with poly-L-lysine (Sigma–Aldrich) at a density of 300–700 neurons/mm^2, and they were cultured in phenol red free Neurobasal supplemented with B-27 and GlutaMAX I (Invitrogen, Crewe, United Kingdom).

2.3. Cell treatments

At 1 or 3 days *in vitro* (DIV) the culture medium was replaced for 2 h by fresh medium devoid of B27 and GlutaMAX I supplement and containing one of the following test compounds, alone or in combination: the GPER agonist G1 (10^{-6} M; Calbiochem, San Diego, CA); the GPER agonist ICI 182,780 (10^{-8} M; Sigma–Aldrich Co., St. Louis, MO); the GPER antagonist G15 (10^{-8} M; Calbiochem), 17β-estradiol (10^{-10} M; Sigma) or the PI3K inhibitor wortmannin (10^{-7} M; Calbiochem). Control cells were incubated with vehicle (control medium). Cells were treated with test compounds for 45 min for Western blotting and for 2 h for quantitative real-time polymerase chain reaction analysis. For immunocytochemistry assays the treatments were performed during 19 h, and cultures were not deprived of B27 and GlutaMAX I supplement.

2.4. Inhibition of GPER expression using small interfering RNAs

ON-TARGET plus SMARTpool small interfering RNA (siRNA) for GPER (L-053623-00) and ON-TARGET plus siControl Non-Targeting siRNA (D-001810-02) were purchased from Dharmacon RNAi Technologies (Dharmacon, Lafayette, CO) and the concentration was 20 nM during electroporation and transfection. Neurons were cotransfected at 2 DIV using the Effectene Transfection Reagent (Qiagen GmbH, Hilden, Germany), in accordance with the manufacturer's instructions using pEGFP-C2 (Clontech, Palo Alto, CA, USA) plus the siRNA oligonucleotide targeted to GPER or the siRNA negative control. After 19 h of treatment, the cultures were processed for immunostaining. The same plasmid and siRNAs were nucleofected into cultured neurons using an Amaxa nucleofector with the Mouse Neuron kit (Amaxa, Gaithersburg, MD, USA) in accordance with the manufacturer's instructions and, after 1 DIV, the neurons were harvested and processed for real-time polymerase chain reaction (PCR) and Western blot analysis.

2.5. Quantitative real-time polymerase chain reaction (q-PCR)

Total RNA was extracted from cultures with illustra RNAspin Mini RNA isolation kit from GE Healthcare (Buckinghamshire, UK). First strand cDNA was prepared from RNA using the First Strand Synthesis kit from Fermentas GMBH (St. Leon-Rot, Germany) following the manufacturer instructions. Quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detector (Applied Biosystems, Weiterstadt, Germany). TaqMan probes and primers for Ngn3 and for the control housekeeping

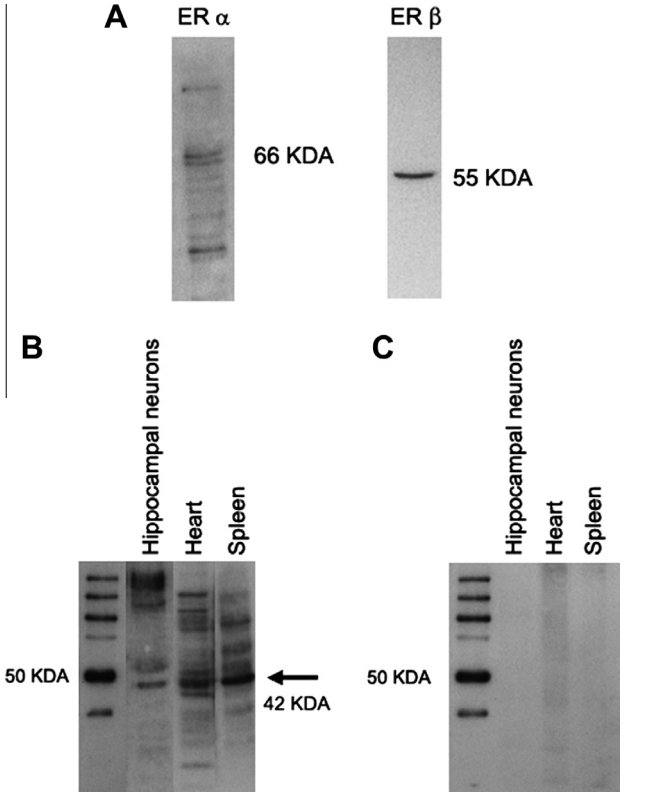

Fig. 1. Primary hippocampal neurons express ERα, ERβ and GPER. (A) Representative Western blots showing the expression of ERα and ERβ in control hippocampal cultures at 3 DIV. (B) Representative Western blot showing the expression of GPER in control hippocampal cultures at 3 DIV, and in the heart and spleen of adult female mice. A band corresponding to the expect molecular weight of GPER (42 kDa) is detected. (C) Preincubation of the primary antibody with the blocking peptide for GPER resulted in the absence of bands.