

## S1

**Oestrogen-related activities in neuronal systems: a novel challenge for old friends**

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Oestrogen is an excellent partner of the brain, protecting this organ against injury and degeneration. The end of this wonderful relationship is marked by the menopause that provokes a dramatic decline of oestrogen levels. One of the consequences of this decline is Alzheimer's disease (AD), a degenerative pathology of the nervous system characterized by a progressive loss of memory and cognitive functions. A prominent feature of AD is the presence of extracellular neuritic plaques mainly formed by amyloid- $\beta$  peptide ( $A\beta$ ) that contribute to dysfunction in septal cholinergic circuits. In an attempt to maintain these friends together, oestrogen replacement therapy administrated to menopausal women has been shown to partially palliate this damage (Brinton, 2001). However, the beneficial effects of this therapy in long-term treatments are still controversial as they need to be quantified after a prolonged follow-up. Oestradiol-mediated prevention of cell degeneration has also been described in cellular paradigms of AD neurotoxicity. These neuro-protective effects have been ascribed to oxidative stress in the injured brain, suppression of intracellular  $Ca^{2+}$  elevation induced by  $A\beta$ , inhibition of toxicity related to acetylcholinesterase- $A\beta$  complexes, modulation of  $A\beta$ -induced apoptosis and increase of  $A\beta$  microglial uptake to decrease  $A\beta$  load in the brain (Garcia-Segura *et al.* 2001). However, the importance of oestrogen receptors (ERs) and the potential mechanisms of action of these proteins in prevention of brain injury are largely unknown.

Here we present recent evidences demonstrating that oestrogen may exert neuroprotection against  $A\beta$ -induced toxicity by modulation of classical ER-mediated mechanisms and alternative membrane-related pathways. These studies have been performed in an SN56 neuronal line. These murine septal cells are considered a good model to study the mechanisms of oestrogenic prevention of neurotoxicity as they show cholinergic, peptidergic and nitrergic properties (Martinez-Morales *et al.* 2001), respond to  $A\beta$ -related toxicity and constitutively express ERs which show transcriptional activity (Marin *et al.* 2001). Using the trypan blue exclusion method, we found that cell death provoked by the 1–40 residue of  $A\beta$  ( $A\beta$ 1–40) was significantly reduced in a dose-dependent manner by long exposure (24 h) to physiological concentrations of oestradiol. Palliation of cell death was blocked in the presence of the specific ER antagonist ICI182,780, suggesting the participation of classical ERs in the modulation of  $A\beta$ -induced toxicity. ER levels of expression were up-regulated by the hormone during injury, as observed by RT-PCR, Western blot and confocal microscopy. Interestingly, part of oestradiol neuroprotection was obtained within 15 min after hormone application and was reproduced with oestradiol coupled to HRP. We propose that, in this model, cell protection by oestradiol also requires the involvement of a putative membrane form of ER (mER) that may be structurally related to classical ER. This affirmation is based first on the fact that, using specific anti-ER $\alpha$  antibodies, we have detected by plasma membrane fractionation and immunocytochemistry what appears to be an ER $\alpha$  at the surface of SN56 neurons. Second, oestrogen protection triggered at the plasma membrane was palliated by both ICI182,780 and a specific antiserum directed to canonical ER $\alpha$ . In addition, affinity cytochemistry employing

two membrane-impermeant oestrogen conjugates (E-HRP and E-BSA-FITC) revealed binding sites on the surface of SN56 cells that were competitively inhibited by oestrogen, anti-oestrogen and ER $\alpha$  antibodies. These results let us to hypothesize that oestrogenic actions in the prevention of  $A\beta$ -induced injury may be initiated via an ER residing at the membrane and co-ordinated to intracellular ERs that may ultimately modulate some of the pathophysiological processes related to AD. Further understanding of the discrete actions by which steroids act in both classical and alternative mechanisms to induce protection of brain integrity may provide alternative targets to maintain this love story in the best terms.

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## S2

**Rapid actions of oestrogen in the anterior pituitary**

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## S3

**Oestrogen receptor  $\beta$  mediates rapid oestrogen actions on GnRH neurons *in vivo***

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The gonadal steroid oestrogen exerts an important modulatory influence upon the activity of multiple neuronal networks. In addition to classical genomic mechanisms of action through the two oestrogen receptors, ER $\alpha$  and ER $\beta$ , oestrogen also exerts poorly understood rapid non-genomic effects on neurones. We have examined whether the gonadotropin-releasing hormone (GnRH) neurones, which regulate fertility, are also influenced by oestrogen in a non-genomic manner. Using a transgenic mouse model in which GnRH neurones are tagged with GFP we undertook gramicidin, perforated-patch recordings of GnRH neurones in the acute brain slice preparation. Oestrogen (100 nM) was found to rapidly depolarize approximately 40% of GnRH neurones. To evaluate whether rapid oestrogen actions may also occur *in vivo*, ovariectomised wild-type mice were given oestrogen (1–10  $\mu$ g) and the phosphorylation status of cAMP response element-binding protein (CREB) examined within the GnRH neurones using immunocytochemistry. An increase in CREB phosphorylation within GnRH neurones was observed as soon as 15 min following oestrogen administration and found to be both time and dose dependent. Studies in both of the ER knockout mice were then undertaken to evaluate whether either of the classical ERs were involved in the rapid oestrogen actions upon GnRH neurones *in vivo*. Whereas the response was maintained in the ER $\alpha$  knockout mouse, the ability of oestrogen

to phosphorylate CREB in GnRH neurones was blocked completely in the ER $\beta$  knockout mouse. Previous studies have established that GnRH neurones express only ER $\beta$  (Herbison & Pape, 2001). A final series of *in vitro* experiments demonstrated that oestrogen acts directly upon GnRH neurones to rapidly phosphorylate CREB and that oestrogen must pass through the cell membrane to achieve this effect. Together, these experiments demonstrate the presence of a functional ER involved in rapid oestrogen signalling within the GnRH neuronal phenotype and provide *in vivo* evidence for a role of ER $\beta$  in mediating non-genomic oestrogen signalling within the brain.

Herbison AE & Pape JR (2001). *Front Neuroendocrinol* **22**, 292–308.

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#### S4

### Control of vascular smooth muscle electrical activity by oestrogens

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#### S5

### Oestrogen actions in the islet of Langerhans

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The endocrine pancreas is not considered a classic oestrogen target, although the effects of 17 $\beta$ -oestradiol in some physiological aspects of the islet of Langerhans have been known for a long time (Sutter-Dub, 2002). On the one hand, the level of plasma insulin is increased in pregnant rats in response to increased levels of sex steroids. Moreover, 17 $\beta$ -oestradiol at concentrations comparable to that of pregnancy enhances insulin secretion in perfused rat pancreas. On the other hand, in glucagon-releasing  $\alpha$ -cells it was described that 17 $\beta$ -oestradiol produces an inhibitory effect on glucagon secretion. In spite of this evidence, the mechanism of action employed by 17 $\beta$ -oestradiol upon  $\alpha$  and  $\beta$  cells is still largely unknown.

Recently, we have described a non-classical membrane oestrogen receptor (ncmER) in pancreatic  $\beta$ -cells (Nadal *et al.* 2000). It is responsible for a rapid insulinotropic effect of 17 $\beta$ -oestradiol when applied at physiological concentrations. Once bound to its membrane receptor, oestrogen triggers the synthesis of cGMP, which in turn activates protein kinase G. Then the ATP-dependent potassium channels (K<sub>ATP</sub>) close in a PKG-dependent manner, causing the plasma membrane to depolarize, enhancing [Ca<sup>2+</sup>]<sub>i</sub> (intracellular calcium) signals (Ropero *et al.* 1999). As a result, insulin secretion is increased and the transcription factor CREB is activated (Quesada *et al.* 2002). This receptor not only exists in pancreatic  $\beta$ -cells, but in  $\alpha$ -cells as well (Ropero *et al.* 2002). When 17 $\beta$ -oestradiol acts through this receptor in  $\alpha$ -cells, it inhibits low-glucose-induced [Ca<sup>2+</sup>]<sub>i</sub> oscillations and therefore will abolish glucagon release.

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