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Oestrogen and progestins differently prevent glutamate toxicity in cortical neurons depending on prior hormonal exposure via the induction of neural nitric oxide synthase

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ABSTRACT

Sex steroids are important for brain function and protection. However, growing evidence suggests that these actions might depend on the timing of exposure to steroids. We have studied the effects of steroid administration on the survival of neural cells and we have partially characterized the possible mechanisms. The effect of a 24 h pre-treatment with 17β-estradiol or 17β-estradiol plus progesterone or medroxyprogesterone acetate on the toxic action of L-glutamate was used to test the experimental hypothesis. Pre-exposure to either steroid combinations turned in enhanced cell survival. Instead, addition of sex steroids together with L-glutamate, in the absence of a pre-exposure had no protective effect. Pre-treatment with the steroid combinations resulted in increased neural NOS expression and activity and blockade of NOS abolished the cytoprotective effects of steroids. These results suggest that NOS induction might be involved in sex steroid-induced neuroprotection. Furthermore, these data supports the hypothesis that prolonged and continued exposure to oestrogen and progesterone, leading to changes in gene expression, is necessary to obtain neuroprotection induced by sex steroids.

1. Introduction

Sex steroid hormones exert prominent regulatory effects on the central nervous system, acting on the neuroendocrine system, mood, behaviour, and nociception [1] and inducing protective effects on brain cells [2–7]. Oestrogen administration counteracts the climacteric neuroendocrine changes including endorphin and neurosteroid synthesis in selected brain areas [8–10]. In perimenopausal women oestrogen therapy positively acts on short-term memory and cognitive function [11]. Whether these actions are linked to the reduced incidence of neurodegenerative diseases is still a matter of debate and recent studies suggest that the alleged neuroprotective effects of oestrogen might be limited to healthy women starting hormone replacement therapy at the time of menopause while oestrogen may induce detrimental actions in women initiating hormone therapy at later stages [12–14]. It is supposed that healthy neural cells, continuously exposed to sex steroids, receive benefit from further steroid administration for both neurological function and survival [7]. In contrast, a further oestrogen exposure to previously sex steroid-deprived neural cells could exacerbate neurological failure [7]. This hypothesis however remains to be proved.

One of the potential mediator of brain cell neuroprotection could be nitric oxide (NO) release induced by oestrogen [3]. In both peripheral and brain tissues oestrogen increases NO synthesis [15,16]. In female hippocampus, 17β-estradiol (E2) induces a rapid increase in NO levels and NOS activity, thus improving hippocampal function [15].

In parallel, the role of natural progesterone (P) or synthetic progestins on brain cells is largely unclear [17]. Recently, P has been demonstrated to increase respiratory function of neural mitochondria, thus reducing oxidative stress and attenuating endogenous oxidative damage in the brain [18]. Furthermore, growing evidence suggests protective effects of P on neural cells [19,20], but the mechanisms of action including inducible effects on NO synthesis or possible differences between P and synthetic progestins are not explored. P modulates the expression of the inducible NOS (iNOS) in response to inflammatory stimuli [21] and the combination of E2 plus P increases the expression of the neural isoform, nNOS, in

Abbreviations: E2, 17β-estradiol; P, progesterone; MPA, medroxyprogesterone acetate; NOS, nitric oxide synthase; nNOS, neural nitric oxide synthase; NO, nitric oxide.

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neural cells [22] but the possible relevance for neuroprotection is not known.

In this paper, we present the different impact of E2 alone or E2 plus P/medroxyprogesterone acetate (MPA) on survival of cortical neurons, depending on the timing of exposure of the cells to the sex steroids. Moreover we demonstrate the involvement of nNOS modulation in these actions.

2. Experimental procedures

2.1. Cell cultures and treatments

Primary cultures of cortical neurons were obtained from Embryonic Day 18 (E18d) rat fetuses as previously described [23–25]. Briefly, cortical cells were dissected and treated with 0.02% trypsin in HBSS (Invitrogen, Grand Island, NY) for 5 min at 37 °C, and dissociated by repeated passage through a series of fire-polished constricted Pasteur pipettes. Cells were plated on poly-D-lysine-coated 60 mm Falcon Petri dishes at a density of $0.5–1 \times 10^5$ cells/cm^2 for biochemical study. Cells were plated on NalgeNunc (Naperville, IL) CC_2-coated four-well chamber slides at a density of $2–4 \times 10^4$ cells/cm^2 for morphological study. Neurons were grown in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 10 U/ml penicillin, 10 μg/ml streptomycin, 0.5 mM glutamine, 25 μM glutamate, and 2% B27 (Invitrogen, Gaithersburg, MD). These culture conditions are well established in multiple laboratories, including our own, to generate cultures that are >95% neuronal and ≤5% glial [24,25]. Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. After that, cells were seeded in 96 wells culture plates, following Fig. 1, and then supplemented with 17β-estradiol, progesterone, medroxyprogesterone acetate alone or in co-administration (Fig. 1A) or starved for 24 h with steroid deprived medium without glutamine and then treated (Fig. 1B). In this low-steroid concentrated medium, steroids concentration were <4.7 pg/ml for oestrogen and <0.12 ng/ml for progesterone.

Neurotoxicity was induced with 100 μM glutamate for 24 h [26].

L-NAME concentration was chosen on the basis of a previous toxicity test on neural cells (data not shown).

2.2. Colorimetric (MTT) assay for cell survival

Cell survival was analyzed by MTT test (Chemicon International Darmstadt, Germany, Catalog N CT02), see manufacturer's data sheets for details. In brief, cortical neurons were counted (around 30,000 cells for each well) and seeded in 96 wells. After the treatment, cells were washed with MEM and exposed to MTT solution for 4 h at 37 °C. Then, cell were analyzed on ELISA plate reader with a test wavelength of 570 nm and a reference of 630 nm.

2.3. Immunocytochemistry

Cortical neurons on gridded coverslips (Fisher scientific, Pittsburgh, PA, USA) were fixed in paraformaldehyde (4%). Fixed neural cells were permeabilized with 0.5%Triton/PBS. Cells were incubated with anti nNOS (AB5380, Chemicon International, Temecula, CA at dilution of 1:200) overnight at 4 °C or anti cyt *c* (1:300 Cell Signalling, USA) for 3 h at RT followed by incubation in fluorescein-conjugated goat anti-rabbit secondary antibody (1:250, Vector Laboratories) and in Texas Red (1:1000, Amersham) for 1 h at RT. Cells were mounted in Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

A semiquantitave imunocytochemistry was performed by assessing the intensity of the nNOS fluorescence after conversion of the coloured pixels to greyscale using the Leica QWin image analysis and processing software. This analysis was performed selecting random boxes including the extra- and intra-cellular space across the membrane, and the linear intensity of the signal was spatially recorded. We sampled five areas per each cell, and we repeated this on 30 different cells per experimental condition

2.4. NOS activity assay

NOS activity was determined as conversion of [^{3}H]arginine to [^{3}H]citrulline in neural cell lysates. [^{3}H]Citrulline was separated using an acidic ion-exchange resin, as described [27]. Extracts incubated with L-NAME (1 mM), served as blank.

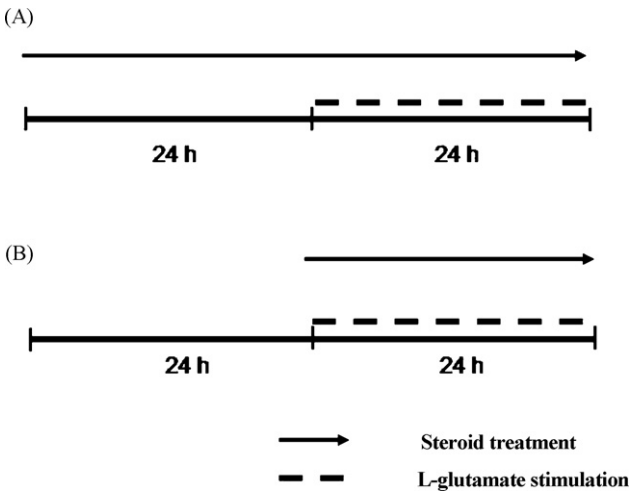

Fig. 1. Two models used: neural cells have been washed in PBS buffer and then seeded in 96 wells plate. Afterwards, cells have been studied for 48 h in different conditions. Treatment (A): E2, P and MPA have been added to neural cells for 48 h. After 24 h sex steroid exposure, neural cells have been treated with E2, P and MPA and L-glutamate simultaneously. Treatment (B): neural cells have been starved for 24 h in steroid deprived medium and E2, P and MPA have been added for only 24 h simultaneously with L-glutamate.

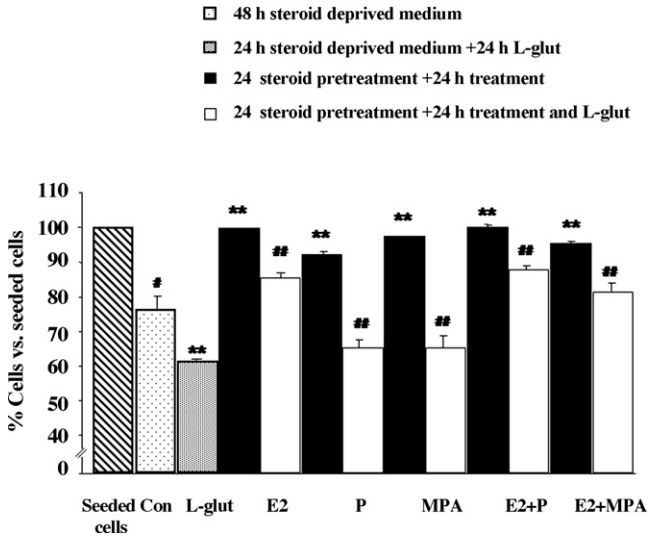

Fig. 2. Sex steroid pre-treatment promotes survival and protects cells from toxic stimuli. 48 h steroid administration significantly induces neural cell survival in comparison to 48 h untreated cells (Con). White bars show cells which have been treated with L-glutamate for 24 h after sex steroid pre-treatment. $^{**}P < 0.01$ vs. Con (48 h steroid deprived cells). $^{\#\#}P < 0.01$ vs. L-glutamate (24 h steroid deprivation plus 24 h L-glutamate). $^{\#}P < 0.01$ vs. seeded cells. Data shown are from a single experiment and are representative of three independent experiments.