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Research Paper

Glial response to 17β-estradiol in neonatal rats with excitotoxic brain injury

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ABSTRACT

White-matter injury is the most common cause of the adverse neurodevelopmental outcomes observed in preterm infants. Only few options exist to prevent perinatal brain injury associated to preterm delivery. 17β-estradiol (E2) is the predominant estrogen in circulation and has been shown to be neuroprotective in vitro and in vivo. However, while E2 has been found to modulate inflammation in adult models of brain damage, how estrogens influence glial cells response in the developing brain needs further investigations. Using a model of ibotenate-induced brain injury, we have refined the effects of E2 in the developing brain. E2 provides significant neuroprotection both in the cortical plate and the white matter in neonatal rats subjected to excitotoxic insult mimicking white matter and cortical damages frequently observed in very preterm infants. E2 promotes significant changes in microglial phenotypes balance in response to brain injury and the acceleration of oligodendrocyte maturation. Maturational effects of E2 on myelination process were observed both in vivo and in vitro. Altogether, these data demonstrate that response of glial cells to E2 could be responsible for its neuroprotective properties in neonatal excitotoxic brain injury.

1. Introduction

Preterm birth is a common condition that affects roughly 1 in every 10 pregnancies, including in developed countries. In 2010, the Global Burden of Disease Study estimated that preterm birth was the most frequent cause of death and disability in children under the age of 5 years (Liu et al., 2012). Indeed, in addition to the immediate problem posed by the care of preterm infants and complications due to the immaturity of their organs, in 25–50% of them, prematurity is associated with long-term neurological disability, cerebral palsy, cognitive impairment and behavioral deficits, presenting enormous challenges to individuals, their families and society. This high rate of disability is partly a consequence of the improved survival of more and more immature infants, thanks to innovations in perinatal medicine over the past 30 years (Marlow et al., 2005). There are currently few treatment options to prevent brain injury associated to preterm delivery (Favrais et al., 2014). The need for novel strategies and care practices to curb the neurodevelopmental sequelae following premature birth is thus urgent.

White-matter injury is the most common cause of the adverse neurodevelopmental outcomes observed in preterm infants (Back and Rosenberg, 2014). In neonatal rodents, excitotoxic injury using glutamate agonists mimics some aspects of the diffuse white and grey matter abnormalities, collectively known as encephalopathy of prematurity, observed in the brain of human preterm neonates (Gressens et al., 1996; Volpe, 2009). Recent evidence suggests the existence of crosstalk between oligodendrocytes and their surroundings, including microglial cells and the hormonal environment. The injury-mediated activation of microglial cells plays a key role in disrupting the developmental maturation of the oligodendroglial lineage (Kaindl et al., 2009), and potentiates white matter injury and cognitive dysfunction into adulthood (Moretti et al., 2015).

Estrogens are lipophilic steroid hormones that can diffuse across the blood-brain barrier. However, the role of estrogens is not limited to the maintenance of female reproductive function and they have remarkable effects at various concentrations on several systems including the cardiovascular, immune, and nervous systems (Arevalo et al., 2015). 17β-

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estradiol (E2), an isomer of estradiol, is the predominant estrogen in circulation and has been shown to be neuroprotective in vitro and in vivo in various models of brain damage (Arevalo et al., 2010; McCarthy, 2008; Gerstner et al., 2007; Gerstner et al., 2009). E2 directly promotes cell survival and synaptic plasticity, prevents axonal and dendritic pruning, and modulates levels of neurotransmitters and their receptors, leading to improved cell survival and neurite branching. However, while E2 has been found to modulate inflammation in adult models of brain damage, whether estrogens also influence the crosstalk between neural cells in the developing brain remains unclear.

The aim of this study was therefore to explore whether E2 could protect the neonatal brain from excitotoxic brain injury through its effects on various glial cell types. To do this, we used both the model of ibotenate-induced brain injury mentioned above (Gressens et al., 1996) and various primary cell cultures.

2. Materials and methods

All experiments were carried out in compliance with the ethical rules of the INSERM. The study and animal protocols were approved by the institutional review board (Bichat-Robert Debré ethics committee, Paris, France, approval number 2010-13/676-0010).

2.1. Animals and model of excitotoxic brain lesions

Rats (Sprague-Dawley, Janvier SAS, Le Genest-St-Isle, France) were housed in a temperature-controlled room (19 °C–23 °C) with a 12 h/12 h light/dark cycle and with food and water ad libitum. Ten micrograms Ibotenate (Tocris, Bristol, UK) diluted in Phosphate Buffered Saline (PBS) was injected intracerebrally (i.c.) in postnatal day 5 (P5) rat pups as previously described (Pansiot et al., 2010). Ibotenate is a glutamate analogue that activates both *N*-methyl-D-aspartate (NMDA) and metabotropic receptors, but does not activate alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) or kainate receptors. Injections were performed using a 26-gauge needle and a 50 μl Hamilton syringe mounted on a calibrated microdispenser. The needle was placed in the frontoparietal area of the right hemisphere, 2.5 μm from the midline in the mediolateral plane, and 4 mm from the bregma in the rostrocaudal plane. Two 1 μl boluses of ibotenate were injected with an interval of 20 s between the two. The needle was left in place for an additional 20 s.

2.2. β-estradiol injections

The animals received a first injection of E2 12 h before the ibotenate injection, and a second at the same time as ibotenate. Pre/per exposure injection regimen was chosen for covering the initial phase of brain injury process. Beta-estradiol (E2) (Sigma, E8875) was first dissolved in DMSO and then diluted in PBS with a final concentration of 5% DMSO, and injected intraperitoneally (i.p., 100 μl each injection) at 4, 40 or 400 μg/kg. Control animals received similar volumes of vehicle (PBS 5% DMSO).

2.3. Dosage of estrogen

Animals were anesthetized with isoflurane and blood collected by cardiac puncture at 3 time points: 3 h after the first E2 injection (H3), 12 h after the first injection (H12) and 3 h after the second injection (H15). The clotted blood was centrifuged (7 min, 4000 rpm) and the serum pooled to obtain 200 μl per time point (n = 4–5), frozen and stored at −80 °C for hormonal measurements. E2 concentrations were measured using the Estradiol HS RIA kit (Immunodiagnostic systems, Paris, France, OD-67031).

2.4. Determination of lesion size

In each treatment group, 10–15 animals were sacrificed by decapitation 5 days after ibotenate injection (i.e. on P10). The brain was harvested, immediately fixed in 4% formalin and kept in this solution for 5 days before paraffin embedding. Coronal sections 16 μm thick were cut and every third section was mounted and stained with cresyl violet. The size of cortical and white matter lesions can be defined by their extent along 3 orthogonal axes: the mediolateral axis (in the coronal plane), the radial axis (also in the coronal plane, from the pial surface to the lateral ventricle), and the fronto-occipital axis (in the sagittal plane). In previous studies using this model (Husson et al., 2002), we observed an excellent correlation among measurements along the 3 axes. Based on these findings, we cut serial sections of the entire brain in the coronal plane for this study. This permitted the accurate and reproducible determination of the fronto-occipital extent of the lesion in the sagittal plane. We used this measurement as an index of lesion volume. All animals, and either males or females injected with 400 μg/kg were analyzed separately for lesion size determination.

2.5. Immunohistochemistry

In each experimental group, we studied 8 to 10 pups on postnatal days P6, P10 depending on experiments.

Paraffin sections were immunolabeled with primary antibodies listed in Table 1 and labeling visualized using the streptavidin-biotin-peroxidase method and the chromogen diaminobenzidine, as previously described (Baud et al., 2003).

2.6. RNA purification and real-time PCR

Four hours after ibotenate injection, at least 8 animals per group (ibotenate ± E2) were sacrificed by decapitation, the brains removed, a sample of the brain cortex and underlying white matter harvested at the i.c. injection site (ipsilateral) and at a similar location in the contralateral hemisphere, immediately snap-frozen in liquid nitrogen and stored at −80 °C. Total RNA from the samples was extracted with the RNeasy mini kit according to the manufacturer's instructions (Qiagen, Courtaboeuf, France). RNA quality and concentration were assessed by spectrophotometry using a NanoDrop™ apparatus (Thermoscientifique, Wilmington, DE, USA). 1 μg of total RNA was subjected to reverse transcription using the Iscript™ cDNA synthesis kit (Bio-Rad, Marnes-la-Coquette, France). qRT-PCR was performed in duplicate for each sample on a CFX384 Real Time System (Bio-Rad), using SYBR Green Supermix (Bio-Rad) for 40 cycles. Amplification specificity was assessed by melting curve analysis. Primers were designed using Primer3 software and manufactured by Eurofins Genomics (Ebersberg, Germany). Primer sequences are summarized in Table 2. The expression of genes of interest was calculated relative to the expression of the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Analyses were performed using Bio-Rad CFX Manager 3.0.

Table 1
Antibodies and markers used in the experiments.

Antibody/marker	Manufacturer	Ref number	Dilution
APC	Calbiochem	OP80	1/500
CD68 (ED1)	Serotec	MCA341B	1/500
MBP	Chemicon	MAB382	1/500
Olig2	IBL	18953	1/200
Tomato lectin	Vector Labs	B-1175	1/800