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Astrocytic reaction to a lesion, under hormonal deprivation

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Abstract

Gonadal hormones can influence the morphology and function of glial cells, particularly astrocytes. Here we explore the hypothesis that 17 β -estradiol (E2) exerts a positive effect on astrocytes within the region of the cholinergic neurons of the basal forebrain, an area heavily implicated in memory and attentional processes. Female rats were ovariectomized at 3 months of age and lesioned with the immunotoxin 192 IgG-saporin before receiving a subcutaneous pellet containing 0.25 mg of estrogen or placebo, released over 60 days. The control, non-ovariectomized group was treated identically. At the end of the treatment, we used image analysis procedures to evaluate changes in the levels of glial fibrillary acidic protein (GFAP) expression in the area of the lesion. Infusion of the immunotoxin induced a slight increase in GFAP expression in some subjects, compared to the contralateral side. However, when differences within animals were factored in, GFAP expression in ovariectomized animals treated with E2 was undistinguishable from intact controls. By contrast, in ovariectomized animals treated with placebo, GFAP expression was significantly higher. These results suggest that E2 deprivation may exacerbate the effects of an immunotoxic lesion, and, more importantly, that E2 administration may contribute to structural recovery of lesioned cholinergic neurons by blocking GFAP expression in the area. These results are particularly relevant in the context of female aging and postmenopausal dementia, and further highlight other potential levels at which to design interventions to preserve an intact cholinergic system, which may be crucial to prevent Alzheimer's disease.

Keywords

Glia; Basal forebrain; Plasticity; Estrogen; Regeneration; Female rat

Estradiol (E2) enhances neuronal response to injury [4] and also exerts an effect on astrocytes, influencing a complex interaction that may be beneficial or detrimental to neuronal recovery. For example, neurite outgrowth can be stimulated by a variety of molecules secreted by astrocytes, but can also be inhibited by the formation of a glial scar. Astrocytes proliferate after injury [11,18], and those closer to the site of injury become hypertrophic and highly immunoreactive for the glial fibrillary acidic protein (GFAP) [6]. GFAP, the major constituent of neuroglial filaments, is mainly localized in fibrous astrocytes [5].

Interestingly, glial cells express receptors for sex steroids [10], and respond to changes in the levels of circulating hormones. Garcia-Estrada et al. [7] have demonstrated that gonadal

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hormones down regulate reactive gliosis and astrocyte proliferation after a penetrating brain injury. A selective immunolesion with 192-IgG saporin induces microglial, but no overt astroglial response in the basal forebrain [1,9], although it does activate astrocytes as shown by induction of c-jun immunoreactivity colocalized with GFAP [14]. Work by Rozovsky and co-workers has shown that E2 can be a mediator of neuronal sprouting through its effects on astrocytes [15,17]. It has also been shown that E2 can repress GFAP expression [3,15,17]. We hypothesized that the E2-induced neuronal outgrowth already described in the cholinergic basal forebrain [16] could be associated with attenuation in the expression of GFAP.

Twenty-eight female Fisher 344 rats, 3 months old, were obtained from the NIA colony at Harlan, 14 ovariectomized (OVX) and 14 intact (NOVX). All experimental procedures were conducted with approved institutional animal care protocols and in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23). Animals were housed individually in an environmentally controlled room with a 12-h light:12-h dark ratio and *ad libitum* access to food and water. One week after arrival, all animals received a 200 nl injection of a solution of 192 IgG-saporin (0.075 mg/ml in PBS) into the HDB, using the following coordinates [13]: AP -0.6; DV -0.8 and RL +2.0. These animals were also included in experiments describing the effect of the lesion on surviving cholinergic neurons [4], and they were allowed to survive 4 weeks to ensure a full development of the lesion. Thirty days after surgery all animals were implanted at the level of the scapula with a subcutaneous pellet containing either 0.25 mg of E2 or placebo (PL), generating four experimental groups: OVX+E2 ($n = 7$), OVX+P ($n = 7$), NOVX+E2 ($n = 7$) and NOVX+P ($n = 7$). These pellets are designed to release 4.167 μ g of E2 daily for 60 days. The composition of the placebo pellets is identical except they lack the hormone. At the end of the treatment the rats were sacrificed by intracar-dial perfusion with 4% paraformaldehyde under deep anesthesia, the brains were extracted, and cut into 40 μ m sections. The sections were immunocytochemically stained using a polyclonal antibody against GFAP made in rabbit (1:25,000, Sigma). After preincubation in a solution of PBS with 0.25% Triton X-100 and 3% H₂O₂ for 30 min to block endogenous peroxidase activity, sections were washed with 5% non-fat dry milk in PBS with Triton X-100 for 60 min at room temperature. Sections were then incubated overnight at 4 °C in the primary antibody, followed by several rinses with PBS, incubation in biotinylated secondary antibody for 60 min, further washes, and a 60-min incubation in the ABC solution (Elite Kit, Vector Labs) at 1:500 dilution. After several additional washes, a peroxidase reaction was carried out with 0.05% 3–3' diaminobenzidine (DAB, Sigma) and 0.01% H₂O₂. Sections were mounted on glass slides, dehydrated in a graded series of alcohol, cleared in xylene and coverslipped for microscopic analysis (Fig. 1).

The density of GFAP immunoreactivity was calculated within the basal forebrain on both the lesioned and intact side (which served as an internal control) from the same three representative sections separated by 480 μ m (Fig. 2A–C). The sections were photographed with a digital camera attached to an Olympus microscope. Then Photoshop software (Version 5.5, Adobe Systems Incorporated) was used to analyze the images, applying a 450 $\times$ 350 pixels frame. Two separate exposures were made for each section, to ensure that density measurements were reproducible. Specific areas containing GFAP immunoreactivity were selected using as reference the adjacent p75-stained sections [16] and the following parameters: the ventral surface of the brain, the angle with the olfactory tubercle, the midline and the ventral edge of the anterior commissure (Fig. 2). Special care was taken to place the frame in exactly the same location on both sides of the brain for each animal using the fiduciary landmarks described above. Frame size was kept constant across the whole study. Each image was converted to grayscale and semi-automatic thresholding was used, comparing the grayscale to the original, to ensure that all relevant staining was included in the analysis. The program then automatically graphed a histogram indicating the number of pixels at each brightness level within the image. The mean value from the histogram, which is a measure of brightness, was used as a quantitative

measure of the density of GFAP immunoreactivity within the area inside the frame. To minimize differences among animals, the data were converted to percentage values using the following formula: $\text{lesioned} \times 100 / \text{control}$. Three percentages were calculated per animal (corresponding to the three sections per animal), and averaged. The average value was used as single data point for each experimental animal, and further corrected with respect to the control values obtained from the unlesioned side in the NOVX+P group. Staining density in the lesioned and control sides was compared using non-parametric statistics, applying Spearman Rank Correlation paired test. Probability level was set at 0.05%.

An initial qualitative assessment showed a slight increase in the number of GFAP immunoreactive astrocytes on the side of the immunotoxic injection (Fig. 1A), compared to the control (intact) side (Fig. 1B). This was particularly noticeable in OVX+P animals, as illustrated in Fig. 2D.

The morphometric analysis revealed a significant interaction between hormone levels and the expression of GFAP in the area of the lesion. E2 treatment, whether from natural sources (the NOVX+P group) or from the pellet (the OVX+E group) was able to maintain GFAP expression on the area of the lesion within levels not significantly different from the unlesioned side. When the results from the OVX groups were compared to the NOVX+P control, there was a small (6%) but significant ($p < 0.05$) increase in GFAP expression in animals totally deprived of E2 (OVX+P) (Fig. 3). By contrast, when E2 was restored after OVX and the immunotoxic lesion, the values of GFAP expression in the lesioned side returned to control levels.

Using the same animals, we have already shown (Fig. 4) that lack of E2 prevents regenerative outgrowth in damaged cholinergic neurons [4], and our present results suggest that astrocytic activation and a concomitant increase in GFAP expression, may be responsible for this effect.

Work by a number of laboratories has shown that E2 affects the morphology of astroglial cells from several brain regions. For example, Brawer et al. [2] demonstrated an increase in the number of astrocytic inclusions in the arcuate nucleus of female rats as a function of E2 treatment, and others [8] have observed changes in the number of glial processes in contact with arcuate nucleus neurons after E2 treatment in female rats. Those changes were concurrent with remodeling of synaptic terminals, suggesting an implication of glial cells in the synaptic changes induced by E2. Compared to untreated OVX controls, the hippocampus and globus pallidus of OVX+E2 rats also reacted with an increase in cell surface density but no change in the number of GFAP expressing cells [19]. Garcia-Segura and co-workers have suggested that these plastic changes in glial surface area are involved in mechanisms of synaptic plasticity driven by gonadal steroids, which they have termed 'phasic synaptic remodeling' [12].

In agreement with previous findings [4], the results from the present investigation suggest that administration of E2 can reverse lesion-induced neurodegeneration in the cholinergic basal forebrain, and this effect may be mediated through the suppression of GFAP activation.

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Fig 1. (A and B) High power brightfield microphotographs (scale bar = 50 μ m for both) illustrating GFAP immunoreactive astrocytes in the lesioned (A) and intact (B) basal forebrain of ovariectomized female rats treated with placebo. There were no overt differences in the morphological features of individual astrocytes, other than a slight increase in staining intensity.

Fig 2.

(A–C) Schematic drawings depicting the three regions of the basal forebrain included in the quantitative analysis. ac, anterior commissure; MS, medial septum; HDB, nucleus of the horizontal limb of the diagonal band of Broca; mfb, medial forebrain bundle; ox, optic chiasm; GP, globus pallidus. (D) Brightfield microphotographs of sections from the basal forebrain region of the HDB (the area represented by a square in A). Sections were stained with an antibody against GFAP, as described in the text.

Fig 3.

Estrogen treatment prevents the over-expression of GFAP after a lesion in the region of the cholinergic basal forebrain, in ovariectomized rats. Pixel density measurements in the lesioned side of all animals were normalized using the contralateral (spared) side (lesion $\times 100$ /spared) and compared to control values ($=1$). Results show that lack of E2 induces a statistically significant increase in GFAP expression (Spearman Rank Correlation test, $\rho = 0.929$; $p = 0.0229$).

Fig 4.

As reported in detail elsewhere [4], morphometric analysis on the same subjects used for the present study indicated that E2 treatment restores dendritic length in lesioned cholinergic neurons of ovariectomized rats. Dendritic measurements in the lesioned side of all animals were normalized using the contralateral (spared) side and compared to control values (=1). We reported that while E2-deprived animals had a statistically significant decrement in dendritic size (ANOVA and post hoc Fisher's PLSD test, $p = 0.0099$), restoring E2 also restored dendritic length to values not significantly different from control.