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Research report

Tamoxifen favoured the rat sensorial cortex regeneration after a penetrating brain injury

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ABSTRACT

A penetrating brain injury produces a glial scar formed by astrocytes, oligodendrocytes, microglia and NG2 cells. Glial scar is a barrier preventing the extent of damage but it has deleterious effects in the regeneration of the axons. Estradiol and tamoxifen reduce gliosis and have neuroprotective effects in the hippocampus and the spinal cord. We evaluated the proliferation of glia and the electrocorticogram in the sensorial cortex in a brain injury model. At seven days post-injury, estradiol, tamoxifen and estradiol plus tamoxifen reduced the number of resident and proliferative NG2 and reactive astrocyte vimentin+ cells. Estradiol and tamoxifen effects on NG2 cells could be produced by the classical oestrogen receptors found in these cells. The glial scar was also reduced by tamoxifen. At thirty days post-injury, the amount of resident and proliferative astrocytes increased significantly, except in the estradiol plus tamoxifen group, whilst the oligodendrocytes proliferation in the glial scar was reduced in treated animals. Tamoxifen promotes the survival of FOX-3+ neurons in the injured area and a recovery in the amplitude of electrocorticogram waves. At thirty days, estradiol did not favour the suvival of neurons but produced a greater number of reactive astrocytes. In contrast, the number of oligodendrocytes was reduced. Tamoxifen could favour brain repair promoting neuron survival and adjusting glial cell number. It seems to recover adequate neural communication.

1. Introduction

Brain injury produces reactive gliosis (Williams et al., 2006) generating a glial scar for avoiding propagation of inflammation and damage. Astrocytes and NG2 cells participate in glial scar formation (Alonso, 2005). Particularly, NG2 cells are progenitor cells, which differentiate into other cell types: astrocytes, oligodendrocytes and neurons (Aguirre et al., 2004; Zhu et al., 2008; Tripathi et al., 2010). NG2 cells express progesterone receptors (Labombarda et al., 2010) and can be modulated by this hormone. After a demyelinating injury, progesterone induces NG2 proliferation and their differentiation to oligodendrocytes (Labombarda et al., 2009) favouring remyelinitation. In a cerebral penetrating injury model, 17β-estradiol (E2) regulates reactive astrocytes and microglia (Barreto et al., 2009), but it is unknown whether it occurres in NG2 cells. Tamoxifen (TAM) is a selective oestrogen receptor modulator (SERM) and, depending on the tissue, can be an agonist or antagonist of the oestrogen receptors (Dutertre and Smith, 2000). In rat brain, TAM exhibits an antagonist action on oestrogen receptor β (ERβ) (Zhao et al., 2005). Glial scar is a barrier for reconnecting axons in the central nervous system (Hatten et al., 1991; Fidler et al., 1999). In the spinal cord, TAM reduces the inhibitor molecules of axonal regeneration, such as Nogo-A, glycoprotein associated to myelin (MAG) and oligodendrocytes myelin glycoprotein (OMgp) (Tian et al., 2009) therefore favouring axonal regeneration. In an injured nervous system, neuron axons and their connections to other neurons are deteriorated. Consequently, the bioelectrical

Abbreviations: BrdU, 5-bromo-2-deoxy-uridine; E2, 17β-estradiol; E2 + TAM, 17β-estradiol plus tamoxifen; CTL, control; ECoG, electrocorticogram; ERα, oestrogen receptor alpha; ERβ, oestrogen receptor beta; GFAP, glial fibrilar acidic protein; MAG, glycoprotein associated to myelin; IP-10, interferon-gamma-inducible protein-10; IL-1β, interleukin 1 beta; IL-6, interleukin 6; OMgp, oligodendrocytes myelin glycoprotein; PBS, phosphate buffer saline; PKC, protein kinase C; SERM, selective oestrogen receptor modulator; 60 mpi, 60 min post-injury; TAM, tamoxifen; VEH-INJ, vehicle injured.

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brain activity is modified, exhibiting changes in its normal physiological oscillatory waves produced by neural cell populations. A normal brain electrocorticogram (ECoG) exhibits waves of several frequencies: alpha (α, 8–13 Hz), beta (β, 13–30 Hz), gamma (γ, 30–130 Hz), delta (δ, 0.1–4 Hz), and theta (θ, 4–8 Hz), changing their amplitude and morphology, depending on brain status (Kekovic et al., 2010). The wave amplitude change post-injury, as well as the change with E2 and/or TAM treatments in rat sensorial cortex have not been established. A model of penetrating injury in sensorial cortex at seven days post-injury was used for analysing the presence of oestrogen receptors in NG2 cells and for the effects of E2 and (or) TAM on the number and proliferation of NG2 cells and astrocytes. The amount of astrocytes, oligodendrocytes and neurons were also evaluated at thirty days post-injury. In addition, the brain sensorial cortex ECoG amplitude waves were studied.

2. Materials and methods

2.1. Animals

Seventy-five adult male Wistar rats (180–250 g), castrated at forty-five day postnal, were used: twenty-five rats for cell counts at seven days post-injury, twenty-five rats at thirty days and twenty-five rats for electrophysiological study. This study was performed according to Mexican Official Norm (NOM-062-ZOO-1999) for the Care and Use of Laboratory Animals.

2.2. Brain injury model

The rats were anaesthetized with xylazine (10 mg/kg body weight) and ketamine (90 mg/kg body weight). The rats were then placed in a stereotaxic frame and a midline incision was made on the head using stereotaxic coordinates located horizontally 3 mm to the left of bregma and −2 to −4 mm vertically from bregma, according to Paxinos and Watson coordinates. A minidrill was used for performing a cleft on the left parietal bone and a tuberculin needle, for causing a 5.5 mm penetrating brain injury.

2.3. Administration of bromodeoxyuridine

5-Bromo-2-deoxy-uridine (BrdU) was administered intraperitoneally for cell proliferation study. Three doses of 100 mg/kg body weight were applied on the same day of the brain damage and the two subsequent post-injury days.

2.4. Study groups and administration of treatments

All treatments were administered intraperitoneally on days 0, 1 and 2 after injury. The rats were divided into five groups of five: one control group (CTL), without injury, and four, of injured rats which received the following treatments: the animals in the vehicle injured (VEH-INJ) group received a vehicle solution diluted in ethanol (100–200 μl); rats in the E2 group were injected with a dose of 5 mg/kg of estradiol (prepared with 18 mg/ml estradiol, mixed with 200 μl ethanol and rediluted in 1 ml castor oil); TAM group rats received a dose of 1 mg/kg tamoxifen and the E2 + TAM group, rats receiving 5 mg/kg of E2 and 1 mg/kg of TAM.

2.5. Tissue processing and cell count

The animals were anaesthetized with sodium pentobarbital (37 mg/kg) at seven or thirty day post-injury and transcardially perfused with a phosphate buffer saline (PBS), followed by a 4% paraformaldehyde solution. The brain was removed and fixed for 24 h at 4 °C. Subsequently, the studied area (−2 to −4 bregma) was cut into 30 μm brain sections and approximately 60 slices were obtained. These sections were divided systematically into six groups of ten slices. Cells were counted using a digital image system (Image pro-plus 6.0 of Media Cybernetics) adapted to an epifluorescence microscope with a 40× objective. Ten microscopic fields were counted per slice, at a distance of 200 μm from the edge of the lesion. The total studied area was equivalent to 0.52 mm^2.

2.6. Haematoxylin–eosin staining

Haematoxylin–eosin staining was used for assessing the extension of the penetrating brain injury. Brain sections were stained with a 5% haematoxylin solution, followed by a 2% eosin solution.

2.7. Immunohistochemistry

Brain tissue sections were pretreated with 2 N hydrochloric acid solution at 37 °C for 30 min for detecting BrdU positive cells. A 10-min neutralization was then performed with 0.1 M borate buffer (pH 8.5). A PBS washing was performed (three times), the non-specific antigen binding sites were blocked for 1 h at room temperature with a normal 10% goat serum. The sections were incubated overnight with primary antibodies at 4 °C: anti-NG2 (Millipore AB5320 1:400, Millipore 05-710, 1:1000), anti-vimentin (Abcam ab45939, 1:1000), anti-oestrogen receptor alpha (ERα), (Millipore MAB447, 1:1000), anti-ERβ (Millipore 06-629, 1:100), anti-glial fibrilar acidic protein (GFAP) for astrocytes (Millipore AB5804, 1:1000), anti-RIP for oligodendrocytes (Millipore MAB1580, 1:50), anti-FOX-3 for neurons, previously known as NeuN (Kim et al., 2009) (Abcam 104225, 1:1000) and anti-BrdU (Hybridoma Bank G3G4, 1:1000). The slices were washed in PBS for 10 min three times between incubations. The slices were simultaneously incubated 2 h, at room temperature, with the following secondary antibodies: anti-IgG Alexa fluor 488 conjugated anti-rabbit, goat polyclonal (invitrogen A-11008, 1:1000), anti-IgG Alexa fluor 594 conjugated anti-mouse goat polyclonal (invitrogen A-11005, 1:1000) and anti-IgG HRP conjugated (Sigma F-2304, 1:1000). The slices were rewashed in PBS for 10 min. Diaminobenzidine was used for identifying slices that were incubated with HRP-conjugated antibody.

2.8. Electrode implant surgery

Animals were anaesthetized intramuscularly with an injection of xylazine (10 mg/kg, body weight) and ketamine (90 mg/kg body weight). They were kept on a heating pad (37° C) during the surgical procedure. The electrodes (stainless steel screws) were attached to the left parietal bone at the onset and at the end of the cleft drilled on the bone, in contact with the dura mater. The skin was sutured and maintained in aseptic conditions throughout the experimental period. To ground the animal, a silver wire electrode was placed beneath the rat skin during electrophysiological recording.

2.9. Brain electrical activity recordings

An ECoG was recorded under ketamine anaesthesia (before the damage, after 60 min and thirty days post-injury). In each case an ECoG was recorded for 70 s using filters ranging from 0.1 to 500 Hz with a 1000× amplification gain. The ECoG was recorded in analogue form and then digitized using an analogue–digital converter card (Digi-Data 1200) and Axoscope 7 software. Different frequency band analysis was carried out using AxoGraph software. It was submitted to digital band pass filters and five frequency intervals were obtained: α, β, γ, δ, and θ waves. The ECoG record was divided into 3 s periods and the ECoG wave amplitude was rectified for evaluating the total amplitude of each band frequency. A 100% value baseline record was established for each rat prior to the injury and