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# Estradiol Decreases Cortical Reactive Astrogliosis after Brain Injury by a Mechanism Involving Cannabinoid Receptors

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**The neuroactive steroid estradiol reduces reactive astroglia after brain injury by mechanisms similar to those involved in the regulation of reactive gliosis by endocannabinoids. In this study, we have explored whether cannabinoid receptors are involved in the effects of estradiol on reactive astroglia. To test this hypothesis, the effects of estradiol, the cannabinoid CB1 antagonist/inverse agonist AM251, and the cannabinoid CB2 antagonist/inverse agonist AM630 were assessed in the cerebral cortex of male rats after a stab wound brain injury. Estradiol reduced the number of vimentin immunoreactive astrocytes and the number of glial fibrillary acidic protein immunoreactive astrocytes in the proximity of the wound. The effect of estradiol was significantly inhibited by the administration of either CB1 or CB2 receptor antagonists. The effect of estradiol may be in part mediated by alterations in endocannabinoid signaling because the hormone increased in the injured cerebral cortex the messenger RNA levels of CB2 receptors and of some of the enzymes involved in the synthesis and metabolism of endocannabinoids. These findings suggest that estradiol may decrease reactive astroglia in the injured brain by regulating the activity of the endocannabinoid system.**

**Keywords:** diacylglycerol lipase, fatty acid amidohydrolase, monoacylglycerol lipase, *N*-acyl phosphatidylethanolamine phospholipase D

## Introduction

Hormones of the gonadal axis regulate the expression of the enzymes involved in the synthesis and metabolism of endocannabinoids in different peripheral tissues (MacCarrone et al. 2000; Xiao et al. 2002; Guo et al. 2005; Grimaldi et al. 2009; Ribeiro et al. 2009). In addition, gonadal hormones, including estradiol, affect the activity of the endocannabinoid system in the brain and the pituitary (Rodríguez de Fonseca et al. 1994; González et al. 2000; Corchero et al. 2001; Bradshaw et al. 2006; Nguyen and Wagner 2006; Hill et al. 2007). In turn, cannabinoids interfere with the activity of the hypothalamo-pituitary gonadal axis at different levels, having a negative impact on sexual behavior and sexual motivation (Ferrari et al. 2000; López 2010; López et al. 2010). In addition, both, cannabinoids and estradiol, have antagonistic actions in the control of nonreproductive functions, including cognition (Daniel et al. 2002) and energy homeostasis (Kellert et al. 2009), while estradiol enhances antinociceptive effects of cannabinoids (Craft and Leitl 2008) and may regulate emotional behavior through an endocannabinoid mechanism (Hill et al. 2007).

Both cannabinoids (Panikashvili et al. 2001; van der Stelt and Di Marzo 2005; Mechoulam and Shohami 2007; Sagredo et al. 2007; Galve-Roperh et al. 2008; Correa, Mestre, et al. 2009) and estradiol (Bourque et al. 2009; DonCarlos et al. 2009; Pike et al. 2009; Simpkins et al. 2009; Suzuki et al. 2009) are neuroprotective in different animal models of neurodegenerative diseases. However, the possible interactions of cannabinoids and estrogens on neuroprotection have not been sufficiently explored. Nevertheless, a recent study has shown that neuroprotective actions of estradiol, in a model of focal cerebral ischemia, are associated to the downregulation of the striatal levels of the endocannabinoid anandamide (AEA), which are increased after ischemia (Amantea et al. 2007). This suggests that endocannabinoids and estrogens may interact in the promotion of neuroprotection.

Glial cells are involved in the neuroprotective actions of cannabinoids and estrogenic compounds. Cannabinoids are known to regulate inflammation in the central nervous system acting on glial and endothelial cells (Eljaschewitsch et al. 2006; Panikashvili et al. 2006; Arévalo-Martín et al. 2008; Correa, Mestre, et al. 2009; De Filippis et al. 2009; Mestre et al. 2009). For instance, AEA, via the activation of CB1 receptors, enhances the synthesis of the cytokine interleukin (IL)-6 and reduces the synthesis of the proinflammatory cytokine tumor necrosis factor (TNF) α in Theiler's virus-infected astrocytes (Molina-Holgado et al. 1997, 1998). In addition, AEA inhibits the release of nitric oxide by astrocytes exposed to lipopolysaccharide (LPS) (Molina-Holgado, Molina-Holgado, et al. 2002). Activation of CB1 receptors also inhibits nitric oxide release by C6 rat glioblastoma cells exposed to neurotoxic stimuli (Esposito et al. 2001, 2002) and decreases amyloid beta–induced reactive astrogliosis, assessed by the levels of glial fibrillary acidic protein (GFAP) and S100B (Esposito et al. 2007). CB2 receptors also mediate anti-inflammatory actions of cannabinoids on astrocytes (Sheng et al. 2005, 2009) and microglia (Correa, Docagne, et al., 2009; Sagredo et al. 2009). The anti-inflammatory effects of cannabinoids on glial cells involve the inhibition of nuclear factor (NF) κB–induced transcription (Curran et al. 2005; Zhang and Chen 2008; Kozela et al. 2010).

Estradiol and different estrogenic compounds also exert anti-inflammatory effects on astrocytes and microglia by mechanisms involving estrogen receptors (Arevalo et al. 2010). For instance, estrogens decrease the expression of nitric oxide and the inflammatory markers TNFα, IL-1β, IL-6, matrix metalloproteinase-9, and interferon-inducible protein-10 in cultured astrocytes incubated with bacterial LPS (Kipp et al. 2007; Tenenbaum et al. 2007; Lewis et al. 2008; Cerciat et al. 2010) by mechanisms that, as for cannabinoids, involve the inhibition of NFκB-induced transcription of proinflammatory chemokines and cytokines (Dodel et al. 1999; Cerciat et al. 2010).

Furthermore, estrogens decrease the number of reactive astrocytes in vivo, after excitotoxic-induced neurodegeneration (Ciriza et al. 2004), in experimental Parkinson's disease (Tripanichkul et al. 2006), in experimental diabetic central neuropathy (Saravia et al. 2006), after lesion of the cholinergic basal forebrain (Martinez and de Lacalle 2007), and after stab wound brain lesions (Garcia-Estrada et al. 1993, 1999; Barreto et al. 2007, 2009). Estrogens also reduce edema in injured brain tissue by the enhancement of aquaporin-4 expression in parenchymal reactive astrocytes and perivascular glial processes (Tomas-Camardiel et al. 2005).

The similitude in the anti-inflammatory actions of cannabinoids and estrogens on astrocytes suggests a possible interaction of these 2 families of molecules in the regulation of reactive astroglia. To test this hypothesis, in this study, we have assessed the effect of estradiol, the cannabinoid CB1 antagonist/inverse agonist AM251, and the cannabinoid CB2 antagonist/inverse agonist AM630 on reactive astroglia in the cerebral cortex of male rats after a stab wound brain injury.

## Materials and Methods

### *Animals and Experimental Treatments*

Wistar albino male rats from the Complutense University animal colony were kept on a 12:12-h light/dark schedule and received food and water ad libitum. Animals were handled in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals, the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience and following the European Union guidelines (Council Directive 86/609/EEC). Experimental procedures were approved by our institutional animal use and care committee. Special care was taken to minimize suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

### *Brain Injury*

Animals were anesthetized with halothane and placed in a stereotaxic apparatus (David Kopf Instruments). In order to relieve postchirurgical pain, animals received one intraperitoneal (i.p.) injection of buprenorphine (1 mg/kg; Buprex, Schering Plough) and one subcutaneous (s.c.) injection of meloxicam (1 mg/kg; Metacam, Boehringer Ingelheim), shortly after anesthesia induction. An incision of the scalp was made and the cranium exposed. Then, a unilateral opening of the skull was made with a dental drill. A 2.5-mm sterile sharp knife was introduced vertically into the right cerebral hemisphere to a depth of 4.5 mm and then removed. The rostral end of the knife was placed 2.5 mm posterior to bregma and 1.1 mm lateral. The caudal end of the knife end was 5.0 mm posterior to bregma and 3.0 mm lateral. Coordinates were determined according to the stereotaxic atlas of Paxinos and Watson (1998). Bleeding was inhibited by compression with a hemostatic gelfoam sponge.

### *Treatments with Estradiol and Cannabinoid Antagonists*

*Experiment 1*

Immediately before brain injury, the animals received one of the following treatments: 1) one i.p. injection of the vehicle for cannabinoid antagonists (ethanol:cremophor EL:saline, 1:1:18; 1 mL/kg), followed by one s.c. injection of the vehicle for 17β-estradiol (corn oil; 1 mL/kg); 2) one i.p. injection of the vehicle for cannabinoid antagonists, followed by one s.c. injection of 17β-estradiol (E2758, Sigma-Aldrich; 1 mg/kg; 1 mL/kg); 3) one i.p. injection of the cannabinoid CB1 antagonist/inverse agonist AM251 (Tocris Bioscience; 1 mg/kg; 1 mL/kg), followed by one s.c. injection of the vehicle for 17β-estradiol; 4) one i.p. injection of the cannabinoid CB2 antagonist/inverse agonist AM630 (Tocris Bioscience; 1 mg/kg; 1 mL/kg), followed by one s.c. injection of the vehicle for 17β-estradiol; 5) one i.p. injection of AM251, followed by one s.c. injection of 17β-estradiol; and 6) one i.p. injection of AM630, followed by one s.c. injection of 17β-estradiol. These treatments were repeated 24 and 48 h after brain injury. Thus, the compounds were administered during the period of glial activation (Garcia-Ovejero et al. 2002). The dose of estradiol selected in this study is known to reduce reactive gliosis after stab wound brain injuries (Barreto et al. 2007). This effect may be at least in part due to the high levels of the steroid achieved shortly following the injections.

*Experiment 2*

In a second experiment, to assess the levels of the enzymes involved in the synthesis and metabolism of cannabinoids and the expression of cannabinoid receptors, sham-operated animals and animals that received a stab wound injury were treated with estradiol or vehicle, as described above. Seventy-two hours after surgery, animals were killed by decapitation and the cerebral cortex around the injured area, and the same cortical region of the right cerebral hemisphere from noninjured controls was dissected and frozen.

### *Tissue Fixation and Immunohistochemistry*

For histological analysis (experiment 1), 1 week after brain injury, animals were deeply anaesthetized with pentobarbital (100 mg/kg, Normon Veterinary Division) and perfused through the left cardiac ventricle, first with 50 mL saline solution (0.9% NaCl) and then with 250 mL fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were removed and immersed overnight at 4 °C in the same fixative solution and then rinsed with phosphate buffer. Coronal sections of the brain, 50 μm thick, were obtained using a Vibratome (VT 1000 S, Leica Microsystems).

Immunohistochemistry was carried out on free-floating sections under moderate shaking. All washes and incubations were done in 0.1 M phosphate buffer pH 7.4, containing 0.3% bovine serum albumin and 0.3% triton X-100. The endogenous peroxidase activity was quenched for 10 min at room temperature in a solution of 3% hydrogen peroxide in 30% methanol. After several washes in buffer, sections were incubated overnight at 4 °C with a monoclonal antibody for vimentin (diluted 1:500; Clone V9, DAKO), a marker of reactive astrocytes, or a monoclonal antibody for GFAP (diluted 1:1000; Clone GA5, Sigma-Aldrich), a marker of reactive and resting astroglia. Sections were then rinsed in buffer and incubated for 2 h at room temperature with biotinylated goat antimouse immunoglobulin G (diluted 1:300; Pierce). After several washes in buffer, sections were incubated for 90 min at room temperature with avidin–biotin–peroxidase complex (diluted 1:250; ImmunoPure ABC peroxidase staining kit). The reaction product was revealed by incubating the sections with 2 μg/mL 3,3′-diaminobenzidine (Sigma-Aldrich) and 0.01% hydrogen peroxide in 0.1 M phosphate buffer. Then, sections were dehydrated, mounted on gelatinized slides, coverslipped, and examined with a Leica DMRB-E microscope.

### *Morphometric Analysis*

Only brains that showed a complete lesion from the dorsal to the ventral limit of the cerebral cortex were selected for morphometry. All morphometric analyses were performed on coded sections. The number of vimentin immunoreactive cells was assessed within a distance of 0–350 μm from the medial border of the wound (Zone I). In addition, the number of GFAP immunoreactive cells was assessed in 3 zones in the injured cortex: within a distance of 0–350 μm from the medial border of the wound (Zone I), within a distance of 350–700 μm from the medial border of the wound (Zone II), and within a distance of 700–1050 μm from the medial border of the wound (Zone III). The number of GFAP immunoreactive cells was also evaluated in the equivalent region of the contralateral noninjured cortex corresponding to Zones I–III. The number of immunoreactive cells was estimated by the optical disector method (Howard and Reed 1998) using total section thickness for disector height (Hatton and von Bartheld 1999) and a counting frame of 55 × 55 μm. A total of 78 counting frames were assessed per animal and per each zone analyzed. Section thickness was

measured using a digital length gauge device (Heidenhain-Metro MT 12/ND221) attached to the stage of a Leitz microscope. Cell nuclei from immunoreactive cells that came into focus while focusing down through the disector height were counted.

As an alternative method to evaluate glial reactivity, the surface density of vimentin and GFAP immunoreactive cell bodies and cell processes was assessed in the injured cerebral cortex in Zone II (350–700 μm from the medial border of the wound). The surface density of immunoreactive material was performed using a stereological grid, according to the point-counting method of Weibel (1979). The ratio of the surface of immunoreactive profiles to the reference volume (surface density, Sv) was calculated by the following formula: $Sv = 2I/L$, where $I$ is the number of points at which the immunoreactive profiles (vimentin or GFAP immunoreactive astroglial cell bodies and processes) cross the test grid lanes and $L$ is the test line length in the tissue (Weibel 1979). The test grid used is based on the C16 grid of Weibel (Weibel 1979) and has 10 × 10 lines of a total length of 2000 μm ($L = 2$ mm). Magnification was calculated with a calibrated slide (100 lines/mm, Leitz). For each animal, a minimum of 2 sections were evaluated. All immunoreactive profiles, whether heavily or less heavily labeled, were considered for quantification.

### *Messenger RNA Levels of the Enzymes Involved in Endocannabinoid Synthesis and Metabolism and Messenger RNA Levels of Cannabinoid Receptors*

Total RNA was obtained from samples of the cerebral cortex (experiment 2) using Trizol reagent (Gibco BRL Life Technologies) according to the manufacturer's instructions. Next, it was purified and concentrated with RNeasy mini kit (Qiagen Hilden) with all RNA samples showing A260/280 ratios between 1.9 and 2.1. One microgram of RNA from each sample was reversed transcribed into complementary DNA (cDNA) using random primer hexamers and a transcriptor reverse transcriptase kit (Roche Applied Science) according to manufacturer's instructions. Negative control included reverse transcription reactions omitting reverse transcriptase. The obtained cDNA was used as the template for real-time quantitative polymerase chain reaction (PCR), which was performed in an iCycler system (Bio-Rad) using the FastStart Universal SYBR Green Mastermix (Roche Diagnostics). Primers for PCR reaction (Sigma-Proligo France SAS) were designed based on NCBI database sequences of rat reference messenger RNA (mRNA; Table 1) and checked for specificity with BLAST software from NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Each reaction was run in duplicate and contained 2.5 μL of cDNA template, 5 mM $Cl_2Mg$, and 0.4–0.5 μM of primers in a final reaction volume of 15 μL. Cycling parameters were 95 °C for 5 min to activate DNA polymerase, then 35–45 cycles of 95 °C for 10 s to denature DNA, annealing temperature for 15 s (see Table 1), and a final extension step of 72 °C for 15 s in which fluorescence was acquired. Melting curves analysis and 1% agarose gel electrophoresis were performed to ensure only a single product was amplified. Gene expression was normalized to the expression of the housekeeping gene β-actin and to the expression of SP1 gene. Similar results were obtained with the 2 genes used for normalization. The data shown in the figures correspond to the normalization using the SP1 gene.

Quantification was carried out with a standard curve that was run at the same time as the sample with each dilution in duplicate. The standards were a purified and quantified PCR product, serially diluted. Briefly, to obtain them, purified RNA from a tissue known to express the target gene was retrotranscribed to cDNA and PCR amplified by using specific primers pair (see Table 1). The obtained PCR product was purified by using a high pure PCR product purification kit (Roche Diagnostic), quantified by 260-nm absorbance, and serially diluted to $10^{-6}$ pg/μL. An aliquot of the undiluted PCR product was run in a 1% agarose gel electrophoresis for checking the fragment size and the absence of other contaminant fragments. Eight 10-fold dilutions ($10^{+1}$ to $10^{-6}$ pg/μL) were checked for optimal cycling on the iCycler system and 5 or 6 of them selected for running the standard curve.

**Table 1**
Oligonucleotides used for quantitative PCR

| Gene | Primer sequences | Annealing temperature (°C) | GenBank accession number |
|---|---|---|---|
| SP1 | 5′-AGCAGGATGGTTCTGGTCAA-3′<br>5′-AGGTGATGTTCCCATTCAGG-3′ | 54.0 | NM_012655.1 |
| β-Actin | 5′-CAGGCTGTGTTGTCCCTGTA-3′<br>5′-GCTGTGGTGGTGAAGCTGTA-3′ | 51.4 | NM_031144.2 |
| DAGLα | 5′-GGGTACCTAATGGCTGCTCA-3′<br>5′-AGGACTGACCATCCAACCTG-3′ | 60.5 | NM_001005886.1 |
| NAPE-PLD | 5′-GGAGCTTATGAGCCAAGGTG-3′<br>5′-ACTCTCCGTGCTTCAGGATG-3′ | 57.0 | AB112351 |
| FAAH | 5′-GTTACAGAGTGGAGAGCTGTC-3′<br>5′-GAGGGTTACTGCAGTCAAAGC-3′ | 46.5 | NM_024132.3 |
| MAGL | 5′-CATGGAGCTGGGGAACACTG-3′<br>5′-GGAGATGGCACCGCCATGGAG-3′ | 58.1 | NM_138502.1 |
| CB1 | 5′-AGACCTCCTCTACGTGGGCTCG-3′<br>5′-GTACAGCGATGGCCAGCTGCTG-3′ | 62.0 | NM_012784.2 |
| CB2 | 5′-GCAGCCTGCTGCTGACTGCTG-3′<br>5′-TGCTTTCCAGAGGACATACCC-3′ | 58.9 | NM_020543.3 |

### *Statistical Analysis*

The $n$ used for statistical analysis was the number of animals and it is indicated in the figure legends. Because the Levene's test revealed that the data do not meet the assumption of normality, the nonparametric Kruskal–Wallis test was used for statistical analysis followed by the Dunn's method, with $P < 0.05$ considered to be significant.

## Results

### *Effect of Estradiol and CB Receptor Antagonists on Reactive Astrogliosis*

The qualitative inspection of the sections immunostained for vimentin revealed a prominent glial scar along the borders of the wound (Fig. 1). Qualitative differences were observed between the different experimental groups. Animals treated with estradiol alone (Fig. 1*B*) showed a glial scar with a lower cellular density compared with control animals (Fig. 1*A*) and with animals injected with estradiol and either the CB1

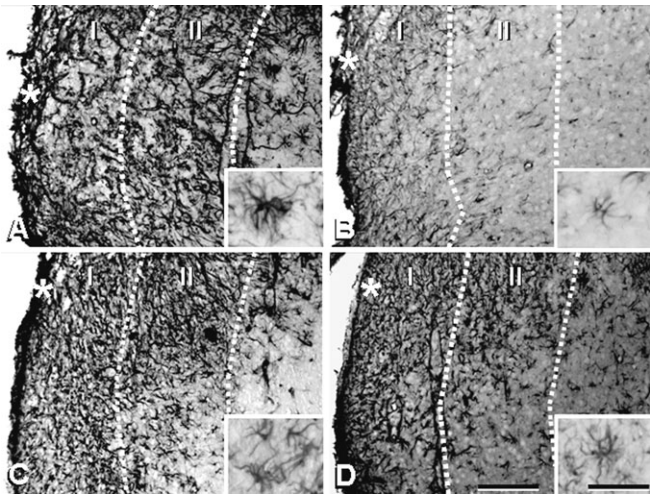


**Figure 1.** Representative images of the injured cerebral cortex showing examples of vimentin immunoreactivity after the administration of vehicles (*A*), estradiol (*B*), estradiol and the CB1 receptor antagonist AM251 (*C*), and estradiol and the CB2 receptor antagonist AM630 (*D*). Asterisks indicate the border of the lesion. Insets show details of vimentin immunoreactive cells at high magnification. Dotted lines delimit the regions of the cerebral cortex in proximity of the border of the wound where the number of vimentin immunoreactive cells (Zone I) and the surface density of vimentin immunoreactive material (Zone II) were evaluated. Scale bar represents 200 μm for main figures and 50 μm for insets.

receptor antagonist (Fig. 1*C*) or the CB2 receptor antagonist (Fig. 1*D*).

By means of the morphometric analysis, estradiol administration resulted in a significant decrease in the number of immunoreactive cells (Fig. 2) and in the surface density of vimentin immunoreactive material (Fig. 3) compared with control values. The cannabinoid receptor antagonists, per se, did not significantly affect vimentin immunoreactivity compared with control values. However, the animals injected with estradiol and either the CB1 or the CB2 receptor antagonist showed a number of vimentin immunoreactive cells and a surface density of vimentin immunoreactive material that was not significantly different from control values (Figs. 2 and 3). This indicates that both cannabinoid receptor antagonists blocked the effect of estradiol on reactive astrogliosis.

Similar results were observed in the sections immunostained for GFAP (Figs. 4 and 5). GFAP immunoreactive cells in the border of the lesion were of smaller size in the animals treated with estradiol alone than in the other experimental groups (Fig. 5). In addition, the morphometric analysis of cortical Zones I–III revealed a significant decrease in the number of GFAP immunoreactive cells (Fig. 6) in the animals treated with estradiol compared with control animals. In control animals, the number of GFAP immunoreactive cells progressively decreased with the distance from the border of the wound. However, the number of GFAP immunoreactive cells was significantly higher in Zones I–III of the injured cortex than in the corresponding region of the contralateral noninjured cortex. In contrast, in the animals treated with estradiol, the number of GFAP immunoreactive cells reached similar values in Zone III than in the contralateral cortex. In agreement with the estimation of the number of GFAP immunoreactive cells, the surface density of GFAP immunoreactive material in Zone II also showed a significant decrease in the animals treated with estradiol (Fig. 7). The cannabinoid receptor antagonists, per se, did not significantly affect the surface density and the number of GFAP immunoreactive cells compared with control values. However, the animals injected with estradiol and either the CB1 or the CB2 receptor antagonist showed a surface density of GFAP immunoreactive material and a number of GFAP immunoreactive cells that was not significantly different from control values (Figs. 6 and 7). None of the treatments induced a significant change in the number of GFAP immunoreactive cells in the noninjured cortex.

### *Effect of Brain Injury and Estradiol Treatment on the mRNA Levels of Enzymes Involved in Endocannabinoid Synthesis and Metabolism*

Brain injury resulted in a significant decrease in the mRNA levels of monoacylglycerol lipase (MAGL; Fig. 8) and *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD; Fig. 9) in the injured cerebral cortex, compared with the control noninjured cortex. However, injured animals injected with estradiol did not displayed decreased mRNA levels of MAGL (Fig. 8) and NAPE-PLD (Fig. 9). Estradiol did not affect the mRNA levels of these enzymes in noninjured cerebral cortex. The mRNA levels of diacylglycerol lipase (DAGL) and fatty acid amidohydrolase (FAAH) in the cerebral cortex were unaffected by brain injury and by estradiol treatment (data not shown).

### *Effect of Brain Injury and Estradiol Treatment on the mRNA Levels of Cannabinoid Receptors*

The mRNA levels of CB1 and CB2 receptors in the cerebral cortex were not significantly affected by brain injury. Estradiol was unable to modify CB1 receptor mRNA levels in the control and the injured cortex (data not shown). In contrast, estradiol treatment resulted in a significant increase in the mRNA levels of CB2 receptors in the injured cortex (Fig. 10).

## Discussion

Previous studies have shown that the systemic administration of estradiol to male rats significantly decreases reactive astrogliosis after a stab wound in the brain (Barreto et al.

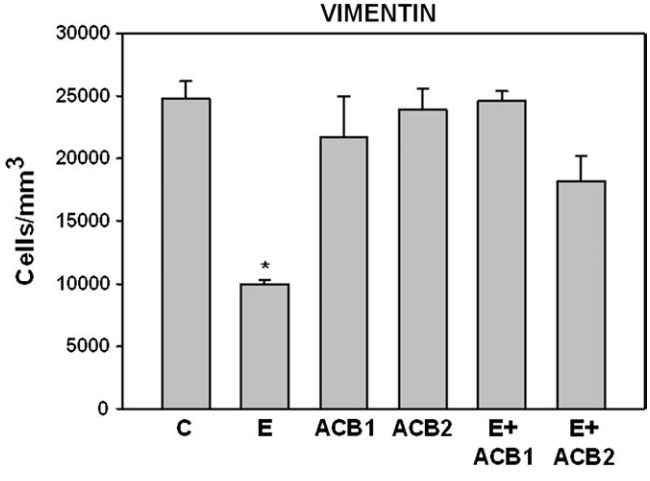


**Figure 2.** Number of vimentin immunoreactive astrocytes per cubic millimeter within a distance of 350 μm from the medial border of the wound (Zone I) in animals injected with vehicles (controls, C; $n = 6$), estradiol (E; $n = 6$), the CB1 receptor antagonist AM251 (ACB1; $n = 6$), the CB2 receptor antagonist AM630 (ACB2; $n = 6$), estradiol and the CB1 receptor antagonist AM251 (E + ACB1; $n = 6$), and estradiol and the CB2 receptor antagonist AM630 (E + ACB2; $n = 6$). Data are mean + standard error of the mean (SEM). *, significant difference versus control values ($P < 0.05$).

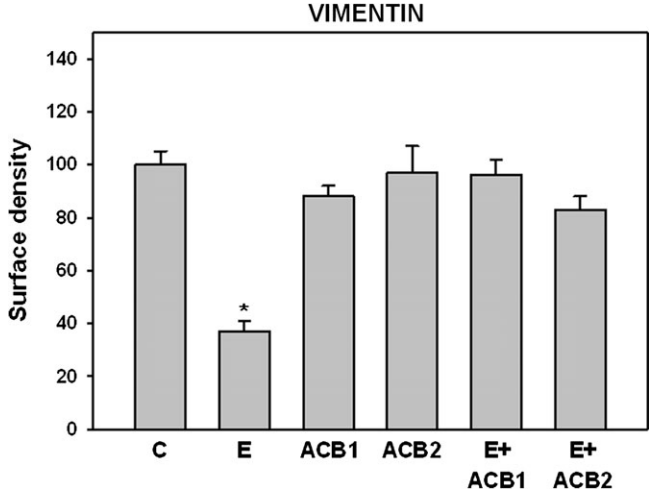


**Figure 3.** Surface density of vimentin immunoreactive material within a distance of 350–700 μm from the medial border of the wound (Zone II) in animals injected with vehicles (controls, C; $n = 6$), estradiol (E; $n = 6$), the CB1 receptor antagonist AM251 (ACB1; $n = 6$), the CB2 receptor antagonist AM630 (ACB2; $n = 6$), estradiol and the CB1 receptor antagonist AM251 (E + ACB1; $n = 6$), and estradiol and the CB2 receptor antagonist AM630 (E + ACB2; $n = 6$). Data are mean + SEM. *, significant difference versus control values ($P < 0.05$).

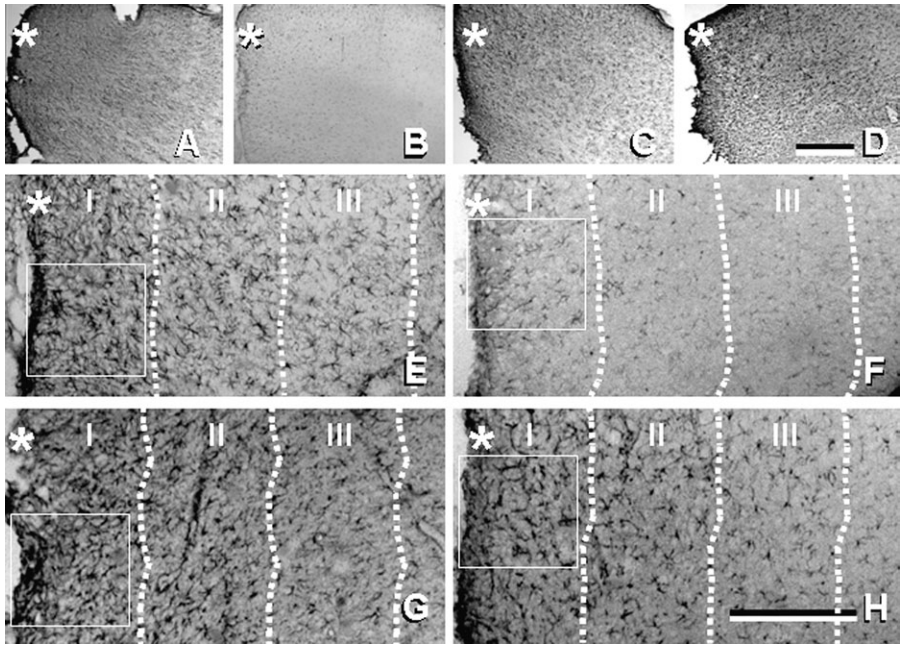


**Figure 4.** Representative images of the injured cerebral cortex showing examples of GFAP immunoreactivity after the administration of vehicles (*A*,*E*), estradiol (*B*,*F*), estradiol and the CB1 receptor antagonist AM251 (*C*,*G*), and estradiol and the CB2 receptor antagonist AM630 (*D*,*H*). The regions demarcated by squares in panels *E–H* are shown at high magnification in Figure 5. Dotted lines delineate the 3 zones in which the number of GFAP immunoreactive cells was evaluated. Asterisks indicate the border of the lesion. Scale bar represents 600 μm for panels *A–C* and 350 μm for panels *E–H*.

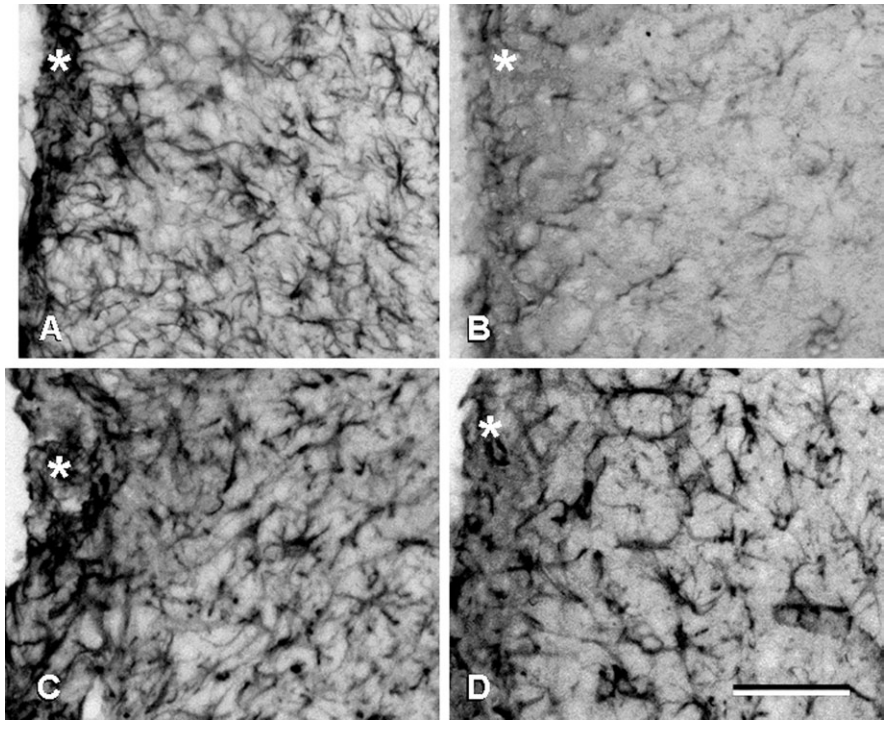


**Figure 5.** Detail of GFAP immunoreactive cells in the proximity of the border of the lesion (the location is indicated in Fig. 4). (*A*) Animal treated with vehicles. (*B*) Animal injected with estradiol. (*C*) Animal injected with estradiol and the CB1 receptor antagonist AM251. (*D*) Animal injected with estradiol and the CB2 receptor antagonist AM630. Asterisks indicate the border of the lesion. Scale bar, 100 μm.

2007). Our present results confirm these previous observations. Estradiol reduced the surface density and the number of astrocytes labeled with vimentin, a marker of reactive astrocytes. These changes were confirmed using GFAP, a marker of resting and reactive astrocytes. Estradiol significantly decreased the surface density and number of GFAP immunoreactive astrocytes in the injured cortex. In addition, in the animals treated with estradiol, the number of GFAP immunoreactive cells reached the levels of the noninjured cortex at a distance of 700–1050 μm from the medial border of

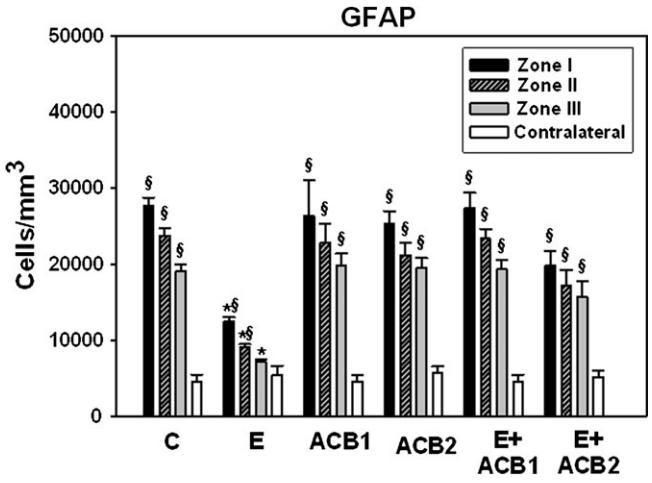


**Figure 6.** Number of GFAP immunoreactive astrocytes per cubic millimeter within distances of 0–350 μm (Zone I), 350–700 μm (Zone II), and 700–1050 μm (Zone III) from the medial border of the wound and in the equivalent region of the contralateral noninjured cortex. C, control animals injected with vehicles ($n = 6$); E, animals injected with estradiol ($n = 6$); ACB1, animals injected with the CB1 receptor antagonist AM251 ($n = 6$); ACB2, animals injected with the CB2 receptor antagonist AM630 ($n = 6$); E + ACB1, animals injected with estradiol and the CB1 receptor antagonist AM251 ($n = 6$); and E + ACB2, animals injected with estradiol and the CB2 receptor antagonist AM630 ($n = 6$). Data are mean + SEM. *, significant differences versus control values ($P < 0.05$) and §, significant differences versus the contralateral cortex ($P < 0.05$).

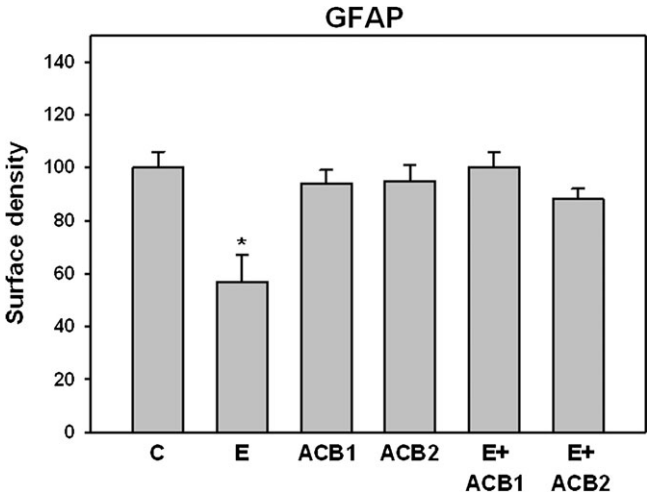


**Figure 7.** Surface density of GFAP immunoreactive material within a distance of 350–700 μm from the medial border of the wound (Zone II) in animals injected with vehicles (controls, C; $n = 6$), estradiol (E; $n = 6$), the CB1 receptor antagonist AM251 (ACB1; $n = 6$), the CB2 receptor antagonist AM630 (ACB2; $n = 6$), estradiol and the CB1 receptor antagonist AM251 (E + ACB1; $n = 6$), and estradiol and the CB2 receptor antagonist AM630 (E + ACB2; $n = 6$). Data are mean + SEM. *, significant differences versus control values ($P < 0.05$).

the wound. In contrast, in control animals, although GFAP immunoreactive cells also progressively decreased with the distance to the injury, their number was still significantly higher at a distance of 700–1050 μm from the medial border of the wound than in the contralateral cortex.

Under physiological conditions, estradiol regulates GFAP expression in astrocytes by a mechanism involving an estrogen response element on the GFAP gene promoter (Stone et al. 1998). In addition, estradiol may indirectly reduce the number

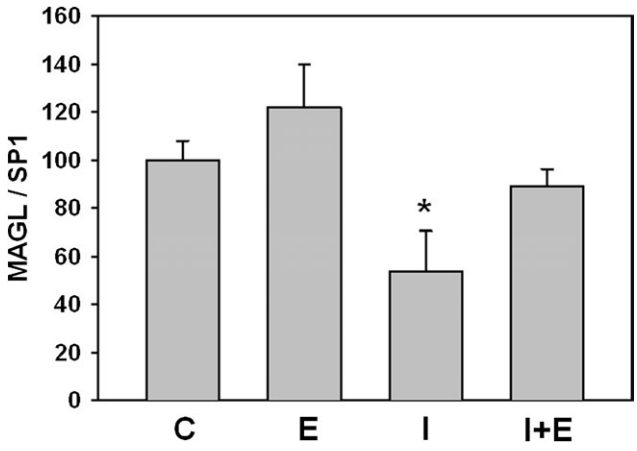


**Figure 8.** Levels of mRNA of MAGL normalized to the levels of mRNA for SP1 gene in the right cerebral cortex from animals that received a stab wound brain injury and from control noninjured animals. C, cerebral cortex from control noninjured animals injected with vehicle ($n = 5$); E, cerebral cortex from control noninjured animals injected with estradiol ($n = 5$); I, injured cortex from animals injected with vehicle ($n = 5$); and I + E, injured cortex from animals that were treated with estradiol ($n = 4$). Data are means + SEM. *, significant difference ($P < 0.05$) versus noninjured cortex.

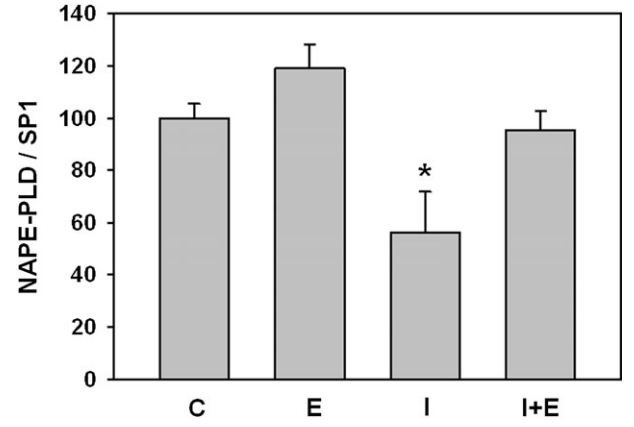


**Figure 9.** Levels of mRNA of NAPE-PLD normalized to the levels of mRNA for SP1 gene in the right cerebral cortex from animals that received a stab wound brain injury and from control noninjured animals. C, cerebral cortex from control noninjured animals injected with vehicle ($n = 6$); E, cerebral cortex from control noninjured animals injected with estradiol ($n = 5$); I, injured cortex from animals injected with vehicle ($n = 5$); and I + E, injured cortex from animals that were treated with estradiol ($n = 4$). Data are means + SEM. *, significant difference ($P < 0.05$) versus noninjured cortex.

of vimentin and GFAP immunoreactive astrocytes after brain injury by affecting the process of glial activation, for instance, by controlling glial cell proliferation (Garcia-Estrada et al. 1993, 1999) and the synthesis and release of proinflammatory cytokines (Arevalo et al. 2010). Therefore, although estradiol directly regulates GFAP expression and may also potentially regulate vimentin expression by direct mechanisms, the possibility exists for an interaction with other factors that regulate reactive gliosis to reduce the number of vimentin and GFAP immunoreactive astrocytes after brain injury. Among these factors, our findings suggest that endocannabinoids may be involved in the antigliotic effect of estradiol, since the antagonism of both cannabinoid CB1 and CB2 receptors reduced the estrogenic effect on reactive astroglia.

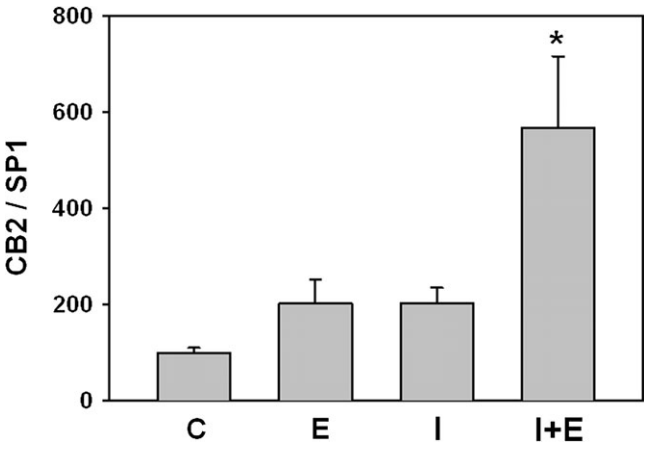


**Figure 10.** Levels of mRNA of CB2 receptors normalized to the levels of mRNA for SP1 gene in the right cerebral cortex from animals that received a stab wound brain injury and from control noninjured animals. C, cerebral cortex from control noninjured animals injected with vehicle ($n = 5$); E, cerebral cortex from control noninjured animals injected with estradiol ($n = 6$); I, injured cortex from animals injected with vehicle ($n = 5$); and I + E, injured cortex from animals that were treated with estradiol ($n = 4$). Data are means + SEM. *, significant difference ($P < 0.001$) versus noninjured cortex.

Although the antagonists of cannabinoid receptors blocked the effect of estradiol, they did not have an effect per se on the number of reactive astrocytes. This suggests that the endogenous cannabinoid tone does not affect reactive astrogliosis in our model. However, our results also suggest that estradiol may reduce reactive astroglia by regulating the activity of the endocannabinoid system in the brain (Rodríguez de Fonseca et al. 1994; Corchero et al. 2001; Bradshaw et al. 2006; Nguyen and Wagner 2006; Amantea et al. 2007; Hill et al. 2007). Indeed, our findings indicate that estradiol blocks the decrease caused by injury in the mRNA levels of MAGL and NAPE-PLD in the cerebral cortex. MAGL is involved in the metabolism of 2-arachidonoylglycerol (2-AG) and NAPE-PLD participates in the synthesis of AEA (Basavarajappa 2007). Therefore, estradiol partially counteracts the effect of brain injury on the enzymes that participate in the synthesis of endocannabinoids. By this action, estradiol may regulate the levels of 2-AG and AEA in the injured cerebral cortex.

The increase in the mRNA levels of CB2 receptors in the injured cerebral cortex of animals treated with estradiol suggests that the hormone may also affect the intercellular signaling of endocannabinoids by the modification of cannabinoid receptors. An interaction may occur in the signaling downstream of cannabinoid receptors as well, since both estradiol and cannabinoids regulate the activity of the mitogen-activated protein kinase and the phosphatidylinositol-3 kinase/Akt pathways, which are involved in the control of the inflammatory response (Molina-Holgado, Molina-Holgado, et al. 2002; Díaz-Laviada and Ruiz-Llorente 2005; Mendez et al. 2006; Marin et al. 2009). Estradiol may also interact with cannabinoid receptors in the regulation of protein kinase A and NO generation in neural cells (Dina et al. 2001; Kim et al. 2006).

Both CB1 and CB2 receptors appear to be involved in the action of estradiol in the control of reactive gliosis, since both cannabinoid receptor antagonists increased the surface density and number of reactive astrocytes in estradiol treated rats to values not significantly different from those of control animals. The interaction of estradiol and cannabinoids may occur directly on reactive astrocytes, since these cells express estrogen receptors and cannabinoid receptors (Garcia-Ovejero et al. 2002, 2009; Stella 2010). Indeed, both cannabinoid receptor agonists and estrogens regulate intracellular $Ca^{2+}$ levels in astrocytes (Chaban et al. 2004; Navarrete and Araque 2008; Bondar et al. 2009) and decrease reactive gliosis by the inhibition of NFκB mediated transcription (Dodel et al. 1999; Curran et al. 2005; Sheng et al. 2005; Zhang and Chen 2008; Leichsenring et al. 2009; Cerciat et al. 2010; Kozela et al. 2010). In addition, cannabinoids and estradiol regulate astrocyte–astrocyte and astrocyte–neuron communication (Venance et al. 1995; Rao and Sikdar 2006, 2007; Navarrete and Araque 2008), which may also participate in the activation of astrocytes.

Cannabinoids and estradiol may also regulate reactive astrogliosis by indirect effects on microglia (Waksman et al. 1999; Puffenbarger et al. 2000; Ramírez et al. 2005; Vegeto et al. 2006; Barreto et al. 2007; Tapia-Gonzalez et al. 2008; Correa, Docagne, et al. 2009; Kreutz et al. 2009; Sagredo et al. 2009; Luongo et al. 2010). Among other possibilities, estradiol may control reactive astrogliosis by the regulation of CB1 receptor signaling in astrocytes and exert an additional control mediated by CB2 receptors expressed in microglia (Correa, Docagne, et al. 2009; Romero-Sandoval et al. 2009; Sagredo et al. 2009; Correa et al. 2010; Luongo et al. 2010). In this regard, the increase in the mRNA levels of CB2 receptors induced by estradiol may be associated with an increase in CB2 receptor signaling, which is known to result in the reduction of the proinflammatory phenotype of microglia (Correa, Docagne, et al. 2009; Romero-Sandoval et al. 2009; Sagredo et al. 2009; Correa et al. 2010; Luongo et al. 2010). Further studies should determine the role of the direct and indirect mechanisms and the cell types involved in the regulation of reactive astrogliosis by estradiol through cannabinoid receptors. In addition, the possible involvement of cannabinoid receptors in the regulation of gliosis exerted by other steroid hormones, such as testosterone and progesterone, merits to be examined.

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