**THE SYNTHETIC STEROID TIBOLONE DECREASES REACTIVE GLIOSIS
AND NEURONAL DEATH IN THE CEREBRAL CORTEX OF FEMALE MICE
AFTER A STAB WOUND INJURY**

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Abstract

Previous studies have shown that estradiol reduces reactive gliosis after a stab wound injury in the cerebral cortex. Since the therapeutic use of estradiol is limited by its peripheral hormonal effects, it is of interest to determine whether synthetic estrogenic compounds with tissue specific actions regulate reactive gliosis. Tibolone is a synthetic steroid that is widely used for the treatment of climacteric symptoms and/or the prevention of osteoporosis. In this study we have assessed the effect of tibolone on reactive gliosis in the cerebral cortex after a stab wound brain injury in ovariectomized adult female mice. By 7 days after brain injury, tibolone reduced the number of glial fibrillary acidic protein (GFAP) immunoreactive astrocytes, the number of ionized calcium binding adaptor molecule 1 (Iba1) immunoreactive microglia and the number of microglial cells with a reactive phenotype in comparison to vehicle injected animals. These effects on gliosis were associated with a reduction in neuronal loss in the proximity to the wound, suggesting that tibolone exerts beneficial homeostatic actions in the cerebral cortex after an acute brain injury.

Keywords: Astrocytes; Microglia; Neuroinflammation; Neuroprotection; Steroid receptors; Brain trauma

Introduction

Different hormonal signals contribute to maintain the homeostasis of brain tissue. Among these, estradiol exerts a variety of regulatory functions on neurons and glial cells and has neuroprotective properties [1-4]. In acute brain lesions, estradiol reduces tissue damage and neurological deficits [5,6]. The mechanisms involved in the neuroprotective actions of estradiol include the regulation of reactive gliosis [7-11] and the decrease in the inflammatory activity of astrocytes and microglia [12-14].

The therapeutic use of estradiol as a neuroprotectant is limited by its peripheral hormonal actions. Therefore, several studies have tested the neuroprotective potency of different estrogenic compounds that imitate the protective actions of estradiol but do not exert undesirable peripheral actions, such as increasing the risk of breast, endometrial and ovarian cancer [15]. These include selective estrogen receptor modulators (SERMs), which exert agonistic, antagonistic or neutral effects on estrogen receptors (ERs) depending on the tissue [16]. Previous studies have shown that some SERMs exert neuroprotective actions and regulate reactive gliosis and neuroinflammation [17-23]. An interesting characteristic of some SERMs, such as tamoxifen and raloxifene, is that in addition to exert neuroprotective actions, they act as ER antagonists in ER positive breast cancers [24], which are one of the risks of estrogen therapy in postmenopausal women.

Estrogen therapy, alone or in combination with progestins, is an effective treatment to alleviate hot flushes and to prevent osteoporosis in postmenopausal women. However, the increased risk of breast and endometrial cancer associated with estrogen therapy promoted the investigation of new alternatives for the treatment of postmenopausal symptoms. This resulted in the introduction of tibolone, a synthetic steroid classified as a

selective tissue estrogenic activity modulator (STEAM) [25-27]. Tibolone is structurally different from estradiol and SERMs. For instance, it lacks the aromatic A ring of estradiol and the aromatic rings of SERMs. In particular, the hydroxyl group in the C3 position of the A ring, which is not present in tibolone, seems to confer antioxidant properties to the estradiol molecule [28].

Like SERMs, tibolone exerts tissue specific actions, but by different mechanisms. Indeed, the tissue selectivity of tibolone depends on its tissue specific local transformation to active metabolites that can exert estrogenic, progestogenic and androgenic activities. Thus, tibolone is converted to its estrogenic metabolites 3 α - and 3 β -hydroxytibolone by the enzymes 3 α - and 3 β -hydroxysteroid dehydrogenase, respectively. Furthermore, tibolone is converted by the enzyme 3 β -hydroxysteroid dehydrogenase-isomerase to its Δ 4-isomer, which activates androgen and progesterone receptors [27,29]. Moreover, tibolone does not have estrogenic actions on ER positive cancers and is now widely used for the treatment of climacteric symptoms and/or the prevention of osteoporosis [30].

Although tibolone is one of the main drugs used for the treatment of postmenopausal symptoms, there is limited information on its effects in the brain. Nevertheless, it is known that tibolone has effects on the nervous system, since it reverses the loss of dendritic spines caused by ovariectomy in rat CA1 hippocampal pyramidal cells [31]. The steroid has also rapid effects in the activity of pro-opiomelanocortin (POMC) hypothalamic neurons, acting on membrane estrogen receptors [32]. In agreement with this direct effect of tibolone on POMC neurons, the steroid increases β -endorphin levels in different brain regions of ovariectomized rats [33]. Furthermore, tibolone decreases the

activity of acetylcholinesterase and choline acetyltransferase in the medial septum/diagonal band of Broca of ovariectomized monkeys [34], suggesting a modulation of cholinergic neurons. It has also been shown that tibolone decreases the hyperphosphorylation of the neuronal protein Tau in the hippocampus and cerebellum [35]. In addition, tibolone reduces anxiety, at least in young female rats [36]. It has also been shown that tibolone reduces oxidative stress and ameliorates cholinergic deficits in the hippocampus of male rats exposed to ozone [37]. Neuroprotective actions of tibolone after an acute brain injury have also been reported. Thus, a high (200 µg) subcutaneous dose of tibolone has been shown to reduce infarct volume in ovariectomized female rats exposed to focal cerebral ischemia [38].

Regulation of reactive gliosis and neuroinflammation may represent a therapeutic approach for the treatment of neurodegenerative diseases. The possible actions of tibolone on gliosis are unknown. Studies on glial cells *in vitro* have revealed that tibolone reduces oxidative damage [39] and exerts anti-inflammatory actions by decreasing NF-κB activation [40,41]. These findings suggest that tibolone may exert anti-inflammatory actions and reduce gliosis in the injured brain *in vivo*. To test this hypothesis, we have assessed the effect of tibolone on the number of astrocytes and microglia after a penetrating cortical injury.

Materials and Methods

Animals

Experiments were performed in 90 day-old C57bL6 female mice (Harlan, Spain). Animals were housed with controlled temperature ($22 \pm 2^\circ \text{C}$), 12 h light/dark cycle and

with food and water *ad libitum*. Animal care and procedures were approved by our institutional animal use and care committee and by the Comunidad de Madrid (PROEX 134/17) and followed the European Parliament and Council Directive (2010/63/EU) and the Spanish regulation (Ley 6/2013, 11th June) on the protection of animals for experimental use.

Cortical Injury and Experimental Treatments

At 90 days of age, animals were bilaterally ovariectomized under isoflurane anesthesia. We have used in this study ovariectomized mice since ovariectomy is a model for human menopause, the main condition in which tibolone is used in clinical practice. Brain lesions were performed 4 days after ovariectomy. Previous studies have shown that in rodents, estradiol levels in plasma are already reduced by 3 days after ovariectomy to the low values detected one month after surgery (to less than 50 pg/ml in rats) [42]. For brain lesions, animals were anesthetized once more with isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments). An incision with the scalp was made and the cranium was exposed. Then, a unilateral opening of the skull was made with a dental drill. Stab wound lesions were generated with a needle by making a sagittal cut through the neocortex and corpus callosum of the left hemisphere, 1 mm lateral from the midline and extending from -1.0 mm to -3.0 mm posterior from bregma. Coordinates were determined according to the stereotaxic atlas of Paxinos and Franklin [43].

Animals received one subcutaneous injection of tibolone (Sigma-Aldrich, T0827; 0.04 mg/Kg) or vehicle (corn oil) immediately after brain injury, a second injection 24 h later and a third injection 48 h later. Tibolone was administered during the period of glial activation. This protocol of administration was selected because it was previously used

to determine the antigliotic effects of estradiol and SERMs in ovariectomized rodents [20,44]. The dose of tibolone was selected on the basis of previous studies in animal models *in vivo* [30] and on the posology used in clinical practice (2.5 mg/day).

Tissue Fixation and Immunohistochemistry

At 3, 7 and 14 days after brain surgery, animals were deeply anaesthetized with pentobarbital (50 mg/kg body weight) and perfused through the left cardiac ventricle, first with pre-warmed (37°C) 0.9% NaCl and then for 5 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4; 37°C). Brains were post fixed for 4 h at 4°C in the same fixative and washed three times with 0.1 M phosphate buffer, pH 7.4 at room temperature. Coronal sections of the brain, 50 µm thick, were obtained using a Vibratome (VT 1000S, Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry was carried out in 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 rabbit antibody against glial fibrillary acidic protein (GFAP; diluted 1:1000; DAKO), a marker of reactive and resting astrocytes, or with a mouse monoclonal antibody against NeuN (diluted 1:1000; Millipore), a marker of neurons. Sections were then rinsed in buffer and incubated for 2 h at room temperature with the corresponding anti mouse or anti rabbit biotinylated secondary antibody (diluted 1:300; Pierce). After several washes in buffer, sections were incubated for 90 min at room temperature with avidin-biotin-peroxidase complex (diluted 1:500; 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 mounted on gelatinized slides, dehydrated, coverslipped, and examined with a Leica DMRB-E microscope.

For immunofluorescence, free-floating sections were washed in 0.1 M phosphate buffer pH 7.4, containing 0.5% triton X-100. Then, 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. After several washes in buffer, sections were incubated overnight at 4°C with rabbit polyclonal antibody for Iba1 (Ionized calcium binding adaptor molecule 1), marker of microglia/macrophages (Wako Chemical Industries, Japan; diluted 1:2000). Sections were then rinsed in buffer and incubated for 2 h at room temperature with a goat anti-rabbit Alexa secondary antibody (diluted 1:1000; 568 nm). Glass coverslip were mounted on slides with Vectashield antifade mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The Z-stack images were visualized on a Leica TCS-SP5 confocal system. 3D images of Iba1+ cells were obtained from stacks of 0.25 µm and the ImageJ program using the 3D viewer plugin (freely available at <https://imagej.nih.gov/ij/plugins/3d-viewer/>).

Morphometric Analysis

Morphometric analysis was performed by an investigator that was unaware of the identity of the experimental groups. The number of NeuN+, GFAP+ and Iba1+ cells was assessed in five consecutive rectangles (lateral frames F1-F5) of 200 µm (horizontal side) x 600 µm (vertical side), starting from the lateral wound edge and moving laterally. In addition,

for GFAP and Iba1 immunoreactive cells, each lateral rectangle was divided in 3 dorsoventral sectors (S1-S3) of 200 μm^2 (Fig. 1).

The number of GFAP, Iba1 and NeuN immunoreactive cells was estimated with the optical disector method, using total section thickness for disector height using a 40 $\times$ objective [45,46] and a counting frame of 200 x 200 μm . Section thickness was measured using a digital length gauge device (Heidenhain-Metro MT 12/ND221; Traunreut, Germany) attached to the stage of a Leitz microscope. In addition, the number of Iba1 immunoreactive cells with different morphologies was assessed by immunofluorescence in a confocal microscope (Leica TCS-SP5 confocal system). Iba1+ cells were classified in two morphological phenotypes, according to previous reports [47-51]. Iba1+ cells with enlarged cell body, short and thick cellular processes and/or amoeboid morphology were considered reactive (Fig. 2a,b and Fig. 3a,b). Those with ramified morphology, with long cellular processes were considered non-reactive (Fig. 2c,d and Fig. 3c,d).

Statistical Analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using SPSS v21.0 software (IBM Corp. Armonk, NY, USA). Main and interactive effects were analyzed by three-way analysis of variance (ANOVA), with time after lesion, treatment and distance from the lesion as independent factors. When justified by the ANOVA analysis, differences between individual group means were analyzed by the Bonferroni post hoc test for homoscedastic data, the Games-Howell test for non-homoscedastic data and the Kruskal-Wallis test for non-parametric data. Differences were considered statistically significant at $P < 0.05$. All the analyses were carried out with an

experimental N of 5-6 animals injected with vehicle and 5-6 animals injected with tibolone for each time after brain injury.

Results

Effects of Tibolone on the Number of Astrocytes and Microglia in the Injured Cerebral Cortex

The number of GFAP+ cells and the number of Iba1+ cells was counted at 3 and 7 days after brain injury. Counts were performed in the 5 lateral zones delimited by frames (F1-F5) and in the 3 dorsoventral sectors (S1-S3) within each frame. Figure 4 shows representative examples of cortical sections immunostained for GFAP. Figure 5 shows representative examples of cortical sections immunostained for Iba1.

Astrocytes

Figure 6 shows the number of GFAP+ cells per mm³ in the 5 lateral zones delimited by frames F1-F5 at 3 and 7 days after brain injury in animals treated with vehicle or tibolone. In figure 6a, data from the 3 dorsoventral sectors (S1-S3) were grouped together. Data sorted by dorsoventral sectors are shown in figure 6b,c,d.

Three-way ANOVA for the number of GFAP+ cells in the 5 lateral zones (Fig. 6a) revealed significant effects of the treatment, the distance to the lesion and the time after injury. Furthermore, significant interactions of the treatment with the time after injury and of the distance to the lesion with the time after injury were detected. Results of the ANOVA analysis are shown in the legend of figure 6. Similar, but not identical results were observed when the number of GFAP+ cells was analyzed separately in each dorsoventral sector (S1-S3; Fig. 6b,c,d).

As shown in figure 6a, a significant decrease in the number of GFAP+ cells was observed in the lateral frames F4 and F5 between 3 and 7 days after brain injury in vehicle injected animals, when counts from the 3 dorsoventral sectors were grouped together. The analysis of the data sorted by dorsoventral sectors (Fig. 6b,c,d) revealed that the decrease in the number of GFAP+ cells between 3 and 7 days in the lateral frames F4 and F5 occurred in both S1, S2 and S3.

In tibolone injected animals, the number of GFAP+ cells dropped down in F2-F5 between 3 and 7 days after brain injury (Fig. 6a). The decrease in F2 was exclusively due to GFAP+ cells located in the third dorsoventral sector, corresponding to a distance between 400 and 600 μm from the dorsal cortical surface (Fig. 6b,c,d).

Compared to vehicle injected animals, treatment with tibolone resulted in a significant decrease in the number of GFAP+ cells in F1 at 3 days after brain injury (Fig. 6a). This decrease was due to GFAP+ cells located at a distance between 200 to 400 μm from the 6dorsal cortical surface (Fig. 6b,c,d). Furthermore, tibolone treatment decreased the number of GFAP+ cells in F1-F4 7 days after brain injury, compared to vehicle injected 6animals (Fig. 6a). The effect of tibolone in F2 and F3 was detected in the 3 dorsoventral sectors analyzed, while the effect in F1 and F4 was detected in the upper and middle dorsoventral sectors (S1 and S2; Fig. 6b,c,d). No significant effect of tibolone was detected in the number of GFAP+ cells in the contralateral hemisphere (data not shown).

Total Microglia/Macrophages Iba1+ cells

Three-way ANOVA for the number of Iba1+ cells in the 5 lateral zones delimited by 7 frames F1-F5 (Fig. 7a) revealed significant effects of the treatment, the distance to the lesion, and the time after injury. Furthermore, significant interactions of the treatment with the time after injury and of the distance to the lesion with the time after injury were detected. Similar, but not identical results were observed when the number of Iba1+ cells was analyzed separately in the 3 dorsoventral sectors (S1-S3; figure 7b,c,d). Results of the ANOVA analysis are shown in the legend of figure 7.

As shown in figure 7a, a significant increase in the number of Iba1+ cells was observed in the lateral frames F1 and F2 between 3 and 7 days after brain injury in vehicle injected animals. This change was detected in the 3 dorsoventral sectors analyzed of the lateral frames F1 and F2 (Fig. 7b,c,d). In addition, a significant increase in the number of Iba1+ cells was also detected in the third dorsoventral sector (S3) of the lateral frame F3 (Fig. 7d).

A significant decrease in the number of Iba1+ cells was observed between 3 and 7 days after brain injury in the lateral frames F4 and F5 of tibolone injected animals (Fig. 7a). In contrast, the number of Iba1+ cells increased between days 3 and 7 after injury in F1 of tibolone injected animals. This increase was due to Iba1+ cells located in the third dorsoventral sector (Fig. 7d).

Compared to vehicle injected animals, treatment with tibolone resulted in a significant decrease in the number of Iba1+ cells within the lateral frames F2 and F5 at 7 days after brain injury (Fig. 7a). The effect of tibolone in F2 was due to Iba1+ cells located in the second and third dorsoventral sectors, while the effect in F5 was due to Iba1+ cells located

in the upper dorsoventral sector. In addition, differences between tibolone and vehicle values at day 7 after injury were observed in the dorsoventral sector S2 of the lateral frame F1 and the dorsoventral sector S3 of the lateral frame F3 (Fig. 7b,c,d). No significant effect of tibolone was detected in the number of Iba1+ cells in the contralateral hemisphere (data not shown).

Microglia with Reactive Phenotype

Iba1+ cells were classified in either reactive or non-reactive (resting) according to their morphology. Three-way ANOVA for the number of Iba1+ cells with reactive phenotype in the 5 lateral zones delimited by frames F1-F5 (Fig. 8a) revealed significant effects of the treatment and the distance to the lesion. Furthermore, significant interactions of the treatment with the time after injury and of the distance to the lesion with the time after injury were detected. Similar, but not identical results were observed when the number of Iba1+ cells was analyzed separately in the 3 dorsoventral sectors (S1-S3; Fig. 8b,c,d). Results of the ANOVA analysis are shown in the legend of figure 8.

As shown in figure 8a, a significant increase in the number of Iba1+ cells with reactive phenotype was observed in the lateral frames F1 and F2 between 3 and 7 days after brain injury. The increase in F1 was detected in the 3 analyzed dorsoventral sectors, but the increase in F2 was exclusively due to Iba1+ cells located in the dorsoventral sector S3 (Fig. 8b,c,d).

In tibolone injected animals, a significant decrease in the number of Iba1+ cells with reactive phenotype was observed in the lateral frames F4 and F5 between 3 and 7 days after brain injury. The decrease in F4 was detected in the dorsoventral sectors S2 and S3.

The decrease in F5 was only detected in the dorsoventral sector S2. In addition, tibolone treatment decreased the number of Iba1+ cells with reactive phenotype between 3 and 7 days after injury in the dorsoventral sector S2 of the lateral frames F3 and F5 (Fig. 8b,c,d).

Compared to vehicle injected animals, treatment with tibolone resulted in a significant decrease in the number of Iba1+ cells with reactive phenotype in the lateral frame F5 at 3 days and in lateral frames F2, F3 and F5 at 7 days after brain injury (Fig. 8a). The effect of tibolone in F2 at 7 days was due to cells located in the dorsoventral sectors S2 and S3. The effect of tibolone in F3 was only detected in the dorsoventral sector S3 and the effect of tibolone in F5 was only detected in the dorsoventral sector S1. In addition, tibolone decreased the number of Iba1+ cells with reactive phenotype in the dorsoventral sectors S1 and S2 of the lateral frame F1 (Fig. 8b,c,d).

Microglia with Resting Phenotype

Three-way ANOVA for the number of Iba1+ cells with non-reactive phenotype in the 5 lateral zones delimited by frames F1-F5 (Fig. 9a) revealed significant effects of the distance to the lesion and the time after injury. Furthermore, a significant interaction of the treatment with the time after injury was detected. Similar results were observed when the number of Iba1+ cells with resting phenotype was analyzed separately in the 3 dorsoventral sectors (S1-S3; Fig. 9b,c,d). Results of the ANOVA analysis are shown in the legend of figure 9.

As shown in figure 9a, the number of Iba1+ cells with resting phenotype increased progressively from the lesion. In tibolone injected animals the number of Iba1+ cells with resting phenotype showed a significant increase in the lateral frames F2, F3 and F4

between days 3 and 7 after injury. This increase was observed in the dorsoventral sectors S1 and S2 of F2, the dorsoventral sectors S1, S2 and S3 of F3 and the dorsoventral sector S1 of F4 and F5 (Fig. 9b,c,d). In addition, in vehicle injected animals the number of Iba1+ cells with resting phenotype showed an increase between days 3 and 7 after injury in the dorsoventral sector S1 of the lateral frame F2 (Fig. 9b). No significant differences in the number of Iba1+ cells with resting phenotype were detected between the animals injected with vehicle and those injected with tibolone.

Effects of Tibolone on the Number of Neurons in the Injured Cerebral Cortex

Cells immunoreactive for the neuronal marker NeuN were counted in the cerebral cortex of the injured and contralateral hemispheres. Neuronal cell counts were performed 14 days after brain injury to have an estimation of the number of remaining neurons after the successive waves of primary and secondary neuronal death [52]. Compared to the contralateral (non-injured) cortex, the injured cortex showed a significant decrease in the number of NeuN+ neurons in the border of the wound (lateral frames F1 and F2; Figs. 10 and 11). Animals treated with tibolone showed a similar reduction in the number of NeuN+ neurons in the close proximity to the wound (frame F1) than those treated with vehicle. However, tibolone resulted in a significant protection for neurons located within frame F2 (Fig. 11). Thus, in control animals injected with vehicle, brain injury resulted in a significant decrease in the number of neurons located within frame F2. In contrast, tibolone prevented neuronal loss in the lateral frame F2 of the injured cortex. Therefore, the number of NeuN+ neurons was significantly higher in this zone in the animals treated with tibolone compared to the animals treated with vehicle (Fig. 11).

Discussion

Reactive gliosis after an acute brain injury follows a precise temporal and regional pattern. In this study, we have observed that the number of GFAP+ cells and the number of Iba1+ cells in the cerebral cortex change depending on the distance to the wound and the time after injury. In addition, reactive gliosis showed differences in function of the distance to the dorsal cortical surface. Changes in reactive gliosis after a stab wound injury in accordance with time after injury and the distance to the border of the injury have been previously reported [7,9,53-58]. However, changes in the number of reactive astrocytes and reactive microglia across cortical depth have been less explored. For example, Miyake et al [53] reported differences in the number of GFAP+ cells between layer I and layers II-VI in the mouse cerebral cortex after a stab wound injury. Our findings indicate that spatial differences in GFAP+ and Iba1+ cells in the cerebral cortex after brain injury not only depend on the distance to the lesion, but also on cortical depth.

Our results indicate that the treatment with tibolone causes a reduction in the number of GFAP+ astrocytes and the number of Iba1+ microglia after an acute cortical injury. The effect of tibolone on GFAP+ and Iba1+ cells also showed differences depending on the time after injury, the distance to the lesion and the position in the dorsoventral cortical axis, suggesting that the action of tibolone on reactive gliosis is regulated in a context-specific manner. The effect of tibolone was observed in cortical regions located in the proximity (0-600 μ m) of the wound, where, in agreement with previous studies [9,56,57], we detected the higher number of astrocytes and microglia with a reactive phenotype after brain injury. In contrast, tibolone did not affect the number of glial cells in the contralateral hemisphere, suggesting that its action on glial cell number is restricted to the damaged tissue. It should be noted, however, that tibolone has effects on healthy brain tissue. For instance, tibolone increases the expression of superoxide dismutase-2 in the

rat hippocampus [37] and regulates synaptic efficacy in hypothalamic POMC neurons [32]. Although tibolone did not reduce the number of glial cells in the contralateral hemisphere, the steroid may regulate other parameters in astrocytes and microglia in the healthy tissue. For instance, another steroid such as estradiol has been shown to reduce reactive gliosis in the injured brain but to promote the growth of astrocytic processes and the expression of GFAP in healthy brain tissue [59]. Further studies are therefore necessary to determine the effects of tibolone on astrocytes and microglia under physiological conditions.

Tibolone induced significant changes in the number of astrocytes and microglia at 7 days post-injury compared to control animals. Thus, while at 3 days no significant differences in the number of GFAP+ or Iba1+ cells were detected between control and tibolone injected animals (with the exception of a reduction by tibolone treatment in the number of GFAP+ cells at 0-200 μm from the wound border and at 200-400 μm from the cortical surface), at 7 days after injury animals injected with tibolone showed a decrease in the number of GFAP+ cells, in the number of total Iba1+ cells and in the number of Iba1+ cells with reactive phenotype. In contrast, tibolone did not affect the number of Iba1+ cells with resting phenotype in comparison to vehicle injected animals. These findings indicate that at 7 days after injury tibolone reduced reactive gliosis.

Reactive gliosis is an adaptive response aimed to restore neural tissue homeostasis [60-63]. However, under certain circumstances reactive astrocytes and reactive microglia may exert detrimental actions [60,64-66]. In the present study, the regulation of reactive gliosis by tibolone was associated with an increased neuronal survival in the injured cerebral cortex at 14 days after injury. In traumatic brain injury, gliosis and neuroinflammation

are associated with a delayed neuronal death that occurs for several days after the primary neuronal loss induced by the injury [52]. Thus, in our study, neuronal survival was assessed 14 days after brain injury, after the two waves of neuronal death. By maintaining a healthy metabolic and functional status in astrocytes and microglia [39-41,67] and by reducing neuroinflammation [41] and gliosis, tibolone may improve the homeostatic function of these cells and increase their neuroprotective actions.

The protective action of tibolone on neurons was limited to the F2 region. The F1 region was probably too close to the injury site and suffered a process of fast primary neuronal death, as the core in ischemic injuries, that could not be protected by tibolone. In contrast, the F2 region may represent a site of secondary neuronal death (penumbra), where tibolone could exert a protective effect. In F3-F5 regions no significant effect of tibolone compared to vehicle was observed, probably since being these regions too far away from the stab wound site, neurons handled the stress situation better.

To compare the effects of tibolone on reactive gliosis with those reported for estradiol and SERMs, in the present study the steroid was injected at 0, 24 and 48 h after brain injury as described in previous protocols [20,44]. This time period corresponds to the phase of initiation of reactive gliosis. Astrocytes react within hours after a cerebral stab wound [68,69], while the response of microglial cells occurs between 3 and 7 days post-injury [70]. In women, tibolone has a plasmatic half-life of seven hours and the levels of their metabolites reach their peak between 60 and 90 minutes after administration [71]. Although these values may be different in our animal model, they suggest that the effects of tibolone on gliosis at 7 days post injury and on the number of neurons at 14 days post

injury are the consequence of the action of the steroid during the first days after brain injury.

The activation of the inflammasome in perilesional neurons and microglia is one of the early events initiated after brain injury [72] and plays a role in the secondary injury mechanisms [73]. Therefore, the anti-inflammatory activity of tibolone, detected in BV-2 microglia [41], may be one of the mechanisms by which the steroid reduces gliosis and reduces secondary neuronal death. Other causes for the secondary wave of tissue damage include oxidative stress and mitochondrial dysfunction [74,75], which are also decreased by tibolone in glial cells *in vitro* [39-41,67]. Furthermore, tibolone may also decrease tissue damage by protecting endothelial cell function [76,77].

The effects of tibolone on primary astrocytes, T98G cells and BV-2 microglia are mediated by estrogen receptors α or β [40,41,78]. It is known that brain injury induces the expression of estrogen receptors α and β in reactive astrocytes [79,80]. Estrogen receptors are also expressed by microglia [17,81-83]. Therefore, it is possible that tibolone may exert direct actions on reactive astrocytes and reactive microglia acting on estrogen receptors α and β expressed by these cells. To our knowledge, there is no information on the possible binding of tibolone to G protein-coupled estrogen receptor 1, which is also expressed by microglia [83] and astrocytes [84]. Furthermore, depending on its tissue specific metabolism, tibolone may also activate androgen and progesterone receptors [26], which are also expressed by glial cells and participate in the regulation of gliosis [59,82,80,85,86-88]. In the cerebral cortex of female ovariectomized monkeys, tibolone is mainly metabolized to 3α and 3β -hydroxytibolone [29,89] which are

metabolites with estrogenic activity [78,90] and to $\Delta 4$ -isomer, which exerts androgenic and progestogenic actions [90].

The effect of tibolone on gliosis after brain injury observed in the present study is similar to that reported after the administration of estradiol or SERMs to ovariectomized rodents [20,44]. This suggests that the mechanism of action of tibolone on gliosis is mediated by estrogen receptors. Acting on estrogen receptors, tibolone could not only reduce neuroinflammation and the activation of astrocytes and microglia, but also decrease the activation of caspase-3 reducing apoptosis [91-93] and promote blood brain barrier integrity and angiogenesis [3,94]. Tibolone could also potentially reduce reactive microglia acting on androgen receptors, since these receptors are known to decrease the activation of microglia after a penetrating brain injury, at least in male rats [8]. Activation of progesterone receptors could also contribute to the effect of tibolone on gliosis, since progesterone also reduces inflammation and oxidative stress after traumatic brain injury [95,96] and increases the respiratory function of mitochondria [97].

A limitation of the present study is that we have used a model of brain injury that is convenient for the quantification of glial cells in the border of the lesion, but that induces very small neural damage, difficulting the determination of functional recovery using behavioral assays. Further studies using other traumatic brain injury models are therefore necessary. This will also allow to assess whether the antigliotic and neuroprotective actions of tibolone reported in the present study are extensible to other types of brain trauma, such as contusion or concussion injury. Since sex differences in the response to traumatic brain injury have been reported [98] it will be also important to assess the effect of tibolone on male animals. Furthermore, it would be interesting to study the effect of

tibolone in gonadally intact females, to determine if endogenous estradiol may potentiate the beneficial homeostatic actions of tibolone.

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Compliance with Ethical Standards All procedures involving animals were approved by the CSIC institutional animal use and care committee and by the Comunidad de Madrid (PROEX 134/17) and followed the European Parliament and Council Directive (2010/63/EU) and the Spanish regulation (Ley 6/2013, 11th June) on the protection of animals for experimental use.

Conflict of Interest The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. Representative histological section of the cerebral cortex at 14 days after a stab wound injury.

a, Panoramic view (reconstructed from microphotographs taken at 10x) of a representative section from a vehicle injected animal which was immunostained for the neuronal marker NeuN. Scale bar, 800 μm . The box is shown at higher magnification (approx. 20x) in panel b.

b, Detail of the injured area. Lateral and dorsoventral frames used for the morphometric analysis are shown overlapped to the section. Scale bar, 400 μm .

Fig. 2. Examples of Iba-1 immunoreactive cells with reactive and resting morphology.

a and b, Examples of Iba1⁺ cells with reactive phenotype, with short and thick cellular processes.

c and d, Examples of Iba1⁺ cells with non-reactive phenotype, with numerous long cellular processes.

Scale bar, 7.5 μm .

Fig. 3. Three-dimensional reconstructions of microglia cells with reactive and resting morphology.

a and b, Examples of Iba1⁺ cells with reactive phenotype, with short and thick cellular processes.

c and d, Examples of Iba1⁺ cells with non-reactive phenotype, with numerous long cellular processes.

Scale bar, 7.5 μm .

Fig. 4. Representative examples of GFAP immunostained sections of the cerebral cortex 7 days after injury.

a, From a vehicle injected animal at 10x.

b, From a tibolone injected animal at 10x.

c, From a vehicle injected animal at 20x.

d, From a tibolone injected animal at 20x.

The asterisk indicates the lesion border. Scale bars, 200 μm .

Fig. 5. Representative examples of Iba1 immunostained sections of the cerebral cortex 7 days after injury.

a, From a vehicle injected animal at 20x.

b, From a tibolone injected animal at 20x.

c, From a vehicle injected animal at 40x.

d, From a tibolone injected animal at 40x.

The asterisk indicates the lesion border. Scale bars, 200 μm .

Fig. 6. Effect of tibolone on the number of GFAP+ astrocytes in the cerebral cortex 3 and 7 days after a stab wound injury. The number of GFAP+ cells was assessed in 5 consecutive lateral frames (F1-F5) of 200 μm x 600 μm , starting from the wound edge and in 3 consecutive dorsoventral sectors (S1-S3) of 200 μm^2 starting from the dorsal cortical surface.

a, Number of GFAP+ cells in the 5 lateral frames when data from the 3 dorsoventral sectors are grouped together. Three-way ANOVA revealed significant effects of the treatment ($F_{(1,19)}=24.012$; $p<0.001$), the distance to the lesion ($F_{(4,19)}=44.964$; $p<0.001$),

and the time after injury ($F_{(1,19)}=43.705$; $p<0.001$). Furthermore, significant interactions of the treatment with the time after injury ($F_{(1,19)}=5.954$; $p=0.017$) and of the distance to the lesion with the time after injury ($F_{(4,19)}=8.785$; $p<0.001$) were detected.

b, Number of GFAP+ cells in sector 1 of the 5 lateral frames. Three-way ANOVA revealed significant effects of the treatment ($F_{(1,19)}=20.827$; $p<0.001$), the distance to the lesion ($F_{(4,19)}=37.328$; $p<0.001$) and the time after injury ($F_{(1,19)}=28.106$; $p<0.001$). In addition, significant interactions of the distance with the time after injury ($F_{(1,19)}=6.786$; $p<0.001$) and of the treatment to the lesion with the time after injury ($F_{(4,19)}=5.725$; $p=0.019$) were detected.

c, Number of GFAP+ cell in sector 2 of the 5 lateral frames. Three-way ANOVA revealed significant effects of the treatment ($F_{(1,19)}=21.901$; $p<0.001$), the distance to the lesion ($F_{(4,19)}=34.373$; $p<0.001$) and the time after injury ($F_{(1,19)}=45.587$; $p<0.001$). In addition, significant interactions of the distance with the time after injury ($F_{(1,19)}=8.724$; $p<0.001$) and of the treatment to the lesion with the time after injury ($F_{(4,19)}=5.365$; $p=0.023$ in S2) were detected.

d, Number of GFAP+ cells in sector 3 of the 5 lateral frames. d.p.i., days post injury. Three-way ANOVA revealed significant effects of the treatment ($F_{(1,19)}=19.950$; $p<0.001$), the distance to the lesion ($F_{(4,19)}=45.124$; $p<0.001$) and the time after injury ($F_{(1,19)}=54.809$; $p<0.001$). In addition, significant interactions of the distance with the time after injury ($F_{(1,19)}=9.058$; $p<0.001$) and of the treatment to the lesion with the time after injury ($F_{(4,19)}=6.960$; $p=0.010$) were detected.

Significant differences with the post-hoc test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$ between vehicle 3 days and vehicle 7 days. Significant differences $^{\$}$ $p<0.05$; $^{\$\$}$ $p<0.01$; $^{\$ \$ \$}$ $p<0.001$ between tibolone 3 days and tibolone 7 days. Significant differences $^{\&}$ $p<0.05$ between

vehicle 3 days and tibolone 3 days. Significant differences [#] $p < 0.05$; ^{##} $p < 0.01$ between vehicle 7 days and tibolone 7 days.

Fig. 7. Effect of tibolone on the number of Iba1+ microglia in the cerebral cortex 3 and 7 days after a stab wound injury. The number of Iba+ cells was assessed in 5 consecutive lateral frames (F1-F5) of 200 μm x 600 μm , starting from the wound edge and in 3 consecutive dorsoventral sectors (S1-S3) of 200 μm^2 starting from the dorsal cortical surface.

a, Number of Iba1+ cells in the 5 lateral frames when data from the 3 dorsoventral sectors are grouped together. Three-way ANOVA revealed significant effects of the treatment ($F_{(1,19)}=8.397$; $p=0.005$), the distance to the lesion ($F_{(4,19)}=38.003$; $p<0.001$), and the time after injury ($F_{(1,19)}=6.659$; $p=0.012$). Furthermore, significant interactions of the treatment with the time after injury ($F_{(1,19)}=26.262$; $p<0.001$) and of the distance to the lesion with the time after injury ($F_{(4,19)}=6.619$; $p<0.001$) were detected.

b, Number of Iba1+ cells in sector 1 of the 5 lateral frames. Three-way ANOVA revealed significant effects of the treatment ($F_{(1,19)}=8.685$; $p=0.004$) and the distance to the lesion ($F_{(4,19)}=21.679$; $p<0.001$). In addition, significant interaction of the treatment with the time after injury ($F_{(1,19)}=17.083$; $p<0.001$) was detected.

c, Number of Iba1+ cells in sector 2 of the 5 lateral frames. Three-way ANOVA revealed significant effects of the treatment ($F_{(1,19)}=3.352$; $p=0.071$) and the distance to the lesion ($F_{(4,19)}=40.384$; $p<0.001$). In addition, significant interactions of the treatment with the time after injury ($F_{(1,19)}=13.430$; $p<0.001$) and of the distance to the lesion with the time after injury ($F_{(4,19)}=4.876$; $p=0.001$) were detected.

d, Number of Iba1+ cells in sector 3 of the 5 lateral frames. Three-way ANOVA revealed significant effects of the distance to the lesion ($F_{(4,19)}=20.828$; $p<0.001$) and the time after

injury ($F_{(1,19)}=8.749$; $p<0.001$). In addition, significant interactions of the treatment with the time after injury ($F_{(1,19)}=18.322$; $p<0.001$) and of the distance to the lesion with the time after injury ($F_{(4,19)}=5.678$; $p<0.001$) were detected.

Significant differences with the post-hoc test: * $p<0.05$; ** $p<0.01$ between vehicle 3 days and vehicle 7 days. Significant differences s $p<0.05$; ss $p<0.01$ between tibolone 3 days and tibolone 7 days. Significant differences $^{\#}$ $p<0.05$; $^{##}$ $p<0.01$ between vehicle 7 days and tibolone 7 days.

Fig. 8. Effect of tibolone on the number of Iba1+ microglia with reactive phenotype in the cerebral cortex 3 and 7 days after a stab wound injury. The number of Iba1+ cells with reactive phenotype was assessed in 5 consecutive lateral frames (F1-F5) of $200\ \mu\text{m} \times 600\ \mu\text{m}$, starting from the wound edge and in 3 consecutive dorsoventral sectors (S1-S3) of $200\ \mu\text{m}^2$ starting from the dorsal cortical surface.

a, Number of Iba1+ cells with reactive phenotype in the 5 lateral frames when data from the 3 dorsoventral sectors are grouped together. Three-way ANOVA revealed significant effects of the treatment ($F_{(1,19)}=9.851$; $p=0.002$) and the distance to the lesion ($F_{(4,19)}=68.720$; $p<0.001$). Furthermore, significant interactions of the treatment with the time after injury ($F_{(1,19)}=27.651$; $p<0.001$) and of the distance to the lesion with the time after injury ($F_{(4,19)}=7.101$; $p<0.001$) were detected.

b, Number of Iba1+ cells with reactive phenotype in sector 1 of the 5 lateral frames. Three-way ANOVA revealed significant effects of the treatment ($F_{(1,19)}=11.615$; $p=0.001$) and the distance to the lesion ($F_{(4,19)}=44.212$; $p<0.001$). In addition, significant interactions of the treatment with the time after injury ($F_{(1,19)}=25.557$; $p<0.001$) and of the distance to the lesion with the time after injury ($F_{(4,19)}=2.928$; $p=0.026$) were detected.

c, Number of Iba1+ cells with reactive phenotype in sector 2 of the 5 lateral frames. Three-way ANOVA revealed significant effects of the treatment ($F_{(1,19)}=10.058$; $p=0.002$) and the distance to the lesion ($F_{(4,19)}=79.885$; $p<0.001$). In addition, significant interactions of the treatment with the time after injury ($F_{(1,19)}=23.316$; $p<0.001$) and of the distance to the lesion with the time after injury ($F_{(4,19)}=7.012$; $p<0.001$) were detected.

d, Number of Iba1+ cells with reactive phenotype in sector 3 of the 5 lateral frames. Three-way ANOVA revealed significant effects of the distance to the lesion ($F_{(4,19)}=36.439$; $p<0.001$). In addition, significant interactions of the treatment with the time after injury ($F_{(1,19)}=22.121$; $p<0.001$ in S3) and of the distance to the lesion with the time after injury ($F_{(4,19)}=6.657$; $p<0.001$ in S3) were detected.

Significant differences with the post-hoc test: * $p<0.05$; ** $p<0.01$ between vehicle 3 days and vehicle 7 days. Significant differences $^{\$}$ $p<0.05$; $^{\$\$}$ $p<0.01$ between tibolone 3 days and tibolone 7 days. Significant difference $^{\&}$ $p<0.05$ between vehicle 3 days and tibolone 3 days. Significant differences $^{\#}$ $p<0.05$; $^{\#\#}$ $p<0.01$ between vehicle 7 days and tibolone 7 days.

Fig. 9. Effect of tibolone on the number of Iba1+ microglia with resting phenotype in the cerebral cortex 3 and 7 days after a stab wound injury. The number of Iba1+ cells with resting phenotype was assessed in 5 consecutive lateral frames (F1-F5) of $200\ \mu\text{m} \times 600\ \mu\text{m}$, starting from the wound edge and in 3 consecutive dorsoventral sectors (S1-S3) of $200\ \mu\text{m}^2$ starting from the dorsal cortical surface. **a,** Number of Iba1+ cells with resting phenotype in the 5 lateral frames when data from the 3 dorsoventral sectors are grouped together. Three-way ANOVA revealed significant effects of the distance to the lesion ($F_{(4,19)}=68.264$; $p<0.001$) and the time after injury ($F_{(1,19)}=35.473$; $p<0.001$). Furthermore,

a significant interaction of the treatment with the time after injury ($F_{(1,19)}=11.423$; $p=0.001$) was detected.

b, Number of Iba1+ cells with resting phenotype in sector 1 of the 5 lateral frames. Three-way ANOVA revealed significant effects of the distance to the lesion ($F_{(4,19)}=65.854$; $p<0.001$) and the time after injury ($F_{(1,19)}=42.293$; $p<0.001$). In addition, a significant interaction of the treatment with the time after injury ($F_{(1,19)}=10.308$; $p=0.002$) was detected.

c, Number of Iba1+ cells with resting phenotype in sector 2 of the 5 lateral frames. Three-way ANOVA revealed significant effects of the distance to the lesion ($F_{(4,19)}=67.564$; $p<0.001$ in S2) and the time after injury ($F_{(1,19)}=24.866$; $p<0.001$). In addition, significant interaction of the treatment with the time after injury ($F_{(1,19)}=4.692$; $p=0.033$) was detected.

d, Number of Iba1+ cells with resting phenotype in sector 3 of the 5 lateral frames.

Significant difference with the post-hoc test: ** $p<0.01$ between vehicle 3 days and vehicle 7 days. Significant differences $^{\$}$ $p<0.05$; $^{\$\$}$ $p<0.01$ between tibolone 3 days and tibolone 7 days. Significant differences ** $p<0.01$ between vehicle 3 days and vehicle 7 days. Significant differences $^{\$}$ $p<0.05$; $^{\$\$}$ $p<0.01$ between tibolone 3 days and tibolone 7 days.

Fig. 10. Representative examples of NeuN immunostained sections of the cerebral cortex 14 days after injury.

a, From a vehicle injected animal.

b, From a tibolone injected animal.

The asterisk indicates the lesion border. Scale bars, 200 μ m.

Fig. 11. Effect of tibolone on the number of NeuN+ neurons in the cerebral cortex at 14 days after a stab wound injury. The number of NeuN+ cells was assessed in five consecutive lateral frames (F1-F5) of 200 μm x 600 μm , starting from the wound edge and moving laterally. In addition, the number of NeuN+ cells was assessed in the contralateral cortex, in regions similar to F1 and F2. Significant difference * $p<0.05$ between tibolone ipsilateral and vehicle ipsilateral in F2. Significant differences ^{##} $p<0.01$; ^{###} $p<0.001$ between vehicle ipsilateral and contralateral. Significant difference ^{\$\$\$} $p<0.01$ between tibolone ipsilateral and contralateral.