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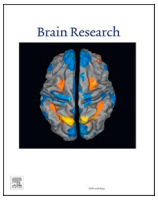

Ginkgo biloba extract modulates astrocytic and microglial recruitment in the hippocampus and hypothalamus of menopause-induced ovariectomized rats

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ABSTRACT

Background: Changes in steroid hormone levels associated with menopause are known to affect body composition, with increased accumulation of visceral fat and impaired actions of appetite-regulating neuropeptides. Anti-obesogenic, antioxidant, anti-inflammatory and neuromodulatory properties have been attributed to *Ginkgo biloba* extract (GbE) oral supplementation.

Hypothesis/purpose: We investigated in menopause-induced ovariectomized rats the effects of GbE oral supplementation on microglial reactivity and astrocyte recruitment in hippocampal and hypothalamic subregions involved in the regulation of feeding behavior and energy homeostasis.

Study design/methods: Ovariectomy (Ovx) or false-Ovx (Sham) surgery were performed in 2-month-old female Wistar rats. Sixty days after surgery, Ovx rats were gavaged daily for 14 days with either saline (Ovx + Veh) or GbE 500 mg/Kg (Ovx + GbE). Rats were subsequently sacrificed, brains harvested and subjected to immunohistochemistry and immunofluorescence analyses.

Results: Ovx increased microglial reactivity in CA1, CA3 and dentate gyrus (DG) in the dorsal hippocampal formation (dHF), as well as in DG in the ventral hippocampal formation (vHF).

Additionally, Ovx reduced astrocyte count in dHF CA3. The disturbances found in Ovx + Veh versus Sham were not found in Ovx + GbE versus Sham. Furthermore, higher astrocyte counts in DG of both dHF and vHF were found in Ovx + GbE as compared to Ovx + Veh. In the hypothalamus, Ovx + Veh showed reduced microglial reactivity in the arcuate (ARC) and ventromedial (VMH) nuclei as compared to Ovx + GbE. Ovx + GbE rats presented higher astrocyte counts in ARC compared to Sham rats.

Conclusion: Our results show for the first time in a rodent model of menopause that GbE supplementation modulates astrocyte and microglial recruitment and reactivity in hippocampal and hypothalamic subregions involved in feeding behavior and energy homeostasis. Future research employing other experimental models may further elucidate whether GbE supplementation possesses therapeutic properties upon glial cell reactivity to potentially alleviate changes in energy homeostasis associated with menopause.

Abbreviations: ARC, Arcuate nucleus; BSA, Bovine serum albumin; CNS, Central nervous system; DG, Dentate gyrus; dHF, Dorsal hippocampal formation; GbE, *Ginkgo biloba* extract; GFAP, Glial fibrillary acidic protein; HRT, Hormone replacement therapy; LH, Lateral hypothalamus; Ovx, Ovariectomy surgery; ROS, Reactive oxygen species; vHF, Ventral hippocampal formation; VMH, Ventromedial hypothalamus.

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1. Introduction

Glial cells actively contribute to the neural control of energy homeostasis (García-Cáceres et al., 2019). In brain regions involved in feeding behavior, such as the hippocampus and hypothalamus (Kanoski and Grill, 2017; Timper and Brüning, 2017), increased glial reactivity – known as gliosis – is triggered in rodents by the consumption of a high-fat diet (Lizarbe et al., 2019; Schmitt and Gaspar, 2023). Furthermore, changes in steroid hormone levels, as seen in menopause, are associated with disruption in glial cell activity, potentially leading to disturbances in brain energy homeostasis (Fuente-Martin et al., 2013; Mishra et al., 2020; Zárate et al., 2017).

Microglia are highly dynamic cells involved in the immune surveillance within the neural environment (Nimmerjahn et al., 2005), and are rapidly recruited in response to neural tissue injury (Douglass et al., 2017). Microglial cells are responsive to changes in brain metabolism, which influence the expression of pro-inflammatory genes within its surrounding tissue (Ghosh et al., 2018). Additionally, microglial function is modulated by estrogens, which in turn control target cell activity and suppress pro-inflammatory responses induced by microglia (Villa et al., 2016).

Astrocytes are functional units of the blood–brain barrier, responsible for regulating the entry of nutrients into the brain. Astrocytes play an essential role in detecting energy deficit, initiating metabolic adjustments within the microenvironment to restore energy supply for neurons (Argente-Arizón et al., 2017; Marina et al., 2018). Astrocytes actively participate in the regulation of energy homeostasis in response to several metabolic signals, including glucose, insulin, leptin and ghrelin (Lyon and Allen, 2022).

Notably, astrocytes are neurosteroidogenic cells, secreting progesterone and estradiol in the neural parenchyma (Yilmaz et al., 2019; Zwain and Yen, 1999). Estrogens and progesterone regulate astrocytic morphology and Glial Fibrillary Acidic Protein (GFAP) expression, promoting astrocytic plastic remodeling in both hypothalamic and hippocampal subregions (Acaz-Fonseca et al., 2016). Dysregulated activity of microglia and astrocytes, which are directly modulated by metabolic signaling and female steroid hormones, may underlie the metabolic changes observed in the brain during menopause.

Current evidence suggests that hormone replacement therapy (HRT) can alleviate some menopause-related changes observed in the central nervous system (CNS) (Gava et al., 2019). HRT has been reported to improve sleep disturbances (Caretto et al., 2019) and mood disorders in postmenopausal women (Gleason et al., 2015). It has been recently reported that HRT is associated with lower risk of negative outcomes in women with higher genetic predisposition for Alzheimer's disease when compared to non-treated matched women (Depypere et al., 2023). In ovariectomized rats, estradiol supplementation treatment was shown to effectively regulate the activity of microglia and astrocyte, also attenuating hypothalamic microgliosis induced by a high fat diet (Butler et al., 2020). Low-dose estradiol therapy suppressed microglial reactivity in a rodent model, leading to a shift from a pro- to an anti-inflammatory phenotype in the hippocampus (Thakkar et al., 2018). Likewise, hippocampal astrocyte reactivity was reduced after estradiol treatment in ovariectomized rats (Barreto et al., 2009). Estradiol regulates gene expression in astrocytes, affording astrocytic remodeling in the hippocampus and hypothalamus (Azcoitia et al., 2010).

Positive health outcomes are clearly demonstrated in HRT. However, inconsistent and controversial findings regarding its risks and benefits have limited its use in specific conditions (Lobo, 2017; Warren, 2004). HRT is contraindicated for women with history of specific gynecologic and non-gynecologic cancers due to the increased chance of recurrence (Deli et al., 2020). Considering the above, investigations into novel and safer approaches to attenuate menopause-related metabolic manifestations become an urgent need. In this context, Ginkgo biloba extract (GbE) supplementation is an approach with potentially therapeutic properties for the management of menopause-associated manifestations.

Our research group has previously reported significant effects of GbE on feeding behavior in ovariectomized rats. GbE improved leptin signaling in the hippocampus, a key mechanism in regulating food intake negative feedback (Machado et al., 2021a). Additionally, GbE restored the appetite-suppressing response induced by serotonin in both hypothalamus and hippocampus (Banin et al., 2017; Machado et al., 2021a). GbE also reduced hippocampal oxidative stress and enhanced depressive- and anxiety-like behaviors (Banin et al., 2021; Machado et al., 2021a). Taken together, the responses attributed to GbE contributed to the amelioration of metabolic disturbances triggered by obesity and to the normalization of feeding behavior in GbE-supplemented ovariectomized rats (Banin et al., 2021, 2017; Machado et al., 2021a). The effects of GbE on neuromodulation do not seem to be specific to female rats. We have demonstrated in adult male Wistar rats that a single dose of GbE was sufficient to stimulate hypothalamic anorexigenic pathways (Machado et al., 2021b).

Upon the knowledge that astrocytic and microglial cell manifestations observed in menopause are associated with disruptions in energy homeostasis, our study investigated the therapeutic impact of GbE supplementation on those cells within specific hippocampal and hypothalamic subregions associated with feeding behavior. GbE was orally administered for two weeks, aiming to assess its potential to influence the activity of astrocytes and microglia in ovariectomized rats. Additionally, we aimed to determine whether GbE supplementation could restore cell recruitment levels to those observed in the non-ovariectomized control group.

2. Experimental procedures

2.1. Source and chemical composition of GbE

The GbE standardized extract used in our experiments was produced by Huacheng Biotech Inc. (Hunan, China), and imported by Galena Pharmaceuticals (lot GK-100708, invoice 0105482, Campinas, SP, Brazil). Its composition consists of 24 % ginkgo-flavoglycosides and 6 % ginkgo-terpenoid lactones (3 % ginkgolides A, B, C, and 2.7 % bilobalides). Our research group has recently described in full the GbE chemical composition utilized in our study by LC-ESI-(+/-)-QTOF MS/MS (Soliani et al., 2023).

2.2. Ethics statement

The Universidade Federal de São Paulo Animal Research Ethics Committee approved the experimental protocol for this study (application register number 7159090317). The present study was conducted following the ethical guidelines determined by the Brazilian National Council for the Control of Animal Experimentation (Conselho Nacional de Controle de Experimentação Animal, CONCEA).

2.3. Study design – Animals

Female Wistar rats (n = 14) were housed in polypropylene cages, three or four rats in each cage, with wood shavings as bedding. All rats had free access to food (Nuvilab®, Brazil, 2.79 kcal/g) and water throughout the experimental period, and were maintained in a temperature-controlled purpose-built animal room (23 ± 1 °C), in a 12:12-h light/dark cycle. On the 60th day of life, having a body weight of approximately 200–220 g, rats were anesthetized with ketamine/xylazine (66/33 mg/kg) intraperitoneally and submitted to either bilateral ovariectomy (Ovx, n = 10) or sham-ovariectomy (Sham, n = 4) surgery following protocol previously established by our group (Boldarine et al., 2021).

2.4. Study design – GbE supplementation

Following a protocol previously established by our group (Banin

et al., 2017), 60 days after surgery the Ovx rats were grouped into two subgroups, and gavaged once daily for 14 days with either 1.5 mL saline (Ovx + Veh, n = 5) or 500 mg/Kg GbE dissolved in 1.5 mL saline (Ovx + GbE, n = 5).

The GbE supplementation protocol adopted in the present study followed the one previously adopted by our research group (Banin et al., 2014, 2017, 2021; Gaiardo et al., 2019; Hirata et al., 2015, 2019a, 2019b; 2023; Machado et al., 2021a, 2021b) to specifically investigate the effects of GbE oral supplementation on energy and lipid homeostasis, and on neural activity and behaviour, in Wistar rats.

The brain samples used in the present study were obtained from animal cohorts previously used in other studies which evaluated peripheral and central effects of GbE supplementation in ovariectomized rats (Banin et al., 2021, 2017; Machado et al., 2021a). The brain samples were kept safely at ultra-low temperature until immunohistochemistry and immunofluorescence analyses. The intention to analyse stored samples is aligned with the ethical principles of reducing the number of animals used in biosciences research.

2.5. Immunohistochemistry

Twenty-four hours after the final gavage, rats were deeply anesthetized with thiopental (80 mg/kg) intraperitoneally, intracardially perfused with 0.01 M phosphate buffer (PBS, pH 7.4, 4 °C), followed with a fixative solution (4 % paraformaldehyde in 0.01 M PBS, pH 7.4, 4 °C (PFA)) perfusion. The brains were carefully harvested, post-fixed in 4 % PFA at 4 °C for 48 h, and subsequently immersed in 30 % sucrose in 0.01 M PBS for cryoprotection for four days. Lastly, brain samples were frozen and stored at −80 °C until sectioning.

The frozen brains were sliced into coronal semi-serial 30-μm-thick sections using a cryostat (Leica Biosystems CM1850UV, Germany). For the hypothalamus, slices from the arcuate nucleus (ARC), ventromedial (VMH) and lateral hypothalamus (LH) were collected from bregma following the coordinates –1.20 to –3.84 mm. For the hippocampus, from dorsal hippocampal formation (dHF) to ventral hippocampal formation (vHF), slices were collected from bregma following the coordinates –2.52 to –5.16 mm. Hippocampus and hypothalamus were sliced serially and collected into sterile 12-well plates, approximately 8 to 11 sections per well, ensuring that each well contained representative equidistant slices of the whole structure. The slices were stored in cryoprotectant solution (30 % ethylene glycol, 15 % sucrose, 0.05 M PBS) at –20 °C until analysis. The immunohistochemistry reaction was performed using all slices from each well, using one well for each marker.

The free-floating immunohistochemistry method was performed following protocol previously employed by our group (Machado et al., 2019). Briefly, brain slices were incubated for 30 min at 50 °C in 0.1 M citrate buffer (pH 6.0) for antigen retrieval. Subsequently, samples were washed, incubated with 0.3 % H2O2 for 30 min at room temperature and blocked with 5 % bovine serum albumin (BSA) for 1 h. Incubation with the primary antibodies anti-Iba-1 (1:500, ab178847, Abcam, United Kingdom) or anti-GFAP (1:1000, G3893, Sigma-Aldrich, USA) was performed overnight at 4 °C. Subsequently, samples were incubated for 2 h at room temperature with the respective biotinylated secondary antibodies anti-rabbit (1:200, BA1000, Vector Laboratories, USA) or anti-mouse (1:200, BA9200, Vector Laboratories, USA). Lastly, slices were incubated with ABC reagent (Vectastain® Elite ABC-peroxidase kit, Vector Laboratories, USA) for 2 h at room temperature followed by incubation with 3,3′-diaminobenzidine (DAB, Sigma-Aldrich, USA). The brain slices were mounted on slides, dehydrated in ascending ethanol solutions, cleared with xylene, and coverslipped.

Iba-1- and GFAP-positive cells were bilaterally photographed at 100 × magnification using an optical microscope Nikon Eclipse 50i (Nikon Instruments Inc., Japan) coupled to a Nikon DXm1200 camera (Nikon Instruments Inc., Japan), and manually counted with ImageJ software (National Institutes of Health, USA) after background subtraction. The total number of astrocytes and microglia was estimated for each slice, and the average for each rat was calculated. Importantly, the Iba-1 and GFAP analyses were performed using slices from the same brain. Based on the integrity of brain slice samples after the free-floating immunohistochemistry processes, the total number of rats for each analysis was not always the same. Iba-1-positive cells were reported as both total and reactive microglia. The criteria adopted to categorize microglia cells as activated or resting was established by Gomes and colleagues (2015). Data are expressed as the percentage of immunoreactive (IR) cells in relation to the Sham group.

2.6. Immunofluorescence

dHF cell distribution was characterized by immunofluorescence. Slices were firstly washed in 0.01 M PBS and blocked in 3 % BSA for 1 h. The second step involved simultaneous incubation at room temperature for 3 h with the following primary antibodies: anti-Iba-1 (1:750, 019–19741, Wako, USA), anti-GFAP (1:750, ab7260, Abcam, United Kingdom), and anti-NeuN (1:1500, MAB377, Millipore, USA). The slices were subsequently incubated for 45 min with the secondary antibodies anti-mouse AlexaFluor 594 (1:400, 715–585-150, Jackson Laboratory, USA) and anti-rabbit AlexaFluor 488 (1:400, 111–545-008, Jackson Laboratory, USA), and mounted with glycerol + DAPI (3:1). Lastly, the slices were photographed at 20 × magnification using a Leica DM5500B fluorescence microscope (Leica Microsystems, Germany) coupled with a Leica DFC345FX camera (Leica Microsystems, Germany). The exposure time was the same for all slices: blue (306.26 s), green (442.12 s), and red (554.78 s). Additionally, the gain was the same for Iba-1 (blue: 2.0, green: 4.0, red: 3.0) and GFAP (blue: 2.0, green: 3.0, red: 5.0).

2.7. Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM). All variables were Shapiro-Wilk-tested for distribution normality. Statistical analyses were performed using one-way ANOVA or Kruskal-Wallis tests followed by Tukey's or Dunn's post hoc tests for multiple comparisons. The significance level was set at $p \leq 0.05$. GraphPad Prism version 8.0.1 was used to run the statistical analyses and produce the graphs.

3. Results

3.1. GbE reverted ovariectomy-induced microglial reactivity in the dorsal hippocampal formation

The hippocampus was an area of interest due to its modulatory activities upon feeding behaviors, differentiation of feeding choices through integration of sensorial cues, energy status, previously learned habits, as well as negative feedback generation of food intake (Kanoski et al., 2011; Kanoski and Grill, 2017). Moreover, visceral signals received by the hippocampus are propagated to the hypothalamus, highlighting the critical role of the hippocampal-hypothalamic circuitry in controlling food intake.

No significant differences were identified in the total number of Iba-1 positive cells, neither in dHF (Fig. 1c-d) nor in vHF (Fig. 2b-d). Statistically significant differences were found in Iba-1 reactive cells in the following dHF regions: CA1 ($F(2,10) = 7.029$; $p = 0.012$), CA3 ($F(2,10) = 17.35$; $p < 0.001$), Dentate gyrus (DG) ($F(2,10) = 8.782$; $p = 0.006$), as well as in the vHF DG ($p = 0.002$) (Fig. 2g).

In all dHF regions analyzed, the Ovx + Veh group showed significantly increased microglial reactivity in comparison to the Sham group (CA1: 111.8 %, $p = 0.021$; CA3: 169.7 %, $p < 0.001$; DG: 211.9 %, $p = 0.004$; Fig. 1f-h). The Ovx + GbE group showed significantly reduced microglial reactivity in CA1 (50.4 %, $p = 0.020$) and in CA3 (50.2 %, $p = 0.002$) compared to Ovx + Veh, while no differences between Ovx + GbE and Sham groups were observed.

In the vHF DG, the Ovx + Veh group showed significantly increased

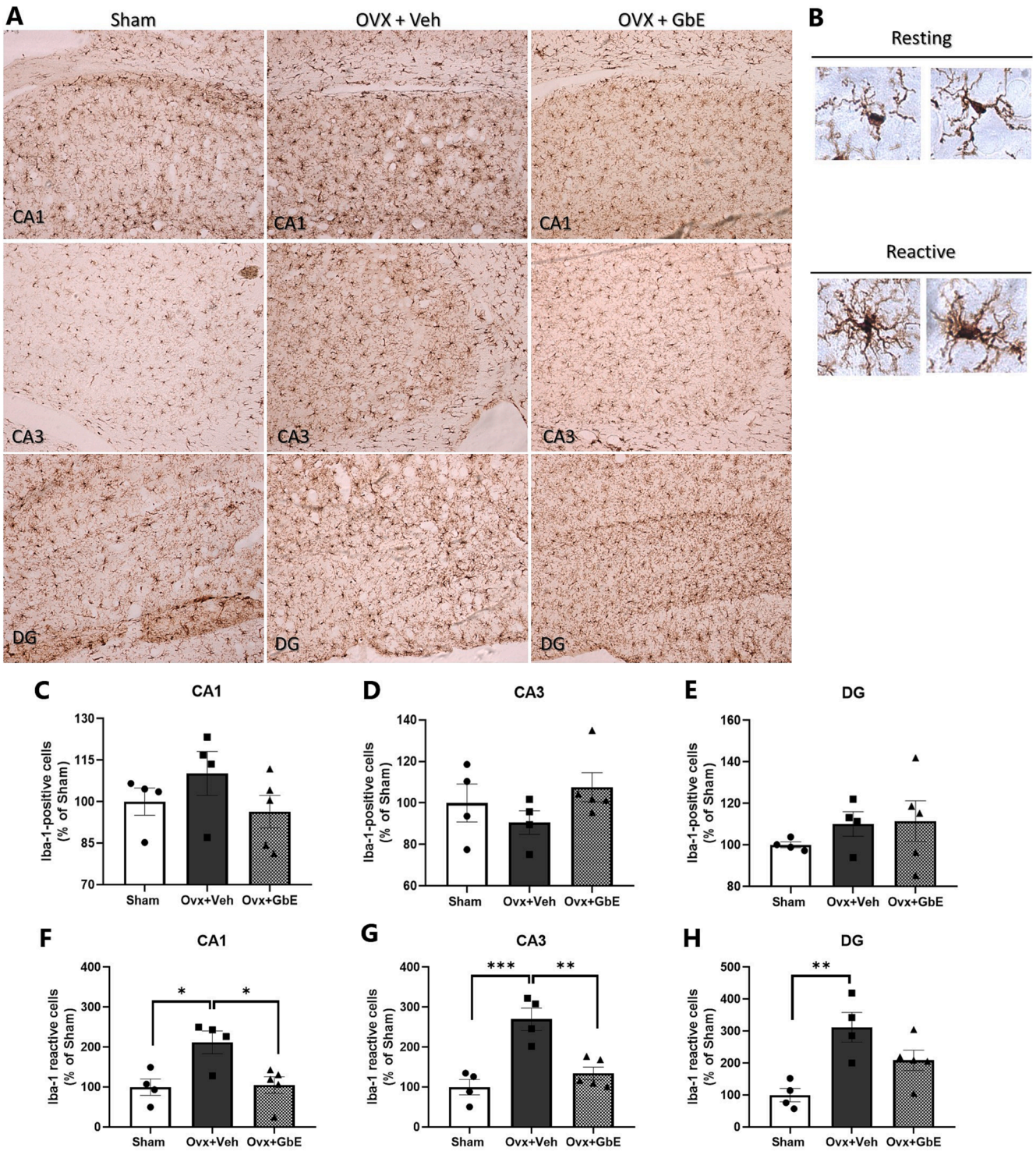

Fig. 1. Microglial reactivity in the CA1, CA3, and dentate gyrus (DG) of the dorsal hippocampal formation. Iba-1 immunostaining (100x magnification) as a microglia marker (a). Examples of cells considered as resting or reactive microglia (b). Percentage (%) of the total-positive cells (c-e) and reactive cells (f-h) in relation to the Sham group (n = 4–5). Data were expressed as mean ± SEM. *p ≤ 0.05, **p < 0.01, ***p ≤ 0.001.

microglial reactivity in comparison to the Sham group (p = 0.026; Fig. 2g). Differently from what was found in the dHF, the Ovx + GbE group did not show statistically significant changes in the vHF when compared to Ovx + Veh. Interestingly, no differences between Ovx + GbE and the Sham group were found in any of the dHF and vHF subregions analyzed. Fig. 3 depicts representative images obtained by immunofluorescence for the Iba-1-positive cells.

3.2. GbE enhanced astrocytic recruitment in dHF and vHF subregions in ovariectomized rats

In the dHF, no significant differences related to GFAP-positive cells were identified in CA1 (Fig. 4b). However, in CA3 (Fig. 4c; [F(2,9) = 7.085; p = 0.014]), the Ovx + Veh group showed a statistically significant reduction of 23.3 % in the number of GFAP-positive cells in comparison to the Sham group (p = 0.039). Furthermore, the Ovx + GbE

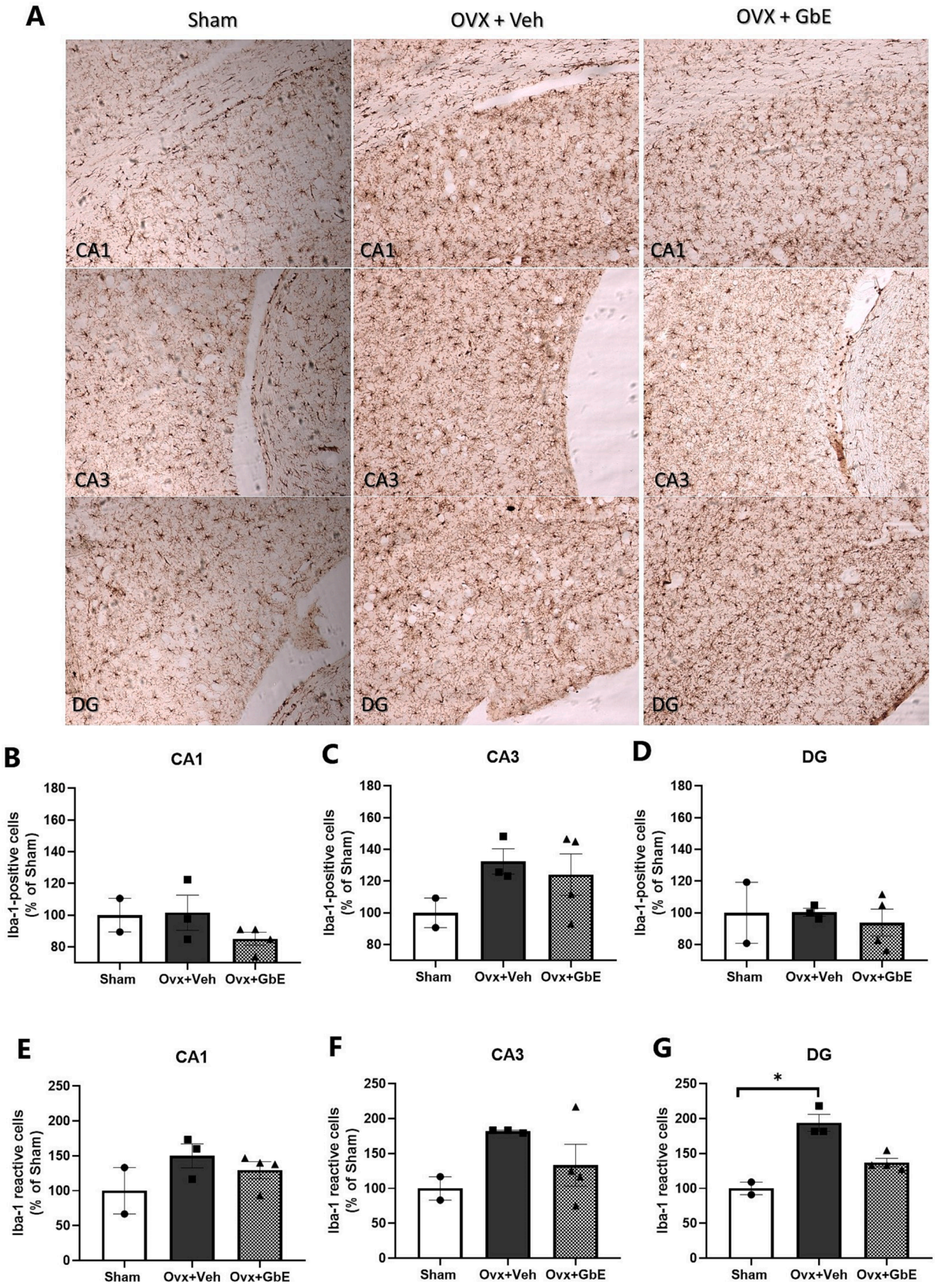

Fig. 2. Microglial reactivity in the CA1, CA3, and dentate gyrus (DG) of the ventral hippocampal formation. Iba-1 immunostaining (100x magnification) as a microglia marker (a). Percentage (%) of the total-positive cells (b-d) and reactive cells (e-g) in relation to the Sham group (n = 2–4). Data were expressed as mean ± SEM. *$p \leq 0.05$.

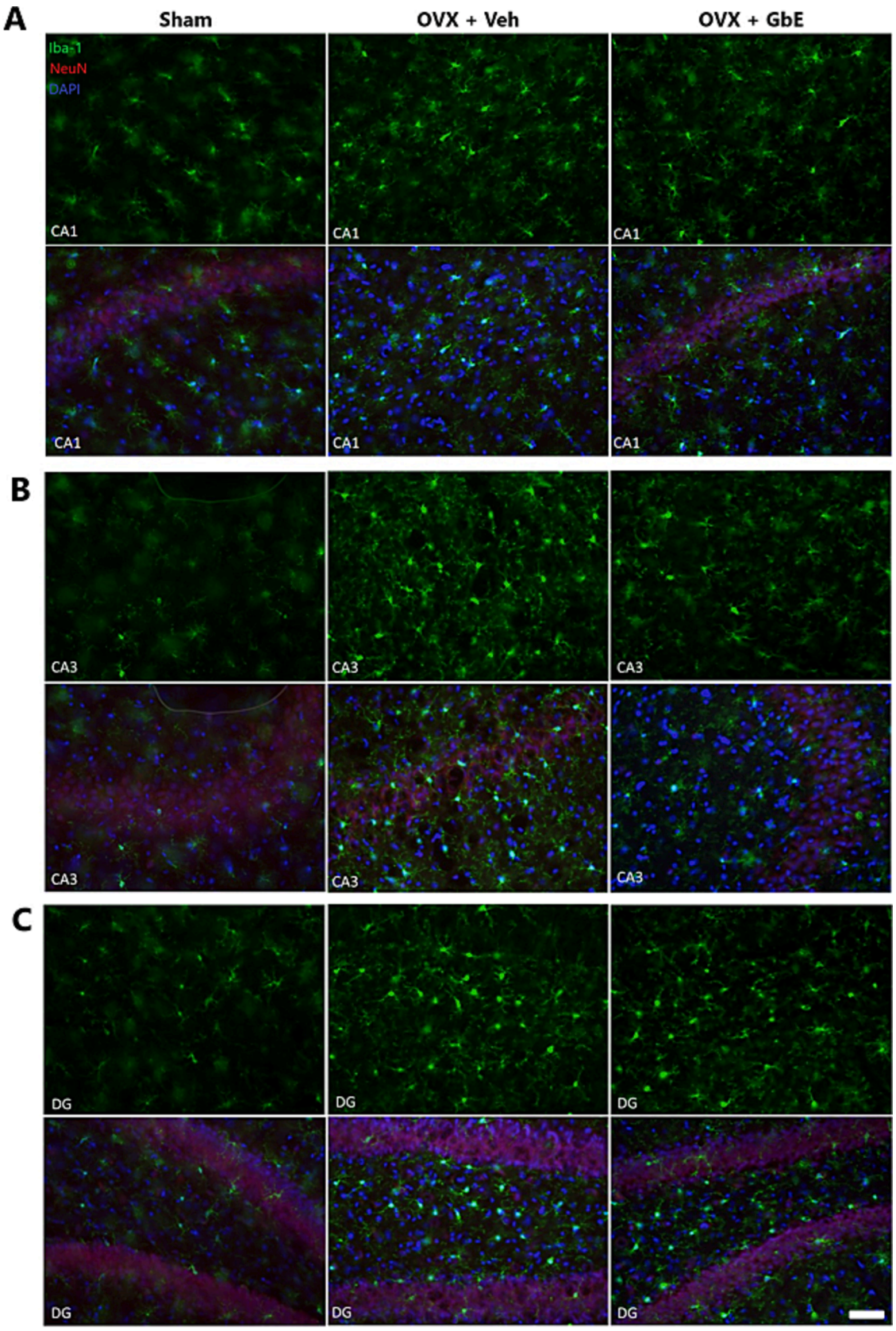

Fig. 3. Representative photomicrographs of Iba-1 in the CA1 (a), CA3 (b), and dentate gyrus (DG; c) of the dorsal hippocampal formation by immunofluorescence (150 μm). Iba-1 (green), NeuN (red), DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

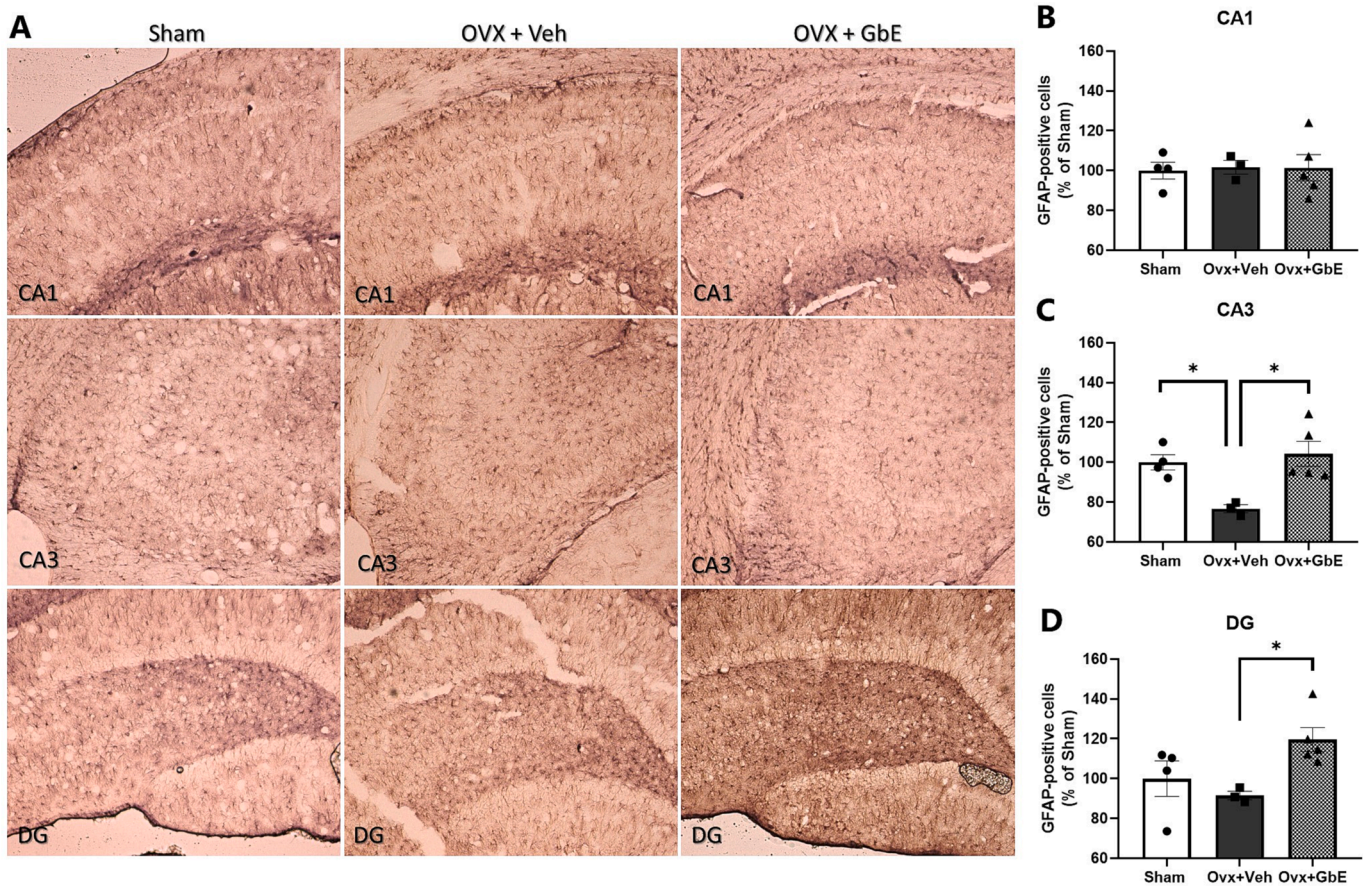

Fig. 4. Astrocytes distribution in the CA1, CA3, and dentate gyrus (DG) of the dorsal hippocampal formation. GFAP immunostaining (100x magnification) as an astrocytic marker (a). Percentage (%) of the total-positive cells (b-d) in relation to the Sham group (n = 3–5). Data were expressed as mean ± SEM. Data were expressed as mean ± SEM. *p ≤ 0.05.

group showed significantly higher counts of GFAP-positive cells in CA3 when compared to Ovx + Veh (36 %, p = 0.013), but no differences were found when compared to the Sham group (p = 0.814) (Fig. 4c). A similar effect was observed in DG (Fig. 4d; [F(2,9) = 4.440; p = 0.045]), where the difference in the number of GFAP-positive cells between Ovx + GbE and Ovx + Veh was 30.6 % (p = 0.050).

In the vHF, no statistically significant effects were detected in the CA1 and CA3 subregions (Fig. 5b-c). However, in DG (Fig. 5d; [F(2,8) = 6.078; p = 0.024]), the Ovx + GbE group showed a significant increase of 52.8 % in the number of GFAP-positive cells in comparison to Ovx + Veh (p = 0.021). Fig. 6 depicts representative images obtained by immunofluorescence for GFAP-positive cells, highlighting hippocampal structures with NeuN immunoreactivity.

3.3. Ovariectomy alters microglia reactivity and astrocytic recruitment in the hypothalamic arcuate nucleus

The hypothalamic nuclei investigated in our study are involved in orexigenic – anorexigenic signaling pathways (Crespo et al., 2014). The arcuate nucleus (ARC), ventromedial (VMH), and lateral hypothalamus (LH) are interconnected and form part of the intrahypothalamic network associated with homeostatic control of food intake (Williams et al., 2001).

Regarding the number of Iba-1 positive cells in the ARC, no differences were found among Ovx + GbE, Ovx + Veh and the Sham group (Fig. 7a, b). However, regarding Iba-1 reactivity, a significant effect was found (Fig. 7c; [F(2,8) = 4.499; p = 0.049]), with a statistically significant difference found between Sham and Ovx + Veh (46.7 %, p = 0.046).

The number of GFAP-positive cells in the ARC was significantly different (Fig. 7d; [F(2,6) = 50.25; p < 0.001]) among groups. Higher astrocyte counts were found in both Ovx + Veh (55.1 %, p < 0.001) and Ovx + GbE (44 %, p < 0.001) groups in comparison to the Sham group.

3.4. GbE restored microglial reactivity impaired by ovariectomy in the ventromedial hypothalamus

The VMH and LH are involved in anorexigenic and orexigenic responses, respectively (García-Cáceres et al., 2019). Those hypothalamic nuclei receive input mainly from ARC, but also from the vHF, more specifically from the vCA1 subregion which, in conjunction with the VMH and LH, process information associated with the feeding behavior (Cenquizca and Swanson, 2006; Kanoski and Grill, 2017).

In the VMH (Fig. 8), although the number of Iba-1 positive cells was not significantly different among groups (Fig. 8b; [F(2,9) = 3.275; p = 0.085]), the number of Iba-1 reactive cells was significantly different (Fig. 8c; [F(2,9) = 16.62; p = 0.001]). While reduced Iba-1 reactive cell counts were observed in Ovx + Veh in comparison to the Sham group (p < 0.001), increased Iba-1 reactive cell counts were observed in Ovx + GbE in comparison to Ovx + Veh (p = 0.0124) (Fig. 8c). The GFAP-positive cell counts in VMH were statistically similar among groups (Fig. 8d).

In the LH (Fig. 9), no differences were found in Iba-1 positive cells or reactivity among groups (Fig. 9b-c). No GFAP-immunoreactivity in the LH was observed, and thus, GFAP-positive cells were not quantified in that hypothalamic nucleus.

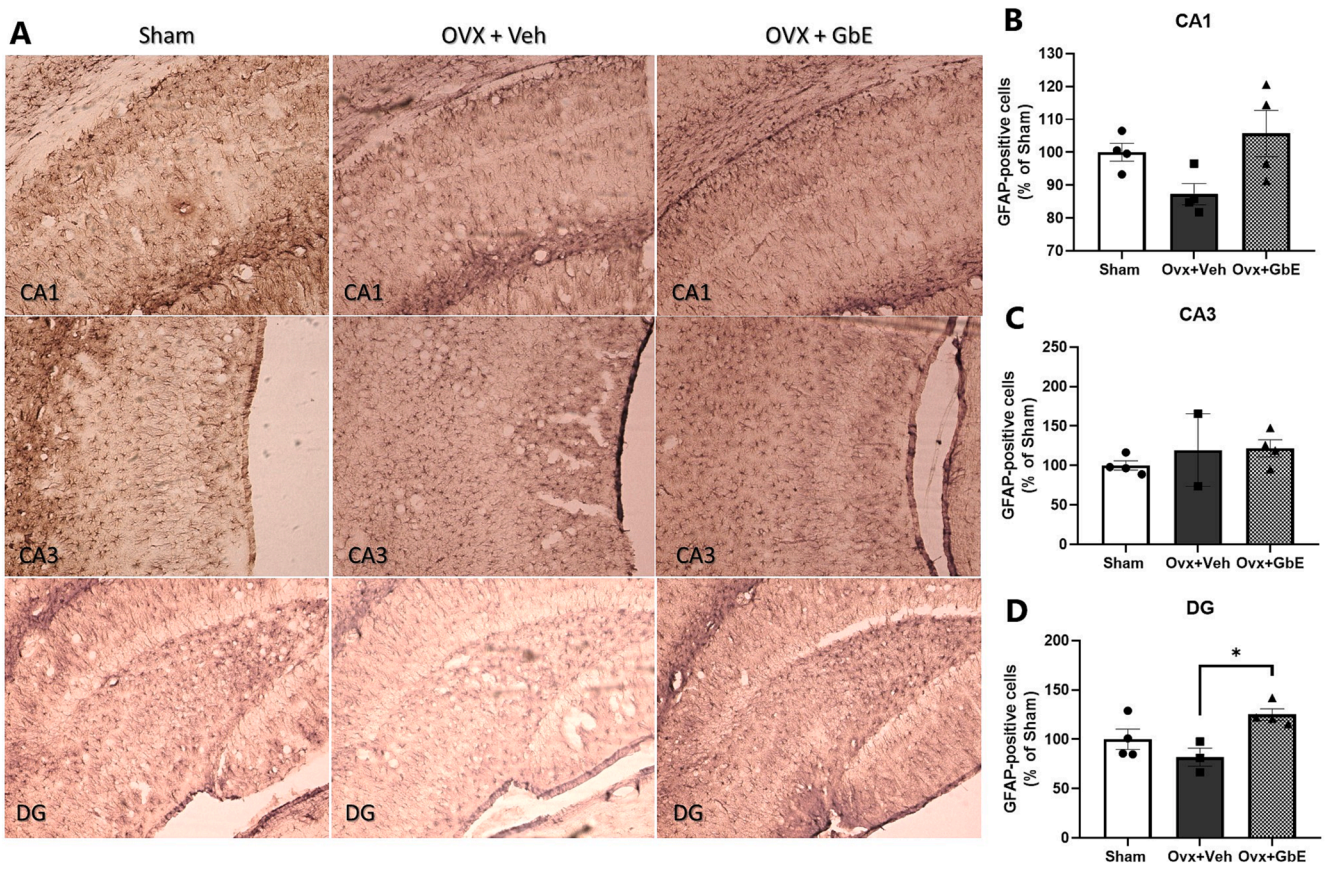

Fig. 5. Astrocytes distribution in the CA1, CA3, and dentate gyrus (DG) of the ventral hippocampal formation. GFAP immunostaining (100x magnification; a). Percentage (%) of the total-positive cells (b-d) in relation to the Sham group (n = 2–4). Data were expressed as mean ± SEM. Data were expressed as mean ± SEM. *$p \leq 0.05$.

4. Discussion

Astrocytes and microglia are known to respond to hormonal variations due to the abundant expression of estrogen and progesterone receptors (Argente-Arizón et al., 2017; Crespo-Castrillo and Arevalo, 2020). Depletion of ovarian hormones induced by menopause may disrupt the activity of astrocytes and microglial cells, potentially contributing to dysregulation of energy homeostasis (Wyse et al., 2020; Zárate et al., 2017). Considering that microglia and astrocytes are major players in the maintenance of the neural milieu (García-Cáceres et al., 2012), the present study investigated in ovariectomized rats the effects of GbE oral supplementation on the recruitment of those cells in hippocampal hypothalamic subregions.

Our findings show that ovariectomy increased microglial reactivity in all dHF subregions investigated (CA1, CA3, and DG), as well as in vHF DG. Remarkably, GbE-supplemented ovariectomized rats showed reduced microglial reactivity in the same subregions as compared to the vehicle-receiving rats, reaching similar levels to those found in Sham rats. The results herein reported are the first to attribute such effect to GbE. Similar findings have been reported in ovariectomized rats treated with estradiol (Sárvári et al., 2014), but not with GbE.

Increased microglial reactivity has been associated with exacerbated neuroinflammation and elevated oxidative stress (Simpson and Oliver, 2020). Such disturbances have been associated with neuropsychiatric (Blank and Prinz, 2013) and neurodegenerative diseases (Hickman et al., 2018), as well as with obesity (Nguyen et al., 2014). Our previous investigations have reported increased oxidative stress in the hippocampus of ovariectomized rats, which was ameliorated after GbE supplementation (Machado et al., 2021a), evidenced by improvement in the activity of endogenous antioxidant enzymes involved in reactive oxygen species (ROS) scavenging.

Previous research has identified a link between GbE activity and ameliorated microglia-induced pro-inflammatory response (Gargouri et al., 2018; Liu et al., 2022). Additionally, the effects of GbE supplementation have been investigated on hippocampal function. GbE supplementation has been associated with behavioral changes and short-term memory improvement (Ribeiro et al., 2016), and also associated with modulation of the proteomic profile in dHF (Gaiardo et al., 2019), increased astrocyte count (Oliveira et al., 2013), and GFAP expression in dHF subregions (Oliveira et al., 2009).

Our findings on astrocyte count and reactivity are novel and well aligned with previous studies involving hormone replacement – rather than phytochemicals – following ovariectomy. We found a significant reduction in the number of GFAP-positive cells in the dHF CA3 subregion in ovariectomized rats, but at the same time a significant increase in astrocyte number in dHF (CA3 and DG) and in vHF (DG) of GbE-supplemented rats, as compared to the vehicle-receiving group. A previous study found reduced microglial reactivity and increased astrocyte count in GFAP-aromatase knockout ovariectomized mice treated with a low dose of exogenous 17β-estradiol (Wang et al.,2020). That same study found in the treated group an improvement in the microglial anti-inflammatory phenotype in the hippocampus after global cerebral ischemia. The gap between our study and the study of Wang and colleagues (Wang et al.,2020) is likely to be bridged by the previously documented affinity of GbE to estrogen receptors (Oh and Chung, 2004).

Our study shows for the first time that the relationship between hippocampal activity and feeding behaviors can be potentially influenced by GbE supplementation following ovariectomy. The vHF receives visceral signals related to energy status and food intake via serotonergic and noradrenergic inputs, specifically in the vDG and vCA1 subregions

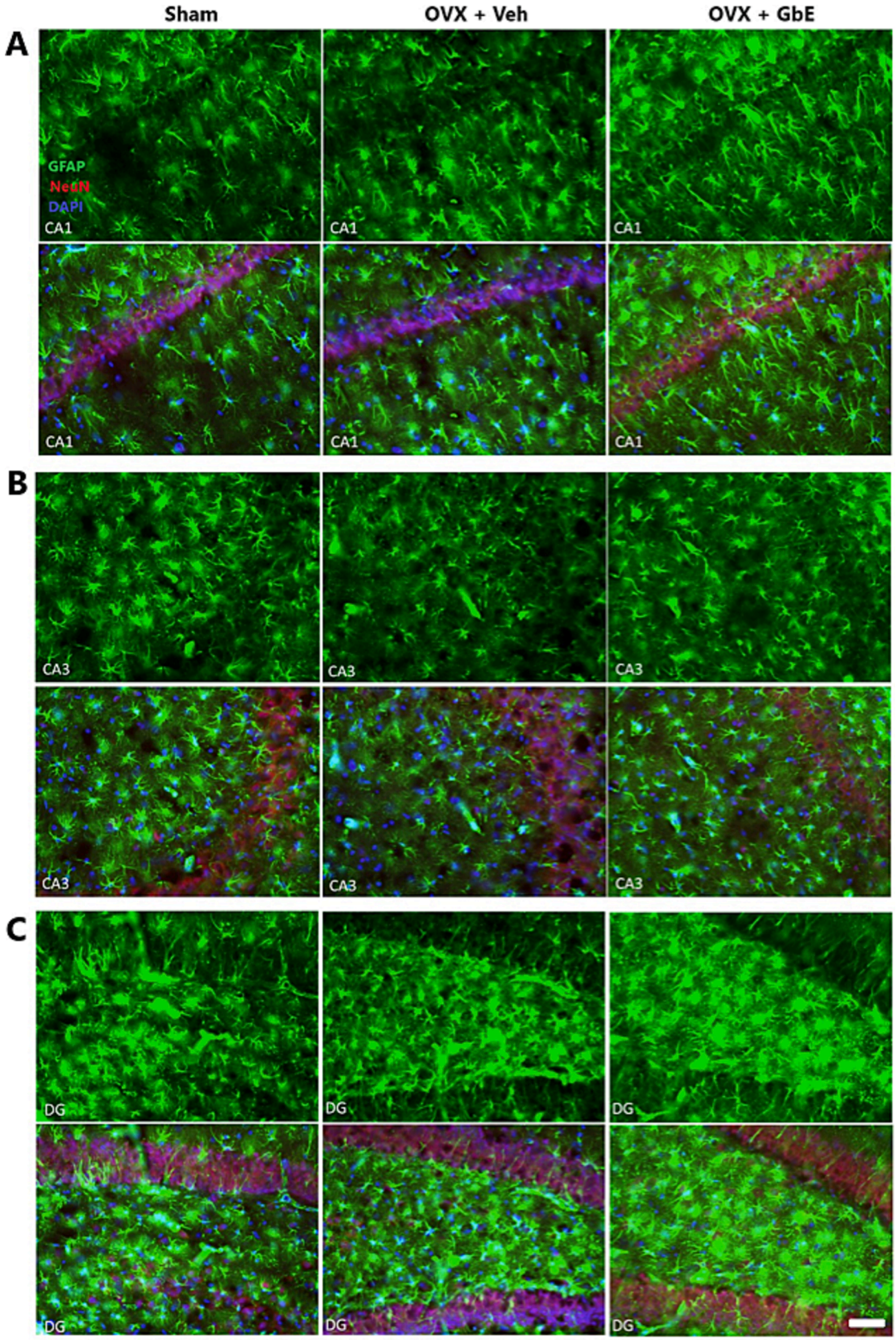

Fig. 6. Representative photomicrographs of GFAP-positive cells in the CA1 (a), CA3 (b), and dentate gyrus (DG, c) of the dorsal hippocampal formation by immunofluorescence (150 μm). GFAP (green), NeuN (red), DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

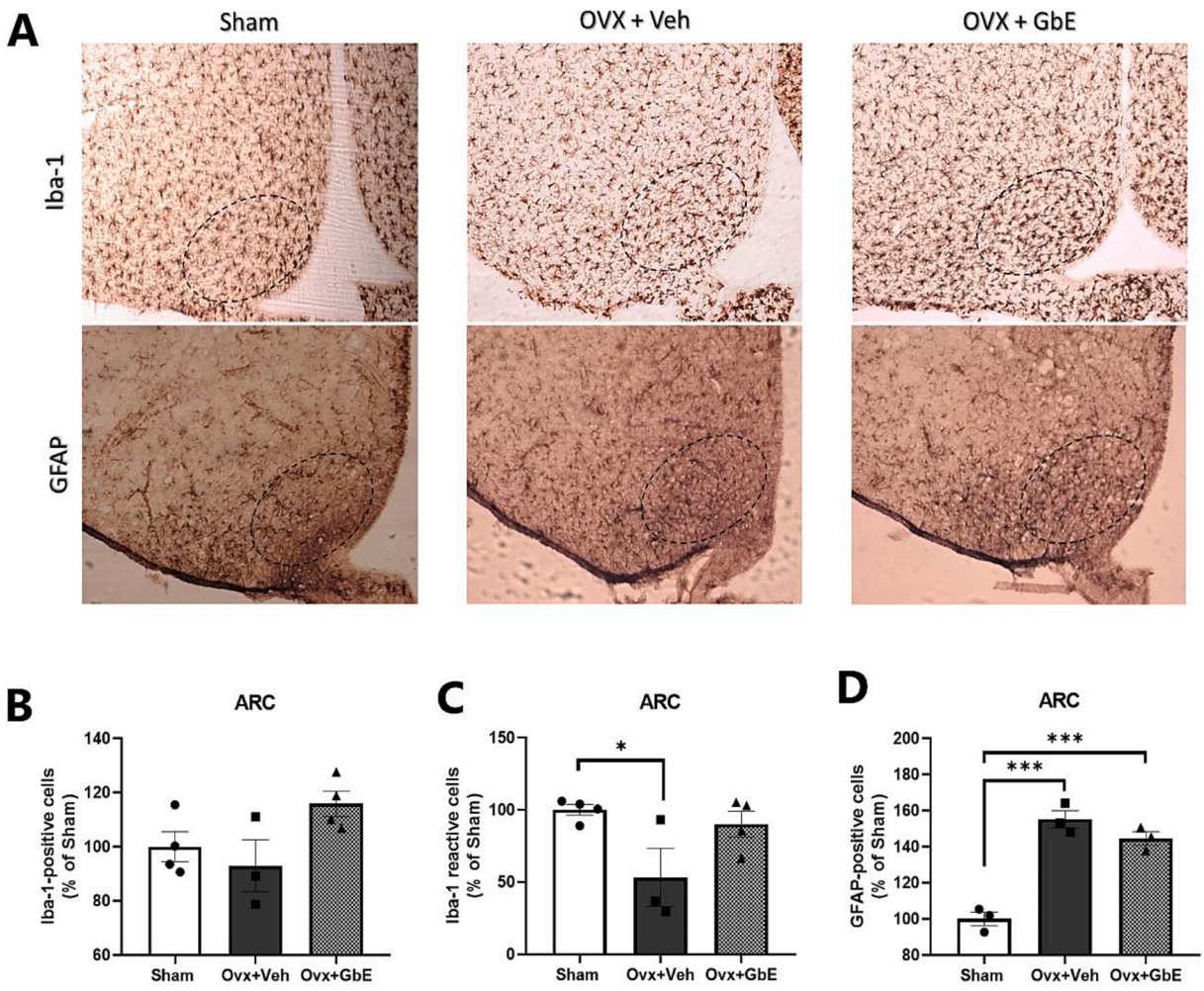

Fig. 7. Microglial reactivity and astrocytic distribution in the hypothalamic arcuate nucleus (ARC). Iba-1 (upper panel) and GFAP (lower panel) immunostaining (100x magnification, a). Percentage (%) of the total-positive cells and reactive cells to Iba-1 (b-c) in relation to the Sham group. % of the GFAP-positive cells in relation to the Sham group (d). Data were expressed as mean ± SEM (n = 3–4). *p ≤ 0.05, ***p ≤ 0.001.

(Kanoski and Grill, 2017). We have previously demonstrated that hippocampal serotonergic and leptinergic signaling were impaired by ovariectomy, and that GbE supplementation was able to reestablish it (Machado et al., 2021a). In a broader context, our latest findings, added to our previous ones, show that GbE supplementation restored the effectiveness of hippocampal systems involved in the recognition of peripheral energy signals, and in the generation of negative feedback to food intake, respectively (Machado et al., 2021a).

The neural networks between the hippocampus and hypothalamus have been well characterized, and so has the role of the hypothalamus in regulating energy homeostasis, responding to variations in energy homeostasis-associated signals, including peripheral hormones and nutrients (Crespo et al., 2014). Through glutamatergic outputs projected from the vHF, the hypothalamus receives feeding-related information transmitted from vCA1 to the LH (Kanoski and Grill, 2017). The VMH also receives input from vCA1, but less densely when compared to LH (Cenquizca and Swanson, 2006). Nonetheless, the main neuronal populations associated with sensing nutritional status and inducing the respective anorexigenic or orexigenic responses are densely expressed in the ARC (Waterson and Horvath, 2015). The ARC in turn appears to rely on communication with other hypothalamic nuclei, such as LH and VMH, to maintain energy homeostasis (Clasadonte and Prevot, 2018).

In the hypothalamus, both astrocytes and microglia can directly regulate neuronal activity involved in the homeostatic control of food intake, modulating synaptic transmission and plasticity (Araque et al., 2014; García-Cáceres et al., 2019). Hypothalamic astrocytes modulate neuroendocrine circuits by regulating extracellular levels of neurotransmitters (Jang et al., 2017). Astrocytes can also stimulate neurons that induce food intake through lactate release (Marina et al., 2018). Additionally, microglia release neurotrophic factors, supporting surrounding networks in the hypothalamus involved in appetite regulation (Urabe et al., 2013). Dysfunctional hypothalamic microglia may potentiate body weight loss (García-Cáceres et al., 2019).

Our results show reduced microglia reactivity without concomitant changes in the number of microglial cells in the ARC and VMH of vehicle-receiving ovariectomized rats. The blood–brain barrier appears to be highly permeable in the ARC, to allow for a rapid detection of variation in energy substrates and hormones (Crespo et al., 2014). At the same time, microglial reactivity is an essential neuroprotective mechanism due to its continuous surveillance within the brain for signals originated from peripheral areas (Léon et al., 2021).

Our findings suggest an impairment in neural immune surveillance in ovariectomy, explained by our findings of reduced microglia reactivity. As the lack of microglial reactivity is known to be detrimental to the neural milieu (Nimmerjahn et al., 2005), if our suggestion turns out to be accurate, ovariectomized rats may be more vulnerable to the penetration of noxious peripheral factors into the brain (Wang and Li, 2021).

Our results on reduced microglia reactivity in ovariectomized rats may also contribute to the understanding of how such response may be

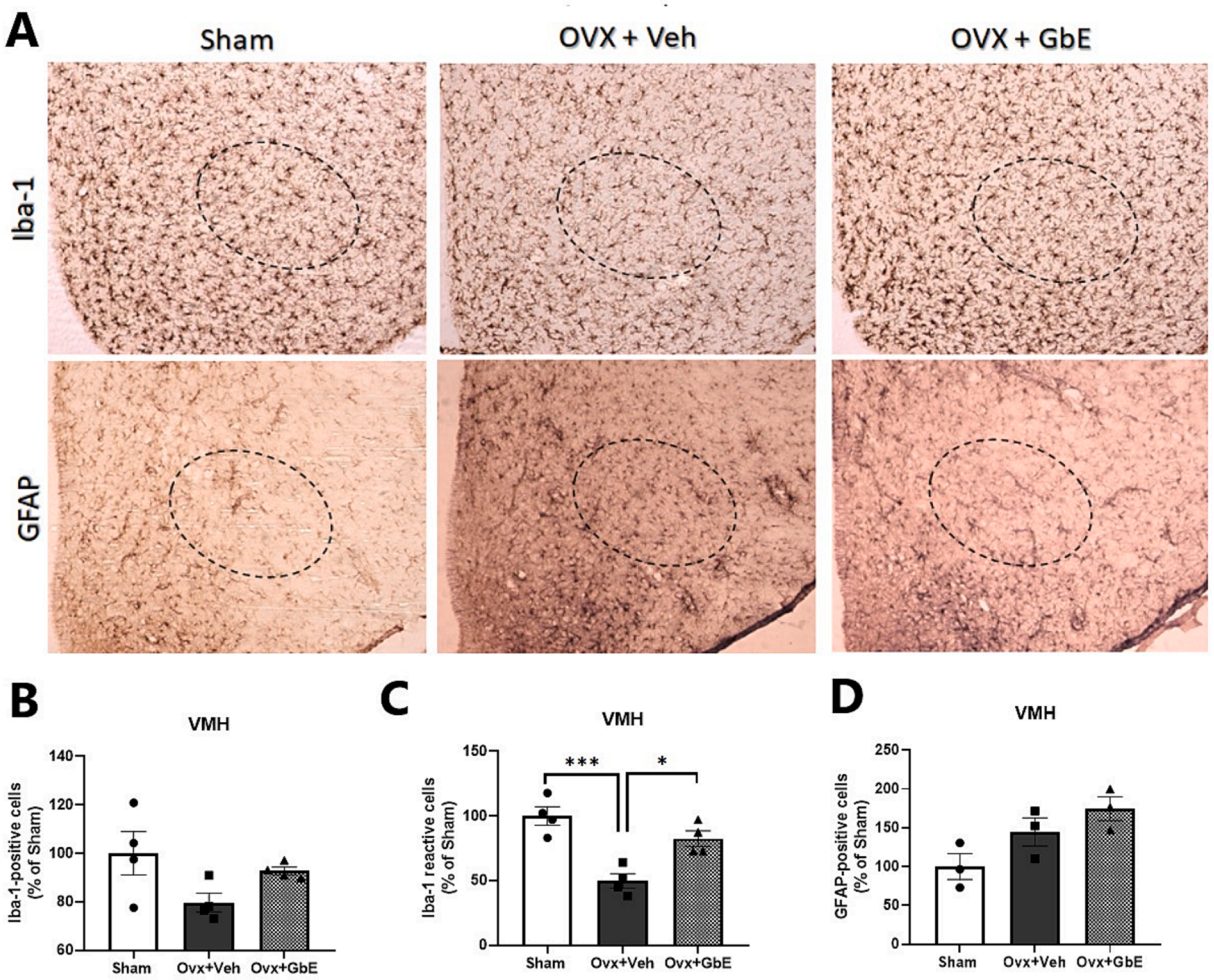

Fig. 8. Microglial reactivity and astrocytic distribution in the ventromedial hypothalamus (VMH). Iba-1 (upper panel) and GFAP (lower panel) immunostaining (100x magnification, a). Percentage (%) of the total-positive cells and reactive cells to Iba-1 (b-c) in relation to the Sham group. % of the GFAP-positive cells in relation to the Sham group (d). Data were expressed as mean ± SEM (n = 3–4). *p ≤ 0.05, ***p ≤ 0.001.

associated with disturbed energy homeostasis and changes in body profile, observed in previous studies. A previous study from our group found higher body weight gain in ovariectomized rats (Banin et al., 2017), and a study from another research group (Gao et al., 2017) found an association between reduced hypothalamic microglia reactivity and increased body weight gain in mice fed a high-carbohydrate high-fat diet.

In our study, GbE-supplemented ovariectomized rats showed levels of microglial reactivity in ARC and VMH nuclei similar to those found in the control sham rats. However, when the GbE-supplemented rats were compared to the vehicle-treated ovariectomized rats, the latter showed lower microglial reactivity in the VHM. To the best of our knowledge, the effects of GbE supplementation on astrocytic and microglia cells in ovariectomized rats unraveled in the present study have not been reported before.

The brain samples examined in the present study were obtained from cohorts of ovariectomized rats assessed in other studies from our group, which investigated various parameters related to feeding behavior and energy homeostasis (Banin et al., 2021, 2017; Machado et al., 2021a). In those studies, GbE supplementation either reverted or ameliorated ovariectomy-induced manifestations such as increased food intake, body mass gain, visceral adiposity, dyslipidemia, and body composition (Banin et al., 2021, 2017). GbE supplementation was also effective in ameliorating anxious- and depressive-like behaviors in ovariectomized rats (Banin et al., 2021). As discussed earlier, the present study shows that GbE supplementation reestablished the effectiveness of hippocampal and hypothalamic signaling pathways responsible for feeding behavior regulation (Banin et al., 2017; Machado et al., 2021a).

5. Conclusion

The present study is the first to show that ovariectomized rats supplemented with GbE show a partial or total amelioration in hippocampal and hypothalamic microglia reactivity and astrocyte recruitment, reaching levels similar to those found in the control group. Collectively, our results corroborate the hypothesis that GbE may exert a modulatory role in maintaining neural homeostasis, which is likely to be explained by the well-documented antioxidant and anti-inflammatory properties attributed to GbE. Microglial cells play different roles in response to the same stimuli, triggering distinctive responses depending on the brain region (Wang and Li, 2021). Such intrinsic heterogeneity characteristic to microglial activity may explain the distinctiveness of our findings between the hippocampus and hypothalamus.

If future studies employing other experimental models of menopause can confirm our findings that GbE supplementation positively influences astrocyte and microglial activity and recruitment, GbE supplementation may become a potential tool to aid in the regulation of energy balance in menopausal conditions. If confirmed, such understanding may ultimately contribute to the development of additional therapeutic strategies that promote healthier body composition and metabolic profile in menopaused individuals.

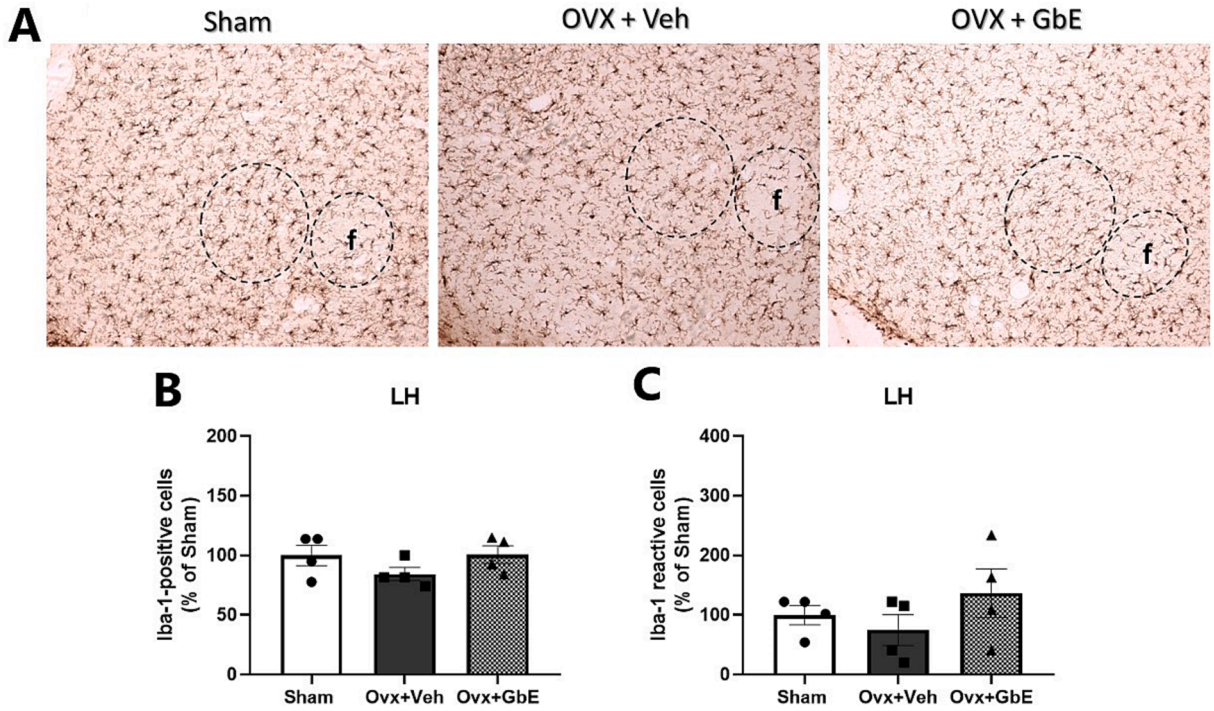

Fig. 9. Microglial reactivity in the lateral hypothalamus (LH). Iba-1 immunostaining (100x magnification, a). Percentage (%) of the total-positive cells and reactive cells to Iba-1 (b-c) in relation to the Sham group (n = 4). Data were expressed as mean ± SEM.

6. Author's contributions

MMFM: Conceptualization, validation, formal analysis, investigation, methodology, data curation, writing – review & editing. EMA, RMB, BKSH, FMT, LMO, PRGK, APP: methodology, investigation, formal analysis. EBR: Conceptualization, visualization, funding acquisition. SMC: Conceptualization, visualization, funding acquisition, supervision. AAB: Critical analysis, writing – review & editing. MMT: Conceptualization, methodology, formal analysis, resources, writing – review & editing, visualization, supervision, project administration, funding acquisition. All authors have read and approved the final version of the manuscript.

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CRediT authorship contribution statement

Meira M.F. Machado: Conceptualization, Validation, Formal analysis, Investigation, Methodology, Data curation, Writing – review & editing. **Esther M. Ático:** Methodology, Investigation, Formal analysis. **Renata M. Banin:** Methodology, Investigation, Formal analysis. **Bruna K.S. Hirata:** Methodology, Investigation, Formal analysis. **Paula R.G. Kempe:** Methodology, Investigation, Formal analysis. **Amanda P. Pedroso:** Methodology, Investigation, Formal analysis. **Fernanda M. Thomaz:** Methodology, Investigation, Formal analysis. **Lila M. Oyama:** Methodology, Investigation, Formal analysis. **Eliane B. Ribeiro:** Conceptualization, Visualization, Funding acquisition. **Allain A. Bueno:** Writing – review & editing. **Suzete M. Cerutti:** Conceptualization, Visualization, Funding acquisition, Supervision. **Mônica M. Telles:** Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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