Macrophages are activated in the rat anterior pituitary under chronic inflammatory conditions

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Macrophages are activated in the rat anterior pituitary under chronic inflammatory conditions

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Abstract

In the anterior lobe of the pituitary gland (AP), non-endocrine cells regulate hormone secretion by endocrine cells. However, the functions of non-endocrine cells in the AP during chronic pain are largely unclear. Here, we show that macrophages, but not folliculostellate (FS) cells, were selectively increased in the AP in the complete Freund's adjuvant (CFA)-induced chronic inflammatory pain model in rats. In addition, IL-1β expression was increased in the AP, and the IL-1β-immunopositive cells were identified as macrophages. On the other hand, increased macrophage density and IL-1β expression were not detected in a neuropathic pain model induced by partial sciatic nerve ligation (PSL). Furthermore, we found c-Fos expression specifically in the somatotrophs under the chronic inflammatory pain condition. Because IL-1β promotes growth hormone (GH) synthesis and release, our results suggest that AP macrophage contributes to GH release through IL-1βduring chronic inflammatory pain.

1. Introduction

In the neuroendocrine system, the pituitary gland plays a central role in homeostatic regulation. The pituitary gland consists of the anterior, intermediate, and posterior lobes. The anterior lobe of the pituitary gland (AP) generally contains two types of cells: endocrine cells, which release a variety of hormones, and glial-like non-endocrine cells, which release growth factors or inflammatory cytokines.

Folliculostellate (FS) cells are a well-characterized non-endocrine cell that directly modulate the activity of endocrine cells through phagocytosis or through interactions via gap junctions [8]. FS cells are also able to regulate the activity of endocrine cells indirectly. For example, lipopolysaccharide (LPS) and IL-1β each stimulate FS cells to release IL-6[4, 18], which induces the secretion of hormones such as adrenocorticotropic hormone (ACTH), growth hormone (GH), prolactin (PRL), luteinizing hormone (LH), and follicular stimulating hormone (FSH) by endocrine cells [24]. Macrophages represent another type of non-endocrine cell in the AP [10, 15, 20]. Macrophages have been identified in the AP using antibodies against macrophage proteins such as F4/80 antigen, OX-42, and Iba-1, but their function in the AP remains largely unclear [35].

Stimulation of endocrine cells in the AP during pain has been reported. For instance, the plasma concentration of corticosterone increased in a chronic pain model in mice [2]. In addition, GH secretion was enhanced in an inflammatory pain model in rats [7]. However, the functional

role of non-endocrine cells such as FS cells and macrophages in the AP under these conditions has not been reported. In this study, we evaluated whether the activity of macrophages and FS cells in the AP is regulated by chronic pain using two different rat models, the complete Freund's adjuvant (CFA) model of inflammatory pain and the partial sciatic nerve ligation (PSL) model of neuropathic pain. We first identified macrophages and FS cells in the AP by immunohistochemistry and evaluated changes in their numbers and activity in the two chronic pain models. These studies demonstrate that macrophages in the AP are selectively activated during chronic inflammatory pain.

2. Materials and Methods

2.1. Animals

Male Wistar rats (280–320 g; Sankyo Laboratories Japan, Inc., Tokyo, Japan) were used. Two rats were housed in the same cage in a temperature-controlled environment with a 12 h light-dark cycle (lights on 9:00 to 21:00) and were allowed food and water *ad libitum*. All experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Tokyo Women's Medical University.

2.2. Animal models of chronic pain

Two chronic pain models were established in the rat. In the inflammatory pain model, CFA (100 μl; Merck, Darmstadt, Germany) was injected subcutaneously into the plantar skin of the right hind paw, as reported previously [16]. In the PSL model of neuropathic pain [27], approximately 1/2 of the right sciatic nerve was tightly ligated with an 8-0 silk suture under isoflurane (1.5%) anesthesia. The sciatic nerve was exposed but not ligated as a sham operation.

2.3. Von Frey tests

To evaluate tactile allodynia, the withdrawal threshold for mechanical stimulation of the rat hind paw was measured using an automated Von Frey type system (dynamic plantar aesthesiometer; Ugo Basile, Lombardia, Italy). The mechanical stimulation, which gradually increases in force from 0 g to 25 g (maximum force), is applied with a plastic filament (0.5 mm diameter). The rate of the force increase was 2.5 g/s. The mouse was allowed to rest for at least 5 mins between measurements. The paw withdrawal threshold was determined as the average of three measurements. The measurements were performed before and 1 week after CFA injection or PSL. At this time, rats showed notable tactile allodynia in the ipsilateral hind paw, similar to previous studies [16, 22, 34].

2.4. Immunohistochemical identification

2.4-1. Non-endocrine cells in the AP

One week after CFA injection or PSL, rats were deeply anesthetized with pentobarbital [150 mg/kg intraperitoneally (i.p.)] and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. The pituitary gland was post-fixed in the same fixative overnight, incubated with 0.05 M phosphate-buffered saline (PBS) containing 25% sucrose for 1 day and PBS containing 40% sucrose for 2 days, frozen in OCT compound (Sakura Finetechnical, Tokyo, Japan), and stored at −80°C until use. Coronal sections (25 μm) throughout the pituitary were obtained using a cryostat (Leica Microsystems Inc., Buffalo Grove, IL, USA). After blocking with 10% normal goat or donkey serum and 0.3% Triton-X 100 in 0.05 M PBS for 1 h at room temperature, the sections were incubated overnight at 4°C with a rabbit polyclonal antibody against ionized calcium binding adaptor molecule 1 (Iba1; 019-19741, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan; 1:1,000) and a mouse monoclonal antibody against S100 (MAB079-1; Merck; 1:1000) in 0.05 M PBS containing 1% normal goat or donkey serum and 0.3% Triton X-100. After washing with PBS, the sections were incubated with Alexa Fluor-conjugated secondary antibodies and mounted with Prolong Gold mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, Waltham, MA, USA).

To identify the cell type that expresses the IL-1ß, we used a goat polyclonal antibody against IL-1β (AF-501NA; R&D Systems, Minneapolis, MN, USA; 1:500). Before conducting the

experiment, the specificity of this antibody was checked by its blocking peptide. The sections of AP were incubated with a rabbit polyclonal antibody against Iba1, a mouse monoclonal antibody against S100, and a goat polyclonal antibody against IL-1β in 0.05 M PBS containing 1% normal donkey serum and 0.3% Triton X-100.

2.4-2. c-Fos positive cells in the AP

Pituitary sections were first incubated in PBS containing 3% hydrogen peroxide for 5 minutes and incubated in a blocking solution containing 10% normal goat serum with 0.3% Triton-X100 for 1 hour at room temperature. The sections were incubated with the primary antibody, rabbit anti-c-Fos (Ab-5, Merck; 1:10000) overnight at 4℃ and then incubated with avidin-biotin-peroxidase complex (Vectastain Elite ABC-Kit, Vector Laboratories; 1:200) for 1 hour. The visualization of the immunohistochemical reaction was achieved by incubation in 0.05% 3,3-diaminobenzidne (DAB; Dojindo Laboratories) solution with nickel intensification for 15 min adding 0.02% hydrogen peroxide.

To identify the cell type that expresses the c-Fos, the sections were incubated with rabbit polyclonal antibody against c-Fos and any one of mouse monoclonal antibody (GH; 1:200, Santa Cruz Biotechnology Inc., ACTH; 1:1000, Chemicon International Inc., or PRL; 1:100, abD Serotec.) in 0.05 M PBS containing 1% normal donkey serum and 0.3% Triton X-100.

2.4-3. Image analysis

Images with a resolution of 512 × 512 pixels (212.55 × 212.55 μm) were acquired at 20X magnification with an LSM 710 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). Each number of Iba1-positive macrophages, S100-positive FS cells or c-Fos positive cells was counted in randomly selected 2 images per each section using ImageJ software (https://imagej.nih.gov/ij/). Three or four pituitary sections were determined per each rat.

2.5. Detection of inflammatory cytokines

Expression of IL-1β and IL-6 in the AP were measured by enzyme-linked immunosorbent assay (ELISA) using Quantikine rat IL-1β or IL-6 immunoassay kits (R&D Systems). Pituitary samples were collected from three rats, placed in 10 volumes of ice-cold lysis buffer (pH 7.4) containing 25 mM HEPES, 1 mM egtazic acid (EGTA), 1.3 mM ethylenediaminetetraacetic acid (EDTA), 5 mM $MgCl_2$, and 0.1 M CHAPS detergent, and homogenized using a Dounce homogenizer. The homogenates were centrifuged at 10,000 x g for 10 min and the supernatant was collected. The protein concentration was quantified by bicinchoninic acid assay using the BCA Protein Assay Kit (Thermo Fisher Scientific), and the same amount of total protein from each sample was analyzed.

2.6. Statistical analysis

Data are represented as the mean ± standard error of the mean (SEM). Wilcoxon tests were used for two-group comparisons. Statistical tests were performed using JMP Pro13 software (SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered statistically significant.

3. Results

Macrophages and FS cells in AP were identified by their specific antibodies, Iba1 and S100, respectively [14, 23]. Iba1-positive macrophages and S100-positive FS cells were abundantly found both in the anterior and posterior lobes of the pituitary gland in normal rats, but few were detected in the intermediate lobe (Fig. 1A). The two types of non-endocrine could be clearly identified in the AP (Fig. 1B). The number of macrophages (2.8 ± 0.19 cells/10^4 μm^2) was smaller than the number of FS cells (7.9 ± 0.48 cells/10^4 μm^2; Fig. 1C).

To determine whether these non-endocrine cell types are activated by chronic pain, we examined these cells in the CFA-induced inflammatory pain model and the PSL-induced neuropathic pain model. At 1 week after the plantar injection of CFA into the hind paw, rats showed severe tactile allodynia (CFA model, ipsilateral side; Pre, 22.54 ± 1.22 g; 1w, 12.67 ± 2.56 g; $p = 0.0294$; Fig. 2A). At that time, the density of macrophages in the AP was increased compared with the control group (saline, 2.58 ± 0.16 cells/10^4 μm^2; CFA, 3.53 ± 0.09 cells/10^4 μm^2; $p = 0.0049$; Fig. 3A). In addition, the shape of macrophages in CFA treated mice was changed from elongated form to round form. (Fig. 3B). However, the density of FS cells was

similar between the saline group (7.86 ± 0.29 cells/10^4 μm^2) and the CFA group (8.15 ± 0.32 cells/10^4 μm^2; p = 0.6093; Fig. 3C and D). We also used PSL to induce neuropathic pain and observed severe tactile allodynia 1 week after surgery, similar to what was observed in the CFA model (PSL model, ipsilateral side; Pre, 22.04 ± 1.16 g; 1 w, 11.5 ± 1.94 g; p = 0.034; Fig. 2A). At that time, in contrast to the CFA model, we found no difference in the density of either macrophages (sham, 2.76 ± 0.14 cells/10^4 μm^2; PSL, 2.75 ± 0.15 cells/10^4 μm^2; p = 1.0000; Fig. 4A and B) or FS cells (sham, 8.66 ± 0.52 cells/10^4 μm^2; PSL, 8.09 ± 0.34 cells/10^4 μm^2; p = 0.2623; Fig. 4C and D) between the sham group and the PSL group.

Activated macrophages and FS cells can release proinflammatory cytokines [18, 29, 31]. We therefore measured the expression of IL-1β and IL-6 in the pituitary tissue by ELISA. IL-1β expression increased in the CFA model (saline, 5.12 ± 0.42 pg/mg; CFA, 7.08 ± 0.73 pg/mg; p = 0.0433) but not in the PSL model (sham, 3.63 ± 0.41 pg/mg; PSL, 4.51 ± 1.13 pg/mg; p = 0.3420; Fig. 5A). In contrast to IL-1β, however, IL-6 expression was unaffected both in the CFA model (saline, 30.45 ± 0.86 pg/mg; CFA, 31.09 ± 1.45 pg/mg; p = 0.5210) and the PSL model (sham, 29.16 ± 0.81 pg/mg; PSL, 30.05 ± 1.30 pg/mg; p = 0.7488; Fig. 5B). To identify which cell type was responsible for IL-1β secretion in the AP of CFA-injected mice, we evaluated IL-1β expression by immunohistochemistry. We observed IL-1β-immunopositive cells in the AP in the CFA model, but not in each control group (saline or sham) or in the PSL model. In the CFA model,

most of the IL-1β-immunopositive cells (89%) also expressed Iba1 and were therefore identified as macrophages (Fig. 5C). By contrast, very few IL-1β-immunopositive cells expressed the FS cell marker S100 (Fig. 5D).

Finally, to identified which hormone-producing cells in the AP were affected under the chronic inflammatory pain condition, we investigated the c-Fos expression, as a cell activity marker, in GH, ACTH, or PRL immunopositive cells, in the AP of the CFA model rats. c-Fos expression was significantly observed in the AP in the CFA model rats (saline, 0.09 ± 0.04 cells/104 μm2; CFA, 5.47 ± 0.41 cells/104 μm2; $p = 0.003$; Fig. 6A). We found c-Fos is expressed specifically in the GH immunopositive cells (Fig. 6B left, arrowhead). Very few c-Fos immunopositive cells were detected, if any, in PRL and ACTH immunopositive cells (Fig. 6B middle and right).

4. Discussion

In this study, we found that CFA treatment which induces chronic inflammatory pain specifically increased the number of macrophages, but not FS cells, in the AP, and these cells produced IL-1β. By contrast, these changes were not detected in a neuropathic pain model induced by PSL.

It is unknown where the increased macrophages derived from. One possibility is proliferation

of resident macrophages in the AP. The other is migration of macrophage due to increased permeability of blood vessels. In the pituitary gland, monocytes and macrophages are candidates for IL-1 producing cell. A recent study has identified CD11c-positive dendritic cells. These dendritic cells were reported to be a subpopulation of S100-positive FS cells [26], and isolated dendritic cells in the AP secreted inflammatory and proinflammatory cytokines such as IL-1β and IL-6 in response to LPS treatment [11]. Although resident macrophage was identified in the AP, whether it secretes IL 1-β was unknown [10]. In the present study, we found that IL-1β expression increased in the AP of rats in the CFA model, and nearly 90% of IL-1β-immunopositive cells were macrophages. Thus, our results suggest that macrophages, including resident macrophage, activated by CFA treatment specifically express IL-1β in the AP during chronic inflammatory pain.

IL-1β acts as an intermediate for communication between the neuroendocrine system and the immune system [13, 25]. Stress increases levels of IL-1β in the AP, which has a paracrine action to promote the secretion of ACTH, β-endorphin, and GH by endocrine cells [1, 3]. Stress induced hormone secretion from the AP contributes to pain hypersensitivity and allodynia [21, 28]. Abnormal function of the hypothalamus-pituitary-adrenal axis has been shown in the chronic inflammatory pain model but not in models of neuropathic pain [5, 6, 30, 32]. Dysregulation of hypothalamic–pituitary–somatotropic axis also reported in chronic inflammatory condition, such

as rheumatic diseases[9, 17]. Thus, paracrine effects of CFA-induced IL-1β is able to change secretion of stress hormones by endocrine cells in the AP, and this process may be involved in the sensing of inflammatory pain. Interestingly, we found GH production cells specifically expressed c-Fos, in the CFA model, suggesting that GH production cells are activated under the chronic pain condition. Several lines of evidence have reported that GH secretion is potentiated in chronic inflammatory pain rats [7, 9, 33]. It has also been reported that IL-beta potentiates GH gene expression and GH release in vitro preparations [12, 19]. Therefore, our results provided the possibility that locally synthesized IL-1β by activated macrophages in the AP promotes the release of GH in chronic inflammatory pain condition.

In conclusion, macrophages, one of the important non-endocrine cell types in the AP, are activated in response to peripheral chronic inflammatory pain. The local synthesis of IL-1β by activated macrophages in the AP may specifically be involved in inflammatory pain and related hormone secretion.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Figure Legends

Fig. 1. Localization of macrophages and FS cells in the rat pituitary gland.

(A) Iba1-positive macrophages (green) and S100-positive FS cells (magenta) in the pituitary gland of a healthy rat. Scale bar, 1 mm. AP, anterior lobe; PP, posterior lobe of the pituitary gland. White dotted lines indicate the intermediate lobe of the pituitary gland, where macrophages and FS cells were rare.

(B) Distinct localization and morphologies of macrophages and FS cells in the AP. Scale bar, 50 μm.

(C) The density of macrophages (green) and FS cells (magenta) in the AP (n = 3 rats). $^{**}p < 0.01$.

Fig. 2 Clear tactile allodynia in the CFA and PSL pain models.

(A) Ipsilateral side paws of CFA-treated rats showed a clear reduction of the withdrawal threshold 1 week after the injection of CFA compared with pre-treatment (n = 4 rats). There was no significant difference in the withdrawal threshold at the contralateral side paw of CFA-treated rats or in the paws of saline-treated rats. $^{*}p < 0.05$. Data are presented as the mean ± SEM. a: Ipsilateral side. b: Contralateral side.

(B) Ipsilateral side paws of rats after PSL showed a clear reduction of the withdrawal threshold 1 week after surgery compared with pre-treatment (n = 4 rats). There was no significant difference

in withdrawal threshold at the contralateral side paw of PSL rats or in the paws of either side in sham-operated rats. *$p < 0.05$. Data are presented as the mean ± SEM. a: Ipsilateral side. b: Contralateral side.

Fig. 3. Macrophages but not FS cells increased in the CFA model.

(A) Macrophages in the AP in the control group (saline, left) and in the CFA group (right). Higher magnification images (lower images) reveal that the macrophages in the CFA group changed morphology from an elongated form to a round form. Scale bar, 50 μm and 10 μm in upper and lower images, respectively.

(B) The density of macrophages in the control group (white bar) and in the CFA group (black bar) (n = 7 rats). **$p < 0.01$.

(C) FS cells in the AP in the control group and in the CFA group. A similar morphology was observed in both groups (lower images).

(D) The density of FS cells in the control group and in the CFA group (n = 7 rats). n.s., not significant.

Fig. 4. The densities of macrophages and FS cells were unaffected in the PSL model.

(A) Macrophages in the AP in the sham group (left) and in the PSL group (right). A similar

morphology was observed in the two groups at higher magnification (lower images). Scale bar, 50 μm and 10 μm in upper and lower images, respectively.

(B) The density of macrophages in the control group (white bar) and in the PSL group (black bar) (n = 6 rats). n.s., not significant.

(C) FS cells in the AP in the control group and in the PSL group. A similar morphology was observed in both groups (lower images).

(D) The density of FS cells in the control group and in the PSL group (n = 6 rats).

Fig. 5. Increased IL-1β expression in the AP in the CFA model.

(A) IL-1β protein levels in the AP in the chronic pain models (n = 4 rats per group). $^{**}p < 0.01$.

(B) IL-6 protein levels in the AP in the chronic pain models (n = 4 rats per group). n.s., not significant.

(C, D) IL-1β-immunopositive cells (green) co-localized with Iba1-positive macrophages (magenta in C) but not S100-positive FS cells (magenta in D) in the CFA model. Scale bar, 20 μm.

Fig. 6. Increased c-Fos expression in the AP in the CFA model.

(A) c-Fos-immunopositive cells in the AP in the control group (left) and in the CFA group (middle). Scale bar, 50 μm. The density of c-Fos positive cells in the control group and in the CFA

group (n = 4 rats) (right). **$p < 0.01$.

(B) The double immunostaining of the c-Fos (magenta) and the hormones (green) in the AP. c-Fos was mainly detected in the nucleus of GH immunoreactive cells. Arrowhead: The cells are GH-immunoreactive in cytoplasm and c-Fos-immunoreactive in the nucleus. Scale bar, 50 μm.

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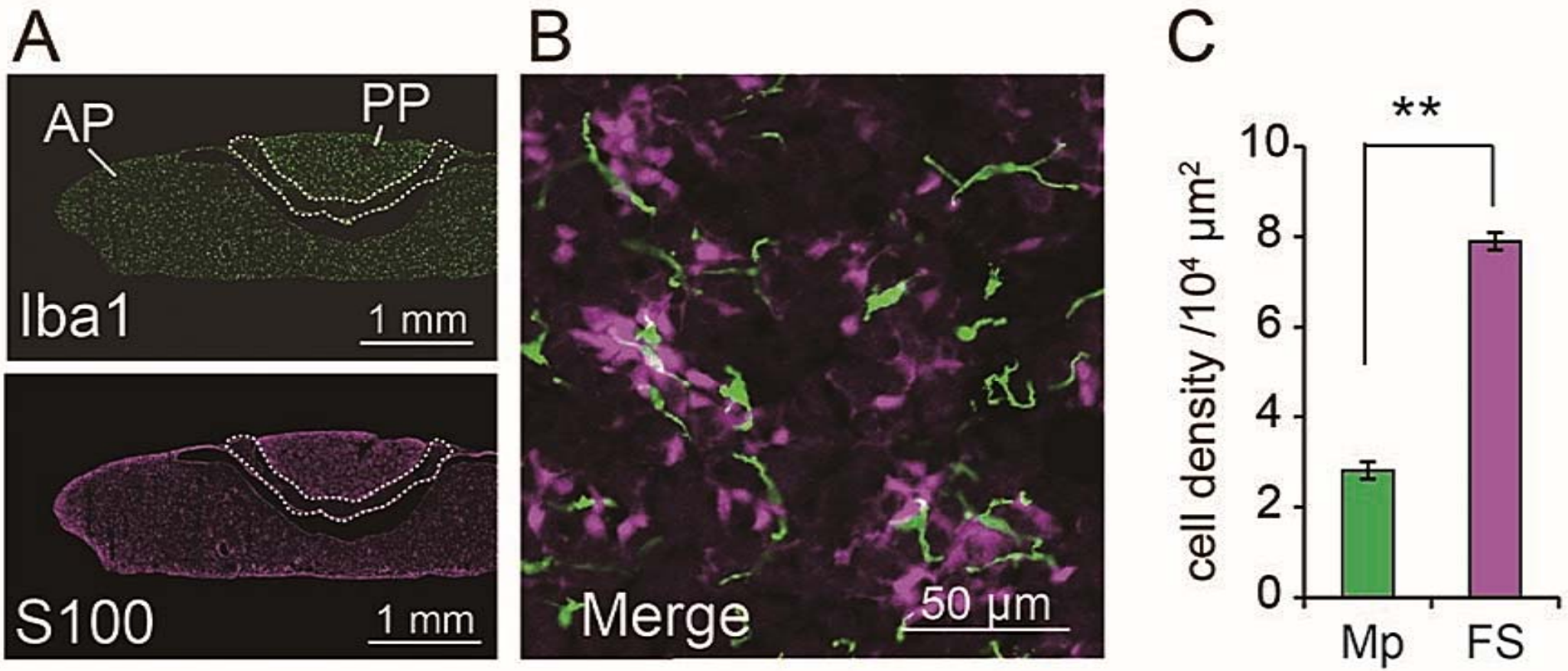
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FS

Fig1

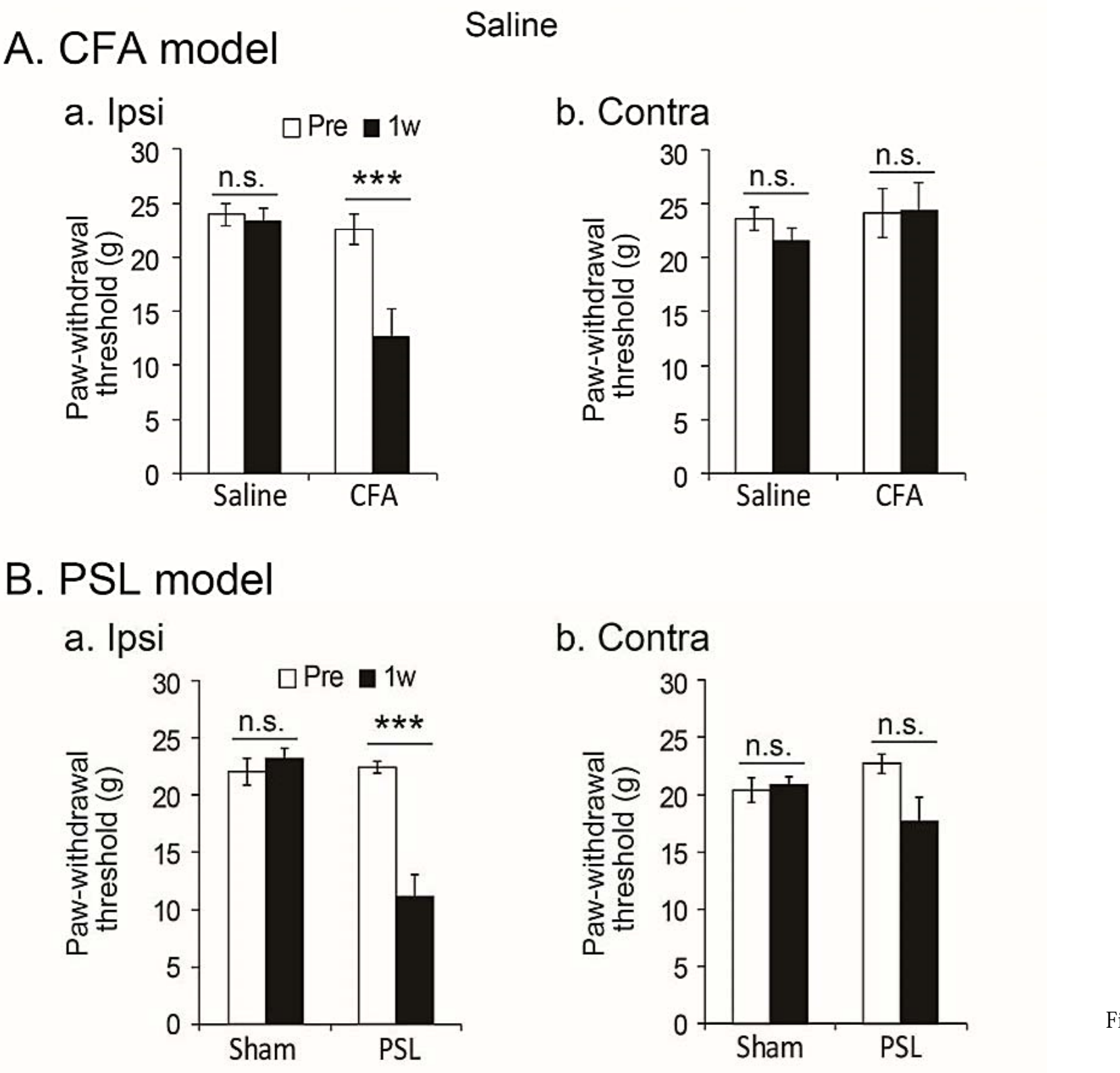

Saline
A. CFA model
a. Ipsi
Pre
1w
Paw-withdrawal threshold (g)
n.s.
***
Saline
CFA
b. Contra
n.s.
n.s.
Saline
CFA
B. PSL model
a. Ipsi
Pre
1w
Paw-withdrawal threshold (g)
n.s.
***
Sham
PSL
b. Contra
n.s.
n.s.
Sham
PSL

Fig2

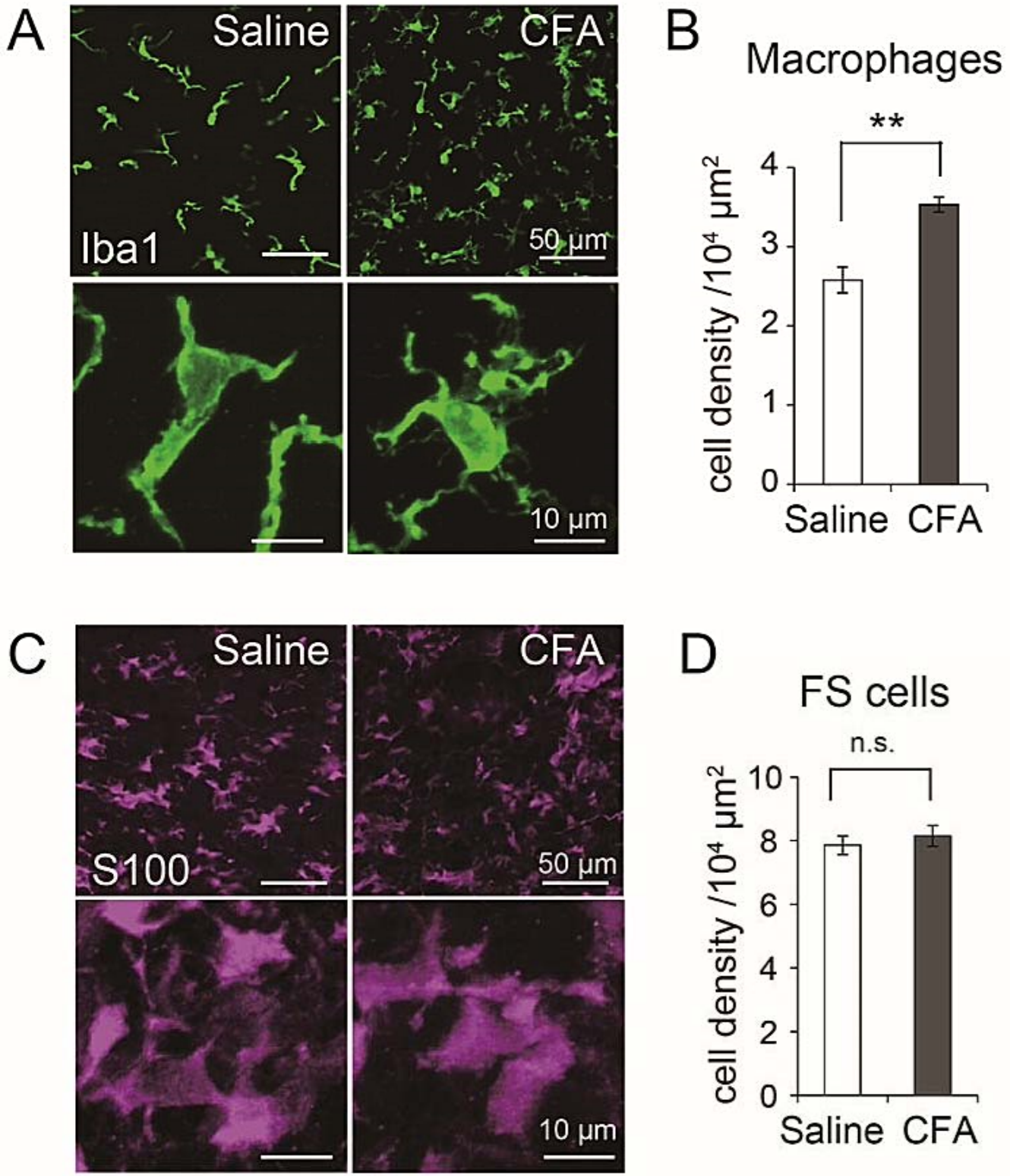
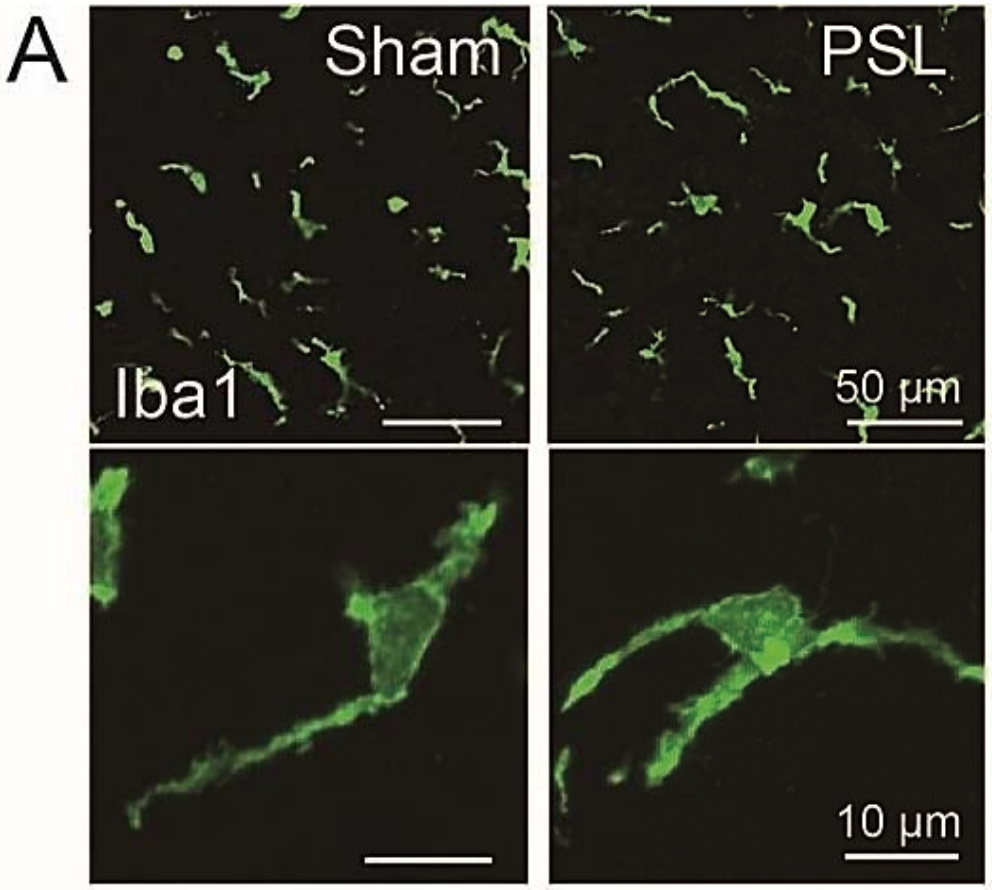
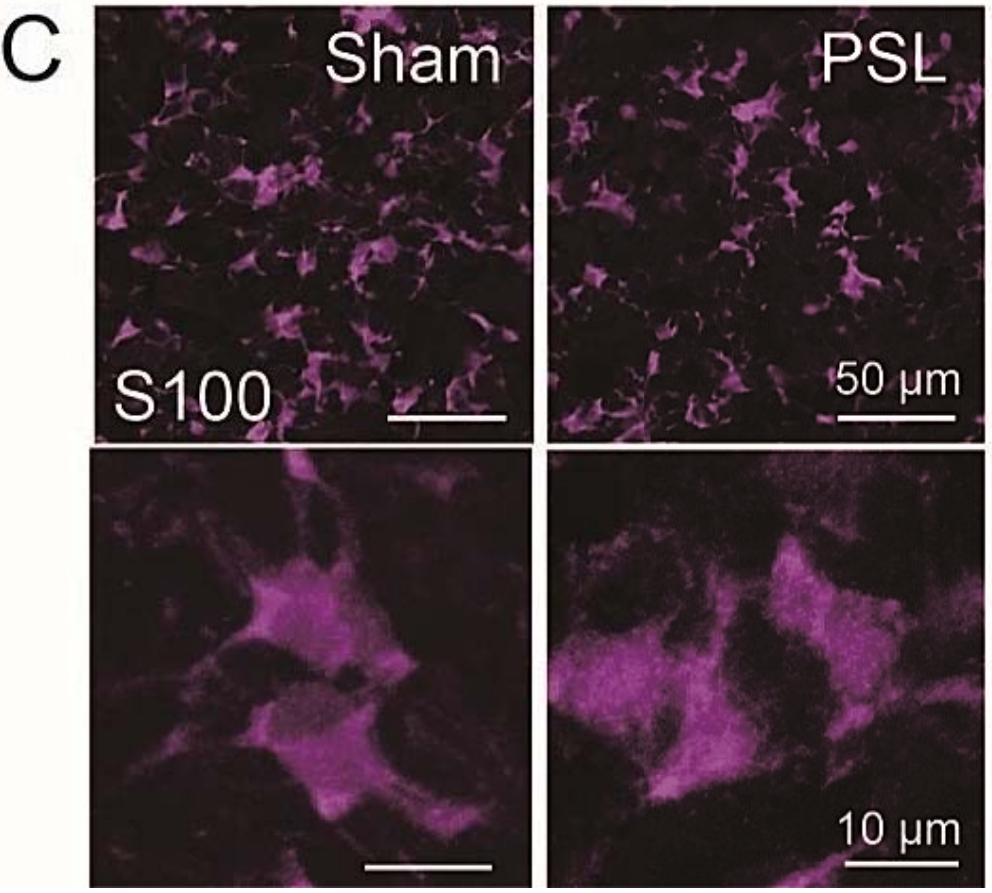
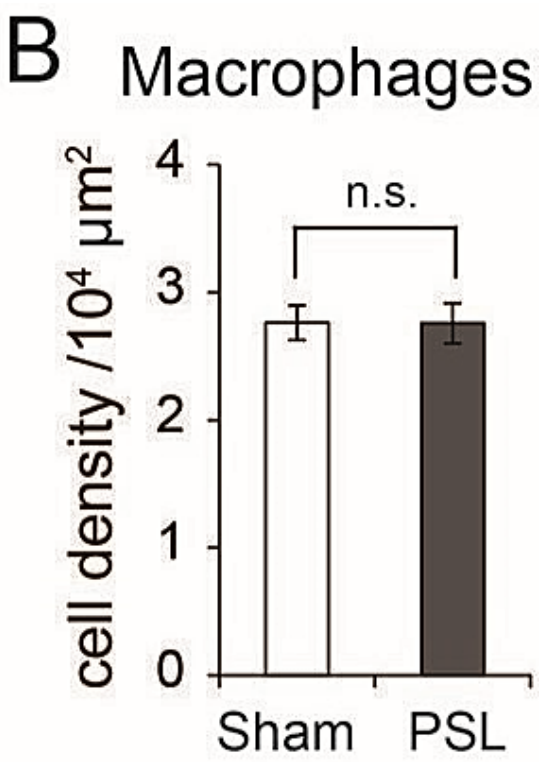

A
Saline
CFA
Iba1
50 μm
10 μm
B
Macrophages
**
cell density /10⁴ μm²
0
1
2
3
4
Saline
CFA
C
Saline
CFA
S100
50 μm
10 μm
D
FS cells
n.s.
cell density /10⁴ μm²
0
2
4
6
8
10
Saline
CFA

Fig3


A
Sham
PSL
Iba1
50 μm
10 μm


C
Sham
PSL
S100
50 μm
10 μm


B
Macrophages
n.s.
cell density /10⁴ μm²
0
1
2
3
4
Sham
PSL

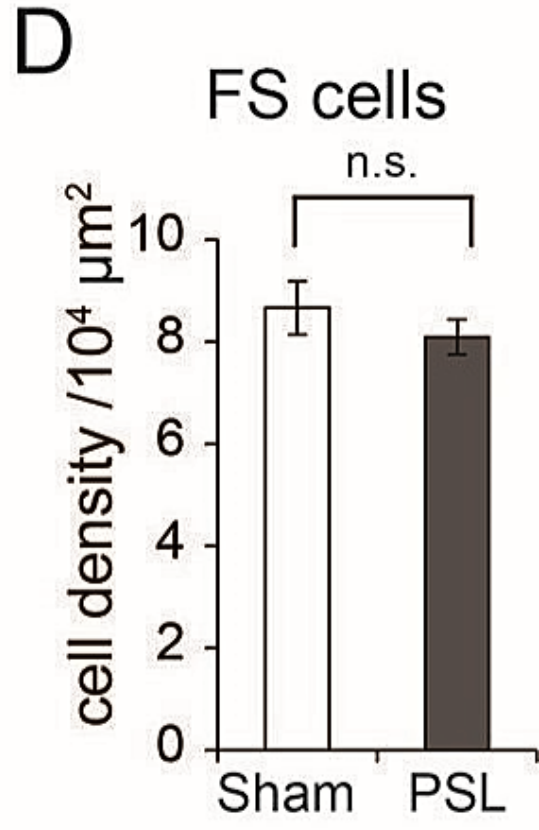

D
FS cells
n.s.
cell density /10⁴ μm²
0
2
4
6
8
10
Sham
PSL

Fig4

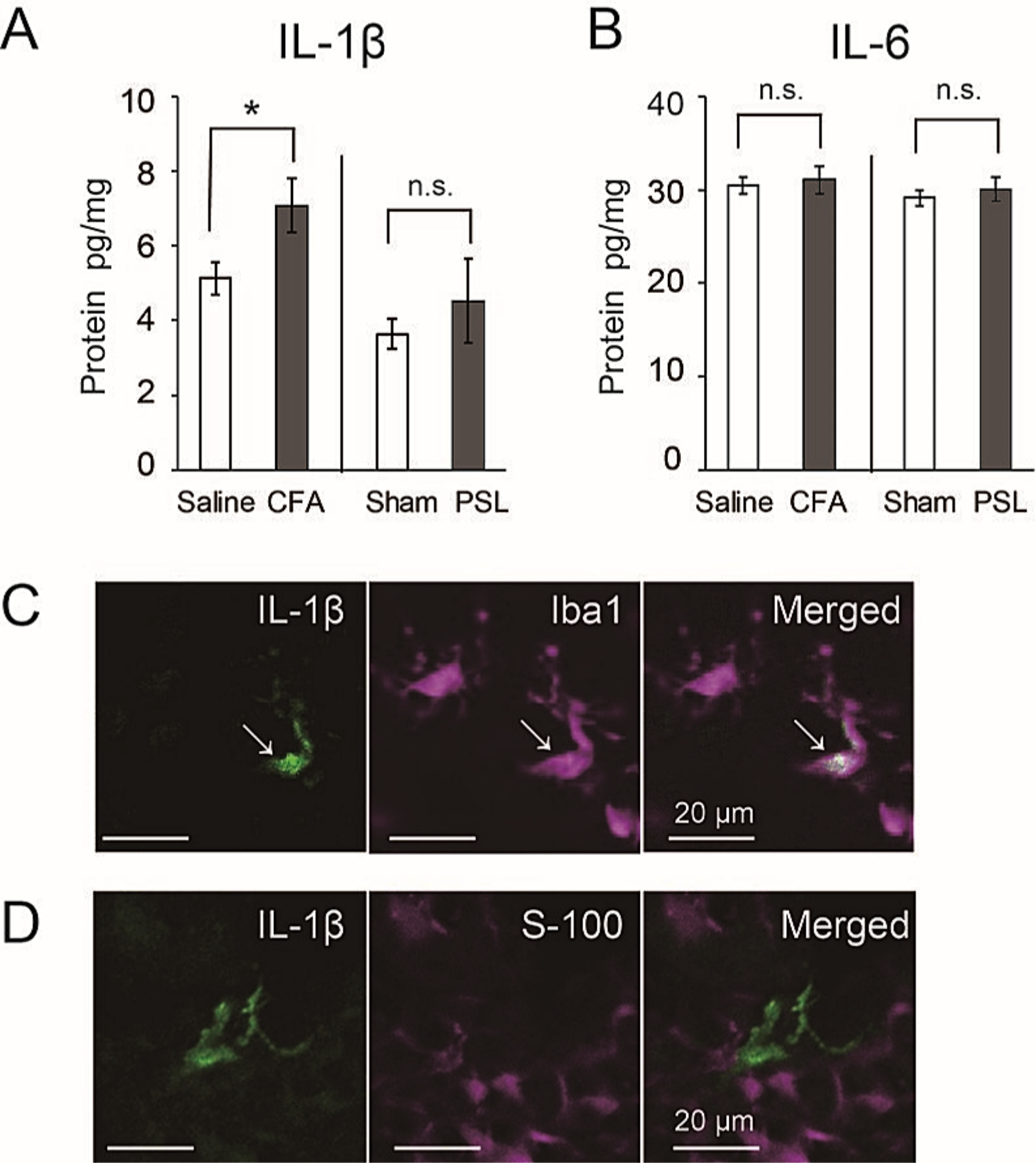

A
IL-1β
Protein pg/mg
10
8
6
4
2
0
*
n.s.
Saline
CFA
Sham
PSL
B
IL-6
Protein pg/mg
40
30
20
10
0
n.s.
n.s.
Saline
CFA
Sham
PSL
C
IL-1β
Iba1
Merged
20 μm
D
IL-1β
S-100
Merged
20 μm

Fig5

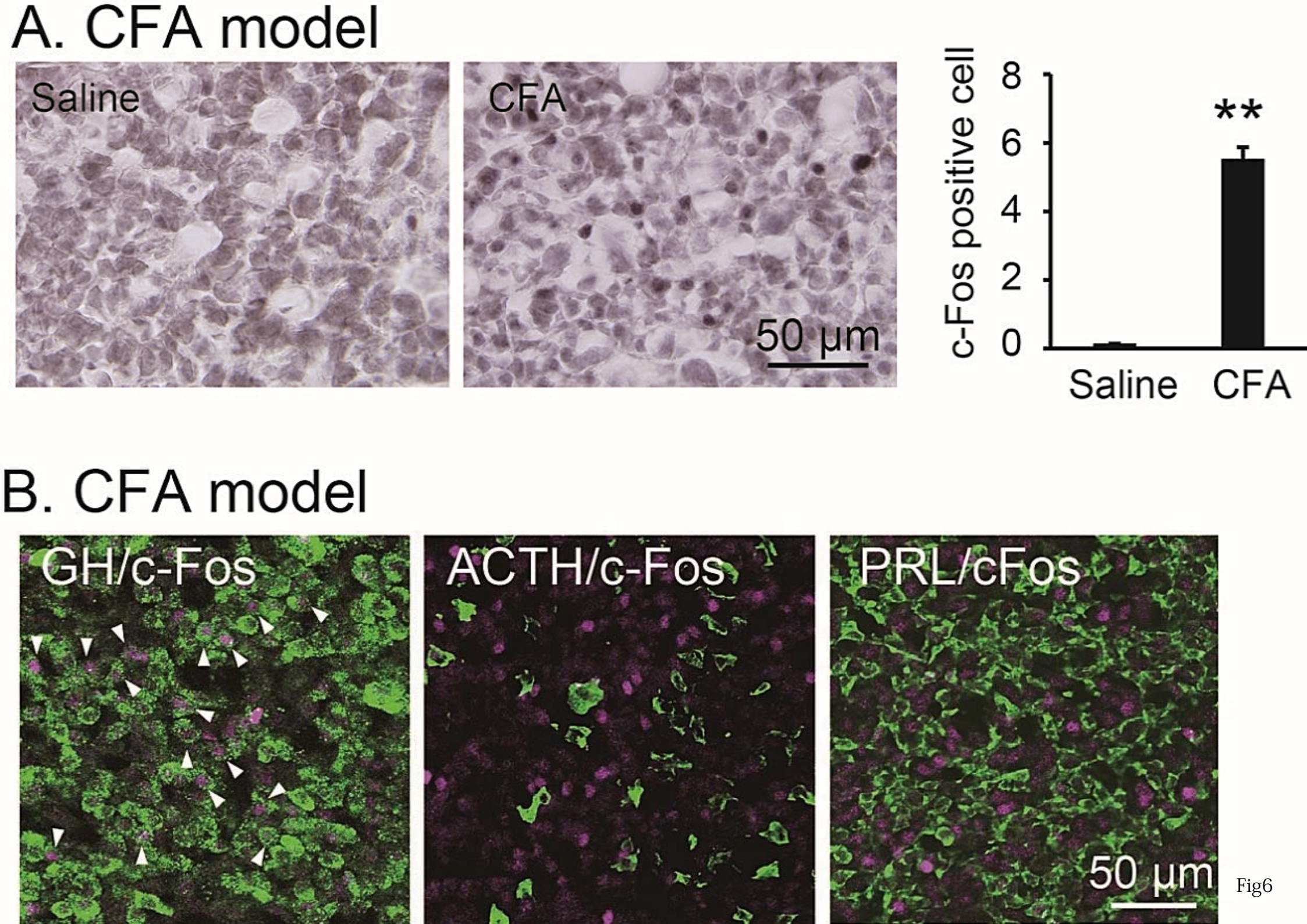

A. CFA model
Saline
CFA
50 μm
c-Fos positive cell
8
6
4
2
0
**
Saline
CFA
B. CFA model
GH/c-Fos
ACTH/c-Fos
PRL/cFos
50 μm

Fig6