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Endothelial immunocytochemical expression of pituitary IL-1β and its relation to ACTH-positive cells is regulated by corticosterone in the male rat

María Carmen Iglesias-Osma[a,b,1], Enrique J. Blanco[b,c], Marta Carretero-Hernández[c], María José García-Barrado[a,b], Leonardo Catalano-Iniesta[a,b], Manuel Carretero[d], Virginia Sánchez-Robledo[a,b], José Carretero[b,c,*,1]

[a] Department of Physiology and Pharmacology, Faculty of Medicine, University of Salamanca, Spain
[b] Laboratory of Neuroendocrinology, Institute of Neurosciences of Castilla y León (INCyL), and Laboratory of Neuroendocrinology and Obesity, Institute of Biomedical Research of Salamanca (IBSAL), University of Salamanca, Spain
[c] Department of Human Anatomy and Histology, Faculty of Medicine, University of Salamanca, Spain
[d] Faculty of Human and Social Sciences, Pontifical University of Salamanca, Spain

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ABSTRACT

Interleukin-1 beta (IL-1β) is a cytokine linking the neuroendocrine system and metabolic homeostasis. We have previously demonstrated the relevance of IL-1β for maintaining the pituitary ACTH-producing cells by immunoblocking its effects in pituitary cultures. However, the morphological characteristics and the intimate relationship of the pituitary cells expressing IL-1β and ACTH remain unknown. For determining pituitary variations of immunoreactivity for IL-1β and its relation with ACTH-positive cells under stress situations, we performed an immunohistochemical analysis of the expression of IL-1β and ACTH in the pituitary gland of adult rats, in the absence or presence of corticosterone, by establishing different groups: untreated, sham-operated, and bilaterally adrenalectomized animals. In the rats subjected to surgery, the glucocorticoid was administered on the same day of the intervention and on the third day post-surgery. Interestingly, it was observed that IL-1β was located in the pituitary endothelial cells at the hypophyseal portal vessels, regardless of the treatment schedule. When comparing the pituitary immunoreactive surface to IL-1β expression without corticosterone, adrenalectomized animals displayed a significantly greater area than the sham-operated animals. Corticosterone significantly inhibited the effect of adrenalectomy depending on the time interval it was administered. By in situ hybridization, IL-1β mRNA expression was also correlated with immnunocytochemical expression of pituitary IL-1β. Our results demonstrate that IL-1β is a constitutive element in endothelial portal pituitary vessels and under stress experimental conditions IL-1β increases its expression and its relation with ACTH-positive cells, suggesting that IL-1β could participate in an autocrine-paracrine fashion thereby modulating the pituitary population of ACTH-positive cells.

1. Introduction

The interleukin-1 family of cytokines encompasses several polypeptides involved in systemic pathways related to the immune and endocrine system, and also participates in cell growth, differentiation and function [1]. Furthermore, the relevance of IL-1β, a cytokine related to the inflammasome, which links the regulation of inflammatory responses and immunomodulation with the neuroendocrine system and metabolic homeostasis, is noteworthy [2–4].

IL-1β is released by different cellular types and, as a proinflammatory cytokine, many of its relevant actions are implicated in the modulation of the HPA axis [5–8].

The pituitary synthesizes both IL-1β and its receptor, as their mRNA and protein expression have been demonstrated [9–12].

Because IL-1β and its specific receptor are present in the pituitary gland [13] autocrine-paracrine effects have been attributed to this

Abbreviations: ACTH, adrenocorticotropic hormone; ADX, adrenalectomized animals without corticosterone; CVO, circumventricular organs; DAB, 3,3′-diaminobenzidine; ECs, endothelial cells; HPA, hypothalamus-pituitary-adrenal; HRP, horseradish peroxidase; ICE, interleukin-1 beta converting enzyme; IL-1β, interleukin-1 beta; IL-1R1, interleukin-1 receptor type 1; LPS, lipopolysaccharide; PAP, peroxidase-antiperoxidase; PBS, phosphate buffered saline; POMC, proopiomelanocortin; TB, trizma-HCl buffer; TBS, trizma-HCl buffered saline

* Corresponding author at: Department of Human Anatomy and Histology, INCyL and IBSAL, Faculty of Medicine, University of Salamanca, Avda. Alfonso X el Sabio s/n, E-37007 Salamanca, Spain.
E-mail address: jcar@usal.es (J. Carretero).

[1] M.C. Iglesias-Osma and J. Carretero contributed equally to this work.

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hypophyseal IL-1β, such as a regulator of hormonal secretion, together with the differentiation, proliferation and apoptosis of several pituitary cells [14–18].

Concerning the regulation of the HPA axis, IL-1β stimulates the secretion of POMC derivatives thus promoting the release of ACTH [16,19–21]. Indeed, the relationship between the hypophyseal IL-1β and the HPA axis follows a bidirectional feedback pattern [5,6,22], including the notorious influence regarding the time course, and correlation with an autocrine-paracrine regulation at the pituitary level [12,23]; although, some aspects concerning the cellular characteristics or local variations still remain unknown.

The question of how this network implicates both the pituitary IL-1β and the HPA axis, considering their bidirectional autocrine-paracrine regulation and their participation in the maintenance of populations of relevant cellular elements involved in these processes, has been of particular interest in our own research [17,18,24].

Therefore, in order to answer questions regarding this and to complement our previous findings, the aim of this study was to elucidate the characteristics and variations of the pituitary cells that might show immunoreaction to IL-1β, by analyzing groups of control, simulated or adrenalectomized adult rats. Additionally, a possible correlation with the pituitary immunocytochemical expression of ACTH was also studied in these animals. Following a brief period after the administration of corticosterone to the groups of rats submitted to their corresponding surgical procedure, and by using double immunohistochemical labeling for ACTH and IL-1β respectively, possible modifications in both the pituitary ACTH-positive cells and IL-1β-positive cells were analyzed.

2. Materials and methods

2.1. Animals and experimental protocol

The experiments were carried out following the protocols and ethical requirements approved by the Committee for the Care and Use of Animals of the University of Salamanca, in accordance with the regulations for the use of animals in investigation procedures from the European Communities Council Directive (2010/63/EU) and the current Spanish legislation (RD 53/2013).

For all the experimental procedures, adult male Sprague-Dawley rats 8 weeks-old and weighing 175–200 g were used. The animals were individually housed under controlled conditions, in a room maintained at 21 ± 2 °C and 50 ± 5% of relative humidity, with a controlled cycle of 14 h light – 10 h darkness. A balanced standard rat diet (Panlab®, Barcelona, Spain) and water were freely available. The groups of animals used for the experiments were as follows (Table 1):

Untreated rats. 5 phenotypically normal rats without any treatment.

Sham-operated (simulated). 40 rats of this group were submitted to surgery although, after laparotomy, the adrenal glands were localized but not removed, and no other surgical procedure was carried out. The animals were sacrificed after 2, 4 or 6 days of surgery, 5 rats per time interval, and the other 25 rats were treated with corticosterone as later described.

Adrenalectomized rats. 40 rats were bilaterally adrenalectomized by a dorsal extraperitoneal laparotomy under ketamine anesthesia (10 mg/kg body weight, administered intraperitoneally); after the surgical procedure, the drinking water was supplemented with 0.9% NaCl and 5% sucrose, as previously described [25]. The animals were sacrificed 2, 4 or 6 days after surgery, 5 rats per time interval, and the other 25 rats were treated with corticosterone as later described.

Rats treated with corticosterone. 50 rats, 25 sham-operated and 25 adrenalectomized, were treated with corticosterone (1.5 mg/kg body weight b.i.d, intramuscularly/12 h). In every surgical circumstance two groups were established. One group was treated starting from the same day of the surgery (day 1) until the day of sacrifice, which occurred on days 2, 4 or 6. In the second group, the treatment with corticosterone started on the 3rd day after surgery (day 3) and animals were sacrificed on days 4 or 6. For every time point, 5 rats were studied.

Table 1
Scheme of the experimental protocol.

Groups of animals	Corticosterone treatment 1.5 mg/kg body weight twice daily (number of rats used is indicated)			Survival time (days after surgery)
	No	Yes[a]		
		Day 1	Day 3	
Untreated	5	–	–	Not applied
Sham-operated	5	–	–	2
	5	–	–	4
	5	–	–	6
Adrenalectomy	5	–	–	2
	5	–	–	4
	5	–	–	6
Sham-operated	5	5	–	2
	10	5	5	4
	10	5	5	6
Adrenalectomy	5	5	–	2
	10	5	5	4
	10	5	5	6

[a] Following the surgical procedure, the glucocorticoid was administered on the same day of surgery (Day 1) and on the third day after the intervention (Day 3) for the indicated groups.

2.2. Sample processing

After the corresponding treatment period, the animals were anesthetized with isoflurane inhalation and were sacrificed by decapitation between 10 am and 11 am. Immediately, the pituitary glands were carefully dissected and fixed in a solution of paraformaldehyde 4% in phosphate buffer (0.1 M, pH 7.4) for 24 h. Then, the samples were dehydrated in ethanol, cleared in xylene, and embedded in paraffin for making serial coronal sections with a thickness of 5 μm.

2.3. Immunohistochemistry

For single immunostaining, the streptavidin-peroxidase method was used; in double immunostaining, the streptavidin-peroxidase method was used followed by the peroxidase-antiperoxidase (PAP) reaction. The slides were previously deparaffinized and rehydrated. The endogenous peroxidase was blocked by incubating with 0.25% H_2O_2 in methanol for 30 min. Afterwards, they were washed three times in TBS (Trizma-HCl buffered saline 0.05 M, pH 7.4, plus 0.9% NaCl, used as the solution for washes and dilutions). The non-specific reaction of the secondary antibody was blocked by incubation in normal goat serum (Dako®, Glostrup, Denmark, diluted 1:30 in TBS) for 30 min.

The single immunostaining method was performed to detect IL-1β expressing cells: the sections were incubated in the primary antibody, polyclonal rabbit anti-rat IL-1β (Endogen®, Endogen Inc., Woburn, MA, USA, diluted 1:120 in TBS) for 24 h at 4 °C in a humidity chamber. After washing, the slides were incubated for 45 min at room temperature with a biotinylated-goat anti-rabbit IgG (Caltag®, San Francisco, CA, USA, diluted at 1:150 in TBS) and then for 30 min at room temperature with streptavidin-horseradish peroxidase complex (Caltag®, diluted at 1:250). Then, the sections were washed again with TBS, and the reaction was revealed using a freshly prepared solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma®, Sigma-Aldrich Inc., St. Louis, MO, USA), at 0.025% in TB (Trizma-HCl buffer 0.05 M, pH 7.4), plus 0.03% H_2O_2. The samples were counterstained with Mayer's hematoxylin.

In order to confirm the specificity of the immune reaction, substitution of the primary antibody by TBS or non-immune rabbit serum,

and preabsorption tests with rat IL-1β (10 ng/ml) were carried out, and in all cases the reaction was abolished.

A double-labeling immunocytochemical method was developed to jointly study of IL-1β- and ACTH-positive cells. The first label applied was for IL-1β in a similar protocol as described for the simple immunolabeling, and then tested for ACTH. In order to avoid cross-reaction among the antibodies employed in both labels, after detection of IL-1β-positive cells elution of IgG was carried out by incubation in glycine buffer (0.2 M, pH 2.2) overnight at 4 °C, followed by two washes in TBS. The peroxidase-antiperoxidase (PAP) reaction was performed for the detection of ACTH by overnight incubation at 4 °C with polyclonal rabbit anti-ACTH antibody (Dako®, diluted 1:800 in TBS) then, a swine anti-rabbit IgG (Dako®, diluted 1:100 in TBS), and the PAP soluble complex (Dako®, diluted 1:100 in TBS) were successively applied at room temperature for 40 and 30 min, respectively. The reaction was finally visualized by using a freshly prepared solution of 4-chloro-1-naphthol (1.7×10^{-3} M in 3% absolute ethanol in TB, containing 0.3% H_2O_2). The control methods included the substitution of the primary antibodies, preabsorption tests, as well as omission of the secondary antibody or PAP complex; after these tests, no immunoreactivity was detected.

2.4. In situ hybridization

To detect mRNA expression of IL-1β in the pituitary gland, in situ hybridization was performed by a non-isotopic method involving the immunocytochemical detection of biotin using the streptavidin-biotin-peroxidase method, as has been previously described [26,27]. For this study there were used as probes the sense biotinylated oligonucleotide 5′Bio-ccacctcaatggacagaacataagccaacaagtggt, and antisense 5′Bio-ggtggagttacctgtcttgtattcggttgttcacca, 100% specific to rat IL-1β according to the NCBI Reference Sequence NM_031512.2.

To avoid RNase contamination in this procedure, all solutions and equipment were treated with diethylpyrocarbonate. The sections were previously deparaffinized and rehydrated, and after postfixation in 1% paraformaldehyde (to avoid the appearance of false positives), they were incubated 15 min twice in phosphate-buffered saline (PBS) and equilibrated for 15 min in standard saline citrate (1xSSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Afterwards, the slides were pre-incubated with a hybridization buffer (Omnibufer®, WAK-Chemie Medical GmbH, Germany) for 2 h at 55 °C using a humidity chamber. Hybridization with the biotinylated-probe (400 ng/ml in Omnibuffer®) was carried out by using a humidified chamber overnight at 55 °C. The reaction was stopped by washing sequentially in high stringency conditions in 2xSSC at room temperature for 30 min, in 2xSSC at 65 °C for 1 h, in 0.1x SSC at 65 °C for 1 h, and equilibrated for 5 min in Tris-buffered saline buffer (100 mM Tris–HCl, 150 mM NaCl, pH 7.5). Biotin was detected using monoclonal anti-biotin antibody (Roche®, Basel, Switzerland, diluted 1:350 in TBS: 0.05 M HCl-Trizma, pH 7.4, plus 0.8% NaCl) overnight at 4 °C in a humidity chamber, followed by biotinylated goat anti-mouse antibody (Abcam®, Cambridge, UK, diluted 1:150 in TBS). The final reaction was developed with DAB (Sigma®, Sigma-Aldrich Inc., St. Louis, MO, USA) in 0.05 M TB buffer, pH 7.4, to which 0.03% H_2O_2 had been added. The slides were counterstained using Mayer's acid hematoxylin. As controls, hybridization with sense probe, omission of the probe and pretreatment with RNase were performed, no reaction being observed in any case.

2.5. Quantification, morphometry and densitometry

By using a Labophot-2 Nikon® microscope, digital images at a final magnification of 400× were taken from 15 fields obtained from 10 different pituitary sections, per animal studied, randomly-selected from all parts of the gland and separated from each other by at least 100 μm. The digital photomicrographs were acquired under homogeneous conditions of color and temperature, without modifications to the voltage during the capture process.

ImageJ 1.49 software (developed by the National Institutes of Health, USA and freely downloaded from http://imagej.nih.gov/ij) was used to select positive elements by densitometric identification, in order to obtain the surface area occupied by IL-1β in the pituitary field analyzed. This protocol was used because most often the IL-1β-positive endothelial cells were bigger than the thickness of the sections. Accordingly, the percentage of the pituitary surface occupied by IL-1β-positive elements was calculated. The densitometrically identified positive-elements were grouped and, after taking a digital mask by automatic segmentation, the area occupied by each element was calculated and the percentage of the field area occupied by the corresponding immunocytochemical reaction was calculated from a field of 47.000 μm^2 of the total pituitary surface.

To determine the percentage of ACTH-positive cells, the number of ACTH-positive cells from 4000 pituitary cells was calculated for every animal by a double-blind method, and the total percentage of ACTH-positive cells was calculated for every group studied. Moreover, ACTH-positive cells contacting IL-1β-positive elements were accounted separately of those ACTH-positive cells no-contacting with IL-1β elements.

2.6. Statistical analysis

The values obtained were analyzed statistically by using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). The results are shown as mean values ± SEM (standard error of the mean) in the text and plots.

The differences between means obtained were contrasted with double-tail ANOVA (analysis of variance), considering statistical significance when $p < 0.05$ in the post hoc Scheffe's F-test.

3. Results

3.1. Immunoreactivity for IL-1β

Adult rats showed immunoreactivity for IL-1β confined mainly but not exclusively to the ECs of hypophyseal vascular sinusoids. The immunocytochemical reaction was usually more intense in the marginal sinusoids than in those at the center, with no significant differences between the untreated and the sham-operated rats (Fig. 1A). After bilateral adrenalectomy the immunoreactivity for IL-1β increased considerably in extent and intensity, affecting the marginal sinusoids on the second (day 2) or fourth day (day 4) following surgery, while both the central and peripheral sinusoids showed a positive reaction on the sixth day (day 6) after the adrenalectomy (Fig. 1A).

After treatment with corticosterone the immunoreactivity for IL-1β declined over time. Although this effect was evident in sham-operated animals, it was more significant in the animals previously adrenalectomized (Fig. 1A), particularly if the adrenalectomized rats had received corticosterone starting from the same day of surgery.

Under high-magnification, as shown in Fig. 1C, the pituitary IL-1β positive cells were brownish and, when the untreated rats were compared with adrenalectomized animals after 6 days, an intense reaction was observed in the latter, which almost disappeared when these animals were treated with corticosterone.

Similar results as described for ECs were observed in some endocrine non-ACTH-positive pituitary cells (Figs. 1C and 3).

3.2. Percentage of pituitary surface occupied by immunoreactivity for IL-1β

Fig. 1B summarizes the variations found in the percentages of pituitary surface occupied by immunoreactivity for IL-1β, in ECs and endocrine positive cells.

A small percentage of the pituitary surface (0.79 ± 0.05%) corresponded to cellular elements reactive for IL-1β in the untreated animals. Sham operated animals sacrificed two days after surgery showed a

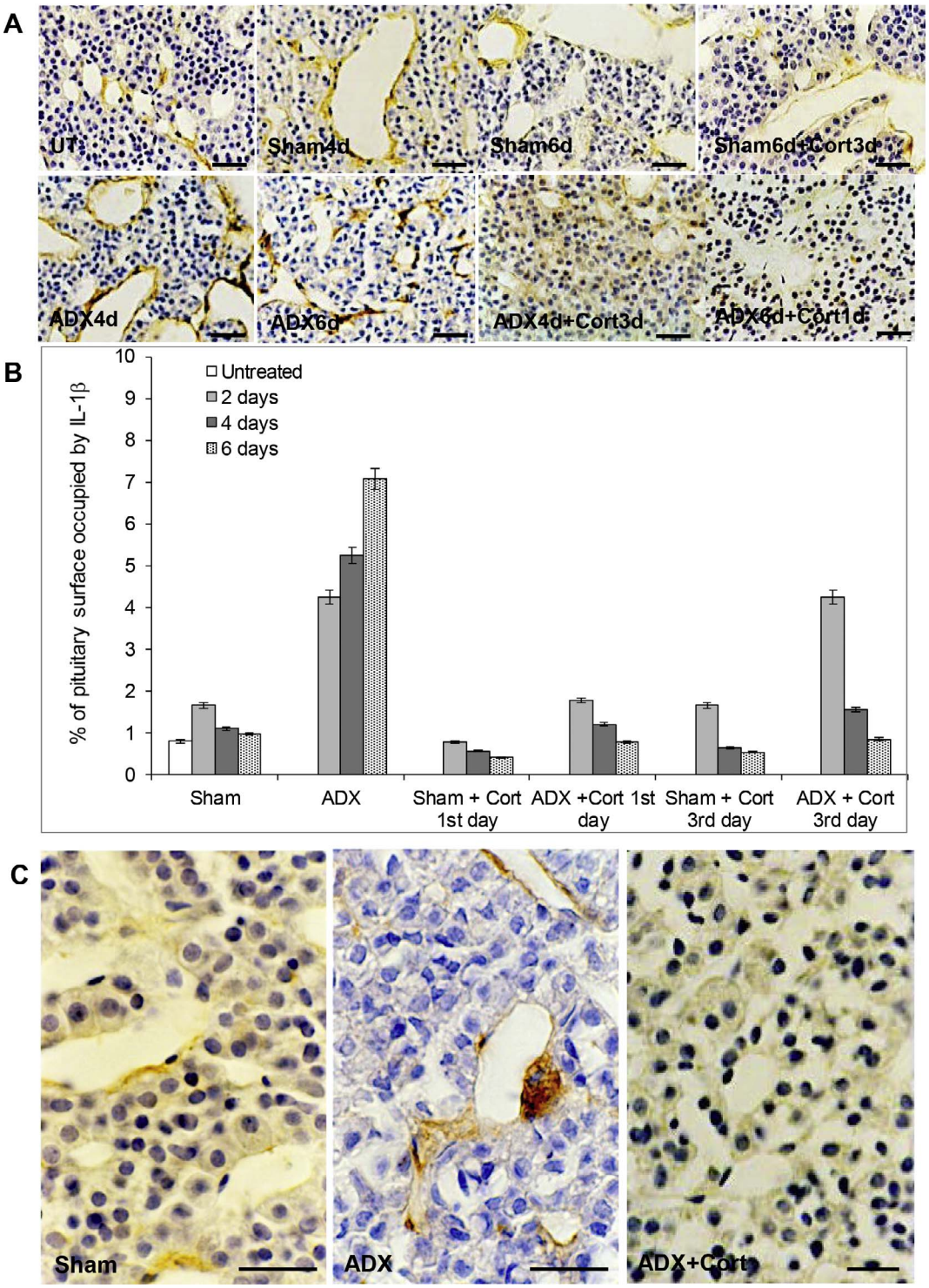

Fig. 1. Pituitary-positive cells to IL-1β. (A) Photomicrographs showing the brown-colored reaction of the immunopositive pituitary cells in untreated (UT), simulated (Sham), and bilaterally adrenalectomized (ADX) animals after the 4th or 6th day of surgery, without and with the administration of corticosterone (Cort) for 1 or 3 days. Scale bar: 50 μm. (B) Plotting showing the percentage of surface of the pituitary gland occupied by immunohistochemically-positive cells to IL-1β, in every group studied. Values represent mean ± SEM. (C) Pituitary-positive reaction to IL-1β (brown-colored) in simulated rats (Sham), bilaterally adrenalectomized (ADX), and bilaterally adrenalectomized and treated with corticosterone from the 1st day after surgery (ADX + Cort). Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significant increase ($p < 0.05$, in relation to untreated animals) in the immunoreactive surface. Later on, the effects of the surgery disappeared and no significant variations were observed at 4 or 6 days after surgery when compared to the untreated animals.

As can be expected, after adrenalectomy the pituitary surface occupied by immunoreactivity to IL-1β increased significantly ($p < 0.01$ in relation to untreated and sham-operated animals sacrificed at the same time interval), and this increase became greater as the time period post-surgery elapsed.

Treatment with corticosterone starting on the first day of surgery prevented the effects of surgery in sham-operated ($p < 0.05$) and adrenalectomized animals ($p < 0.01$). However, significantly larger surfaces ($p < 0.05$) were observed for the adrenalectomized rats when compared to sham-operated animals at the same time interval.

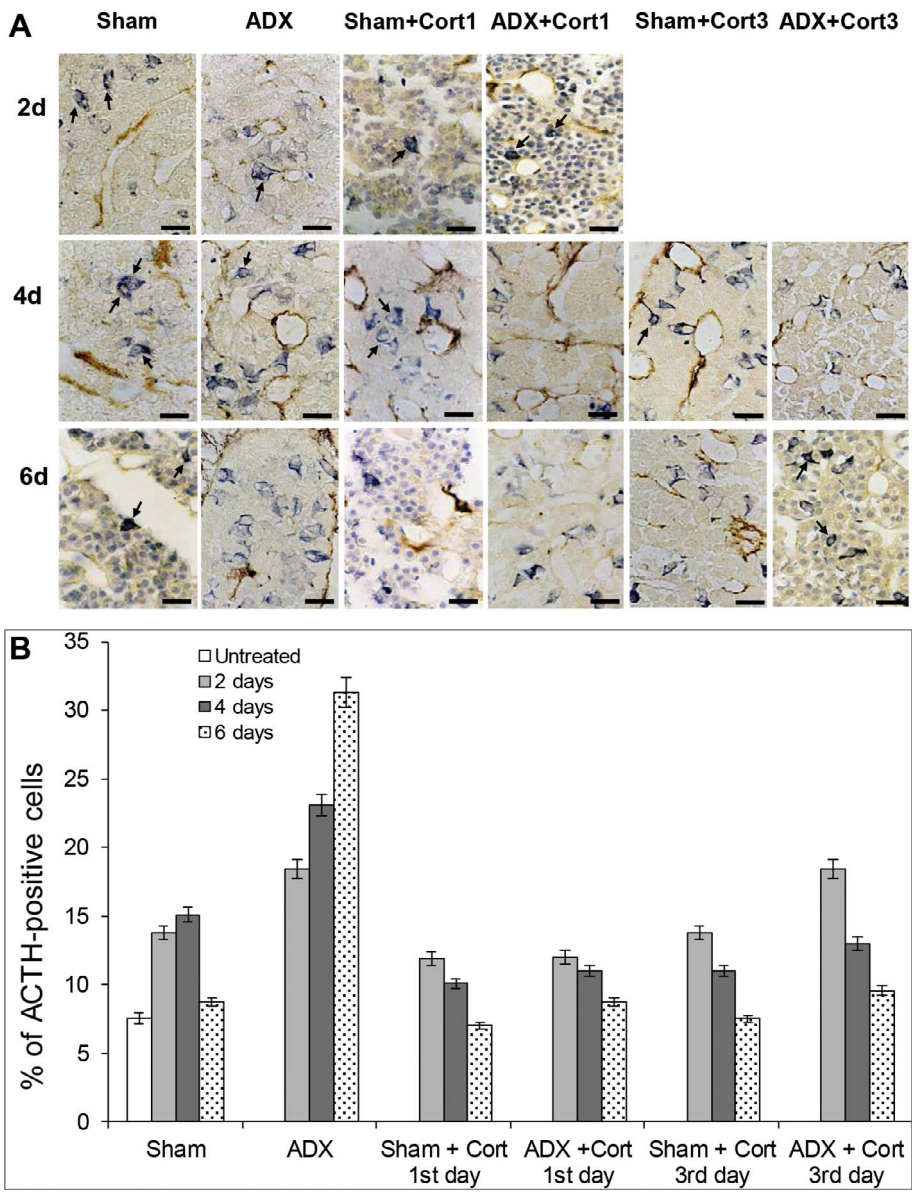

Fig. 2. Pituitary-positive cells to ACTH. (A) Morphology and immunoreaction of ACTH-positive cells (blue-colored, some indicated with arrows), and its relation with positive immunoreaction to IL-1β (brown-colored), in simulated (Sham) and bilaterally adrenalectomized (ADX) rats, at 2, 4 or 6 days after surgery, and the effects of corticosterone (+Cort) administration at day 1 or after 3 days of the corresponding surgical procedure. Scale bar: 50 μm. (B) Effects of corticosterone administration on simulated (Sham) or adrenalectomized (ADX) animals, related to the percentage of ACTH-positive cells. Values represent mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

When corticosterone was administered on day 3 after surgery, it blocked the effects of the sham surgery and the adrenalectomy, and the pituitary surface immunoreactive to IL-1β was significantly lower when compared to the corresponding animals that had not received the corticosterone treatment ($p < 0.05$ for sham-operated animals and $p < 0.01$ for adrenalectomized animals). Significant differences among sham-operated and adrenalectomized animals were detected at day 4 and day 6 intervals ($p < 0.05$). The immunoreactive surface was discretely larger in animals treated with corticosterone on day 3 than those observed in the adrenalectomized animals treated with the glucocorticoid starting from the first day of surgery ($p < 0.05$). The animals were sacrificed on day 4 after the adrenalectomy.

3.3. ACTH-positive cells and immunoreactivity for IL-1β

As expected, surgical stress on the sham-operated and the adrenalectomized animals significantly increased the percentage of pituitary ACTH-positive cells (Fig. 2A, blue cells, and B). However, surgical stress only increased this percentage on days 2 and 4 post-surgery ($p < 0.01$ in relation to untreated animals), while the percentage of ACTH-positive cells increased significantly ($p < 0.01$ in relation to sham animals, and $p < 0.005$ in relation to untreated animals) and linearly from day 2 to day 6 (Fig. 2B). Significant differences ($p < 0.01$) were observed between day 2 and day 4, or from day 4 to day 6. Treatment with corticosterone blocked or reverted these changes ($p < 0.05$ in sham-operated animals and $p < 0.005$ in adrenalectomized animals).

ACTH-positive cells (blue cells in Figs. 2 and 3) were irregular or stellate cells with cytoplasmic prolongations. Double immunolabeling revealed that two different populations of ACTH-positive cells could be considered: ACTH-contacting IL-1β and ACTH-non contacting IL-1β (Figs. 2A and 3A). The results observed when the percentage of ACTH-contacting IL-1β, from the total percentage of ACTH-positive cells analyzed, are summarized in Fig. 3B. On day 2 ($p < 0.05$) and day 4 ($p < 0.01$) post-surgery, the percentage of ACTH-contacting IL-1β increased significantly in sham-operated animals in comparison to the untreated animals. This result was not observed for day 6. After adrenalectomy a marked increase in the percentage of cells was observed ($p < 0.001$ in relation with untreated animals). When sham-operated and adrenalectomized animals were compared, the adrenalectomy increased the percentage of ACTH-contacting IL-1β on day 2 ($p < 0.005$), day 4 ($p < 0.005$) and day 6 ($p < 0.001$).

Corticosterone decreased linearly from day 2 to day 6 in all of the groups treated within the study ($p < 0.05$ in sham-operated animals and $p < 0.001$ in adrenalectomized animals).

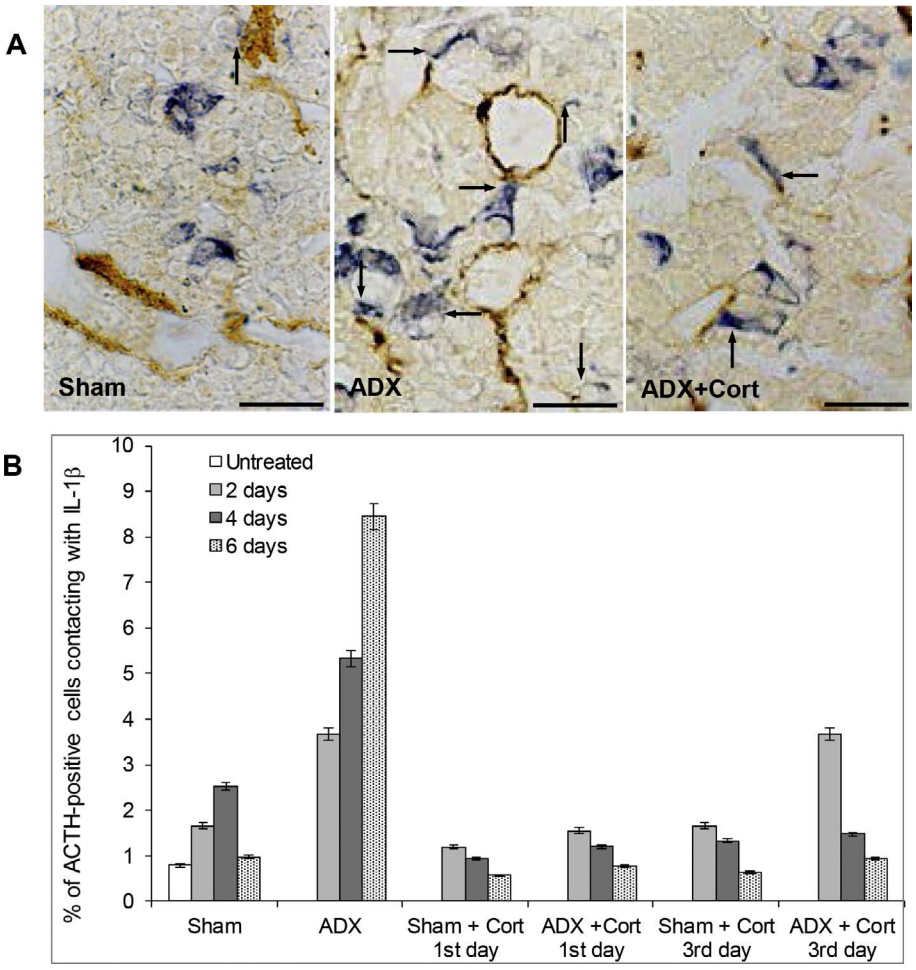

Fig. 3. Pituitary ACTH-positive cells contacting IL-1β-positive cells in untreated, simulated (Sham) and bilaterally adrenalectomized (ADX) rats, with or without corticosterone (+Cort) treatment. (A) High magnification photomicrographs showing the population of ACTH-positive cells (blue-colored) contacting IL-1β (arrows). Scale bar: 50 μm. (B) Percentage of ACTH-positive cells contacting IL-1β from the total percentage of ACTH-positive cells, for all the groups of animals studied; the glucocorticoid was administered at day 1 or after 3 days of the surgical intervention. Values represent mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. In situ hybridization for pituitary IL-1β mRNA in the pituitary gland

In situ hybridization showed that pituitary IL-1β mRNA was weakly expressed in untreated rats (Fig. 4A). Surgical stress in both the sham-operated animals (Fig. 4B) and those adrenalectomized (Fig. 4C) markedly increased the pituitary expression of IL-1β mRNA, as was found by immunocytochemistry. In situ hybridization was more sensitive than immunocytochemistry to detect pituitary IL-1β in ECs and endocrine cells. Positivity for IL-1β mRNA was observed in endocrine and endothelial cells, these latest mainly in adrenalectomized animals (Fig. 4C, arrow). Endothelial expression of pituitary IL-1β mRNA in adrenalectomized rats treated with corticosterone (Fig. 4D) showed similar changes as those described above for the endothelial immunocytochemical reaction to the pituitary IL-1β protein.

4. Discussion

The cellular expression of IL-1β has been mainly attributed to cells of the monocyte-macrophage system, although it has been reported that diverse conditions may also induce the protein and mRNA expression of this cytokine in vascular ECs in different sites and among diverse mammalian species [28–33]. The results of the present study show a constitutive expression of the IL-1β in ECs of the adult rat pituitary gland and to our knowledge this is the first report providing evidence regarding the immunohistochemical expression of IL-1β protein and its mRNA in some of the rat pituitary ECs and endocrine cells.

As it is well known, the pituitary is vascularized mainly by portal vessels that act as capillary sinusoids with fenestrated endothelium that facilitates a bidirectional permeability for molecules of different characteristics [34]. Despite the original publication regarding hypophyseal immunoreactivity for IL-1β [9], and evidence supporting the expression of IL-1 receptor type 1 (IL-1R1) mRNA within the pituitary [11], a possible peripheral origin for this cytokine was proposed [35].

Regardless of this controversy, it was later reported that in adrenalectomized mice the administration of lipopolysaccharide (LPS) enhanced the expression of IL-1β within the pituitary at both the protein and mRNA level [22]. Additionally, the pituitary production of the IL-1β converting enzyme (ICE) and the constitutive and inducible forms of the cytokine were also identified [36]. This evidence supported the idea of the in situ production of functionally active IL-1β in the pituitary.

Furthermore, stress induces IL-1β in the brain which is inhibited by corticosterone administration [37]. Remarkably, in certain situations, IL-1β mRNA expression in rodent brain followed a time-course pattern, principally at the glial level; while the capillary endothelium has also been suggested as another possible source of IL-1β in the brain [12,23,38,39].

Interestingly, it was described that the peripheral administration of LPS in rats induces *de novo* synthesis of bioactive IL-1β in the ECs of their CVO [40], whose endothelium is arranged in a fenestrated layer like the portal pituitary sinusoids. In addition, the presence of IL-1R1 protein and mRNA in rat brain vascular cells was also demonstrated by the same authors [41].

In streptozotocin-diabetic rats, a stressor condition as is hyperglycemia induces the expression of IL-1β from the isolated retinal vascular endothelium, and the cytokine is also stimulated in an autocrine and paracrine fashion resulting in an increased synthesis of IL-1β, both in the glial and endothelial cells [32].

Indeed, it has been demonstrated that the response of the HPA-axis and the release of ACTH and corticosterone, elicited by exogenous administration of IL-1β in mice, are mediated by brain ECs expressing

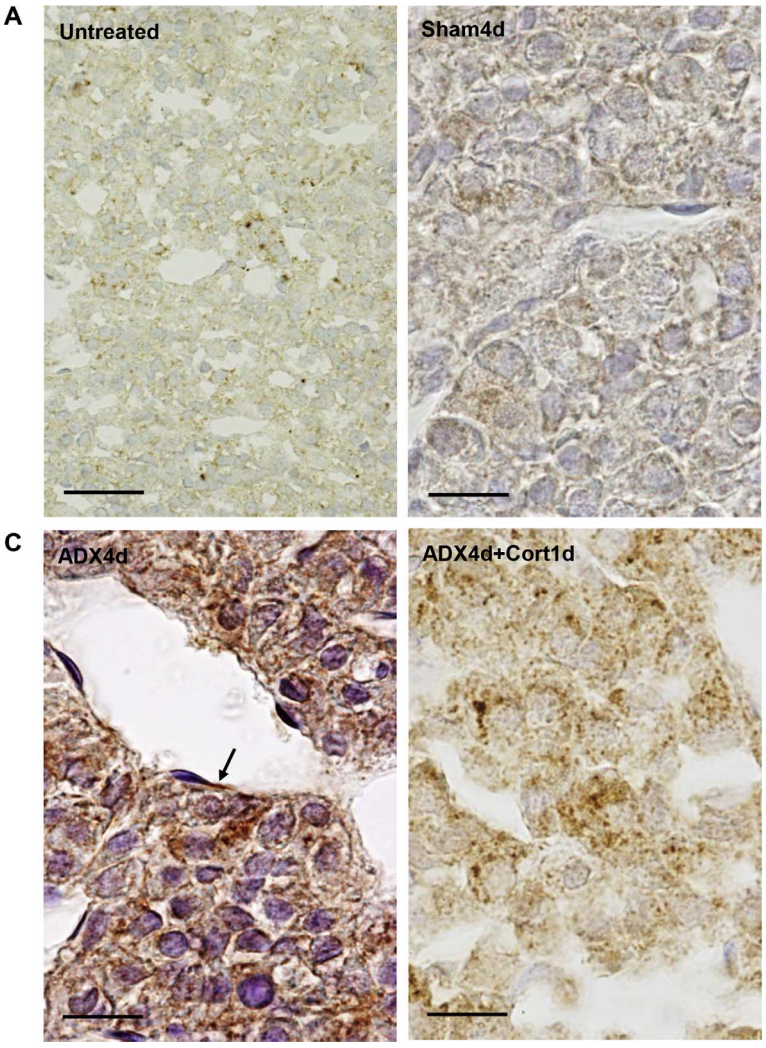

Fig. 4. Photomicrographs showing IL-1β mRNA-positive pituitary cells detected by in situ hybridization (being developed with DAB and counterstained with hematoxylin). (A) Untreated rats. (B) Sham-operated animals at 4 days after surgery, (C) Bilaterally adrenalectomized (ADX) rats after the 4th day of surgery, without the administration of corticosterone (Cort), where an IL-1β mRNA-positive endothelial cell is indicated (arrow), and (D) Bilaterally adrenalectomized (ADX) animals after the 4th day of surgery, with the administration of corticosterone (Cort) for 1 day. Scale bars: (A) 25 μm; (B, C and D): 10 μm.

the IL-1β receptor [42]. Thus, taking into account the support of the aforementioned evidence (expression of both IL-1β and its receptor IL-1R1 in the brain ECs of rodent), together with the recognized autocrine-paracrine actions of IL-1β in the HPA axis, the expression of IL-1β at the pituitary vascular cells might well be possible.

In our study, an increased expression of IL-1β in the pituitary endothelium was observed in the groups of rats subjected to surgical intervention without any other type of procedure, indicating an activation of the HPA axis related to the surgical stress. Furthermore, the adrenalectomized rats showed a maximal expression of the cytokine, both morphologically and in the percentage of area, that was directly proportional to the number of days post-surgery. Our findings of increased IL-1β expression in the vascular pituitary sinusoids of rat suggest that some of the hypophyseal endothelial cells are also a source of IL-1β, and that IL-1β expression is highly related to stress and the HPA axis.

In the analysis of human umbilical vein and human aortic ECs, after administering physiological doses of cortisol or dexamethasone, it has been reported changes in the morphology of ECs and the inhibition of some functional aspects associated to their relationships with the neighboring cellular elements [43]. Likewise, in our study the administration of corticosterone in simulated or adrenalectomized animals, following a time-course, significantly reduced both the IL-1β morphological expression and percentage of the immunoreactivity area of some of the pituitary ECs. Hence, this suggests that the inhibitory actions of the glucocorticoid on the release of IL-1β also occur within the pituitary endothelium.

The need for using classical markers to identify ECs could be proposed. However, this is unnecessary considering that the absence of these indicators is a particularity of the sinusoidal ECs of the pituitary portal vessels [44].

The morphometry and the densitometric assessment used the NIH ImageJ software, a computer-assisted image analysis widely accepted, provides objectivity, accuracy, reproducibility, and time efficiency compared to manual cell counts [45,46].

The pituitary vascular sinusoids exhibited an irregular shape, with ECs of irregular shape and spatial disarrangement, and as a consequence were located in the same plane of section. As a result, we found nucleated and non-nucleated IL-1β-positive cellular fragments that made it impossible to distinguish if they were from the same or different cells. Therefore, we chose to calculate the pituitary surface occupied by IL-1β rather than calculate the percentage of ECs cells.

IL-1β mRNA expression was observed in endocrine and endothelial cells. Certainly, determining the mRNA expression of IL-1β in the pituitary endothelium has been of particular interest, especially considering that it has been reported its presence in other vascular endothelial cells [28,31]. Indeed, in the present work we have also demonstrated by in situ hybridization that endothelial expression of pituitary IL-1β mRNA goes in parallel and is in accordance with the variations detected in this study by immunocytochemistry for the endothelial expression of pituitary IL-1β protein.

The results obtained in the present study after analyzing the percentage of ACTH-positive cells are in agreement with the findings observed in previous studies from our laboratory where effects of

adrenalectomy with or without treatment with corticosterone on cellular proliferation and apoptosis were studied [24].

5. Conclusions

As demonstrated by our results, we were unable to find, in all groups of the rats studied, a co-localization of the immunostaining for ACTH or IL-1β. We only observed relationships among the cells that were immunopositive for the cytokine or the hormone within the same regions. The changes observed by surgical stress, adrenalectomy or treatment with corticosterone demonstrated that the variations of ACTH-positive cells are associated to similar variations of the pituitary surface occupied by IL-1β, and variations in the contact among ACTH- and IL-1β-positive cells. This suggests an important relationship between increases of endothelial pituitary IL-1β and the pituitary responses of the HPA to stress. Therefore, this might involve a pituitary release of IL-1β under stress and/or a paracrine regulation of ACTH by IL-1β. Moreover, as was reported using similar experimental conditions [28,32], endothelial pituitary IL-1β could exert an autocrine effect on ECs.

Conflict of interest

The authors declare no actual or potential conflicts of interest.

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References

[1] J.E. Sims, D.E. Smith, The IL-1 family: regulators of immunity, Nat. Rev. Immunol. 10 (2) (2010) 89–102, http://dx.doi.org/10.1038/nri2691.
[2] C.A. Dinarello, A clinical perspective of IL-1beta as the gatekeeper of inflammation, Eur. J. Immunol. 41 (5) (2011) 1203–1217, http://dx.doi.org/10.1002/eji.201141550.
[3] V.A. Rathinam, S.K. Vanaja, K.A. Fitzgerald, Regulation of inflammasome signaling, Nat. Immunol. 13 (4) (2012) 333–342, http://dx.doi.org/10.1038/ni.2237.
[4] R. Zhao, H. Zhou, S.B. Su, A critical role for interleukin-1beta in the progression of autoimmune diseases, Int. Immunopharmacol. 17 (3) (2013) 658–669, http://dx.doi.org/10.1016/j.intimp.2013.08.012.
[5] A.V. Turnbull, C.L. Rivier, Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action, Physiol. Rev. 79 (1) (1999) 1–71.
[6] J.J. Haddad, N.E. Saade, B. Safieh-Garabedian, Cytokines and neuro-immune-endocrine interactions: a role for the hypothalamic-pituitary-adrenal revolving axis, J. Neuroimmunol. 133 (1–2) (2002) 1–19.
[7] A. Gadek-Michalska, J. Bugajski, Interleukin-1 (IL-1) in stress-induced activation of limbic-hypothalamic-pituitary adrenal axis, Pharmacol. Rep. 62 (6) (2010) 969–982.
[8] A. Gadek-Michalska, J. Tadeusz, P. Rachwalska, J. Spyrka, J. Bugajski, Effect of prior stress on interleukin-1beta and HPA axis responses to acute stress, Pharmacol. Rep. 63 (6) (2011) 1393–1403.
[9] J.I. Koenig, K. Snow, B.D. Clark, R. Toni, J.G. Cannon, A.R. Shaw, C.A. Dinarello, S. Reichlin, S.L. Lee, R.M. Lechan, Intrinsic pituitary interleukin-1 beta is induced by bacterial lipopolysaccharide, Endocrinology 126 (6) (1990) 3053–3058, http://dx.doi.org/10.1210/endo-126-6-3053.
[10] E.B. De Souza, E.L. Webster, D.E. Grigoriadis, D.E. Tracey, Corticotropin-releasing factor (CRF) and interleukin-1 (IL-1) receptors in the brain-pituitary-immune axis, Psychopharmacol. Bull. 25 (3) (1989) 299–305.
[11] E.T. Cunningham Jr., E. Wada, D.B. Carter, D.E. Tracey, J.F. Battey, E.B. De Souza, In situ histochemical localization of type I interleukin-1 receptor messenger RNA in the central nervous system, pituitary, and adrenal gland of the mouse, J. Neurosci. 12 (3) (1992) 1101–1114.
[12] M.L. Wong, P.B. Bongiorno, V. Rettori, S.M. McCann, J. Licinio, Interleukin (IL) 1beta, IL-1 receptor antagonist, IL-10, and IL-13 gene expression in the central nervous system and anterior pituitary during systemic inflammation: pathophysiological implications, Proc. Natl. Acad. Sci. USA 94 (1) (1997) 227–232.
[13] M.N. Silverman, B.D. Pearce, C.A. Biron, A.H. Miller, Immune modulation of the hypothalamic-pituitary-adrenal (HPA) axis during viral infection, Viral Immunol. 18 (1) (2005) 41–78, http://dx.doi.org/10.1089/vim.2005.18.41.
[14] H. Stepien, G. Zerek-Melen, S. Mucha, K. Winczyk, J. Fryczak, Interleukin-1 beta stimulates cell proliferation in the intermediate lobe of the rat pituitary gland, J. Endocrinol. 140 (3) (1994) 337–341.
[15] P.J. Simons, F.G. Delemarre, H.A. Drexhage, Antigen-presenting dendritic cells as regulators of the growth of thyrocytes: a role of interleukin-1beta and interleukin-6, Endocrinology 139 (7) (1998) 3148–3156, http://dx.doi.org/10.1210/endo.139.7.6110.
[16] B.L. Spangelo, D.D. Farrimond, M. Pompilius, K.L. Bowman, Interleukin-1 beta and thymic peptide regulation of pituitary and glial cell cytokine expression and cellular proliferation, Ann. N. Y. Acad. Sci. 917 (2000) 597–607.
[17] J.A. Moro, J. Carretero, M.I. Alonso, C. Martin, A. Gato, L. Mano, Ade, Prenatal expression of interleukin 1beta and interleukin 6 in the rat pituitary gland, Cytokine 44 (3) (2008) 315–322, http://dx.doi.org/10.1016/j.cyto.2008.08.005.
[18] J. Carretero, M.C. Montero, G. Vazquez, E. Blanco, M. Rubio, J.M. Riesco-Lopez, J.J. Herrero, M. Carretero, J.M. Riesco, E. Perez, R. Vazquez, Auto-paracrine regulation of the proliferation of pituitary ACTH-producing cells demonstrated by immunoblockade of IL-1ß, Eur. J. Anat. 10 (1) (2006) 7–13.
[19] S.M. Parsadaniantz, E. Batsche, P. Gegout-Pottie, B. Terlain, P. Gillet, P. Netter, B. Kerdelhue, Effects of continuous infusion of interleukin 1 beta on corticotropin-releasing hormone (CRH), CRH receptors, proopiomelanocortin gene expression and secretion of corticotropin, beta-endorphin and corticosterone, Neuroendocrinology 65 (1) (1997) 53–63.
[20] M.R. Haedo, J. Gerez, M. Fuertes, D. Giacomini, M. Paez-Pereda, M. Labeur, U. Renner, G.K. Stalla, E. Arzt, Regulation of pituitary function by cytokines, Horm. Res. 72 (5) (2009) 266–274, http://dx.doi.org/10.1159/000245928.
[21] A. Paoletta, G. Arnaldi, R. Papa, M. Boscaro, G. Tirabassi, Intrapituitary cytokines in Cushing's disease: do they play a role? Pituitary 14 (3) (2011) 236–241, http://dx.doi.org/10.1007/s11102-010-0285-9.
[22] E. Goujon, P. Parnet, S. Laye, C. Combe, R. Dantzer, Adrenalectomy enhances pro-inflammatory cytokines gene expression, in the spleen, pituitary and brain of mice in response to lipopolysaccharide, Brain Res. Mol. Brain Res. 36 (1) (1996) 53–62.
[23] N. Quan, M. Whiteside, M. Herkenham, Time course and localization patterns of interleukin-1beta messenger RNA expression in brain and pituitary after peripheral administration of lipopolysaccharide, Neuroscience 83 (1) (1998) 281–293.
[24] M.C. Montero, R. Vazquez, M. Rubio, E. Blanco, J.M. Riesco, J.J. Herrero, G. Vazquez, J.M. Riesco-Lopez, R. Gonzalez, M. Carretero, M. Basco, J. Carretero, Apoptosis is involved in the anti-proliferative effect of corticosteroid in non-tumoral ACTH pituitary cells, Eur. J. Anat. 10 (3) (2006) 143–149.
[25] F. Sanchez, M. Rubio, V. Hernandez, M. Santos, J. Carretero, R.J. Vazquez, R. Vazquez, NADPH-diaphorase activity and vasopressin in the paraventricular nucleus of the hypothalamus following adrenalectomy, Neuropeptides 30 (6) (1996) 515–520.
[26] D. Fu, Y.K. Ng, P. Gan, E.A. Ling, Permanent occlusion of the middle cerebral artery upregulates expression of cytokines and neuronal nitric oxide synthase in the spinal cord and urinary bladder in the adult rat, Neuroscience 125 (4) (2004) 819–831, http://dx.doi.org/10.1016/j.neuroscience.2004.02.012.
[27] M.J. Garcia Barrado, E.J. Blanco, M. Carretero Hernandez, M.C. Iglesias Osma, M. Carretero, J.J. Herrero, D. Jane Burks, J. Carretero, Local transformations of androgens into estradiol by aromatase P450 is involved in the regulation of prolactin and the proliferation of pituitary prolactin-positive cells, PLoS ONE 9 (6) (2014) e101403, http://dx.doi.org/10.1371/journal.pone.0101403.
[28] P. Libby, J.M. Ordovas, K.R. Auger, A.H. Robbins, L.K. Birinyi, C.A. Dinarello, Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult human vascular endothelial cells, Am. J. Pathol. 124 (2) (1986) 179–185.
[29] S.K. Clinton, J.C. Fleet, H. Loppnow, R.N. Salomon, B.D. Clark, J.G. Cannon, A.R. Shaw, C.A. Dinarello, P. Libby, Interleukin-1 gene expression in rabbit vascular tissue in vivo, Am. J. Pathol. 138 (4) (1991) 1005–1014.
[30] J. Galea, J. Armstrong, P. Gadsdon, H. Holden, S.E. Francis, C.M. Holt, Interleukin-1 beta in coronary arteries of patients with ischemic heart disease, Arterioscler. Thromb. Vasc. Biol. 16 (8) (1996) 1000–1006.
[31] Z. Zhang, M. Chopp, A. Goussev, C. Powers, Cerebral vessels express interleukin 1beta after focal cerebral ischemia, Brain Res. 784 (1–2) (1998) 210–217.
[32] Y. Liu, M. Biarnes Costa, C. Gerhardinger, IL-1beta is upregulated in the diabetic retina and retinal vessels: cell-specific effect of high glucose and IL-1beta autostimulation, PLoS ONE 7 (5) (2012) e36949, http://dx.doi.org/10.1371/journal.pone.0036949.
[33] M. Alfaidi, H. Wilson, M. Daigneault, A. Burnett, V. Ridger, J. Chamberlain, S. Francis, Neutrophil elastase promotes interleukin-1beta secretion from human coronary endothelium, J. Biol. Chem. 290 (40) (2015) 24067–24078, http://dx.doi.org/10.1074/jbc.M115.659029.
[34] G. Fink, 60 Years of neuroendocrinology: memoir: Harris' neuroendocrine revolution: of portal vessels and self-priming, J. Endocrinol. 226 (2) (2015) T13–T24, http://dx.doi.org/10.1530/JOE-15-0130.
[35] T. Nakamori, A. Morimoto, K. Yamaguchi, T. Watanabe, N.C. Long, N. Murakami, Organum vasculosum laminae terminalis (OVLT) is a brain site to produce interleukin-1 beta during fever, Brain Res. 618 (1) (1993) 155–159.
[36] S. Laye, E. Goujon, C. Combe, R. VanHoy, K.W. Kelley, P. Parnet, R. Dantzer, Effects of lipopolysaccharide and glucocorticoids on expression of interleukin-1 beta converting enzyme in the pituitary and brain of mice, J. Neuroimmunol. 68 (1–2) (1996) 61–66.
[37] K.T. Nguyen, T. Deak, S.M. Owens, T. Kohno, M. Fleshner, L.R. Watkins, S.F. Maier, Exposure to acute stress induces brain interleukin-1beta protein in the rat, J. Neurosci. 18 (6) (1998) 2239–2246.
[38] M. Buttini, H. Boddeke, Peripheral lipopolysaccharide stimulation induces interleukin-1 beta messenger RNA in rat brain microglial cells, Neuroscience 65 (2) (1995) 523–530.
[39] T.R. Sairanen, P.J. Lindsberg, M. Brenner, A.L. Siren, Global forebrain ischemia results in differential cellular expression of interleukin-1beta (IL-1beta) and its

receptor at mRNA and protein level, J. Cereb. Blood Flow Metab. 17 (10) (1997) 1107–1120, http://dx.doi.org/10.1097/00004647-199710000-00013.
[40] J.P. Konsman, K. Kelley, R. Dantzer, Temporal and spatial relationships between lipopolysaccharide-induced expression of Fos, interleukin-1beta and inducible nitric oxide synthase in rat brain, Neuroscience 89 (2) (1999) 535–548.
[41] J.P. Konsman, S. Vigues, L. Mackerlova, A. Bristow, A. Blomqvist, Rat brain vascular distribution of interleukin-1 type-1 receptor immunoreactivity: relationship to patterns of inducible cyclooxygenase expression by peripheral inflammatory stimuli, J. Comp. Neurol. 472 (1) (2004) 113–129, http://dx.doi.org/10.1002/cne.20052.
[42] T. Matsuwaki, A. Eskilsson, U. Kugelberg, J.I. Jonsson, A. Blomqvist, Interleukin-1beta induced activation of the hypothalamus-pituitary-adrenal axis is dependent on interleukin-1 receptors on non-hematopoietic cells, BrainBehav. Immun. 40 (2014) 166–173, http://dx.doi.org/10.1016/j.bbi.2014.03.015.
[43] J.J. Logie, S. Ali, K.M. Marshall, M.M. Heck, B.R. Walker, P.W. Hadoke, Glucocorticoid-mediated inhibition of angiogenic changes in human endothelial cells is not caused by reductions in cell proliferation or migration, PLoS ONE 5 (12) (2010) e14476, http://dx.doi.org/10.1371/journal.pone.0014476.
[44] C. Denef, Paracrinicity: the story of 30 years of cellular pituitary crosstalk, J. Neuroendocrinol. 20 (1) (2008) 1–70, http://dx.doi.org/10.1111/j.1365-2826.2007.01616.x.
[45] J. Carretero, M. Angoso, M. Rubio, E.J. Blanco, E. Sierra, J.J. Herrero, E. Perez, D.J. Burks, In vitro immunoblockade of VIP inhibits the proliferation of pituitary prolactin cells, Anat. Embryol. (Berl.) 211 (1) (2006) 11–18, http://dx.doi.org/10.1007/s00429-005-0058-9.
[46] K. Prasad, G.K. Prabhu, Image analysis tools for evaluation of microscopic views of immunohistochemically stained specimen in medical research-a review, J. Med. Syst. 36 (4) (2012) 2621–2631, http://dx.doi.org/10.1007/s10916-011-9737-7.