EM66-containing neurones in the hypothalamic parvicellular paraventricular nucleus of the rat: No plasticity related to acute immune stress

Fatima-Zohra El Yamani [1], Laurent Yon [2], Marlène Guérin [2], Seloua El Ouezzani [1], Abdelilah Alaoui [1], Nicolas Chartrel [2], Youssef Anouar [2], Rabia Magoul [1]

1 Laboratoire de Neuroendocrinologie et Environnement Nutritionnel et Climatique, Faculté des Sciences Dhar Mehrez, Université Sidi Mohamed Ben Abdellah, Fès, Morocco.
2 Inserm U982, Laboratoire Différenciation et Communication Neuronale et Neuroendocrine (DC2N), IFRMP 23, Université de Rouen, Mont-Saint-Aignan, France.

Correspondence to: Dr. Youssef Anouar,
Lab DC2N, INSERM U982, EA4310, IFRMP23, Université de Rouen,
76821 Mont-Saint-Aignan, France.
TEL: +33-235146661; E-MAIL: youssef.anouar@univ-rouen.fr

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Abstract

OBJECTIVES AND METHODS: Neuropeptides, as the main neuroendocrine system effectors, regulate notably the response to different stressors via a secretory plasticity within their respective hypothalamic neuronal populations. The aim of the present study was to explore by immunocytochemistry the occurrence and the potential expression plasticity of the novel neuropeptide EM66 in the CRH neurones of stressed rats.

RESULTS: The secretogranin II (SgII)-derived peptide EM66 is strongly expressed within hypothalamic neuroendocrine areas such as the parvocellular aspect of the paraventricular nucleus (pPVN) as well as the median eminence, suggesting a probable hypophysiotropic effect of this peptide. As a first approach to investigate such a role, we evaluated by immunohistochemistry EM66 expression within the pPVN following acute immune stress induced by lipopolysaccharide (LPS) or interleukin-1β (IL-1β) injection in rat. This study showed that EM66 is present in the pPVN but the number of EM66 immunolabeled cells did not fluctuate in this structure following LPS peripheral injection. In line with this observation, an intracerebroventricular injection of IL-1β did not provoke any significant variation of the number of intraparaventricular EM66 neurones.

CONCLUSION: The present data revealed for the first time that EM66 expression would be insensitive to the central and peripheral cytokines within the neurosecretory hypothalamic pPVN. This result indicates that EM66 does not participate to the phenotypic plasticity of hypothalamic parvicellular neurones in response to acute inflammatory stress.

ORIGINAL ARTICLE

INTRODUCTION

The neuropeptide EM66 arises from the proteolytic processing of secretogranin II (SgII), a member of the granin acidic secretory protein family (Anouar *et al.* 1998; Montero-Hadjadje *et al.* 2003; Yon *et al.* 2003). EM66 has been first characterized in the adult and fetal human adrenal gland, as well as in the rat pituitary and specific hypothalamic nuclei, suggesting putative endocrine and/or neuroendocrine roles for this peptide (Anouar *et al.* 1998; Boutahricht *et al.* 2007; Montero-Hadjadje *et al.* 2008). High-performance liquid chromatography analysis of jerboa hypothalamic extracts combined with a radioimmunoassay of EM66 revealed a single peak of immunoreactive material exhibiting the same retention time as recombinant EM66, thus demonstrating the occurrence of EM66 also in the brain (Boutahricht *et al.* 2005). The localization of EM66 within neurones of the jerboa hypothalamic structures, including the suprachiasmatic, supraoptic, parvocellular paraventricular and arcuate nuclei, and the lateral hypothalamus has been established (Boutahricht *et al.* 2005). Similarly, EM66 was found within hypothalamic structures of the rat, such as the parvocellular paraventricular, preoptic and arcuate nuclei, as well as the lateral hypothalamus. However, unlike in jerboa, the suprachiasmatic and supraoptic nuclei of the rat hypothalamus were devoid of EM66 neurones. Thus, the peptide EM66 may exert common neuroendocrine activities in rat and jerboa, e.g. control of food intake, and species-specific roles such as the regulation of biological rhythms and hydromineral homeostasis in jerboa (Boutahricht *et al.* 2007). Indeed, food deprivation induced a significant increase in the number of EM66-containing neurones within the arcuate nucleus and the parvocellular aspect of the paraventricular nucleus (pPVN) in jerboa, suggesting that EM66 could be involved in the control of feeding behaviour (Boutahricht *et al.* 2005). These data revealed a physiological plasticity of the EM66 system in the hypothalamus and suggested the implication of this peptide in the regulation of neuroendocrine functions.

Because EM66 was strongly expressed within pPVN neurones, it may play a role in the response to stress. The PVN is involved in the neuroendocrine adaptative response to stress through parvicellular effector neurones synthesizing and releasing into the pituitary portal blood the corticotropin-releasing hormone (CRH) (Harbuz et al 1990; Herman *et al.* 1995; Karalis *et al.* 1997). The CRH neurones have a high potential for phenotypical plasticity, allowing them to modify their neuroendocrine output depending upon circumstances (nature and intensity of a stimulus). Indeed, in addition to CRH, they elaborate several other neuropeptides, such as vasopressin (VP), cholecystokinin (CCK), enkephalin or neurotensin, each probably subserving a complementary function to CRH in the control of the pituitary and each depending on different regulatory mechanisms for its expression. For example, VP is a powerful pituitary corticotropin (ACTH) releaser and VP mRNA is overexpressed in CRH neurones in response to various stressful stimuli (Makino *et al.* 1995). The neuropeptide CCK is also an accessory neuropeptide which is overexpressed within CRH neurones following immune and immobilization stress (Juaneda *et al.* 2001; Barakat *et al.* 2006).

The aim of the present study was to explore the possible involvement of EM66 as an accessory neuropeptide in the response to stress, particularly immune stress induced by the endotoxin lipopolysaccharide (LPS) in rat. Immune system activation by LPS induces the release of the proinflammatory cytokine interleukin-1 (IL-1) that centrally activates PVN CRH neurones, leading to ACTH discharge in order to trigger glucocorticoid secretion from the adrenal, which in turn modulates the immune response (Rivest *et al.* 2000). Injection of LPS is known to induce an array of peripheral and central responses associated with increased activity of the hypothalamic–pituitary–adrenal axis by increasing PVN CRH mRNA and circulating corticosterone levels (Sapolsky *et al.* 1987; Crestani *et al.* 1991; Harbuz *et al.* 1992; Ericsson *et al.* 1994; Tilders *et al.* 1994; Rivest and Lacroix 1995). We explored here the impact of an acute injection of LPS or IL-1β (an intermediary in LPS action) on the EM66 intraneuronal expression within pPVN which was evaluated by immunocytochemistry.

MATERIALS AND METHODS

Animals

Male Wistar rats, weighing 250–300 g, were maintained under controlled conditions of temperature (24 °C) and natural photoperiod. The diet was supplied *ad libitum*. Rats were anesthesized and sacrificed by intracardiac perfusion. Animal manipulations were performed according to the recommendations of the Local Ethical Committee.

LPS treatment

Rats were randomly assigned to control (n=8) or experimental (n=8) groups which received, respectively, an intraperitoneal (i.p.) injection of vehicle (0.9%) or LPS (250 µg/kg; from *E. coli* Serotype 0127:B8 (Sigma-Aldrich, St. Louis, MO)) 16 h after intracerebroventricular (icv) injection of 100 µg/10 µl volume of colchicine (necessary to visualize EM66 immunoreactive cell bodies) (AP: –0.9 mm, length: 1.8 mm, height:–4.5 mm, according to Paxinos and Watson (1986)). Rats were sacrificed 8 h post-treatement.

Intracerebroventricular injection of IL-1β

Anesthetized rats (sodium pentobarbital 35 mg/kg i.p.) were stereotaxically implanted into the right lateral cerebral ventricle with a 21-gauge stainless steel guide cannula. The placement coordinates were obtained from

Paxinos and Watson (1986) as previously described. Cannulas were fixed to the skull surface with a screw and dental acrylic cement. Animals were left during 5 days to recover from the surgical procedure before beginning the icv injections. Correct placement of the cannulas was confirmed by histological examination after trypan blue injection. The injections were given using a Hamilton syringe attached to polyethylene tubing. Two groups of rats received an injection of 100 µg of colchicine in a 10-µl volume 16 h before another injection of vehicle (0.9%) for the control group (n=5) or IL-1β (125 ng/rat, human recombinant expressed in *E. coli*, Sigma) for the experimental group (n=5). The rats were sacrificed 8 h later.

Tissue Processing

The occurrence of EM66 within hypothalamic cells and fibers was studied by immunohistochemistry using polyclonal antibodies raised in rabbits against the recombinant fusion protein MBP–EM66 (Anouar *et al.* 1998; Montero-Hadjadje *et al.* 2003). Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (35 mg/kg) and perfused through the aorta with 50 ml of a saline solution followed by 300 ml of a fixative solution containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB), pH 7.4. The brains were dissected, cut into 5 mm thick slabs and post-fixed for 48 h at 4 °C with the same fixative. Brain sections (60 µm) were cut on a vibratome and rinsed with phosphate-buffered saline (PBS).

Immunohistochemical Procedures

The tissue sections were rinsed several times in PBS and processed for indirect immunohistochemistry. Tissue slices were then incubated overnight at 4°C with the primary EM66 antiserum diluted 1:2000 in PBS containing 0.3% Triton X-100. The sections were rinsed in PBS for 30 min and incubated for 2 h at room temperature with a biotinylated goat anti-rabbit antiserum (Vector Laboratories, Paris, France) diluted 1:400. Finally, brain sections were incubated for 2 h at room temperature with standard avidin–biotin peroxidase (Vector Laboratories) diluted 1:400 in PBS. The peroxidase activity was revealed according to the method of Shu *et al.* (1988) using diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich) as chromogen intensified with nickel ammonium sulphate (Sigma–Aldrich). The reaction was stopped by rinsing in PB. Sections were deposited on gelatin-coated slides, air-dried, dehydrated in graded alcohol and mounted in Eukit (O. Kindler GmbH & Co, Freiburg, Germany).

Tissue Examination and Statistics

The sections were examined under a Nikon Microphot-FX microscope (Tokyo, Japan) and photomicrographs were taken with a Leitz microscope (Leica, Heidelberg, Germany). The specificity of the immunoreactions was verified as previously described (Montero-Hadjadje *et al.* 2003) by substitution of the primary antiserum with PB or non-immune serum, and liquid phase preabsorption of the antiserum with purified recombinant EM66 peptide (10^{-6} M). Quantification of EM66-immunoreactive cell bodies within the pPVN of control and stressed rats was performed on four sections per animal and an average value was calculated for each animal. The results are expressed as means ± SEM. Statistical significance was determined by using Student's unpaired t-test.

RESULTS

The pattern of distribution of EM66 neurones within hypothalamic PVN in control rats confirmed the data previously reported (Boutahricht *et al.* 2007). A high density of EM66-IR cell bodies occurred in the parvicellular subdivisions of the PVN (Figure 1 A,B). Most of the EM66 neurones were concentrated within the medial nuclei of the pPVN along its rostrocaudal extent.The median eminence (ME), the site of neurohormone release, exhibited a discrete pattern of EM66-immunoreactive fibers in its internal layer and a dense plexus of fibers in its external zone with a preferential distribution around capillaries (Figure 1 C,D).

In spite of various experimental conditions (Control vs LPS- or IL-1β-injected groups), the pattern of distribution of EM66-IR neurones in the pPVN was unchanged. Intraperitoneal injection of LPS induced an apparent increase of EM66-IR cell bodies (Figure 2A, B). However, the quantitative analysis showed no significant increase in the number of EM66-IR cell bodies within the pPVN of stressed compared to control animals (Figure 3A). Similarly, ICV injection of IL-1β provoked no significant effect on the number of the EM66-IR cell bodies and therefore on the expression of EM66-IR in the pPVN of immune stressed rats compared to controls (Figure 2C,D; Figure 3B).

DISCUSSION

Consistent with a previous report (Boutahricht *et al.* 2007), the results described here show that the pPVN of the rat displays an important EM66-immunopositive neuronal population. Such hypothalamic site is known to play a critical role in the neuroendocrine control of the stress response (Paulmyer-Lacroix *et al.* 1995). Indeed, the occurrence of a high density of EM66-immunoreactive fibers in the external zone of the median eminence suggests that EM66 could be released into the capillaries of the pituitary portal plexus and thus may act as a hypophysiotropic factor by regulating notably the stress response in rat.

The immune challenge is one form of systemic stressors which activates the hypothalamo-pituitary-adrenal axis (Herman & Cullinan 1997; Hayley & Anisman 2005; Anisman *et al.* 2007). It represents a stressful stimulus inducing an increase in parvicellular CRH

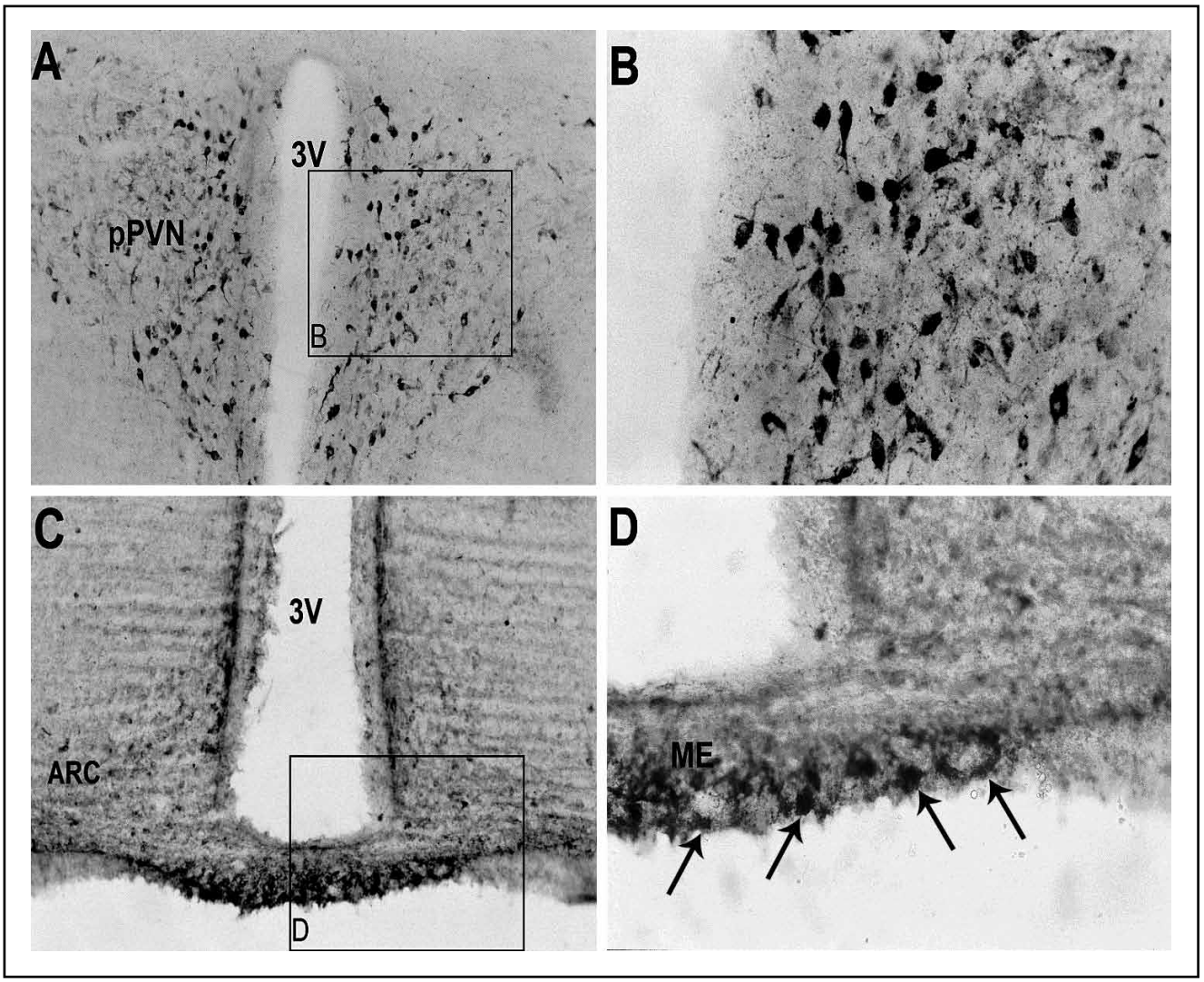

Fig. 1. Frontal sections of rat colchicine-treated brains showing EM66 immunoreactivity at the level of the hypothalamus. EM66-immunoreactive cell bodies were detected within the parvocellular paraventricular nucleus (pPVN; **A**, **B**), and at the medial part of the arcuate nucleus (ARC; **C, D**). The median eminence (ME) displayed a dense plexus of EM66-immunopositive nerve fibers (arrows) clearly visible in its external zone around capillaries. Magnification (**A, C**) ×100, (**B, D**) ×250. 3V, third ventricle.

mRNA expression and ACTH hypersecretion followed by hypercorticosteronemia.

The present work showed that immune system mobilization by LPS did not affect the EM66 immunocytochemical expression evaluated by the number of EM66-immunoreactive neurones within the pPVN. This result is reminiscent of data previously obtained with VP. Indeed, 8 h after LPS treatment, VP mRNA expression remained unchanged in CRH neurones of pPVN, suggesting that VP may not be essential in this short-term adaptation of CRH neurones. However, the same stimulus provoked an increase in the expression levels of CCK and neurotensin mRNAs in CRH neurones, indicating a role for CCK and neurotensin in the neuroendocrine response to acute immune stimulation (Juaneda *et al.* 2001). Other paradigms such as chronic stress as well as adrenalectomy were reported to induce a sharp increase in parvicellular VP mRNA (de Goeij DC *et al.* 1991; de Goeij et al 1992; Makino et al 1995). Although analysis of EM66 expression should be also performed under these alternative experimental conditions, it should be mentioned that repeated injections of LPS (three successive days) did not affect the number of EM66-immunoreactive neurones within the pPVN (*data not shown*).

These data provide an additional evidence for the complexity of the phenotypical plasticity of neurones within the pPVN (Pirnik & Kiss 2005) and thus of the regulatory mechanisms of the stress response. Moreover, the stressor stimulus plays a critical role in the control of the phenotypical plasticity of pPVN neurones. The data highlight differences in neurosecretory behaviour of rat pPVN neurones which would be dependent on the stressor.

Intraperitoneal injection of LPS is known to increase cytokines in brain and plasma (Elmquist *et al.* 1997; Tilders *et al.* 1994) associated with HPA axis activation. Peripheral IL-1 can influence the brain via the

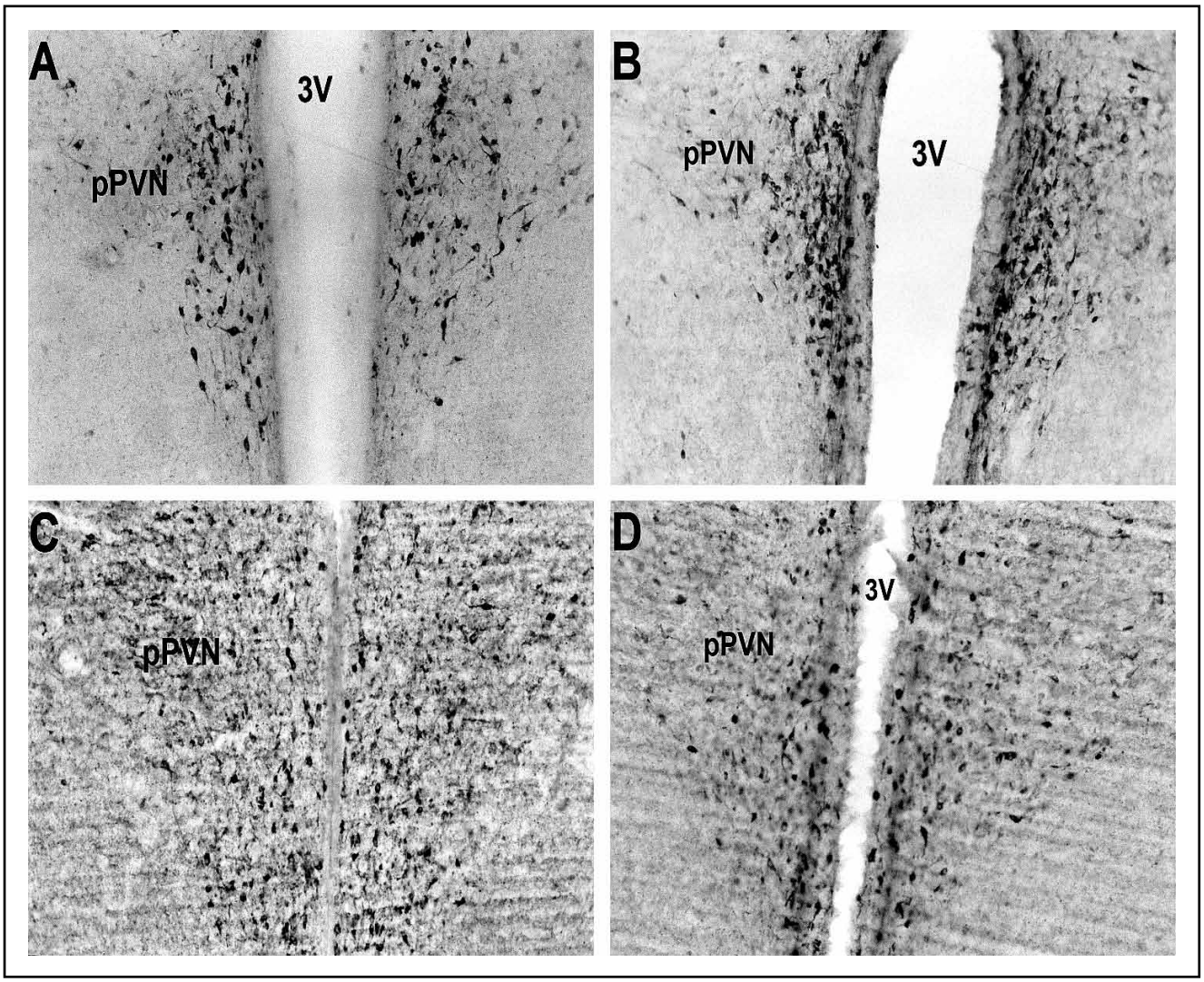

Fig. 2. Frontal sections of colchicine-treated brains of control (**A**) and LPS-injected (**B**) rats showing an apparent increase in the density of EM66-immunoreactive neurones within the parvocellular paraventricular nucleus (pPVN). In contrast, coronal sections of colchicine-treated brains of control (**C**) and IL-1β treated rats (**D**) showed no apparent difference in the density of EM66-immunoreactive neurones within the pPVN. Magnification ×100. 3V, third ventricle.

direct activation of CRH neurones or an indirect effect through vagal afferent fibers. In this context, vagotomy blocks many centrally-mediated effects of peripheral immune activation (Gaykema *et al.* 2000; Watkins *et al.* 1995), and attenuates the behavioral effects of peripherally administered IL-1 (Bluthe *et al.* 1996). In line with these data, it has been reported that icv injections of IL-1 can cause the release of ACTH. For instance, IL-1 β produced an immediate increase in plasma corticosterone and ACTH (Brown *et al.* 1991; Cragnolini et al 2004). To further explore the possible immune stress-EM66 interaction, we evaluated the effect of acute central injection of IL-1β on the expression of neuronal EM66 in the pPVN. In accordance with the data obtained with LPS, IL-1β had no effect on such expression since the number of EM66-containing cell bodies displayed no significant change following IL-1β treatment.

In conclusion, our work confirmed the occurrence of EM66 in hypothalamic parvicellular PVN neurones of the rat and revealed for the first time that EM66 within the neurosecretory parvicellular system of the hypothalamic PVN would be insensitive to central and peripheral cytokine action. These data show that EM66 does not participate to the phenotypic plasticity of hypothalamic parvicellular neurones in response to inflammatory stress. They constitute an additional evidence for the occurrence of several phenotypically different neurone groups within PVN, each one characterized by its capacity to regulate (or not) the neuropeptide expression which would be function of the stressor. Additional investigations will be conducted to determine the possible regulation of EM66 expression in the PVN in response to other stressful circumstances such as acute and chronic immobilization, anorexia induced by repeated immobilization and thermal stress.

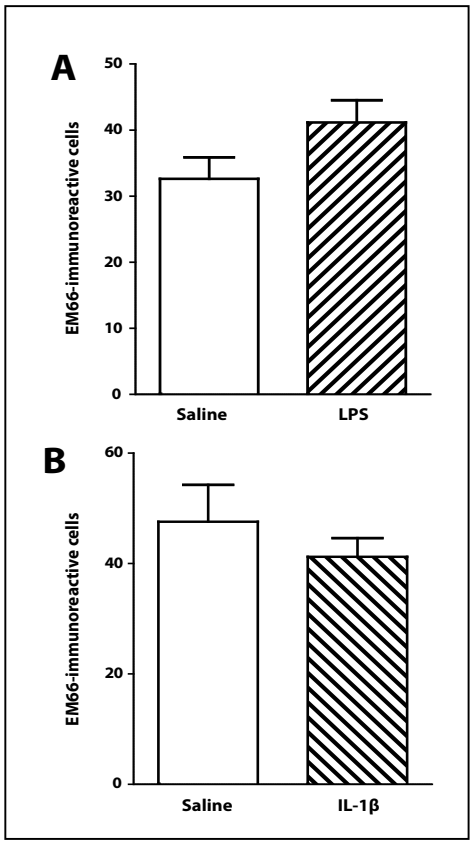

Fig. 3. A: Comparison of the number of EM66-immunoreactive neurones within the PVN of control and LPS-injected rats. The results are expressed as the number of EM66-immunoreactive cells per four sections per experimental group (control vs. LPS-treated animals; n=8 in both groups). Values are shown as mean ±SEM. Student's t-test revealed no significant change in the average number of EM66-immunoreactive cells within the pPVN. **B:** Comparison of the number of EM66-immunoreactive neurons within the pPVN of control and IL-1β treated rats. The results are expressed as number of EM66-immunoreactive cells per four PVN sections per experimental group (n=5 in both groups). The histograms show no significant difference in the number EM66-IR neurones in the pPVN between control and treated animals (t=0.42, $p<0.05$).

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REFERENCES

1 Anouar Y, Desmoucelles C, Yon L, Leprince J, Breault L, Gallo-Payet N, Vaudry H (1998). Identification of a novel secretogranin II-derived peptide (SgII (187–152)) in adult and fetal human adrenal glands using antibodies raised against the human recombinant peptide. J Clin Endocrinol Metab. **83**: 2944–2951.
2 Montero-Hadjadje M, Pelletier G, Yon L, Guillemot J, Li S, Magoul R, Tillet Y, Vaudry H, Anouar Y (2003). Biochemical characterization and immunocytochemical localization of EM66, a novel peptide derived from secretogranin II, in the rat pituitary and adrenal glands. J Histochem Cytochem. **51**: 1083–1095.
3 Yon L, Guillemot J, Montero-Hadjadje M, Grumolato L, Leprince J, Lefebvre H, Contesse V, Plouin PF, Vaudry H, Anouar Y (2003). Identification of the secretogranin II-derived peptide EM66 in pheochromocytomas as a potential marker for discriminating benign versus malignant tumors. J Clin Endocrinol Metab. **88**: 2579–2585.
4 Boutahricht M, Guillemot J, Montero-Hadjadje M, Barakat Y, El Ouezzani S, Alaoui A, Yon L, Vaudry H, Anouar Y, Magoul R (2007). Immunohistochemical distribution of the secretogranin II-derived peptide EM66 in the rat hypothalamus: A comparative study with jerboa. Neuroscience Lett. **414**: 268–272.
5 Montero-Hadjadje M, Vaingankar S, Elias S, Tostivint H, Mahata SK, Anouar Y (2008). Chromogranins A and B and secretogranin II: evolutionary and functional aspects. Acta Physiol. (Oxf) **192**: 309–324.
6 Boutahricht M, Guillemot J, Montero-Hadjadje M, Bellafqih S, El Ouezzani S, Alaoui A, Yon L, Vaudry H, Anouar Y, Magoul R (2005). Biochemical characterisation and immunohistochemical localization of the secretogranin II-derived peptide EM66 in the hypothalamus of the jerboa (Jaculus orientalis): modulation by food deprivation. J Neuroendocrinol. **17**: 372–378.
7 Harbuz MS, Nicholson SA, Gillham B, Lightman SL (1990). Stress responsiveness of hypothalamic corticotropin-releasing factor and pituitary pro-opiomelanocortin mRNAs following high-dose glucocorticoid treatment and withdrawal in the rat. J Endocrinol. **127**: 407–415.
8 Herman JP, Adams D, Prewitt C (1995). Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. Neuroendocrinology. **61**: 180–190.
9 Karalis K, Muglia LJ, Bae D, Hilderbrand H, Majzoub JA (1997). CRH and the immune system. J Neuroimmunol. **72**: 131–136.
10 Makino S, Smith MA, Gold PW (1995). Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels. Endocrinology. **136**: 3299–3309.
11 Juaneda C, Lafon-Dubourg P, Ciofi P, Sarrieau A, Wenger T, Tramu, G Corio M (2001). CCK mRNA expression in neuroendocrine CRH neurons is increased in rats subjected to an immune challenge. Brain Res. **901**: 277–280.
12 Barakat Y, Pape JR, Boutahricht M, El Ouezzani s, Alaoui A, Chaigniau M, Tramu G, Magoul R. (2006). Immunocytochemical detection of CCK and CRH neuropeptides in the hypothalamic paraventricular nucleus of the jerboa (Jaculus orientalis): modulation by immobilization stress. J Neuroendocrinol. **18**: 767–775.
13 Rivest S, Lacroix S, Vallieres L, Nadeau S, Zhang J, Laflamme N (2000). How the blood talks to the brain parenchyma and the paraventricular nucleus of the hypothalamus during systemic inflammatory and infectious stimuli. Proc Soc Exp Biol Med. **223**: 22–38.
14 Sapolsky R, Rivier C, Yamamoto G, Plotsky P, Vale W (1987). Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor. Science. **238**: 522–524.
15 Crestani F, Seguy F and Dantzer R (1991). Behavioural effects of preferentially injected interleukin-1: role of prostaglandins. Brain Res. **542**: 330–335.
16 Harbuz MS, Stephanou A, Sarlis N, Lightman SL (1992). The effects of recombinant human interleukin (IL)-1α, IL-1β or IL-6 on hypothalamo-pituitary–adrenal axis activation. J Endocrinol. **133**: 349–355.
17 Ericsson A, Kovacs KJ and Sawchenko PE (1994). A functional anatomical analysis of central pathways subserving the effects of interleukin-1 on stress-related neuroendocrine neurons. J Neurosci. **14**: 897–913.
18 Tilders FJH, DeRijk RH, Dam A-M V A M, Schotanus K, Persoons J H A (1994). Activation of the hypothalamus-pituitary-adrenal axis by bacterial endotoxins: routes and intermediate signals. Psychoneuroendocrinology. **19**: 209–232.
19 Rivest S, Lacroix S (1995). Influence of cytokines on neuroendocrine functions during immune response. Mechanisms involved and neuronal pathways. Ann Endocrinol. **56**: 159–167.

20 Paxinos G, Watson C (1986). The Rat Brain in Stereotaxic Coordinates. Second Edition. New York: Academic Press. p: 21.
21 Shu S, Ju G, Fan L (1988). The glucose-oxidase DAB-nickel method in peroxydase histochemistry of the nervous system. Neurosci Lett. **85**: 169–171.
22 Paulmyer-Lacroix O, Guillaume V, Anglade G, Grino M, Oliver C (1995). Regulation of corticotropic function in stressful situations. Ann Endocrinol. **56**: 245–251.
23 Herman JP, Cullinan WE (1997). Neurocircuitry of stress: central control of hypothalamo-pituitary-adrenocortical axis. Trends Neurosci. **20**: 78–84.
24 Hayley S, Anisman H (2005). Multiple mechanisms of cytokine action in neurodegenerative and psychiatric states: neurochemical and molecular substrates. Curr Pharm Des. **11**: 947–962.
25 Anisman H, Poulter MO, Gandhi R, Merali Z, Hayley S (2007). Interferon-α effects are exaggerated when administered on a psychosocial stressor backdrop: cytokine, corticosterone and brain monoamine variations. J Neuroimmunol. **186**: 45–53.
26 de Goeij DC, Kvetnansky R, Whitnall MH, Jezova D, Berkenbosch F, Tilders FJ (1991). Repeated stress-induced activation of corticotropin-releasing factor neurons enhances vasopressin stores and colocalization with corticotropin-releasing factor in the median eminence of rats. Neuroendocrinology. **53**: 150–159.
27 de Goeij DCE, Jezova D and Tilders FJH (1992). Repeated stress enhances vasopressin synthesis in corticotropin releasing factor neurons in the paraventricular nucleus. Brain Res. 577: 165–168.
28 Pirnik Z, Kiss A (2005). Fos expression variances in mouse hypothalamus upon physical and osmotic stimuli: co-staining with vasopressin, oxytocin, and tyrosine hydroxylase. Brain Res Bull. **5**: 423–431.
29 Elmquist K, Scammell TE, Saper CB (1997). Mechanisms of CNS response to systemic immune challenge: the febrile response. Trends Neurosci. **20**: 565–570.
30 Gaykema RP, Goehler LE, Hansen MK, Maier SF, Watkins LR (2000). Subdiaphragmatic vagotomy blocks interleukin-1beta-induced fever but does not reduce IL-1beta levels in the circulation. Auton Neurosci. **85**: 72–77.
31 Watkins LR, Maier SF, Goehler LE (1995). Cytokine-to-brain communication: a review & analysis of alternative mechanisms. Life Sci. **57**: 1011–1026.
32 Bluthe RM, Michaud B, Kelley KW, Dantzer R (1996). Vagotomy attenuates behavioural effects of interleukin-1 injected peripherally but not centrally. Neuroreport. **7**: 1485–1488.
33 Brown R, Li Z, Vriend CY, Nirula R, Janz L, Falk J, Nance DM, Dyck DG, Greenberg AH (1991). Suppression of splenic macrophage interleukin-1 secretion following intracerebroventricular injection of interleukin-1β: evidence for pituitary–adrenal and sympathetic control. Cell Immunol. **132**: 84–93.
34 Cragnolini AB, Perello M, Schiöth HB, Scimonelli TN (2004). α-MSH and γ-MSH inhibit IL-1β induced activation of the hypothalamic–pituitary–adrenal axis through central melanocortin receptors. Regul Pept. **122**: 185–190.