# Immunocytochemical distribution of EM66 within the hypothalamic parvocellular paraventricular nucleus: Colocalization with CRH and TRH but no plasticity related to acute stress and thyroidectomy in the rat

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## 1. Introduction

The hypothalamic paraventricular nucleus (PVN) is known to be involved in the neuroendocrine adaptive response to stress through parvocellular effector neurons synthesizing and releasing into the pituitary portal blood the corticotropin-releasing hormone (CRH) [1–3]. The CRH-containing neurons of the parvocellular PVN (pPVN) have a high potential for phenotypical plasticity, allowing them to modify their neuroendocrine output depending upon circumstances [4]. Indeed, in addition to CRH, they are able to elaborate several other accessory neuropeptides, such as vasopressin, cholecystokinin, enkephalin, neurotensin or oxytocin, each probably subserving a complementary function to CRH in the control of the pituitary and each depending on different regulatory mechanisms for their expression [5–9].

The pPVN neurons also contain the hypophysiotropic thyrotropin-releasing hormone (TRH), the central regulator of the hypothalamus–pituitary–thyroid (or thyrotropic) axis [10–14] which plays an essential role in the maintenance of metabolic homeostasis in response to alterations in metabolism and external environment. The TRH neurons have a primary role in the regulation of the thyroid hormone (TH) production through release of TRH at the median eminence to stimulate pituitary TSH release. TSH then stimulates the thyroid gland to produce T4 and T3. TSH, in turn, regulates the activity of TRH neurons in the hypothalamus by a classical negative feedback pathway [15–17]. TRH neurons represent a neuron population completely distinct from the CRH neurons [18]. However, they express glucocorticoid receptors indicating that they may be under corticotrope axis control [19].

The secretogranin II-derived peptide EM66 is among the neuropeptides strongly expressed within the pPVN as well as in the external

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median eminence (ME), suggesting a probable hypophysiotropic role for this neuropeptide in the control of the corticotrope axis [20,21]. As a first approach to investigate such a role, our previous results showed that EM66 does not participate to the phenotypical plasticity of pPVN neurons in response to acute inflammatory stress in rat [21]. The patterns of distribution of the EM66-containing neurons and the CRH neurosecretory cells within the pPVN and their terminals in the ME are very similar, suggesting an eventual colocalization of these two neuropeptides in this hypothalamic neuroendocrine area [21] and thus a possible functional interaction between CRH and EM66 within the pPVN in the regulation of the corticotrope axis.

To explore a possible EM66-CRH coexpression within the PVN as well as the ME (site of CRH release) of rat, we used in the present work a double immunofluorescence procedure followed by confocal microscope analysis. In order to investigate also an eventual neuroendocrine plasticity involving EM66 in response to stress and since the stress-induced neuroendocrine response seems to be stressor-specific [22], we analyzed by immunohistochemistry EM66 expression within the pPVN neurons of rats submitted to different stressful circumstances such as acute immobilization and thermal stress. Such stressors are known to activate the corticotrope axis and represent interesting stressful stimulus inducing an increase in parvocellular CRH mRNA expression and ACTH hypersecretion followed by hypercorticosteronemia [23].

The similarity of the distribution pattern of TRH-containing neurons and the EM66 neuronal system within pPVN as well as ME suggests their possible coexpression in this neuroendocrine area and thus an eventual interaction in the regulation of the thyrotrope axis. Thyroidectomy is known to induce a significant increase in the number of TRH-containing neurons within the parvocellular aspect of the PVN and plasma TSH concentration [24]. In this context, we evaluated by double immunofluorescence EM66-TRH coexpression within the PVN and the ME and studied the effect of short-term thyroidectomy on EM66 expression in the rat pPVN.

## 2. Material and methods

### 2.1. Animals

Male Wistar rats weighing 250–300 g were maintained under controlled conditions of temperature (24 °C) and photoperiod (12 h light/12 h dark). The diet was supplied ad libitum. Animal manipulations were performed according to the recommendations of the Local Ethical Committee.

### 2.2. Stress procedures

Rats were randomly assigned to three groups. A control group consisted of six animals (n = 6) sacrificed 24 h after intracerebroventricular (icv) injection of 100 μg/10 μl volume of colchicine (necessary to visualize EM66, CRH and TRH-immunoreactive cell bodies) (AP: −0.9 mm, length: 1.8 mm, height: −4.5 mm, according to Paxinos and Watson [25]). The second group of rats (n = 6) was exposed to acute immobilization stress for 2 h according to the method of Kvetnansky and Mikulaj [26] by fixing all four limbs to a board with adhesive tape. The heads were also fixed at the neck area, thus limiting their motion. This was followed by colchicine icv injection as described above. Rats of the third group (n = 5) were exposed to acute cold stress by keeping animals at 5 °C for 4 h, followed by colchicine treatment, and sacrificed 24 h after.

### 2.3. Thyroidectomy procedure

The surgical procedure was performed under pentobarbital anesthesia (35 mg/kg ip) as previously described [24]. Briefly, in thyroidectomized rats (n = 6), thyroid glands were surgically removed. In the sham-control animals (n = 6), the thyroid glands were just exposed. All the experimental rats were kept alive during 6 days with food and water available ad libitum, and treated with colchicine 24 h before sacrifice.

### 2.4. Tissue processing

The expression of EM66, TRH, and CRH within hypothalamic cells and fibers was studied by immunohistochemistry, using respectively, rabbit polyclonal antibodies raised against recombinant EM66 [27,28], and a TRH synthetic peptide (13482, Abnova Taïwan) and sheep polyclonal antibodies raised against a CRH synthetic peptide (110-81721, Novus Biologicals). The 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) at the hypothalamic level were cut on a vibratome and rinsed with phosphate-buffered saline (PBS).

### 2.5. Immunohistochemical (IHC) procedures

#### 2.5.1. Single IHC labeling

The tissue sections were rinsed several times in PBS and processed for indirect immunohistochemistry. Tissue slices were 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 (GAR) 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. [29] using diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) as chromogen, and intensified with nickel ammonium sulfate (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). The sections were examined under a Nikon Microphot-FX microscope (Tokyo, Japan) and photomicrographs were taken with a Leitz microscope (Leica, Heidelberg, Germany).

#### 2.5.2. Double IHC labeling

Tissue slices were treated for 90 min with normal donkey serum diluted at 1:100 in a solution containing 1% BSA and 0.3% Triton X-100 in PBS, to reduce non-specific labeling. For EM66-CRH double-immunolabeling, sections were then incubated for 2 h at room temperature with a mixture of the two primary antibodies diluted 1:800 and 1:100, respectively. The immunoreactivities were revealed with a mixture of two secondary antibodies, Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen) diluted 1:100 and Alexa Fluor 594-conjugated donkey anti-sheep IgG (Invitrogen) diluted 1:100. For EM66-TRH double-immunolabeling, a sequential approach [30] was used since both antibodies were raised in rabbit. Indeed, the saturation step was performed with normal goat serum, diluted 1:100 for 90 min at room temperature, before incubation with the TRH primary antisera (1:100, 2 h at room temperature). Tissue sections were rinsed in PBS and then incubated for 90 min at room temperature with Alexa Fluor 488-conjugated GAR IgG (Invitrogen) diluted 1:100. An intervening 15 min paraformaldehyde (2%) treatment was used between the first (TRH) and the second (EM66) immunolabeling procedures to allow fixation of the TRH-GAR immunoreactive complexes. The sections were then washed and treated with normal goat serum, diluted 1:100 for 90 min at room temperature. Subsequently, they were incubated overnight at 4 °C with the EM66 primary antibody diluted 1:800 and then incubated for 90 min with Alexa Fluor 594-conjugated GAR IgG

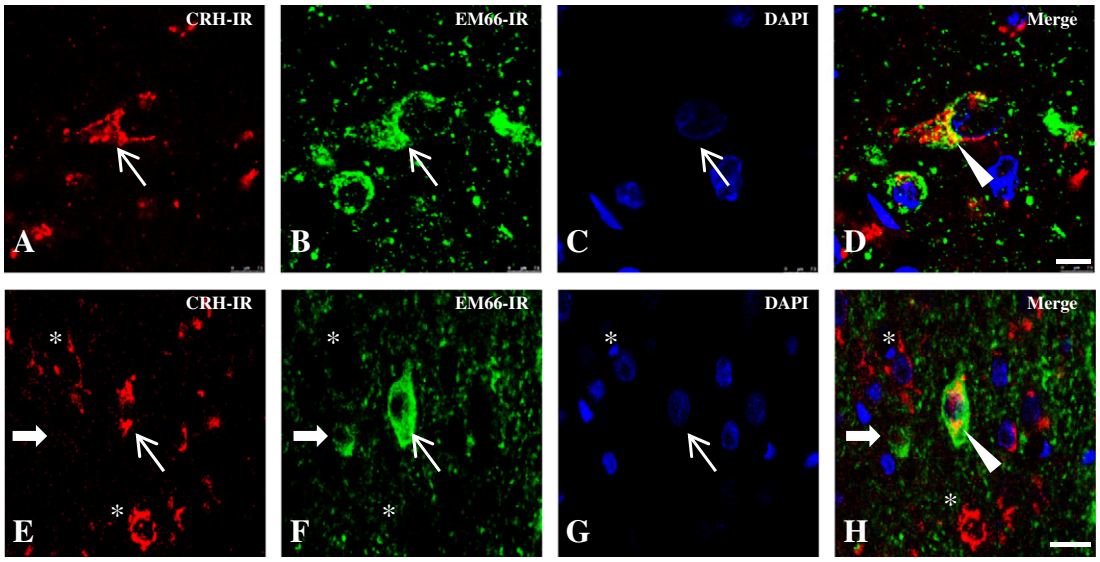


**Fig. 1.** Confocal microscopy of CRH- and EM66-IR neurons in rat pPVN. CRH and EM66 cell bodies respectively immunolabeled with Alexa fluor 594 (red color, A and E, thin arrows) and Alexa fluor 488 (green color, B and F, thin arrows) and nuclei stained by DAPI (blue color, C and G, thin arrows) were visualized in the same sections. CRH and EM66 colocalization (merge, D and H, arrowheads) appeared to concern only some cytoplasmic compartments. Neurons containing only either CRH (E–H, asterisk), or EM66 (A–D, thick arrows) were also observed. Scale bar, (A–D) 7.5 μm and (E–H) 10 μm.

(Invitrogen) diluted 1:100. Selected sections for double immunolabeling were incubated for 2 min with 1 μg/ml 4,6-diamino-2-phenylindole (DAPI, Sigma) to stain nuclei. Immediately after, tissue slices were rinsed in PBS, fixed again and buffer/glycerol (1:1) coverslipped to be examined using a Leica SP2 upright confocal laser scanning microscope (DMRAX-UV) equipped with the Acousto-Optico Beam Splitter system (Leica).

To verify the specificity of the immunoreactions, control tests were performed including omission of primary antibodies (substitution of the primary antibodies with PB) incubation with non-immune serum instead of the primary antibodies, and preincubation of the EM66 antiserum (diluted 1:800 or 1:2000 for respectively immunofluorescent or immunoenzymatic methods) with purified recombinant EM66. For the IHC procedure of TRH-EM66 labeling, omission of the primary EM66 antisera was performed in order to check the absence of unspecific Alexa Fluor 594-conjugated GAR IgG immunoreactive signal.

### 2.6. Statistical analysis

Quantification of EM66-IR cell bodies within the pPVN of control, stressed or thyroidectomized rats was performed on all PVN sections per animal and an average value was calculated for each animal. The magnification used for such measurements was ×100 and ×250. The results are expressed as mean ± SEM. Statistical significance was determined using Student's unpaired *t*-test.

## 3. Results

### 3.1. EM66-IR neurons in the rat pPVN and ME

The distribution pattern of EM66-IR neurons within the pPVN of control rats confirmed previously reported data [20,21]. Indeed, most of the EM66-IR neurons were concentrated within the medial nuclei of the pPVN along its rostrocaudal extent (data not shown). As previously reported [20,21], the median eminence (ME) exhibited a dense plexus of EM66-IR fibers in its external zone with a preferential distribution around capillaries.

### 3.2. Colocalization of EM66 and CRH-IR neurons within the pPVN and the ME

Co-incubation of hypothalamic slices with the anti-EM66 and the anti-CRH antibodies revealed that CRH-IR neurons within the pPVN contain EM66 immunoreactivity (Fig. 1A–H), indicating that the EM66 peptide occurs in corticotrope neuroendocrine cells. CRH and EM66 co-localization appears in cytoplasmic compartments (Fig. 1D and H, arrowhead). However, the CRH-EM66 colocalization is not observed in all cells since some CRH-IR cells did not exhibit any EM66-IR (Fig. 1E–H, asterisk) and some EM66-IR neurons did not display any CRH-IR (Fig. 1E–H, thick arrow). In the external zone of the ME, site of neurohormone release, numerous nerve terminals were positively labeled for EM66 and CRH (Fig. 2A and B). Moreover, it is clear that EM66 is

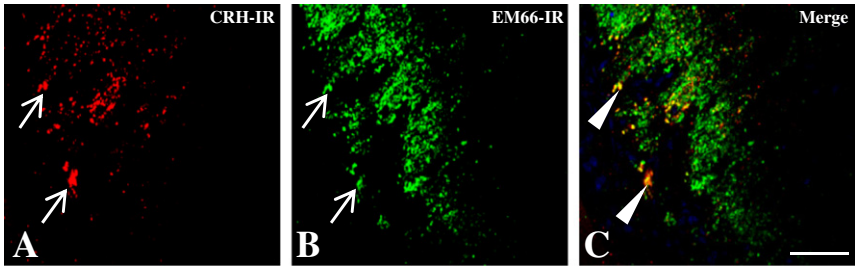


**Fig. 2.** Confocal microscopy of CRH- and EM66-IR axon terminals in the external layer of the median eminence. Colocalizations of CRH (A, thin arrows) and EM66 immunoreactivities (B, thin arrows) are shown in the merged photomicrograph (C, arrowheads). Scale bar 25 μm.

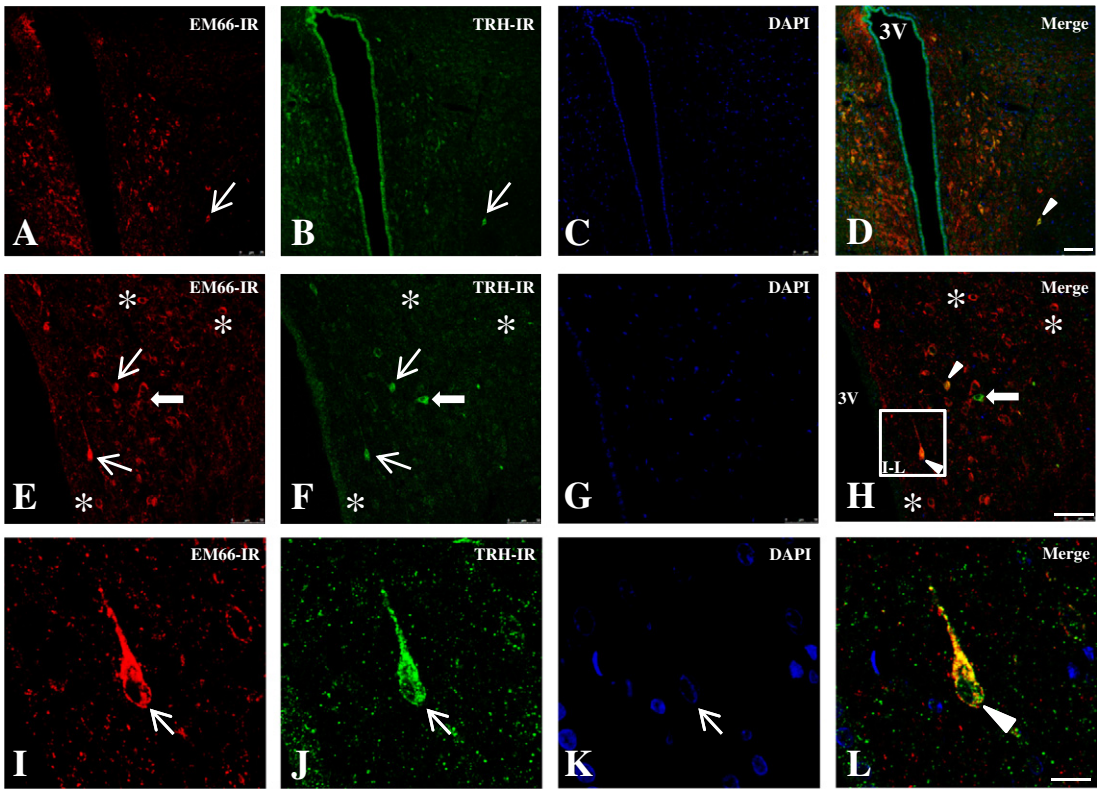


**Fig. 3.** Confocal microscopy of TRH- and EM66-IR neurons in pPVN of rat. EM66 immunolabeling with Alexa fluor 594 (A, E and I), TRH immunostaining with Alexa fluor 488 (B, F and J) and DAPI nuclei fluorescence (C, G and K) were visualized in the same sections. Colocalization of TRH and EM66 is shown in the merged photomicrograph (D, H and L, arrowheads). High-powered magnification clearly evidenced EM66 and TRH colocalization within pPVN neurons (inset H, L). Neurons containing either TRH only (E–H, thick arrow) or EM66 (E–H, asterisk), were also observed. Scale bars, (A–D) 75 μm, (E–H) 50 μm and (I–L) 10 μm.

coexpressed with CRH in some axon terminals, with a privileged distribution around capillaries (Fig. 2C, arrowhead). No immunoreaction was observed when primary antibodies were omitted, replaced by non-immune serum or pre-incubated with the EM66 peptide in the case of the EM66 antibody.

### *3.3. Colocalization of EM66 and TRH-IR neurons within the pPVN and the ME*

Co-incubation of hypothalamic sections with the anti-EM66 and anti-TRH antibodies revealed that numerous TRH-IR neurons within the pPVN were also immunolabeled for EM66 (Fig. 3D, H and L, arrowhead), indicating that the EM66 peptide is expressed in thyrotrope neuroendocrine cells. However, some TRH immunopositive cells were devoid of EM66-IR (Fig. 3E–H, thick arrow). Conversely, some EM66-IR neurons did not display any TRH immunoreactivity (Fig. 3E–H, asterisk). Furthermore, the external zone of the ME displayed intense colabelings of EM66 and TRH in fiber terminals, with a privileged distribution around capillaries (Fig. 4A–D). No immunoreactivity was observed when the EM66 antibody was omitted, replaced by non-immune serum or pre-incubated with the EM66 peptide. In contrast, non-specific labeling of cells bordering the third ventricle was observed in the presence and absence of the TRH antibody (Fig. 3B and D, and data not shown).

### *3.4. Effect of acute immobilization or cold stress on EM66 neuronal population in the rat pPVN*

The distribution pattern of EM66-IR neurons within the pPVN remained unchanged in the various experimental conditions (control vs stressed groups). Indeed, acute immobilization (2 h) as well as acute cold stress (4 h) had no apparent effect on the EM66

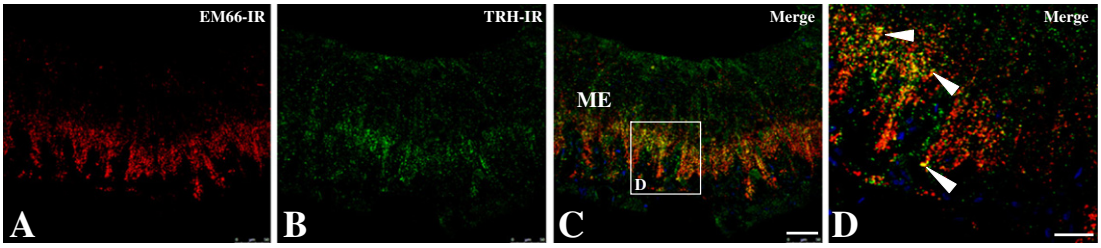


**Fig. 4.** Confocal microscopy of axon terminals immunoreactive to TRH and EM66 in the external layer of the median eminence (ME). Low-powered magnification at the level of the ME showing immunoreactivity for EM66 (A), TRH (B) and DAPI nuclei staining. A dense plexus of TRH- and EM66-IR nerve fibers is shown in the merged photomicrograph (merge, C). High magnification indicates colocalization of EM66 and TRH within axon terminals of the external layer of the ME with a privileged distribution around capillaries (inset, D, arrowheads). Scale bars (A–C) 50 μm and (D) 10 μm.

immunoreactivity within the pPVN compared to control rats (data not shown). A quantitative analysis confirmed these observations showing no significant change in the number of EM66-IR cell bodies within the pPVN of immobilization stressed compared to control animals (Fig. 5A). Similarly, rats exposed to acute cold stress showed no significant variation in the number of EM66-IR cell bodies compared to control animals (Fig. 5B).

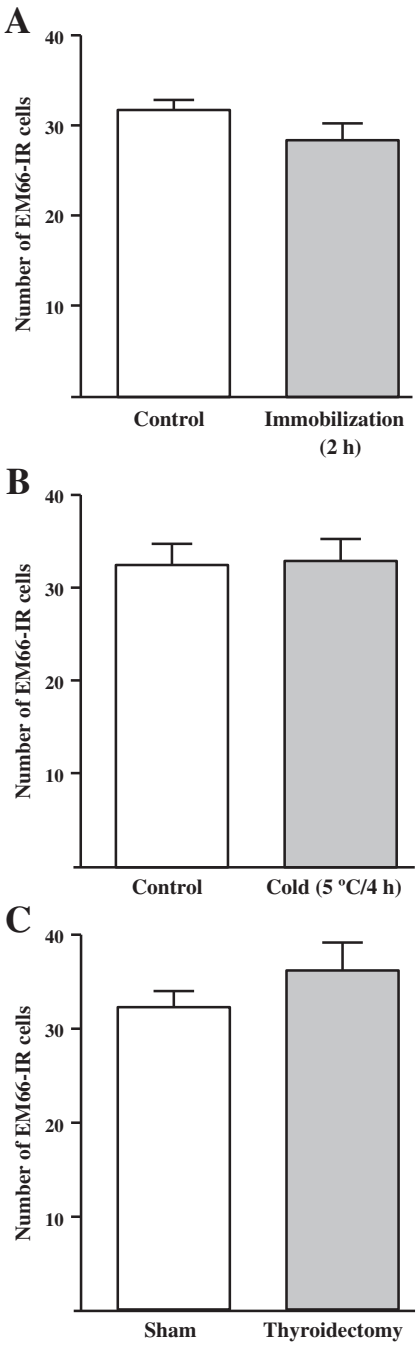


**Fig. 5.** Effects of acute stresses or thyroidectomy on EM66 pPVN neuronal populations. (A) Comparison of the number of EM66-IR neurons within the pPVN of control vs experimental animals (rats immobilized for 2 h; n = 6 in both groups). Student *t*-test revealed no significant change in the average number of EM66-IR cells between the two groups. (B) Comparison of the number of EM66-IR neurons within the pPVN of control and cold stressed rats (5 °C for 4 h). The histograms showed no significant difference in the number of EM66-IR neurons between control and stressed groups (n = 5 in both groups). (C) Comparison of the number of EM66-IR neurons within the pPVN of sham-operated vs thyroidectomized rats (n = 6 in both groups). No significant change in the average number of EM66-IR cells was noted. Values are shown as mean ± SEM.

### *3.5. Effect of thyroidectomy on EM66-IR neurons in the rat pPVN*

Six days after thyroidectomy, no apparent change in the density of EM66-immunopositive cell bodies was observed between sham and thyroidectomized rats (data not shown). A statistical analysis confirmed the lack of difference in the number of EM66-IR cell bodies within the pPVN of sham versus operated animals (Fig. 5C).

## 4. Discussion

In line with previously published data [20,21], the present results show that in colchicine-treated rats EM66 immunoreactivity is concentrated within pPVN cell bodies as well as in terminals of the external layer of the median eminence, suggesting a hypophysiotropic role for this peptide. Indeed, these hypothalamic structures are well known to be involved in multiple neuroendocrine regulations, in particular the control of stress and the thyroid axis [31,32]. One argument in favor of such roles for EM66 is that EM66-IR is colocalized with CRH and TRH within pPVN hypophysiotropic neurons. CRH- and TRH-containing axon terminals located around capillary vessels of the ME also contained EM66-IR. These data suggest that EM66 may be co-released with CRH and TRH into the portal circulation and therefore, that EM66 could be an accessory peptide which modulates the effects of CRH and TRH on anterior pituitary cells, and thus participates to the control of corticotrope and thyrotrope axis activity in rat. Taking into account the fact that the TRH neuronal population of the pPVN is completely distinct from the CRH neurosecretory system [18], colocalization of EM66 with CRH and TRH within parvocellular neurons argues for the occurrence of at least two distinct EM66 neuronal populations in the rat pPVN.

To evaluate a possible hypophysiotropic role for the EM66 neuropeptide in relation with the corticotrope and/or thyrotrope axis in rat, we studied the effect of acute stressors and thyroidectomy on the distribution of EM66 immunoreactivity in the pPVN of the rat hypothalamus. There are indications that various experimental stress paradigms may evoke different neural and neuroendocrine responses [33]. We analyzed the effects of some of these paradigms such as acute immobilization stress and cold exposure which represent strong neurogenic stressors, and thyroidectomy which has extensively been studied in rat [24,34–36]. Immobilization and cold exposure represent interesting stressful stimuli inducing an array of peripheral and central responses associated with increased activity of the hypothalamic–pituitary–adrenal (HPA) axis [23,36] which is evidenced by an increase in parvocellular CRH mRNA and ACTH hypersecretion followed by hypercorticosteronemia [37–39]. The same acute stressors are also known to up-regulate the expression of other pPVN neuropeptides such as vasopressin which may play a role in the regulation of pituitary–adrenal responses to immobilization and cold stress [37,39].

Using protocols thoroughly validated previously [21,40–43], the present work showed that acute immobilization (2 h) or cold exposure (5 °C, 4 h) did not affect the EM66 immunoreactivity evaluated by the number of EM66-IR neurons within the pPVN. Consistent with these results, other paradigms such as acute immune stress induced by an ip injection of LPS, known to increase cytokines in brain and plasma [44,45], or central injection of cytokines as IL-1β known to be associated with HPA axis activation, did not affect the number of EM66-IR neurons of the pPVN [21]. Similarly, vasopressin mRNA expression remained unchanged in CRH neurons of pPVN after acute immune stress [21,46]. Our data suggest that EM66, like vasopressin, does not participate to the phenotypic plasticity of CRH parvocellular neurons in response to acute stress; thus it may not be essential in this short-term adaptation of PVN neurons. Since chronic stress as well as adrenalectomy were reported to induce a sharp increase in parvocellular vasopressin mRNA [47–49], we cannot exclude that the stressor stimulus duration plays a critical role in the control of the phenotypical plasticity of PVN neurons and neuropeptide expression. It will be useful to study an eventual impact of

chronic stressors on EM66 expression within the pPVN. These data provide an additional evidence for the complexity of the phenotypical and/or secretory plasticity of neurons within the pPVN [50] and thus of the regulatory mechanisms of the stress response in rat.

In fact, multiple regulations take place in the paraventricular nucleus of the hypothalamus, which is known to be the feedback site for several peripheral hormones, including thyroid hormones [15]. As soon as hypothyroidism develops, a dramatic increase in TRH mRNA expression is observed, which is reversed by T4 administration [34,51]. The plasma TSH concentration following thyroidectomy is also increased, suggesting that plasma levels of TSH correlate directly with activation of TRH neurons located in the pPVN [24]. Since our results showed an important EM66 expression within TRH-containing parvocellular cells and ME terminals, EM66 may represent an accessory peptide which could regulate the thyrotrope activity and which could be sensitive to peripheral hormones of this axis. The present study showed that thyroidectomy (1 week) did not affect the number of EM66-IR neurons in the pPVN, as it is also the case for CRH and Enk [24]. However, it is known that 4 to 7 weeks of hypothyroidism provokes a decrease in CRH mRNA expression within pPVN and that T4 restoration reverses this effect, suggesting that the thyrotrope axis regulates the corticotrope function after prolonged hypothyroidism [34,52]. A reciprocal action of the corticotrope axis on the thyroid activity is also possible since cold stress is known to induce an increase in TSH release in rat [53]. In addition to TRH and CRH, other hypothalamic peptides are affected by thyroid hormone levels, and their expression depends on short- or long-term hypothyroidism. For instance, 4 and 7 weeks of hypothyroidism elicits a dramatic decrease in galanin mRNA in the pPVN. Likewise, VIP mRNA which is undetectable in the PVN of normal animals, is upregulated after 4 and 7 weeks of thyroidectomy [34]. However, other pPVN peptides such as neurotensin, enkephalin and vasopressin are not altered by 4 and 7 weeks of hypothyroidism [34]. It remains to be determined whether EM66 neuropeptide expression could be altered following a long-term duration of hypothyroidism, in order to unravel a potential role of EM66 in the control of the thyrotrope axis and/or in a thyrotrope–corticotrope interaction.

## 5. Conclusion

The present study revealed for the first time the occurrence of EM66 in the CRH- and TRH-containing neurons of the rat pPVN as well as in their terminals at the external layer of the ME. Since the TRH neuronal population of the pPVN is known to be completely distinct from the CRH neurosecretory system, our data strongly suggest the existence of at least two distinct EM66 neuronal populations in the rat pPVN. Functionally, EM66 levels do not depend on acute immobilization or cold exposure stresses, as we have previously shown also for an acute immune challenge. In addition, EM66 expression is insensitive to short term hypothyroidism induced by one-week thyroidectomy. These data indicate that EM66 does not participate to the phenotypic plasticity of hypothalamic parvocellular neurons in response to acute stress and to a short duration hypothyroidism. Additional investigations will be conducted to determine the putative regulation of EM66 expression in the pPVN in response to chronic stressors and to long-term hypothyroidism.

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