Cold Stress Induces Metabolic Activation of Thyrotrophin-Releasing Hormone-Synthesising Neurones in the Magnocellular Division of the Hypothalamic Paraventricular Nucleus and Concomitantly Changes Ovarian Sympathetic Activity Parameters

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Abstract

Recent studies suggest thyrotrophin-releasing hormone (TRH) serves as a neurotransmitter and thereby provides a functional vegetative connection between the brain and the ovary. In the present study, magnocellular neurones of the paraventricular nucleus (PVN) in animals subjected to cold exposure were studied to determine the hypothalamic origin of the TRH involved in this pathway. *In situ* hybridisation analysis of hypothalamic tissue showed that cold exposure causes a two-fold increase in the total number of neurones expressing TRH mRNA in the PVN. Immunohistochemical studies showed that TRH peptide is localised to the magnocellular PVN and that the number of TRH immunoreactive cells increases two-fold following 64 h of cold exposure. Double-immunostaining for MAP-2 and TRH revealed that TRH peptide is localised in the perikarya of the magnocellular neurones. TRH release was measured *in vivo* from the magnocellular portion of the PVN using push–pull perfusion. Although controls exhibited a very low level of TRH release, animals subjected to cold showed a pulsatile-like TRH release profile with two different patterns of release: (i) low basal level with small bursts of TRH release and (ii) a profile with an up to seven-fold increase in TRH release compared to controls. The colocalisation of TRH with the specific somato-dendritic marker MAP-2 in processes of the magnocellular neurones suggested a local release of TRH. Additional studies demonstrated a reduction in ovarian noradrenaline content after 48 h of cold exposure, a feature indicative of nerve activation at the terminal organ. After 64 h of cold exposure, the ovarian noradrenaline returned to control values but the noradrenaline content of the coeliac ganglia was increased, suggesting a compensatory effect originating in the cell bodies of the sympathetic neurones that innervate the ovary. The correlation between the local release of TRH from dendrites within the magnocellular PVN in conditions of cold and the activation of the sympathetic nerves supplying the ovary raises the possibility that TRH contributes to the processing regulating sympathetic outflow and may thereby impact on the functional activity of the ovary.

It is well known that hypothalamic thyrotrophin-releasing hormone (TRH) plays an important part in mediating the body's response to cold by serving both as a neurohormone (1–5) and a neurotransmitter substance. In accordance with these data, we recently reported that 64 h of cold exposure induces an increase in the activity of ovarian sympathetic nerves in the rat and postulated that thyrotrophin releasing hormone (TRH) is a neurotransmitter involved in this response (6). Whereas the origin of neurohormonal TRH has been localised to the medial parvocellular and periventricular subdivisions of the middle zone of the paraventricular nucleus (PVN) (7–8), the hypothalamic origin of TRH as a neurotransmitter in the vegetative system has not yet been well-defined. Cells linking the PVN to sympathetic structures have been considered classically to be located in the parvocellular region of the mid-PVN (9) where several peptidergic perikarya, including those expressing TRH, are present (10). However, there is additional evidence suggesting that the magnocellular region of the mid-PVN is also involved in sympathetic control (11–13).

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Under some experimental conditions, such as colchicine treatment (14), the arginine-vasopressin (AVP) cells of the magnocellular PVN region coexpress immunoreactive TRH. Because AVP cells exhibit anatomical sympathetic projections (12, 15), it is possible that at least part of the TRH produced in this region is sympathetic in nature and is released either alone or together with AVP. Because AVP release from the magnocellular region is sensitive to cold stress (16), cold exposure might be the common stimulus for both peptides. However, less attention has been paid to the role of parvicellular TRH neurones present in the magnocellular region of the PVN (17) because the TRH produced by these neurones is unlikely to fulfil a hypophysiotrophic role. We hypothesised that the TRH produced by AVP/TRH or TRH synthesising cells located in the magnocellular region of the PVN could fulfil a neurotransmitter function, possibly being released from secretory dendritic process, as demonstrated for oxytocin and vasopressin (AVP) peptides (18, 19).

In situ hybridisation (ISH) was used to examine the expression of TRH synthesising activity in the magnocellular region of PVN in rats subjected to a cold stimulus and corresponding controls. Parallel measures of TRH-peptide were made by immunocytochemistry. To determine if TRH exhibits a somato-dendritic localisation, double-staining immunocytochemistry was performed using an antibody against the specific somato-dendrite marker, MAP-2. Finally, to correlate localisation with function, we carried out push–pull perfusion experiments to monitor TRH release *in vivo* from the PVN magnocellular region under basal and cold exposure conditions. Peripheral sympathetic activity in the same animals was assessed by measure of noradrenaline content in the coeliac ganglia and in the ovary (20).

Materials and methods

Animals

Adult female Sprague-Dawley rats weighing approximately 200 g with regular 4-day oestrous cycles were obtained from a stock at the University of Chile. They were maintained in individual cages at 23 °C under 12 : 12 h light/dark cycles and food and water were available *ad lib*. These rats were separated into three experimental groups, each comprising four to eight animals. Untreated groups served as controls and the second and third groups were cold-stressed for 48 h or 64 h, respectively. There were no differences in oestrous cyclicity between the experimental groups. All animal procedures followed protocols approved by the Institutional Ethic Committee of the Faculty of Chemistry and Pharmaceutical Sciences. The experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction.

Rats undergoing cold stress were placed in individual cages in a room maintained at 4 °C for 48 h or 64 h and maintained with regular 12 : 12 h light/dark cycles. Control and experimental animals were killed by decapitation 1 h after the stress session terminated; the brains were frozen immediately in liquid nitrogen and kept at −80 °C until used for TRH *in situ* hybridisation or immunohistochemistry. To correlate the changes observed in the PVN with other parameters of sympathetic ovarian activation, ovary and coeliac ganglia were also collected, and frozen at −80 °C for catecholamine determination.

Determination of noradrenaline by high-performance liquid chromatography

Frozen ovaries or ganglia were homogenised in 300 μl (ovary) or 1000 μl (ganglia) 0.2 N perchloric acid using a glass-glass homogeniser and centrifuged at 15 000 ***g*** for 10 min. Aliquots (50 μl) of the supernatant were purified by adsorption in alumina. The catecholamines were then separated by high-performance liquid chromatography and quantified with an electrochemical detector adjusted to an oxidation potential of + 0.7 V, as previously described (21). Dihydroxibenzil amine (200 pmol) was added as an internal standard to correct recovery during alumina adsorption.

In situ *hybridisation*

Oligonucleotide probes labelled with digoxigenin were used (22, 23). A 48-mer oligonucleotide sequence complementary to bases 319–366 of TRH mRNA (24) (access number NM_013046) and obtained from SIGMA-Genosys Ltd. (London, UK) was used a probe for TRH mRNA. It was labelled with the Dig-dUTP tailing kit (Roche Diagnostics Corporation, Indianapolis, IN, USA) precipitated in ethanol and re-suspended in DEPC-treated water. Coronal cryostat sections (12 μm) of the brains were collected on adherent slides and fixed in 4% p-formaldehyde in phosphate-saline buffer (PBS) for 30 min at 4 °C. The hybridisation was conducted as described by Koller *et al.* (25) with minor modifications. In brief, after washing in PBS, the sections were permeabilised with 0.001% proteinase K (Molecular Biology grade, Merck, Darmstad, Germany) and treated with 0.25% acetic anhydride in 0.1 M triethanolamine. After dehydration in increasing concentrations of ethanol, the sections were delipidated in chloroform for 10 min, rinsed in ethanol, and air-dried. Hybridisation was carried out (37 °C for 16 h in a humidity chamber) with 40 pmol/ml of TRH digoxigenin-oligonucleotide in the presence of 4 × SSC, 50% formamide, 1% Denhart's solution, 50 μg/ml sheared salmon sperm DNA, 10% dextran sulphate and 125 μg/ml tRNA. The hybridisation was stopped in 2 × SSC and the slides were washed in 4 × SSC for 10 min, 2 × SSC (three times, 15 min each) and finally in 2 × SSC at 50 °C for 15 min. To visualise the digoxigenin hybridised probe, the slides were incubated for 2 h in a solution of 0.1 M maleic acid, 150 mM NaCl, pH 7.4 (buffer 1) in the presence of 0.03% Triton-X100, 3% foetal calf serum, antidigoxigenin Fab fragments conjugated with alkaline phosphatase (Roche Diagnostics Corporation, diluted 1 : 500) and 1% blocking reagent for nucleic acid hybridisation (Roche Diagnostics Corporation). After a quick rinse in buffer 1, sections were incubated in 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris (pH 9.5) in the presence of the alkaline phosphatase substrate NBT-BCIP and 0.024% levamizole for 16 h at room temperature. The reaction was stopped by a 30-min wash in 10 mM Tris, 1 mM EDTA, pH 8.0. Controls for specific hybridisation were made with the antisense digoxigenin-oligonucleotide in the presence of 4 nmol/ml of unlabelled probe.

For cellular counting and digital image analysis of the sections, final photomicrographs of the PVN (− 1.8 mm from Bregma, from Paxinos Atlas) (26) were acquired using a computer connected to a microscope and digital camera (Coolpix 995, Nikon, Tokyo, Japan).

To minimise potential overlap between the cells of the parvocellular and magnocellular division of PVN, we took advantage of the clear differences in cell size in the cross-sectional areas (27). Thus, all stained cells in the magnocellular area were scored. However, this procedure allowed inclusion of the parvocellular neurones for TRH, which are found in the magnocellular division of the PVN (17). At least three sections per animal were scored bilaterally by two independent investigators using Scion Image for Windows (Scion Corporation, Frederick, MD, USA).

Immunohistochemistry for TRH

Adjacent coronal sections from the same animal were taken to compare the localisation of TRH mRNA with TRH protein. Sections (12 μm) were fixed in 4% p-formaldehyde in PBS for 30 min at 4 °C. After two washes in PBS, endogenous peroxidase activity was blocked by incubation in 0.1% H_2O_2 in PBS for 15 min. The sections were then permeabilised with 0.3% Triton-X-100-PBS and nonspecific binding blocked by subsequent incubation in 3% normal goat serum (NGS), for 30 min. Then sections were incubated for 24 h at 4 °C in a humidified chamber with the primary rabbit anti-TRH (CRR4B72, kindly provided by Dr V. D. Ramirez from University of Illinois) (28) diluted 1 : 500 in 3% NGS-PBS. The specificity of the antibody was tested for 15 related peptides and six neurotransmitters, all of which showed less than 0.0005% of cross reactivity (28). After three washes in 0.1% Triton-X 100-PBS, the tissue was incubated in biotinylated affinity purified anti-rabbit IgG (diluted 1 : 200, Vector Laboratories, Burlingame, CA, USA) in the presence of 3% NGS, 0.1% Triton-X100 in PBS for 4 h. After several washes in PBS, tissues were incubated in the avidine-peroxidase complex (diluted 1 : 100) (ABC, Vector kit, Vector Laboratories) for 1 h. The colour

development was conducted with a peroxidase substrate kit. A negative control was obtained in the absence of primary antibody. A similar result was obtained with TRH antiserum kindly provided by Dr D.Grouselle (Centre Paul Broca, Inserm, Paris) (29) and used at a dilution of 1 : 1000. Cell counting was conducted as described for ISH experiments.

Double immunohistochemistry for MAP-2 and TRH

After 50 min fixation in 4%-p-formaldehyde and washing in PBS, the sections were incubated for 30 min in 100% methanol at 4 °C, rinsed in PBS for 10 min and blocked for 1 h in 5% NGS in 0.3% Triton-X100-PBS. They were then incubated in primary rabbit anti-TRH (CRR4B72) diluted 1 : 500 in 3% NGS in 0.3% Triton-X100-PBS for 24 h at 4 °C in a humidified chamber. After rinsing in PBS, the sections were incubated in antirabbit Alexa fluor (Molecular Probes, Eugene, OR, USA) diluted 1 : 100 in 3% normal goat serum-PBS) for 2 h, washed twice with PBS and postfixed in 4% p-formaldehyde (15 min at 4 °C). Following several washes in PBS, the brain sections were incubated in mouse anti-MAP-2 (M4403, Sigma Chemicals, St Louis, MO, USA) diluted 1 : 100 in 3% normal goat serum-PBS (24 h at 4 °C), washed three times in PBS and incubated for 2 h with Texas Red (Molecular Probes) diluted 1 : 100 in 3% normal goat serum-PBS. Finally, the sections were washed in PBS and mounted with DAKO antifade. Fluorescence was observed with a confocal microscope (LSM-410 Axiovert 488, Zeiss, Jena, Germany). Positive double-staining for TRH and MAP-2 cells was defined as fluorescence overlapped at least at nine levels through section in the Z-axis (1 μm). Controls for immunostaining specificity consisted of: (i) omission of the primary antibody and application of the secondary antibody alone and (ii) the use of inappropriate excitation wavelengths.

Push–pull perfusion

After a 7-day acclimation period in the laboratory 14 rats were anaesthetised with sodium pentobarbitone (40 mg/kg). A stainless steel cannula (0.5 mm outer diameter) fitted with a stylette was implanted stereotaxically into the magnocellular portion of the hypothalamic PVN according to Paxinos and Watson coordinates (AP, 1.8 mm; L, 0.7 mm; DV, 8.0 mm) (26). After surgery, the animals were caged separately and handled daily for 1 week by which time they had recovered their preoperative body weight. They were then separated into two groups. One group was exposed to cold-stress for 64 h and the other was kept at room temperature. The stylet was removed from the cannula and replaced by a thin push cannula (outer diameter 0.23 mm) to minimise tissue damage in the perfused bed. Briefly, push–pull perfusion was performed on unanaesthetised animals according to published experimental procedures described elsewhere (1). Artificial cerebrospinal fluid was infused at a flow rate of 16 μl/min, a flow rate equivalent to that of capillary vessels in the region (30). Samples of 240 μl were collected every 15 min. Perfusates were acidified (hydrochloric acid, 0.1 M final concentration), centrifuged and evaporated in a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and stored immediately at −20 °C for TRH radioimmunoassay (RIA). In the control ($n = 7$) and the experimental groups ($n = 7$), samples were collected over a period of at least 90 min. Control and experimental animals were perfused at the same time of the day. At the end of the experiments, all animals were killed and their brains removed and fixed for histological examination. Rats with unsuitable cannula location (i.e. outside of the magnocellular portion of PVN) were excluded from the study (two controls and one stressed rat).

TRH assay

TRH was quantified by a specific RIA (31) using a TRH antiserum provided by Dr D. Grouselle (Centre Paul Broca, Inserm, Paris) (29). Samples and standards (2–1000 pg) were dried and evaporated as detailed above, and dissolved in 100 μl assay buffer (saline-phosphate buffer, 0.05% BSA, pH 7.0) and mixed with 100 μl of a 1 : 45 000 dilution of TRH antiserum. Approximately 10 000 c.p.m. of [^{125}I]-TRH (Perkin-Elmer Life Sciences, Inc., Boston, MA, USA) in 100 μl assay buffer were added to each sample and the final volume was adjusted to 300 μl with assay buffer. After incubation for 24 h at 4 °C, the bound hormone was separated by addition of 500 μl 40% polyethyleneglycol and 100 μl 0.5% bovine gamma globulin fraction II (ICN ImmunoBiologicals, Lyon, France) in PBS. The tubes were centrifuged and the precipitates counted in a Packard scintillation spectrometer. The results are given as pg/tube of TRH immunoreactivity and calculations considered the flow rate for each fraction sample to express the results as pg/15 min.

Statistical analysis

Control and cold-stressed groups were compared through ANOVA (Statview 4.5; SAS Institute, Cary, NC, USA) followed by Fisher's PLSD test ($P < 0.05$ was considered statistically significant). To evaluate the effect of cold stress on TRH release at PVN level, the mean values obtained during the cold-stress perfusion period were compared with the mean basal values obtained from the control group using ANOVA (Statview 4.5), followed by paired Student's *t*-test ($P < 0.05$ was considered statistically significant).

Results

Effect of cold exposure on the number of cells expressing TRH mRNA in the PVN

An adapted *in situ* hybridisation technique was used to visualise TRH mRNA changes using digoxigenin-labelled TRH oligonucleotide probe. This technique combines good probe penetration (small size of the oligonucleotide) (32) with excellent spatial cell resolution (22).

Figure 1(A) shows the positive cells for TRH mRNA in PVN of control rats. Specificity was demonstrated by the addition of excess unlabelled probe (Fig. 1B). In control rats, the number of cells expressing TRH mRNA in the PVN was 186.6 ± 20.2 cells (Fig. 1A) ($n = 6$), in agreement with the literature (33). Cold exposure induced a significant increase in the number of TRH mRNA positive cells compared to controls (Fig. 1B). Thus, the number of PVN cells expressing TRH mRNA increased by 70% (330.4 ± 31.6 cells) versus the control group after 48 h of cold exposure ($n = 4$) (Fig. 1C) and by 100% after 64 h of cold exposure (397.5 ± 17.9 cells, $n = 7$). However, the apparent increase in cell number observed at 48 h did not reach significance. We also examined the topographic distribution of labelling within the magnocellular region of the mid-PVN: approximately 50 cells expressing TRH mRNA were detected in the magnocellular region under control conditions. After 48 h or 64 h of cold exposure (Fig. 1D), a 50% increase in the number of positive cells was observed in this area (at 48 h: 70.2 ± 8.7; at 64 h: 70.5 ± 3.3 cells).

Effect of cold exposure on immunoreactive TRH levels in the PVN

Immunohistochemical studies were undertaken to correlate the changes in TRH mRNA with those of TRH itself. Figure 2(A) shows TRH immunoreactive cells were readily detected in the PVN of control rats. The number of positive cells was increased by 64 h of cold exposure (Fig. 2B) giving a total of 779 ± 154 ($n = 3$) compared to 384 ± 144 ($n = 3$) in controls. This change occurred in the magnocellular region (220 ± 56 cells versus 100 ± 32 cells in control rats) (Fig. 2D). Similar results were obtained irrespective to the TRH antiserum used (28, 29). The immunolabelling specificity was confirmed using goat normal serum in the absence of primary antibody.

Indirect immunofluorescence for TRH peptide also demonstrated an increase in the PVN TRH immunoreactive cells

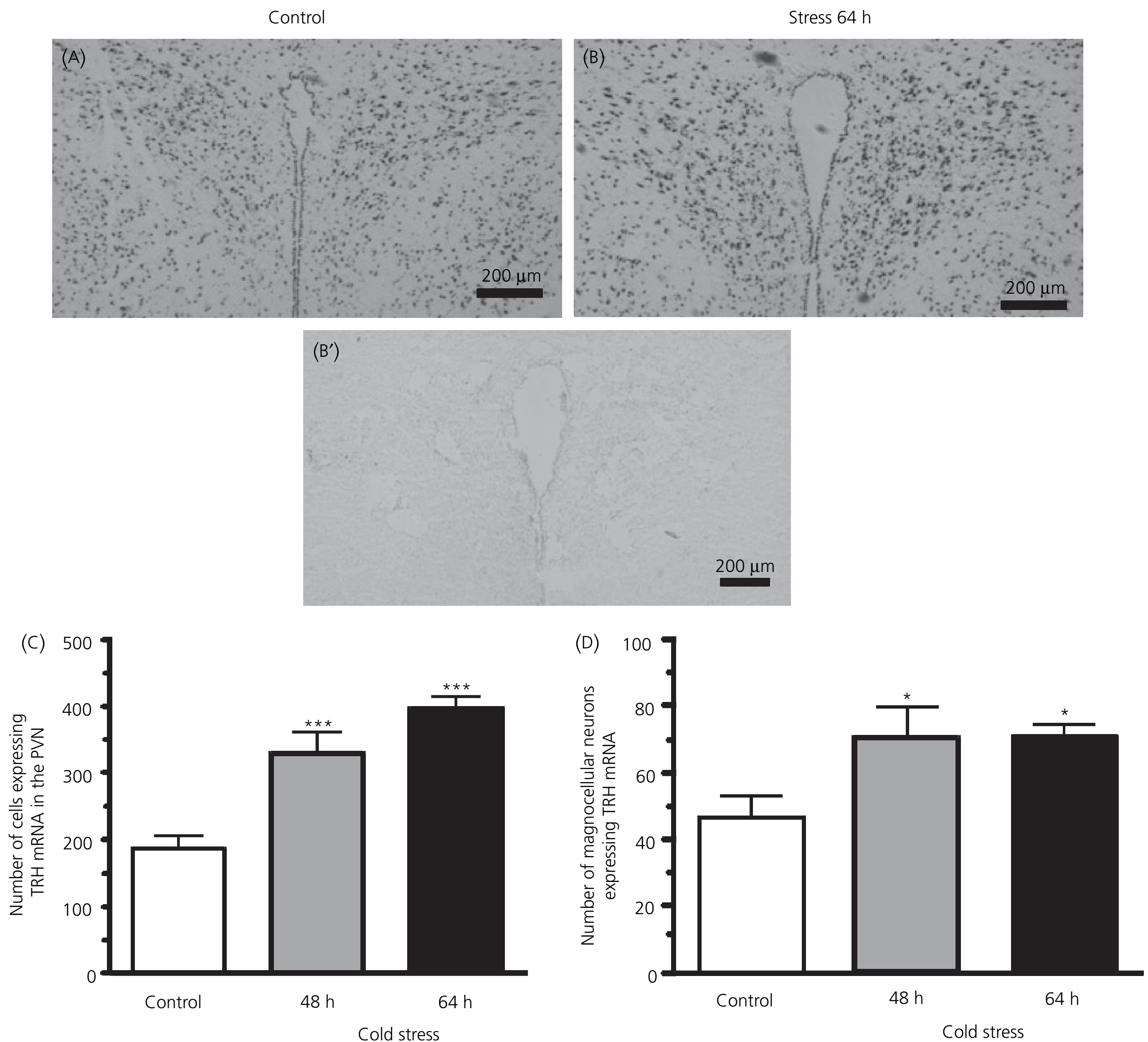

Fig. 1. Cold exposure increases thyrotrophin-releasing hormone (TRH) mRNA levels in the paraventricular nucleus (PVN). Control rats were kept at 22 °C and cold stress rats were maintained at 4 °C for 48 h or 64 h. Frozen coronal sections (−1.8 Bregma) of 12 μm were fixed in p-formaldehyde and hybridised with a 48-mer TRH probe oligonucleotide complementary to bases 319–366 of TRH mRNA [24] labelled with digoxigenin. The hybridisation for TRH in control rats is displayed in (A) and 64 h of cold exposure rats in (B). The negative control of TRH hybridisation was performed in the excess of unlabelled digoxigenin probe (B′). (C) Shows the total number of neurones expressing TRH mRNA in the PVN and (D) shows the number of cells located in the magnocellular region of the PVN. The quantification of the number of positive cells was performwd using NIH Scion Image for Windows. Scale bar = 200 μm. Data shown are the mean ± SEM (control, n = 6; 48 h of cold exposure, n = 4; and 64 h of cold exposure, n = 7). *P < 0.05 versus control ***P < 0.001 versus control.

in rats subjected to 64 h of cold stress compared to control rats (Fig. 3A). Double immunostaining with MAP-2 somatodendritic marker (32) showed that TRH is localised to the perikarya and dendritic compartment of PVN neurones in cold-exposed rats (Fig. 3B). Higher magnification indicated that, unlike MAP-2, TRH is not localised to the cytoplasm, but is contained within an intracellular organelle. The nature of this organelle was not defined, but it could represent a vesicular compartment (Fig. 3C).

Effect of 64 h of cold stress on TRH release from the PVN

Basal TRH release was detected in control animals perfused for 2 h at room temperature (Fig. 4A). Cold-stress produced a marked increase in TRH release (Fig. 4A). The pattern of release appeared to be pulsatile with small and larger (sevenfold the level of spontaneous release) peaks of TRH release (Fig. 4B). The statistical analysis including all animals used is shown in Fig. 4(C). The cannula location was always checked

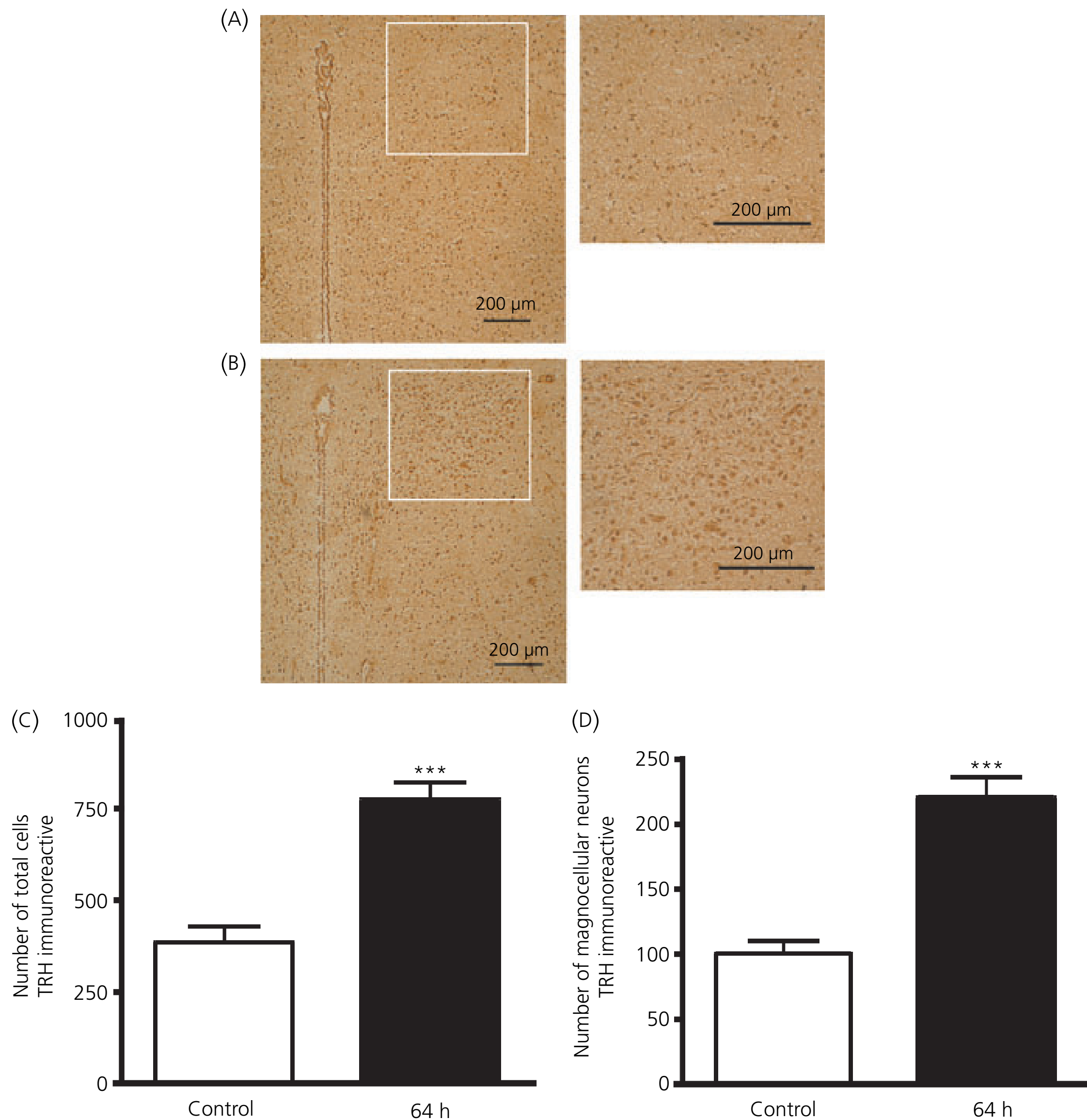

Fig. 2. Cold exposure increases thyrotrophin-releasing hormone (TRH) immunoreactivity in the paraventricular nucleus (PVN). Control rats were kept at 22 °C and cold stress rats were maintained at 64 h. Frozen coronal sections (−1.8 Bregma) of 12 μm were fixed in p-formaldehyde and incubated with (CRR 4B72) TRH antibody. The immunoreactivity for TRH in control rats is displayed in (A) and 64 h of cold exposure rats in (B). (C) Shows the total number of positive neurones for TRH antibody in PVN and (D) shows the number of positive neurones located in the magnocellular region of PVN. The quantification of the number of positive cells was performed using NIH Scion Image for Windows. Scale bar = 200 μm. Data shown represent the mean ± SEM (control, n = 3 and 64 h of cold exposure, n = 3). ***$P < 0.001$ versus control.

to ensure that it was placed in the magnocellular region of the PVN (Fig. 4D).

Sympathetic activity following cold exposure

We previously reported that 64 h of cold exposure induces changes in ovarian sympathetic activity (6). Cold exposure (48 h) did not change the noradrenaline content of the coeliac ganglia, a region where the cell-bodies of ovarian sympathetic nerves are located (control = 67.2 ± 5.6 ng noradrenaline/ganglia versus 73.8 ± 5.6 ng noradrenaline/ganglia at 48 h) (Fig. 5A). However, a 50% increase in noradrenaline content of the ganglia was found after 64 h of cold exposure (101.9 ± 11.8 ng noradrenaline/ganglia) (Fig. 5A). Analyses of ovarian noradrenaline content (Fig. 5B) showed an initial decrease in noradrenaline after 48 h of cold exposure (48 h = 5.6 ± 0.8 ng noradrenaline/ovary versus control = 8.4 ± 0.6 ng noradrenaline/ovary), followed by a recovery to control values after 64 h of cold (8.9 ± 1.3 ng noradrenaline/ovary), and these data paralleled the increase of noradrenaline content at the coeliac ganglion.

Discussion

The major finding of the present study is the expression of TRH mRNA and protein in cells of the magnocellular region

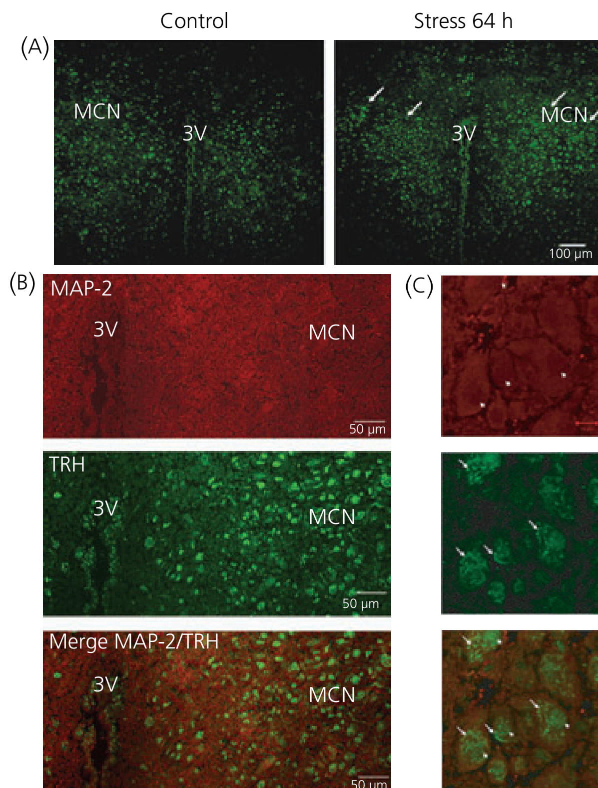

Fig. 3. Colocalisation of thyrotrophin-releasing hormone (TRH) and MAP-2 immunoreactivity in the paraventricular nucleus (PVN) and changes after 64 h of cold stress. (A) Confocal microscopic image showing the increase in TRH immunoreactive cells after 64 h of cold stress. (B) Confocal microscopic image showing the colocalisation of MAP-2 and TRH immunoreactivities in cells of the magnocellular region of the PVN. (C) Higher magnification of the magnocellular region of the PVN to show that MAP-2 is mainly located in cytoplasm of cells but TRH is located in intracellular constituents.

of the hypothalamic PVN, a region thus far considered classical for magnocellular vasopressin and oxytocin-body neurone location. We also studied the TRH mRNA response to cold stimulus and thus extended the study of Zoeller *et al.* (33), which analysed the effects of cold stress on the TRH mRNA expression in the thyrotrophic area of PVN and that of Sanchez *et al.* (34), which examined pro-TRH mRNA expression in the rostrocaudal zones of the PVN. Although other studies have reported a small number of TRH neurones in the magnocellular subdivision of the PVN (33), we believe that our success in localising TRH mRNA to this region was due to the use of the digoxigenin-labelled oligonucleotide probe, which shows good probe penetration and excellent spatial resolution (32) and is as sensitive as the radioactive method (22, 23).

The fact that the changes in TRH mRNA expression occurred concomitantly with biochemical correlates of sympathetic activity in the ovary (20) raises the possibility that activation of TRH pathways arising from PVN provides a functional vegetative link between the brain and the ovary (36). Indeed, several reports have indicated that some TRH activity is neurovegetative in nature (i.e. non-neurohormonal) and that it could be involved in the control of various physiological functions such as thermoregulation (3), gastric acid secretion (37) or ovarian activity (6). In accordance with this view, local administration of a viral tracer into the ovary produces intense cell-body labelling in the hypothalamic PVN (36, 38). In addition, TRH is present in sympathetic structures and pathways linking the autonomic nervous system to the brain (39, 40). Sympathetic hypothalamic function has been classically attributed to the parvocellular neurones located in the mid-PVN (9). However, sympathetic functions have been also attributed to AVP derived from the magnocellular portion of the middle PVN (12) but the localisation of TRH cells in this region has only been incidentally observed (14, 17). Because magnocellular AVP neurones are sensitive to cold (16), the increase in the number of cells expressing TRH mRNA in the magnocellular PVN following 48 h and 64 h of cold exposure might be in keeping with earlier findings of AVP and TRH cellular colocalisation (14). Indeed, the cold-induced increase TRH mRNA appears to be specific as other brain regions, such as the hippocampus, did not show a marked increase (data not shown).

The increase in TRH mRNA observed in the thyrotrophic area of PVN after 48 h of cold exposure had already been reported using a radioactive method (33). Similarly, Sanchez *et al.* (34) found increased pro-TRH mRNA levels in neurones residing in the rostral, medial and caudal zones of the PVN in rats exposed to 1 h of cold stress. We chose 64 h of cold exposure because, within this time period, there is a specific increase in tyrosine hydroxylase in sympathetic targets, such as the adrenal glands and in the superior cervical ganglia (41). Under these experimental conditions, we observed both alterations in sympathetic markers in both the ovary and the coeliac ganglia (i.e. the origin of sympathetic nerves that innervate the ovary) (33). It should be noted that, at the same time, neuroendocrine TRH production is also activated, as judged by raised plasma TSH levels (42), which suggests a good temporal correlation between neuroendocrine and vegetative events using this particular regulatory molecule (43). Furthermore, our data show that the increase in TRH mRNA in the magnocellular region of the PVN is accompanied by an increase in TRH protein. These data strongly suggest that both markers are located in the same cells in the magnocellular region of the PVN, not in axonal projections coming from the parvocellular region as previously suggested (44).

Attempts to demonstrate peptide localisation in the magnocellular portion of the PVN by immunological methods have been reported (14, 17, 35) using colchicine (14), a drug that disrupts microtubules, leading to accumulation of intracellular material, including TRH-storing vesicles. Our observations clearly show that immunohistochemical staining for TRH is more intense in cold-exposed animals than in controls. We found similar results using two different but previously characterised TRH antibodies (28, 29). Similarly, Anraku *et al.* (45) also visualised perikarya exhibiting TRH immunoreactivity in the absence of colchicine treatment and PVN TRH-containing neurones have been also described in the magnocellular subdivision of the human PVN (35). Although the subcellular localisation of TRH was not determined, it is possible the TRH mRNA induced by cold-

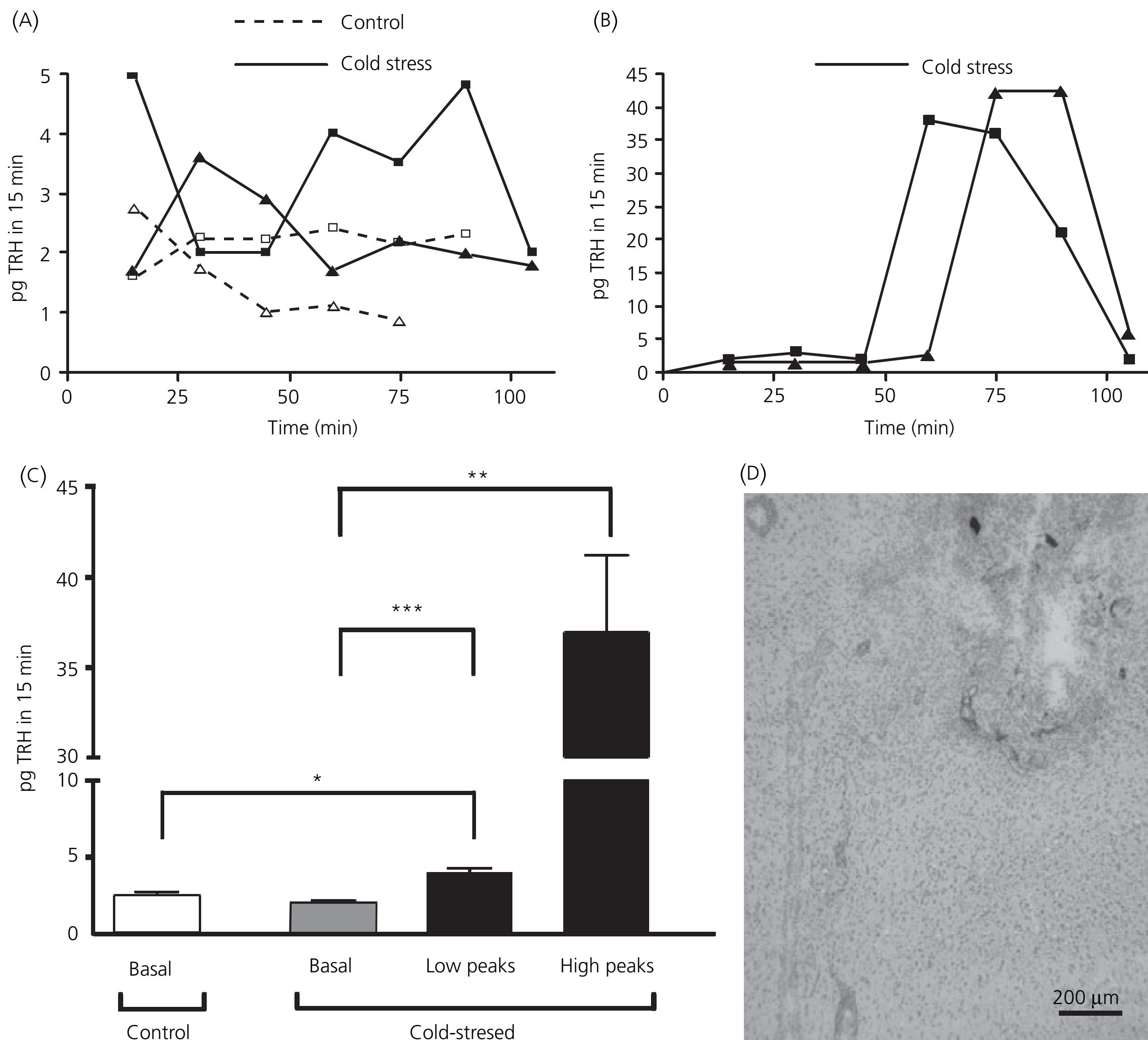

Fig. 4. Cold stress for 64 h increases thyrotrophin-releasing hormone (TRH) release from the magnocellular portion of hypothalamic paraventricular nucleus (PVN) analysed by *in vivo* push–pull perfusion in unanaesthetised animals. (A) TRH release by push–pull perfusion in two illustrative experiments from control (dashed lines) and in two experiments of animals in response to 64 h of cold exposure (continuous lines). Note that, although of low magnitude, TRH secretion from control and cold-stressed rats exhibit basal release but, in addition, cold-stressed rats present a peak-like secretion. (B) TRH release of high magnitude frequently seen after cold stress. (C) Represents the statistical analysis of TRH release obtained from all rats studied in control (n = 5) and stressed conditions (n = 6). Data for control rats exhibit the mean release of TRH calculated from the samples obtained by push–pull perfusion at room temperature (control spontaneous release). For cold-stressed rats, we calculated three means values of TRH release: one represents the cold-stressed basal release; the second represents the mean values for the low magnitude peak-like secretion and the third corresponds to the high magnitude peak-like secretion. The data are expressed as the mean ± SEM (control, n = 5 and 64 h of cold stress, n = 6). Microphotograph insert (D) shows the histological control of the push–pull cannula implanted in the magnocellular subdivision of the PVN. *$P < 0.05$ versus Control. **$P < 0.02$ versus Cold basal release. *** $P < 0.001$ versus Cold basal release.

stress was translated and the product processed and stored in intracellular organelles for peptide release. Cold stimulus could thus trigger TRH synthesis and release, in the magnocellular PVN.

Data obtained from push–pull perfusion experiments support the possibility that dendritic TRH is released from the magnocellular portion of the PVN. However, caution is necessary when interpreting data obtained through push–pull perfusion, because a certain degree of diffusion in the perfusion system could be responsible for inaccuracy in determining the exact locus of TRH secretion (46) (i.e. cells of the magnocellular subdivision or cells corresponding to the parvicellular subdivision of PVN, whose axons could project to the magnocellular region) (44). Thus, the push–pull perfusate could be contaminated with TRH released from parvicellular neurones, which is classically stimulated by cold exposure (1, 2). However, this fact does not invalidate the hypothesis that cold stress also stimulates magnocellular TRH secretion, especially considering that the stereotaxic implantation of a perfusion cannula in this discrete region was rigorously controlled by histological procedures (Fig. 4D).

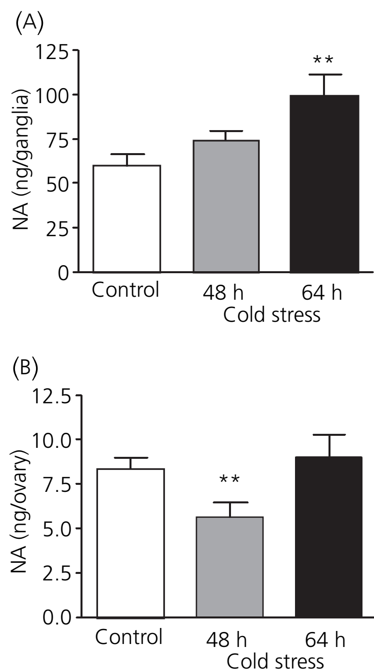

Fig. 5. Effect of cold exposure on sympathetic activity. Rats were maintained at 4 °C for 48 h or 64 h; controls were kept at 22 °C. (A) Changes in total noradrenaline (NA) content in celiac ganglia expressed as ng of NA/ganglion. (B) NA content in the ovary expressed as ng NA/ovary. Each bar represents the mean ± SEM (control, n = 6; 48 h of cold exposure, n = 4; and 64 h of cold exposure, n = 7). **P < 0.02 versus control.

Local TRH release may result from either depolarising events that favour dendritic peptide release in the magnocellular region (47) or alterations in gene expression (48) and may have an autoregulatory role (49–51). Increasing evidence suggests that dendritic peptide release occurs in a manner independent of axonal release (52, 53), thus indicating that it does not necessarily accomplish a neurohormonal role (54). Thus, for example, the dendritic release of AVP and oxytocin neurones can occur without increased electrical activity and independently of axonal release (18) through a process that mobilises intracellular Ca^{2+} (55). Because AVP and oxytocin also exert important vegetative functions (3, 37, 56) in addition to their classical endocrine roles, it is possible that hypothalamic magnocellular dendritic TRH release is a process linked to vegetative rather than endocrine activity. Dendritic depolarisation appears in general to make an important contribution to spontaneous neuronal firing (57), a feature observed in cells possessing vegetative tonic activity (58). In addition, it was recently reported that a continued excitatory synaptic input is required to sustain phasic bursts in rat magnocellular neurosecretory cells (59). Similarly, in our hands, cold-induced TRH release *in vivo* within the magnocellular PVN, exhibited spontaneous bursts of TRH release.

Dendritically-released TRH could arise from sympathetic neurones, whose axons project to vegetative centres before reaching their peripheral target (e.g. the ovary in this study). Such a view is supported by evidence that neurones sensitive to lithium chloride and located in the magnocellular division of the PVN form descending sympathetic projections (60, 61). Intermediate steps in the vegetative pathways arising from the forebrain to the spinal cord, such as the dorsal motor nuclei, the medullary raphe nuclei and the dorsal vagal complex, also use TRH as a neuromediator (43).

In the present study, we demonstrate that one of the peripheral effects of cold exposure is activation of ovarian sympathetic terminals expressed as an initial (48 h), as indicated by the decrease in ovarian noradrenaline content which is probably consequent upon increased noradrenaline efflux from the ovary. Similarly, we have shown that chronic intermittent cold stress activates ovarian sympathetic nerves. Evidence that TRH contributes to the ovarian responses to cold has been provided by attempts to characterise the role of TRH during the cold-induced ovary stimulation by studies using microinjections of TRH into the third ventricle (6).

Taken as a whole, the present study provides the first morphofunctional evidence that the magnocellular portion of PVN is a source of TRH mRNA, which is translated locally to TRH peptide. It also reveals that TRH is associated with dendritic processes in the PVN. Our functional studies suggest that TRH is released from magnocellular neurones within the PVN, particularly in conditions of cold stress. Indeed, the correlation between intrahypothalamic TRH release and biochemical changes in the sympathetic nerves which project to the ovary raises the possibility that TRH has a role in the regulation of the sympathetic drive to the ovary.

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