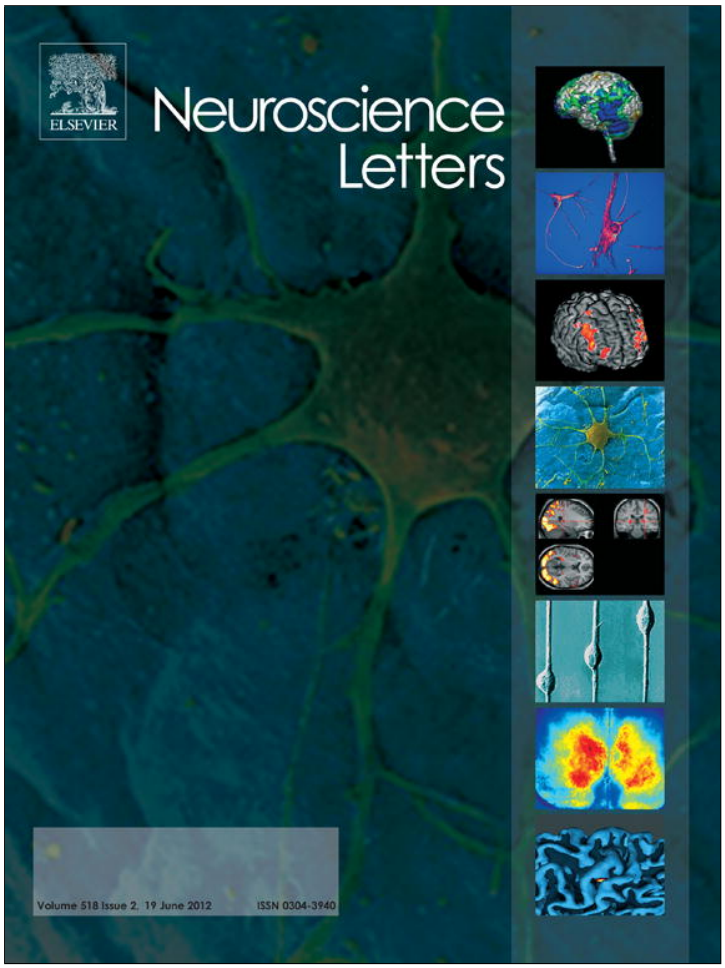

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Short-term cold exposure activates TRH neurons exclusively in the hypothalamic paraventricular nucleus and raphe pallidus

Agustina Cabral[a,1], Spring Valdivia[a,1], Mirta Reynaldo[a], Nicole E. Cyr[b], Eduardo A. Nillni[b,c], Mario Perello[a,*]

[a] Laboratory of Neurophysiology, Multidisciplinary Institute of Cell Biology (CONICET/CICPBA), La Plata, Argentina
[b] Division of Endocrinology of Brown University/Rhode Island Hospital, Providence, USA
[c] Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, USA

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ABSTRACT

The neuropeptide thyrotropin releasing hormone (TRH) is necessary for adequate cold-induced thermogenesis. TRH increases body temperature via both neuroendocrine and autonomic mechanisms. TRH neurons of the hypothalamic paraventricular nucleus (PVN) regulate thermogenesis through the activation of the hypothalamic–pituitary–thyroid axis during cold exposure. However, little is known about the role that TRH neurons play in mediating the sympathetic response to cold exposure. Here, we examined the response of TRH neurons of rats to cold exposure in hypothalamic regions including the PVN, the dorsomedial nucleus and the lateral hypothalamus along with areas of the ventral medulla including raphe obscurus, raphe pallidus (RPa) and parapyramidal regions. Our results using a double immunohistochemistry protocol to identify TRH and c-Fos (as a marker of cellular activity) followed by analysis of preproTRH gene expression demonstrate that only TRH neurons located in the PVN and the RPa are activated in animals exposed to short-term cold conditions.

1. Introduction

The neuropeptide TRH controls thermogenic responses via the regulation of both neuroendocrine and autonomic functions [16,18]. The neuroendocrine role of the TRH produced in hypophysiotropic TRH neurons has been extensively studied [13,15]. These TRH neurons, located in the hypothalamic parvocellular paraventricular nucleus (PVN), regulate the hypothalamic–pituitary–thyroid (HPT) axis via the release of TRH in the median eminence. This TRH stimulates the secretion of thyroid stimulating hormone in the pituitary, that, in turn, stimulates secretion of the thyroid hormones [13,15]. Cold exposure activates the hypophysiotropic TRH neurons and, as a consequence, plasma thyroid hormone levels increase [24,32,35]. Thyroid hormones increase thermogenesis by accelerating ATP turnover and expenditure in peripheral tissues, especially the brown adipose tissue (BAT) [4]. Also, TRH is produced in brain nuclei involved in regulating autonomic nervous system tone such as the PVN itself, the pre-optic area (POA), dorsomedial nucleus (DMN), the lateral hypothalamus (LH) and areas located in the ventral medulla including raphe obscurus (ROb), raphe pallidus (RPa) and parapyramidal regions (PPR) [16,18]. TRH increases thermogenesis via up-regulation of the sympathetic branch of the autonomic nervous system [2,18]. In this case, norepinephrine released by sympathetic terminals mediates a potent increase in energy expenditure [3]. Interestingly, intra-cerebro-ventricular (icv) administration of an anti-TRH antibody induces hypothermia while TRH peptide given icv induces hyperthermia, each in a manner independent of circulating thyroid hormone levels [5,25,29]. Despite the importance of sympathetic mechanisms in regulating thermogenesis, it is currently unknown which population of TRH neurons participate in these circuitries.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (250–270 g) were provided with regular rodent diet and water ad libitum. The Institutional Animal Care and Use Committee of the IMBICE approved all the protocols. For the study, rats were divided in two groups: control rats, maintained at controlled room temperature (22 °C) and cold-exposed rats, transferred to an environmental chamber at 4 °C.

* Corresponding author at: Calle 526 entre 10 y 11-PO Box 403 1900 La Plata, Buenos Aires, Argentina. Tel.: +54 2214210112.
E-mail address: marioperello@yahoo.com (M. Perello).
[1] These authors contributed equally to this work.

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2.2. Samples and procedures

Animals were processed for analysis after being either 1 or 2 h under temperature controlled environment. Animals exposed for 1 h to either cold or room temperature were sacrificed by decapitation. Blood was collected for the thyroid hormone T4 analysis. Brains were dissected and sectioned into 1 mm coronal slices using a brain matrix. This protocol was adapted from similar protocols published by us in the past [21]. Micro-dissections of tissue were identified by comparing the coronal slices to a rat brain atlas [20] and collected for RNA isolation. Cold-exposed and control groups contained 8 and 6 animals, respectively. Animals exposed to either cold or room temperature for 2 h were perfused to perform neuro anatomical studies as described [24]. Brains were frozen and cut coronally at 20 μm into four equal series on a sliding cryostat. Each group contained 5 animals.

2.3. Immunohistochemistry

Brain sections were used to perform double c-Fos/TRH prohormone (proTRH) staining similarly as described [6,23,24]. First, brain sections were incubated with anti-c-Fos antibody (Calbiochem cat#PC38, 1:5000) for 2 days at 4 °C. Then, sections were treated with biotinylated donkey anti-rabbit antibody for 1 h, and with avidin–biotin complex for 1 h. Sections were exposed to 3-3′-diaminobenzidine (DAB)/nickel solution that produced a black/purple precipitate. Then, sections were consecutively washed and incubated overnight with a anti-proTRH antibody (1:3000). Rabbit anti-proTRH antibody was generated against C-terminal preproTRH239-255 sequence (KQSPQVEPWDKEPLEE) plus a tyrosine added at the N-terminal end [22]. The next day, sections were sequentially incubated with biotinylated anti-rabbit antibody and avidin–biotin complex as detailed above. Finally, sections were incubated with DAB solution that produced a brown precipitate. This double immuno-staining is very specific and no cross reactivity is observed. Anti-proTRH antibody has been well characterized in previous studies [22], and we have an extensive experience using it to perform double immuno-staining [23,24]. The c-Fos antibody was also specific as no staining was observed when the antiserum was pre-absorbed with excess of the synthetic immunogen (Calbiochem, Cat#PP10, 10 μM). Furthermore, proTRH and c-Fos antibodies label distinctly separated cellular location in both single- and double-immuno-staining: c-Fos is seen exclusively in the nucleus and proTRH in the perikarya and proximal dendrites. This differential distribution of brown proTRH-immunoreactive (proTRH-IR) signal easily allows determination whether the nucleus contained the previously developed dark purple label for c-Fos. Of note, the tonality of dark purple label for c-Fos was not affected by further incubations with biotinylated anti-rabbit antibody and development with DAB reagent for proTRH staining. Double-labeled brain sections were visualized using bright-field light sources. Bright-field images were acquired with an Eclipse 50i microscope and a DS-Ri1 digital camera. The software program Adobe PhotoShop 7.0 was used to combine the photomicrographs into plates and adjust levels, contrast and brightness in the images.

2.4. Quantitative analysis

For each brain region under study, we quantified the total number of proTRH-IR neurons and the number of proTRH-IR neurons that were positive for c-Fos. Anatomical limits of each brain region were identified using a rat brain atlas [20]. To determine the total number proTRH-IR cells in each brain region, we quantified cells containing distinct cytoplasmic light brown chromogen reaction product for proTRH in one out of four complete series of 20 μm coronal sections through the whole nuclei. Then, these numbers were summed and multiplied by four. The data were corrected for double counting, according to the method of Abercrombie [1], where the ratio of the actual number of neurons to the observed number is represented by $T/T+h$, where T = section thickness, and h = the mean diameter of the neuron. For this, proTRH-IR cell diameter was quantified, of at least 40 cells in each brain area and experimental group, using the software Image J-1.44. To estimate the proportion of activated proTRH-IR cells, we quantified all proTRH-IR cells positive for c-Fos in the same brain regions and with the same procedure. Only cells with intense dark purple density of nuclear labeling for c-Fos were counted as double-labeled cells. The proportion of activated proTRH-IR cells was expressed as a percentage, which represents proTRH-IR cells positive for c-Fos compared to the total number of proTRH-IR cells observed in each brain region.

2.5. Gene expression analysis

Total isolated RNA was quantified and treated with DNase I. cDNA synthesis was generated using random hexamer primers and reverse transcriptase. Quantitative real-time PCRs were conducted using the SYBR Green® PCR Core Reagents and the ABI 7500 Fast-Real time PCR system. Averaged levels of preproTRH and c-Fos were normalized to the housekeeping gene hypoxanthine phosphoribosyl transferase (hprt1). Values were calculated by the comparative threshold cycle (Ct) method [28]. Primers sequences were: upstream preproTRH, 5′-GGAGAGGGTGTCTTAATGCCT-3′; downstream preproTRH, 5′-GGCCTGTTTGACCACAAGTCC-3′; upstream c-Fos, 5′-TGACCTCCCTGGACTTGACT-3′; downstream c-Fos, 5′-ATGATGCCGGAAACAAGAAG-3′; upstream hprt1, 5′-GCAGACTTTGCTTTCCTTGG-3′; downstream hprt1, 5′-GTCTGGCCTGTATCCAACACT-3′. Standard curves for preproTRH, c-Fos and hprt1 transcript levels were generated using hypothalamic rat cDNA with ABI 7500 Fast System SDS Software v1.3.1.

2.6. RIA analysis

Plasma T4 levels were measured using commercial RIA kit from MP Biomedicals Diagnostic Division.

2.7. Statistical analyses

Data, expressed as the mean ± SEM, were analyzed by Student's *t*-test for comparison of different mean values. Significant differences were considered when $p < 0.05$.

3. Results

3.1. Cold exposure activates c-Fos in proTRH-IR neurons located in the hypothalamic PVN and RPa of the medulla

We found a significant increase of plasma T4 levels in cold exposed as compared to control animals (4.83 ± 0.31 vs. 6.73 ± 0.54 μg/dL in control and cold-exposed animals, respectively, $p < 0.01$) [24]. To determine which populations of TRH neurons could be activated by cold exposure, we performed double immunohistochemistry for c-Fos and proTRH. Fig. 1 shows representative photomicrographs and quantitative analysis of this experiment. In the hypothalamus, we found proTRH-IR cells in the PVN, DMN and LH, as previously described [16,18]. In the PVN, we estimated a total of 1994 ± 213 proTRH-IR neurons/side, located in sections between bregma −0.92 and −2.12 mm of rostral-to-caudal axis of the nucleus. Cold exposure failed to affect total number of proTRH-IR cells in the PVN (2113 ± 253 neurons/side);

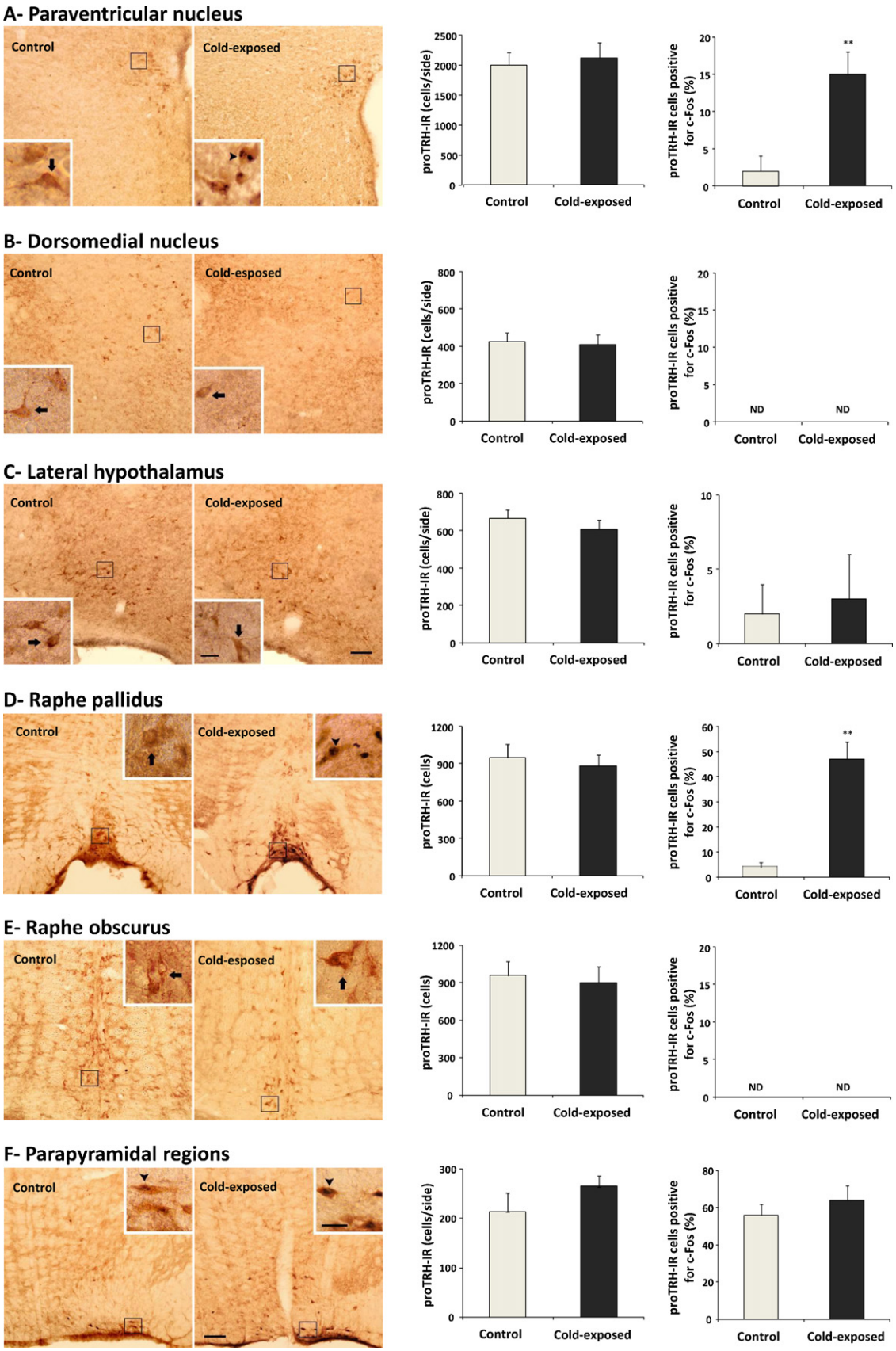

Fig. 1. Representative microphotographs and quantitative analysis of proTRH and c-Fos staining in specific brain regions of control and cold-exposed rats. Regions include: PVN (A), DMN (B), LH (C), RPa (D), ROb (E) and PPR (F). Representative microphotographs show c-Fos (black/purple signal) and proTRH (brown signal) double immuno-staining of control (left) and cold-exposed (right) animals. Arrowheads point to proTRH-IR neurons positive for c-Fos, and arrows point to proTRH-IR neurons negative for c-Fos. Each panel shows images in low (20×, scale bar: 100 μm) and high (120×, scale bar: 20 μm) magnification. Histograms depict the total number of proTRH-IR neurons (left) and the percent proTRH-IR neurons positive for c-Fos (right) for each experimental group. ND: no detectable. $^{*}p < 0.05$ vs. control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Relative mRNA levels of preproTRH and c-Fos in control and cold-exposed animals.[a]

	preproTRH		c-Fos	
	Control	Cold exposed	Control	Cold exposed
Hypothalamus				
Paraventricular nucleus	100.0 ± 4.5	226.4 ± 29.1*	100.0 ± 30.0	222.5 ± 18.4*
Dorsomedial nucleus	100.0 ± 7.2	114.4 ± 12.4	100.0 ± 35.0	184.3 ± 48.3
Lateral hypothalamus	100.2 ± 9.1	106.3 ± 9.1	100.0 ± 16.4	214.7 ± 51.3*
Medulla				
Raphe pallidus	99.9 ± 9.0	187.9 ± 15.2*	100.0 ± 11.9	153.5 ± 21.6*
Raphe obscurus	100.2 ± 9.5	109.3 ± 10.5	100.0 ± 18.0	164.9 ± 15.2*
Parapyramidal region	99.9 ± 7.4	108.9 ± 8.9	100.0 ± 6.8	190.4 ± 30.7*

[a] All mRNA values are normalized to the levels of the housekeeping gene hprt1, which were not affected significantly by cold exposure. Data is presented as a percentage of levels observed in the same brain areas of control animals.
* $p < 0.05$.

however, it induced a significant increase in the percentage of proTRH-IR neurons positive for c-Fos (2 ± 2 vs. 15 ± 3% in control and cold-exposed animals, respectively, $p < 0.01$). In each DMN, we estimated a total of 426 ± 45 proTRH-IR neurons/side located in sections between bregma −2.56 and −3.30 mm of rostral-to-caudal axis of the nucleus. Total number of proTRH-IR cells (409 ± 52 neurons/side) was not affected by cold-exposure. We did not detect proTRH-IR cells positive for c-Fos in the DMN of neither control nor cold-exposed animals. The proTRH-IR neurons in the LH were located in sections between bregma −1.88 and −3.30 mm of rostral-to-caudal axis of the nucleus. In control animals, we estimated 666 ± 72 proTRH-IR neurons within each LH. In cold-exposed rats, total number of proTRH-IR cells (607 ± 77 neurons/side) and the percentage of proTRH-IR cells positive for c-Fos in the LH were not significantly affected as compared to control animals (2 ± 2 vs. 3 ± 3% in control and cold-exposed animals, respectively). We also detected proTRH-IR cells in the POA; however, the intensity of the staining was weak and the number of cells was small and variable limiting a strict analysis on this hypothalamic region.

We found proTRH-IR cells in the RPa, ROb and PPR of the ventral medulla. In the RPa, we estimated a total of 950 ± 109 proTRH-IR neurons located in sections between bregma −11.30 and −14.10 mm of rostral-to-caudal axis of the nucleus. Cold exposure failed to affect total number of proTRH-IR cells in the RPa (882 ± 89 neurons); however, it induced a significant increase in the percentage of proTRH-IR neurons positive for c-Fos (4 ± 2 vs. 47 ± 7% in control and cold-exposed animals, respectively, $p < 0.01$). In the ROb of control animals, we estimated 959 ± 113 proTRH-IR neurons, which were located in sections between bregma −11.30 and −13.80 mm of rostral-to-caudal axis of the nucleus. In cold-exposed rats, the total number of proTRH-IR cells in the ROb (903 ± 127) was similar to control animals. We did not detect proTRH-IR cells positive for c-Fos in the ROb of neither control nor cold-exposed animals. In the PPR, proTRH-IR neurons were located in sections between bregma −10.52 and −14.08 mm of rostral-to-caudal axis of the nucleus. In control animals, we estimated 213 ± 37 proTRH-IR neurons within the each PPR, where 56 ± 6% of them were positive for c-Fos. In the PPR of cold-exposed rats, the total number of proTRH-IR cells (265 ± 21 neurons/side) and the percentage of proTRH-IR cells positive for c-Fos (64 ± 8%) were not significantly affected as compared to control animals.

3.2. Cold exposure increases the mRNA levels of preproTRH in the PVN and RPa of the medulla

To further examine the impact of cold exposure on the transcriptional activity, we analyzed the c-Fos and preproTRH mRNA levels in the above-mentioned brain regions (Table 1). We found that cold exposure increased c-Fos mRNA levels in the PVN, RPa, LH, Rob and PPR regions compared to controls. Among the hypothalamic regions examined, the PVN showed the highest preproTRH mRNA levels (CT = 20.7), whereas DMN and LH showed the lowest levels of preproTRH mRNA (CT = 23.2 and CT = 22.7, respectively). Cold exposure induced a significant increase of preproTRH mRNA levels exclusively in the PVN, where it increased ∼2.3 times in cold exposure animals as compared to control animals. In the ventral medulla, the RPa presented the highest level of preproTRH mRNA (CT = 22.3), whereas ROb and PPR had the lowest levels of expression (CT = 23.4 and CT = 24.8, respectively). Cold exposure induced a significant increase of preproTRH mRNA levels only in the RPa, where it increased ∼1.9 times in cold exposure rats as compared to control animals.

4. Discussion

Both neuroendocrine and autonomic actions of TRH are essential for body temperature homeostasis. In fact, Cpe(fat/fat) mice, deficient in hypothalamic TRH due to a defect in the proteolytic processing of proTRH, cannot sustain a cold challenge [19]. Also, TRH-knockout mice are unable to maintain body core temperature when exposed to cold, and this defect cannot be fully restored with thyroid hormone supplementation [34]. Hypophysiotropic TRH neurons of the PVN are known to regulate the HPT axis [13,15]. However, it is unclear which populations of TRH neurons regulate sympathetic-mediated thermogenesis. The goal of this study was to identify TRH neurons that are recruited to presumably activate early thermogenic responses. We found that TRH neurons located in the PVN and RPa are the main candidates to mediate thermogenic mechanisms in response to cold exposure.

We used neuroanatomical studies in combination with gene expression analysis to assess which populations of TRH neurons are sensitive to short-term cold exposure. Cold-induced c-Fos expression was studied within a few hours after cold exposure because TRH-dependent thermogenic mechanisms are activated quickly [18]. Gene expression analysis was performed in animals exposed to cold for 1 h, when an increase of preproTRH and c-Fos mRNA levels can be detected [24]. The c-Fos-IR within proTRH-IR neurons was studied 2 h after cold-exposure, which presumably is the shortest time period required for c-Fos gene expression, protein biosynthesis and mobilization from cytoplasm to the cell nucleus [11,14]. Both strategies indicated that TRH neurons of the PVN and the PRa are quickly activated in cold-exposed animals. Of note, the data indicated a doubling in the increase of preproTRH and c-Fos gene expression in the PVN of cold-exposed animals with only ∼15% of proTRH-IR neurons positive for c-Fos. Sanchez et al. also reported similar c-Fos activation in TRH neurons of the PVN when animals were exposed to cold for 1 h [26]. In the past, we used the phospho-cAMP response element binding protein as a marker for activation of hypophysiotropic TRH neurons since beta

adrenoreceptors signaling mediates the cold-induced activation of these neurons [24]. With this strategy, we found that ~50% of TRH neurons in the PVN were activated in animals exposed to cold for 45 min [24]. c-Fos gene transcription is also downstream of cAMP signaling [10]. Thus, it is possible that a longer exposure to cold is required for a full induction of nuclear c-Fos in TRH neurons of the PVN. Despite these considerations, we were able to successfully map early-activated TRH neurons in the brain of short-term cold-exposed animals.

We confirmed that TRH neurons in the PVN and the HPT axis are activated in animals exposed to cold. This thermogenic mechanism exerted by the HPT axis is vital to maintain body temperature as demonstrated by the fact that hypothyroid animals do not survive when exposed to cold stress [24,35]. Hypothalamic TRH is mainly produced in the PVN, where the largest population of TRH neurons exists according to our quantitative analysis. The hypophysiotropic TRH neurons are found primarily in the medial and periventricular parvocellular subdivisions of the PVN. However, TRH neurons also exist in the anterior and dorsal parvocellular subdivisions of this nucleus [15]. The physiological role of these non-hypophysiotropic TRH neurons present in the PVN is unclear [33]. Some PVN neurons innervate autonomic nervous system centers including the dorsal vagal complex, spinal cord and DMN of the hypothalamus [27]. In fact, viral retrograde transynaptic transport studies have showed that the parvocellular subdivision of the PVN contain premotor neurons innervating the spinal sympathetic circuit controlling BAT thermogenesis [7]. Thus, some TRH neurons in the PVN could directly regulate BAT thermogenesis via autonomic mechanisms.

Our understanding about TRH neurons located in the DMN and LH regions of the hypothalamus is very limited [16,18]. TRH neurons in the LH produce mature TRH, which is not affected by the thyroid status [21]. Evidence links hypothalamic DMN and LH with the control of thermogenesis. For instance, the rostral medulla receives excitatory inputs from the DMN, which is tonically inhibited by POA neurons in thermo-neutral conditions [17]. Cold exposure results in a de-inhibition of the DMN neurons and, as a consequence, an activation of thermogenesis-promoting neurons in the RPa [17]. Also, activation of LH neurons stimulates BAT thermogenesis via an activation of DMN and RPa neurons [8]. However, we were not able to detect activation of these TRH neurons in animals exposed to short-term cold conditions. Thus, it seems that TRH neurons in these areas are not involved in neuronal circuits mediating the acute cold-induced thermogenesis and that other neurons of these nuclei participate in the short-term response to cold.

The POA is a key hypothalamic center for thermoregulation. Temperature information is detected by peripheral thermoreceptors and transmitted to the POA, where temperature-sensitive neurons also exist [17]. The TRH administration into the POA increases thermogenesis in an autonomic-dependent fashion [9,12]. A subpopulation of TRH-producing neurons is present in the POA [16,21], and preproTRH gene expression in this area can be transiently affected by cold exposure [32]. However, we detected modest levels of preproTRH mRNA and proTRH-IR neurons under our experimental conditions. These observations suggest that the production of TRH in the POA is low and, likely, that TRH from other origins are more relevant for thermoregulation.

The RPa, ROb and PPR are not only important regions of the rostral medulla that control autonomic outputs but also contain the largest group of TRH neurons outside the hypothalamus [17,31]. Of note, our quantitative analysis indicates the RPa and the ROb contain significantly more TRH neurons than the PPR. These medullary TRH neurons innervate the dorsal vagal complex, which belongs to the parasympathetic branch of the autonomic system [31]. Evidence supports a role for medullary TRH in mediating the vagal stimulation of gastric transit and secretion induced by cold exposure [30]. The RPa also contains premotor neurons that innervate the intermedio-lateral cell column containing sympathetic preganglionic neurons that control BAT functions [7]. The above-mentioned study using virally labeled neurons following inoculations of BAT provided strong evidence for a key role of RPa in controlling BAT thermogenesis [7]. Here, we did not detect any sign of activation in the Rob and the PPR. Although we found an increase of c-Fos mRNA in the PPR and the Rob, we failed to detect any increase of preproTRH mRNA levels or proTRH-IR neurons positive for c-Fos. Thus, it seems that other non-TRH neuronal populations of these brain regions may be activated in response to cold-exposure. In contrast, the increase of preproTRH mRNA levels and proTRH-IR neurons positive for c-Fos observed in the RPa suggests that these TRH neurons, in addition to TRH neurons of the PVN, are activated in response to short-term cold exposure. Overall, our data is consistent with the possibility that TRH neurons in the RPa regulate BAT sympathetic premotor neurons and, as a consequence, BAT thermogenesis.

In summary, we found that TRH-producing neurons located in the PVN and RPa participate in the neuronal circuits activated in response to short-term cold exposure. In contrast, TRH neurons located in other central areas failed to show signs of activation in our experimental conditions. Future studies will be needed to elucidate the role of these TRH-producing neurons.

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