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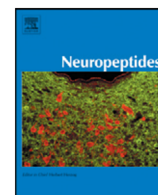

Mct8 and trh co-expression throughout the hypothalamic paraventricular nucleus is modified by dehydration-induced anorexia in rats

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ABSTRACT

Thyrotropin-releasing hormone (TRH) is a neuropeptide with endocrine and neuromodulatory effects. TRH from the paraventricular hypothalamic nucleus (PVN) participates in the control of energy homeostasis; as a neuromodulator TRH has anorexigenic effects. Negative energy balance decreases PVN TRH expression and TSH concentration; in contrast, a particular model of anorexia (dehydration) induces in rats a paradoxical increase in TRH expression in hypophysiotropic cells from caudal PVN and high TSH serum levels, despite their apparent hypothalamic hyperthyroidism and low body weight. We compared here the mRNA co-expression pattern of one of the brain thyroid hormones' transporters, the monocarboxylate transporter-8 (MCT8) with that of TRH in PVN subdivisions of dehydration-induced anorexic (DIA) and control rats. Our aim was to identify whether a low MCT8 expression in anorexic rats could contribute to their high TRH mRNA content. We registered daily food intake and body weight of 7-day DIA and control rats and analyzed TRH and MCT8 mRNA co-expression throughout the PVN by double *in situ* hybridization assays. We found that DIA rats showed increased number of TRHergic cells in caudal PVN, as well as a decreased percentage of TRH-expressing neurons that co-expressed MCT8 mRNA signal. Results suggest that the reduced proportion of double TRH/MCT8 expressing cells may be limiting the entry of hypothalamic triiodothyronine to the greater number of TRH-expressing neurons from caudal PVN and be in part responsible for the high TRH expression in anorexia rats and for the lack of adaptation of their hypothalamic-pituitary-thyroid axis to their low food intake.

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1. Introduction

Thyrotropin-releasing hormone (TRH) is a peptide that has both, neuroendocrine and neuromodulatory effects. As a neurohormone, it is responsible for the control of the hypothalamic-pituitary-thyroid (HPT) axis and thermogenesis, through the stimulation of thyrotropin (TSH) release from the anterior pituitary, which in turn increases thyroid hormones (TH: triiodothyronine T_3, thyroxine T_4) concentration in serum by activating their synthesis and release from the thyroid gland. By controlling TH levels, TRH regulates metabolic rate and energy homeostasis. TRH-expressing neurons involved in HPT axis regulation are exclusively those from the periventricular, medial (mPVN) and caudal (cPVN) parvocellular subdivisions of the hypothalamic paraventricular nucleus (PVN) (Ishikawa et al. 1988; Merchenthaler and Liposits 1994).

HPT axis function regulation involves a classic negative feedback loop directed by TH levels: i.e. hyperthyroideal animals show a decreased expression of TRH in the PVN; it is recognized that T_3, which is the biologically active thyroid hormone, is able to inhibit TRH biosynthesis only in hypophysiotropic TRHergic cells (Kakucska et al. 1992; Segerson et al. 1987; Sugrue et al. 2010).

During negative energy balance, as occurs in fasting or food restriction, animals have reduced TRH synthesis in the PVN (Blake et al. 1991), in spite of the reduced T_3 serum levels characteristic of low nutrient availability. Among the possible factors responsible for the low TRH mRNA levels during negative energy balance are the decrease in circulating leptin levels along with high corticosterone concentration (Coppola et al. 2005), which favor an increase in type-2 deiodinase (D2) activity in the mediobasal hypothalamus (MBH). This enzyme converts T_4 into active T_3, increasing its local content in the hypothalamus that when transported inside the TRHergic neurons, is able to down-regulate TRH expression (Freitas et al. 2010; Kallo et al. 2012).

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There are different types of transporters for TH: organic anion transporter polypeptides (OATP), large neutral amino acid transporters (LAT), sodium/taurocholate co-transporting polypeptide (NTCP) and monocarboxylate transporters (MCT), amongst which MCT8 is the only one known to carry T_3, T_4 and their metabolites, reverse T_3 (rT_3) and diiodothyronine (T_2) (Friesema et al. 2003) as exclusive substrates, whereas the others may transport other substances like aminoacids and steroids in addition to TH (Bernal et al. 2015; Friesema et al. 2001; Friesema et al. 2005; Taylor and Ritchie 2007; Westholm et al. 2008). In the rat, MCT8 protein is expressed in nerve terminals from the median eminence, allowing T_3 transport into TRH-expressing neurons (Kallo et al. 2012). It is known that most of the T_3-dependent genes expression is similar between MCT8 KO and WT animals (Friesema et al. 2003; Friesema et al. 2004), however the uptake of T_3 specifically in TRHergic neurons has been suggested to be altered in the hypothalamic PVN (Muller and Heuer 2012) of KO mice. Importantly, the only pathogenic mutation of thyroid transporters in humans is described in the MCT8 gene, *SLC16A2* (Schwartz et al. 2005).

Dehydration-induced anorexic (DIA) rats present an aberrant adaptation of the HPT axis to negative energy balance. In this model of anorexia, rats are offered a hypertonic saline solution (2.5% NaCl) as drinking water, and after the first day of the experiment, due to the dehydration, animals display a marked inhibition of food ingesting behavior and a consequent body weight loss (Watts et al. 1999). DIA animals show low serum leptin levels along with high corticosterone concentration (Jaimes-Hoy et al. 2008) similar to those found during food restriction and fasting. Regarding the HPT axis, these animals present elevated TRH expression in the PVN -and not the expected reduction due to their negative energy balance- along with high TSH and low T_3 circulating levels (Alvarez-Salas et al. 2012; Jaimes-Hoy et al. 2008), which indicates the presence of primary hypothyroidism (altered thyroid gland function) that contrasts with the tertiary nature (due to hypothalamic alteration) of the one evident in starvation (Rondeel et al. 1992; van Haasteren et al. 1995).

In DIA rats, TRHergic neurons from distinct subdivisions of the PVN respond differently to the amount of T_3 produced by MBH tanycytes (specialized ependymal cells that line the floor and infralateral walls of the third ventricle). D2 activity and expression are enhanced in MBH of DIA rats vs. *ad libitum* fed controls, suggesting the development of a hypothalamic hyperthyroid state. Thus, in spite of the elevated local T_3 concentration, a high expression of TRH is maintained in the cPVN (Alvarez-Salas et al. 2012). Increased TRH expression in the caudal subdivision of the PVN occurs despite the hypophysiotropic nature of its TRH-synthesizing cells, which are the unique type where the expression of the peptide is down-regulated by TH (Perello et al. 2006; Segerson et al. 1987). Since mPVN (with hypophysiotropic TRHergic cells) shows the expected decrease in TRH expression due to the negative energy balance (Alvarez-Salas et al. 2012), it is possible that the activation of the TRHergic neurons of cPVN may account for the higher release of the peptide and for the elevated TSH serum levels found in the anorexic rats (Jaimes-Hoy et al. 2008).

In this study, we analyzed the co-expression pattern of MCT8 and TRH mRNAs throughout the PVN of DIA animals, and compared them to those of *ad libitum* fed controls in order to elucidate whether an altered co-expression pattern could be involved in the differential regulation of TRH expression in subdivisions of the PVN in DIA animals.

2. Experimental procedures

2.1. Animals

All experiments were approved by the local committee of ethics on animal experimentation and complied with the Mexican official norm NOM-062-ZOO-1999 related to experiments with laboratory animals. Fourteen male Wistar rats of 250 ± 10 g (ten weeks old) from National Institute of Psychiatry (INPRFM) animal house facilities were used for the experiment. Animals were housed individually and maintained in controlled light (12 h–12 h, lights on at 8:00) and temperature conditions (23 ± 1 °C). After one week of acclimation, rats were randomly divided in two groups, controls (C, n = 7) with *ad libitum* access to food (Lab rodent diet #5001, PMI feeds) and tap water, or dehydration-induced anorexic (DIA, n = 7) animals with *ad libitum* access to food and a 2.5% NaCl solution as drinking water (Watts 1999); their body weight, liquid and food intake were registered daily by obtaining the difference between the grams or milliliters of the offered food or drinking solution one day minus the weight or volume of the remaining pellets or liquid on the next day. The experiment had a 7-day duration after which, rats were sacrificed by decapitation; brains were excised and frozen on dry ice until analyses were performed.

2.2. Tissue preparation

Rostro-caudal coronal slices (14 μm thick) corresponding to aPVN (−1.08 to −1.44 mm from bregma), mPVN (−1.44 to −1.8 mm from bregma) and cPVN (−1.8 to −2.16 mm from bregma) (Paxinos and Watson 2005) were obtained by cutting brains with a cryostat (Microm HM500 OM, Walldorf, Germany) at −20 °C and thaw-mounted onto APTS-coated (3-aminopropylyltriethoxysilane; Sigma-Aldrich, St. Louis, MO, USA) slides. Every ninth 14 μm section was Nissl-stained with 0.1% cresyl violet (Sigma-Aldrich, St. Louis, MO, USA), dehydrated in an increasing ethanol gradient, defatted in xylene and mounted with Entellan (Merck KGaA, Darmstadt, Germany). Representative sections of the starting area of aPVN (Fig. 1A), mPVN (Fig. 1B), and cPVN (Fig. 1C) are shown.

2.3. Hybridization probes

Oligonucleotides were complementary to the following base sequences of MCT8 mRNA [NM_147216.1]: 359 to 403, 802 to 846 and 1419 to 1463, probes were independently labeled at their 3′-end using [α-^{33}P] dATP (3000 Ci/mmol; New England Nuclear, Boston, MA, USA). TRH mRNA [NM_013046.2] sequences used were complementary to the following bases: 85 to 129, 407 to 471 and 568 to 612, these probes were independently labeled with digoxigenin-11-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) as previously reported (Schmitz et al. 1991). All oligonucleotides were synthesized and High Performance Liquid Chromatography-purified by Isogen Bioscience BV (Maarsden, The Netherlands). Evaluation of oligonucleotide sequences with basic local alignment search tool (BLAST) of GenBank database indicated that probes showed no significant similarities with mRNAs others than their corresponding targets in the rat. For *in situ* hybridization histochemistry (ISHH) experiments, all oligonucleotides were labeled using terminal deoxynucleotidyltransferase (Oncogene Research Products, San Diego, CA, USA).

As negative controls of ISH, we used sense (non-complementary) probes directed against the same TRH and MCT8 mRNA nucleotide sequences as the complementary ones, which were also radioactively and non-radioactively labeled.

2.4. Double in situ hybridization histochemistry (dISHH)

Radioactive and non-radioactive labeling of oligonucleotides and dISHH procedures are reported elsewhere (de Gortari and Mengod 2010). Briefly, frozen tissues sections were thawed, fixed for 20 min at 4 °C in 4% paraformaldehyde in phosphate-buffered saline (PBS; 1× PBS: 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 136 mM NaCl, 2.6 mM KCl), washed for 15 min in PBS at room temperature and incubated for 2 min at 21 °C in pre-digested pronase solution (Calbiochem, San Diego, CA, USA) at a final concentration of 24 U/mL in 50 mM Tris–HCl pH 7.5, 5 mM EDTA. Enzymatic activity was stopped by 2 mg/mL glycine in 1× PBS immersion for 30 s. Tissues were rinsed in 1× PBS and dehydrated with ethanol. Radioactively and non-radioactively labeled

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