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A screen for modulators reveals that orexin-A rapidly stimulates thyrotropin releasing hormone expression and release in hypothalamic cell culture

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ABSTRACT

In the paraventricular nucleus of the mammalian hypothalamus, hypophysiotropic thyrotropin releasing hormone (TRH) neurons integrate metabolic information and control the activity of the thyroid axis. Additional populations of TRH neurons reside in various hypothalamic areas, with poorly defined connections and functions, albeit there is evidence that some may be related to energy balance. To establish extracellular modulators of TRH hypothalamic neurons activity, we performed a screen of neurotransmitters effects in hypothalamic cultures. Cell culture conditions were chosen to facilitate the full differentiation of the TRH neurons; these conditions had permitted the characterization of the effects of known modulators of hypophysiotropic TRH neurons. The major end-point of the screen was *Trh* mRNA levels, since they are generally rapidly (0.5–3 h) modified by synaptic inputs onto TRH neurons; in some experiments, TRH cell content or release was also analyzed. Various modulators, including histamine, serotonin, β-endorphin, met-enkephalin, and melanin concentrating hormone, had no effect. Glutamate, as well as ionotropic agonists (kainate and *N*-Methyl-D-aspartic acid), increased *Trh* mRNA levels. Baclofen, a $GABA_B$ receptor agonist, and dopamine enhanced *Trh* mRNA levels. An endocannabinoid receptor 1 inverse agonist promoted TRH release. Somatostatin increased *Trh* mRNA levels and TRH cell content. Orexin-A rapidly increased *Trh* mRNA levels, TRH cell content and release, while orexin-B decreased *Trh* mRNA levels. These data reveal unaccounted regulators, which exert potent effects on hypothalamic TRH neurons *in vitro*.

1. Introduction

The rodent hypothalamus contains neurons involved in the control of energy balance. Among these, parvocellular neuroendocrine thyrotropin releasing hormone (TRH) neurons of the paraventricular nucleus of the hypothalamus (PVN) control the secretion of thyrotropin from the anterior pituitary, and thus metabolic rate and thermogenesis. These neurons integrate multiple inputs related to energy balance, including those arising from neurons of the arcuate nucleus (ARC). ARC proopiomelanocortin (POMC)/cocaine and amphetamine regulated transcript (CART) and neuropeptide Y (NPY)/agouti related peptide neurons sense peripheral clues about nutrients and energy balance, and control PVN TRH neurons. Hypophysiotropic TRH neurons are also controlled by catecholaminergic inputs from the brain stem, which convey signals activated by a drop in ambient temperature, and control TRH neuronal activity. Furthermore, hypophysiotropic TRH neurons are regulated by negative feedback from thyroid hormones, and may also respond directly to leptin and glucocorticoids (Ghamari-Langroudi et al., 2010; Fekete and Lechan, 2014; Sotelo-Rivera et al., 2014; Joseph-Bravo et al., 2015; Hollenberg, 2008, Kakucska et al., 1992; Kim et al., 2000). Retrograde communication by endocannabinoids mediates the rapid non-genomic action that glucocorticoids exert on glutamate inputs onto PVN, including TRH neurons (Deli et al., 2009; Di et al., 2003). Other neuronal stimuli to these hypophysiotropic neurons have been suggested by *in vivo* approaches, generally based on the effect of pharmacological interventions and read out of serum thyrotropin (TSH) levels; these include dopaminergic and serotoninergic inputs, albeit their effect may be indirect (Männistö, 1983). Medio-basal hypothalamic explants containing the median eminence have also allowed the identification of additional regulators of TRH secretion such as histamine (Joseph-Bravo et al., 1979).

The role of many additional subtypes of TRH neurons in the hypothalamus is still poorly understood. Non-hypophysiotropic PVN TRH neurons populate the anterior, dorsal and ventral (pre-sympathetic) parts of the rat PVN; they have many targets, and may be involved in diverse aspects of energy balance regulation (Wittmann et al., 2009; Fekete and Lechan, 2014; Joseph-Bravo et al., 2015). Magnocellular

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TRH neurons have also been described (Fiedler et al., 2006; Ghamari-Langroudi et al., 2010), although their physiological role is poorly understood. Outside the PVN, some TRH neurons are localized to the lateral hypothalamus; they receive NPY and alpha melanocyte stimulating hormone (α-MSH) inputs from the ARC (Horjales-Araujo et al., 2014). Other TRH neurons are localized in the dorsomedial nucleus (DMN); some of them project into the lateral hypothalamus (Chou et al., 2003), where they may directly or indirectly control orexin and melanin concentrating hormone (MCH) neurons (González et al., 2009; Hara et al., 2009; Zhang and van den Pol, 2012). A significant population of TRH neurons is also present in the preoptic area (Simerly et al., 1986); these TRHergic neurons are involved in blood pressure control and are thermo-responsive (García and Pirola, 2005; Dolan et al., 1992). Some TRH neurons map into part of the hypothalamic attack area (Hrabovszky et al., 2005a).

The identification of afferents requires labor-intensive methodologies. Primary cultures of hypothalamic cells are a simplified model useful for the study of neuronal properties. In conditions that allow the full differentiation of hypothalamic neurons, various modulators exert effects consistent with their *in vivo* effect on PVN TRH biosynthesis and release in adult animals. For example, thyroid hormones reduce *Trh* mRNA levels (Carreón-Rodríguez et al., 2009; Díaz-Gallardo et al., 2010). Noradrenaline rapidly enhances *Trh* mRNA levels and peptide release (Cote-Vélez et al., 2005; Perello et al., 2007). CART increases the cell content of TRH (Fekete et al., 2000), while leptin enhance TRH biosynthesis and release (Nillni et al., 2000), in agreement with *in vivo* data in the PVN. A nitric oxide donor rapidly reduces TRH cell content, consistently with *in vivo* data in the PVN and mediobasal hypothalamus (MBH) (Uribe et al., 2011). Short-term stimulatory effects of corticosterone, dependent on the nuclear glucocorticoid receptor, have also been evidenced in hypothalamic cell cultures (Pérez-Martínez et al., 1998, Cote-Velez et al., 2008); they are consistent with *in vivo* data that show that PVN *Trh* mRNA levels are rapidly enhanced by an injection of corticosterone (Sotelo-Rivera et al., 2014). However, although *in vitro* responses of hypothalamic TRH neurons to modulators tend to mirror *in vivo* responses of PVN TRH neurons, a substantial proportion of the TRH neurons included in primary cultures of hypothalamus is likely from other nuclei (Guerra-Crespo et al., 2001; Ubieta et al., 2007).

To identify new extracellular modulators of hypothalamic TRH neurons activity, we performed a screen of neurotransmitter, neuropeptide and endocannabinoid effects in fully differentiated hypothalamic cultures. We chose modulators that had not been tested previously in culture, but that have been implicated, directly or indirectly, in the control of hypothalamic TRH neurons. The end-points of this screen were *Trh* mRNA levels, and in some experiments TRH cell content and/or release, surrogate markers of TRH neuronal activity since peptide metabolism can be coupled to electrical activity in response to synaptic or hormonal inputs (Watts and Khan, 2013); we chose to analyze various early time points, to facilitate the identification of responses likely coupled to electrical activity. The results demonstrate the existence of unaccounted regulators, which may exert potent effects on hypothalamic TRH neurons.

2. Materials and methods

2.1. Animals

Wistar rats raised at the animal facility of the Instituto de Biotecnología (Ibt), Universidad Nacional Autónoma de México (UNAM), and naïve to any previous treatment were maintained under artificial illumination in a 12 h light-dark cycle (lights on at 7:00 AM), temperature 21 ± 2 °C, food (Purina chow 5001, Lab diet) and water *ad libitum*. Animal care and protocols were approved by the Bioethics Committee of the Institute; animals were used according to the Guide for the Care and Use of Laboratory Animals, National Research Council, USA.

2.2. Reagents

Insulin, MCH, met-enkephalin, β-endorphin, orexins A and B, somatostatin, amines, (*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone (WIN 55,212-2), 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide (AM-251), 1-(2-methylbenzoxazol-6-yl)-3-(Arufe et al., 2002; Chomczynski and Sacchi, 1987)naphthyridin-4-yl urea (SB-334867), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), glucose, glutamine, cytosine arabinoside, charcoal, ethidium bromide, kainate, *N*-methyl-D-aspartic acid (NMDA), glutamic acid, bicuculline methiodide, baclofen, muscimol, γ-aminobutyric acid (GABA), guanidine thiocyanate, TRIS base, boric acid and EDTA were from Sigma-Aldrich. Fetal bovine serum (FBS), antibiotic-antimycotic mix, vitamin solution, deoxynucleotides, phenol, and MMLV reverse transcriptase were from Invitrogen. Taq DNA polymerase was from Biotecnologias Universitarias (México), and agarose from Biorad. Oligodeoxynucleotides were made at the "Unidad de Síntesis de Macromoleculas" (Ibt, UNAM).

2.3. Primary cultures of hypothalamic cells and treatment with drugs

Fetal (E17) rat hypothalamic cells were cultured in serum-supplemented medium as described (Joseph-Bravo et al., 2002). 6×10^5 viable cells were plated in 11 mm ø dishes (Corning) (for RNA purification), or 2.4×10^6 cells in 35 mm ø dishes (Corning), pretreated with poly-D-lysine (30 μg/ml) (to measure TRH cell content and release), in 10% FBS and 0.25% glucose, 3.8 μg/ml insulin, 1% antibiotic-antimycotic mix, 2 mM glutamine and 1% vitamin solution. Cells were treated with 10^{-4} M cytosine arabinoside at day *in vitro* (DIV) 4, and half of the medium substituted for fresh medium at each medium change. Consistent with previous results (Cote-Vélez et al., 2005, Cote-Velez et al., 2008), 14–18 DIV primary cultures expressed *Trh*, *Crh*, glucocorticoid receptor (*Nr3c1*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*), and cyclophilin A (*Ppia*) mRNAs. They also expressed orexin receptor 1 (*Hcrtr1*) (not shown); the expression of other receptors was not tested. The spontaneous release of TRH increased with time in culture, higher at DIV 14 than at earlier time points (not shown).

Only dishes with high cell density and adequate morphological appearance (phase contrast microscopy) were selected for treatment. Treatments with neuromodulators were initiated at DIV 14 or 18 (indicated in legends), coincident with a full medium change. DIV 14 cultures were used to test modulators effects on TRH cell content, or TRH release; DIV 14 or 18 cultures were used to test modulators effects on *Trh* mRNA levels; *Trh* mRNA levels were regulated similarly by second messenger agonists whether cells were tested at DIV 14 or at DIV 18 (not shown). Amines were dissolved in ascorbic acid 10^{-4} M, and protected from light before and during use; orexins were dissolved in DMSO, and other reagents in water. Controls contained the appropriate vehicle. For kinetic experiments, control dishes were incubated with vehicle for as long as for the latest treatment time point. Treatments were performed in medium supplemented with 10% FBS (most data), or 2% FBS (where indicated). There was no effect of treatments on phase contrast morphology (not shown). Various separate independent experiments were performed and numbered from 1 and up for each neurotransmitter tested.

2.4. Trh mRNA quantification

After incubation, cell medium was removed, cells rinsed with PBS, and frozen onto dry ice. Total RNA was isolated as described (Chomczynski and Sacchi, 1987). RNA was quantified by spectroscopy, and integrity examined by gel electrophoresis and staining with

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