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# LATERAL HYPOTHALAMIC THYROTROPIN-RELEASING HORMONE NEURONS: DISTRIBUTION AND RELATIONSHIP TO HISTOCHEMICALLY DEFINED CELL POPULATIONS IN THE RAT

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**Abstract—The lateral hypothalamic area (LHA) constitutes a large component of the hypothalamus, and has been implicated in several aspects of motivated behavior. The LHA is of particular relevance to behavioral state control and the maintenance of arousal. Due to the cellular heterogeneity of this region, however, only some subpopulations of LHA cells have been properly anatomically characterized. Here, we have focused on cells expressing thyrotropin-releasing hormone (TRH), a peptide found in the LHA that has been implicated as a promoter of arousal. Immunofluorescence and *in situ* hybridization were used to map the LHA TRH population in the rat, and cells were observed to form a large ventral cluster that extended throughout almost the entire rostro-caudal axis of the hypothalamus. Almost no examples of coexistence were seen when sections were double-stained for TRH and markers of other LHA populations, including the peptides hypocretin/orexin, melanin-concentrating hormone and neurotensin. In the juxtaparaventricular area, however, a discrete group of TRH-immunoreactive cells were also stained with antisera against enkephalin and urocortin 3. Innervation from the metabolically sensitive hypothalamic arcuate nucleus was investigated by double-staining for peptide markers of the two centrally projecting groups of arcuate neurons, agouti gene-related peptide and α-melanocyte-stimulating hormone, respectively; both populations of terminals were observed forming close appositions on TRH cells in the LHA. The present study indicates that TRH-expressing cells form a unique population in the LHA that may serve as a link between metabolic signals and the generation of arousal.** 

**Key words: neuropeptide, immunofluorescence, *in situ* hybridization, arcuate nucleus, coexistence, arousal.**

## INTRODUCTION

The lateral hypothalamic area (LHA) occupies a large portion of the diencephalon (Bernardis and Bellinger, 1993, 1996), yet has remained difficult to define in functional as well as anatomical terms. In a seminal study, Anand and Brobeck (1951) described the hypophagia that ensues in rats and cats following electrolytic ''*. . .small, bilateral lesions. . .in the extreme lateral portion of the lateral hypothalamus. . .*''. This phenotype was interpreted primarily as a loss of motivation to feed that has been speculated to involve diminished arousal. Concurrent lesion studies by Ranson (1937) and Nauta (1946) had indicated that the integrity of the LHA is required for wakefulness. More recent work aimed at elucidating the network basis for LHA control of vigilance has focused on the hypocretin (Hcrt) peptides (also known as orexins; de Lecea et al., 1998; Sakurai et al., 1998) that are exclusively expressed in this brain region. A loss-of-function mutation in the Hcrt receptor 2 gene in dogs results in the sleep disorder narcolepsy (Lin et al., 1999), whereas human narcoleptic patients exhibit an almost complete loss of Hcrt-immunoreactive (-ir) cells (Peyron et al., 2000; Thannickal et al., 2000). These observations and others (see de Lecea and Sutcliffe, 2005) have identified LHA Hcrt neurons as essential for the maintenance of normal arousal. A population of LHA neurons separate from the Hcrt cells express another peptide, melanin-concentrating hormone (MCH; Skofitsch et al., 1985; Elias et al., 1998; Broberger et al., 1998a). Contrary to Hcrt cells, the MCH population is at its most active during sleep (Hassani et al., 2009) and stimulation of these cells promotes sleep (Jego et al., 2013; Konadhode et al., 2013). Thus, available information suggests that the LHA plays a key role in contributing arousal to motivated behavior.

Yet, the Hcrt- and MCH-expressing neurons account for only a minority of the total LHA cells (see Broberger

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*Abbreviations:* 3v, third ventricle; α-MSH, alpha-melanocyte stimulating hormone; AGRP, agouti gene-related peptide; Arc, arcuate hypothalamic nucleus; CART, cocaine and amphetamine regulated transcript; DA, dorsal hypothalamic area; EDTA, ethylenediaminetetraacetic acid; Enk, enkephalin; f, fornix; Hcrt, hypocretin; LPO, lateral preoptic area; MCH, melanin-concentrating hormone; MPA, medial preoptic area; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide tyrosine; NT, neurotensin; Opt, optic tract; PACAP, pituitary adenylate cyclase-activating polypeptide; PLH, peduncular part of lateral hypothalamus; POMC, pro-opiomelanocortin; ppTRH, preproTRH; RCh, retrochiasmatic area; SCh, suprachiasmatic nucleus; Som, somatostatin; Sox, supraoptic decussation; TH, tyrosine hydroxylase; TRH, thyrotropin-releasing hormone; TuLH, tuberal region of lateral hypothalamus; UCN3, urocortin 3.

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and Hökfelt, 2005). Indeed, the LHA is a large region encompassing some quarter of a million neurons (Palkovits and Van Cuc, 1980), whose borders are relatively poorly defined. Recent efforts to define subregions of the LHA based on cytoarchitectonics and connectivity have provided new tools to understand the structure of this region (Swanson, 2004; Swanson et al., 2005; Hahn and Swanson, 2010). Ideally, however, this information needs to be complemented with histochemical approaches. The existence of cells expressing thyrotropin-releasing hormone (TRH) has earlier been described in the LHA (Johansson and Hökfelt, 1980; Lechan and Jackson, 1982; Nishiyama et al., 1985; Lechan et al., 1986a,b; Segerson et al., 1987; Tsuruo et al., 1987; Merchenthaler et al., 1988; Heuer et al., 2000). Interestingly, administration of TRH to animals results in reduced sleep time and electroencephalographic signs of arousal (Breese et al., 1974; Nishino et al., 1997). Here, we have studied the topographical distribution of TRH cells in the rat LHA, the relationship of these cells to other transmitter-defined populations and their innervation by hypothalamic neurons sensitive to the metabolic state.

## EXPERIMENTAL PROCEDURES

### Animals

Rats were purchased from Charles River (Kisslegg, Germany) and maintained on a 12/12-h light/dark cycle (lights on at 6 a.m.) in a temperature-controlled environment with free access to standard rodent chow and tap water. All animal experiments had received prior approval by the local ethics board, *Stockholms Norra Djurförsöksetiska Nämnd*, and were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were done to minimize the number of animals used and their suffering.

### *In situ* hybridization

For *in situ* hybridization experiments, male Sprague–Dawley rats ($n$ = 6; 2–8 w) were deeply anesthetized, decapitated and brains rapidly dissected out and frozen. Coronal 14-μm-thick sections were cut on a cryostat (Microm, Heidelberg, Germany) and thaw-mounted onto SuperFrost® Plus slides (Thermo Fisher Scientific, Pittsburg, PA, USA). Oligonucleotide probes complementary to nucleotides 464–502, 716–761 and 894–940 of the mouse preproTRH (ppTRH) mRNA (Satoh et al., 1992; GenBank accession no. NM_013047.3) were synthesized (CyberGene, Solna, Sweden). Probe sequences were controlled against other sequences in the GenBank database, and no homologies exceeding 75% were found. The oligonucleotides were labeled at the 3′-end with 1:10 ratio digoxigenin-11-dUTP:dATP using terminal deoxynucleotidyl transferase (Fermentas, Burlington, Ontario, Canada) for 45 min at 37 °C, precipitated with 133 mM LiCl in 100% ethanol for 16 h at −80 °C and re-suspended in Tris–EDTA buffer.

Tissue sections were air-dried and incubated for 16 h at 42 °C with 120–200 ng of each of the labeled probes. The probes were diluted in a hybridization solution containing 50% deionized formamide (Baker, Deventer, the Netherlands), 4× standard saline citrate (SSC, 1X SSC = 0.15 M NaCl, 0.015 M tri-Na-citrate dehydrate), 1× Denhardt's solution (0.02% bovine serum albumin, 0.02% Ficoll [Pharmacia, Uppsala, Sweden], 0.02% polyvinylpyrrolidone) 0.02 M $NaPO_4$ (pH 7.0), 1% N-lauroylsacrosine, 10% dextran sulfate (Pharmacia), 500 mg/L denatured salmon testis DNA (Sigma, St. Louis, MO, USA) and 200 mM dithiothreitol (DTT; Sigma). After hybridization, the sections were rinsed in 1× SSC, four times for 15 min at 55 °C and left in SSC for 90 min to cool to room temperature.

The sections were next processed with direct immunohistochemistry; all reactions in room temperature unless otherwise stated. Sections were washed 2 × 10 min in Buffer A (0.1 M Tris pH 7.5, 1 M NaCl, 2 mM $MgCl_2$), blocked in 0.8% BSA in Buffer A for 30 min and incubated in alkaline phosphatase-conjugated anti-digoxigenin $F_{ab}$-fragments (1:5000; Roche, Basel, Switzerland) diluted in Buffer A for 16 h at 4 °C. After 2 × 10-min wash in Buffer A and 2 × 10-min wash in Buffer C (0.1 M Tris pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$), sections were incubated with NBT-BCIP substrate (375 μg/ml nitro blue tetrazolium chloride and 188 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate; Roche) in Buffer C containing 0.5 mM levamisole (Sigma) for 3 days. The alkaline phosphatase reaction was stopped by washing in 10× PBS for 16 h. Slides were rinsed in water and air dried.

Bright field micrograph montages were generated automatically by taking consecutive pictures with a Zeiss Axio Imager M1 (Carl Zeiss, Jena, Germany) that were merged using MBF Neurolucida (Williston, VT, USA) computer software. For final images, only brightness, contrast and sharpness were adjusted digitally.

### Immunofluorescence

Male Sprague–Dawley rats ($n$ = 8; 6–8 w) were used. Prior to perfusion, animals received a unilateral intracerebroventricular (i.c.v.) colchicine infusion (90 μg in 15 μl 0.9% NaCl) over a period of 20 min, and the needle was slowly retracted 10 min after infusion. Colchicine injections were performed to interrupt axonal transport (Hökfelt and Dahlström, 1971). This treatment results in cell soma accumulation of peptides and other transported proteins targeted toward the axon terminals, providing for better immunohistochemical identification of cell bodies. Twenty-four hours after colchicine injections, the rats were anesthetized with an intra-peritoneal injection of 1 ml of sodium pentobarbital (60 mg/ml; Apoteksbolaget, Umeå, Sweden) and perfused as previously described (Hökfelt et al., 1989) via the ascending aorta with 50 ml of Tyrode's $Ca^{2+}$-free solution (37 °C) followed by 50 ml fixative solution containing 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid in 0.16 M phosphate buffer, pH 6.9 (37 °C), followed by 300 ml of the same, but ice-cold, fixative. The brains were dissected out, immersed in the same fixative for 90 min, and rinsed for 15 min in 0.1 M phosphate buffer (pH 7.4)