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ScienceDirect
Neuropeptides 43 (2009) 13–19
Neuropeptides
www.elsevier.com/locate/npep

Distribution and genesis of the RFRP-producing neurons in the rat brain: Comparison with melanin-concentrating hormone- and hypocretin-containing neurons

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Received 22 October 2008; accepted 6 November 2008
Available online 19 December 2008

Abstract

Prepro-RFRP-containing neurons have recently been described in the mammalian brain. These neurons are only found in the tuberal hypothalamus. In this work, we have provided a detailed analysis of the distribution of cells expressing the RFRP mRNA, and found them in seven anatomical structures of the tuberal hypothalamus. No co-expression with melanin-concentrating hormone (MCH) or hypocretin (Hcrt), that are also described in neurons of the tuberal hypothalamus, was observed. Using the BrdU method, we found that all RFRP cell bodies are generated between E13 and E14.

Thus, RFRP neurons form a specific cell population with a complex distribution pattern in the tuberal hypothalamus. However, they are generated in one peak. These observations are discussed with data concerning the distribution and genesis of the MCH and Hcrt cell populations that are also distributed in the tuberal hypothalamus.

Keywords: RFRP; Dorsomedial hypothalamic nucleus; Ventromedial hypothalamic nucleus; Lateral hypothalamic area

1. Introduction

Kriegsfeld et al. (2006a,b) have recently characterized in the Syrian hamster brain a RFamide-related peptide named RFRP or gonadotropin-inhibitory hormone (GnIH) according to its role in the inhibition of gonadotropin release (observed in birds and mammals – Bentley et al., 2006; Tsutsui et al., 2007). In many species, including human, three peptides (RFRP1–3) can putatively be produced from the prepro-RFRP. GnIH corresponds to the RFRP2. In rats and mice, RFRP2 is lacking; nevertheless, an inhibitory role for RFRP peptides on the gonadotropic axe is reported. A mammalian hypothalamic distribution of RFRP has emerged and revealed a unique RFRP cell body condensation in the dorsomedial capsule of the ventromedial nucleus and in the dorsomedial nucleus of the hypothalamus, whereas RFRP-positive fibers were observed in many brain regions, but mostly in the hypothalamus. Exact mechanisms involved in the GnIH function are unknown, but direct synaptic contacts on GnRH-containing neurons in the preoptic area are described. The distribution pattern of the RFRP-producing neurons is peculiar as many of them are observed outside the borders of cytoarchitectonically defined hypothalamic nuclei. However, this feature is shared by other neuron

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doi:10.1016/j.npep.2008.11.001

populations distributed outside well-defined structures in the caudal hypothalamus. For example, neurons producing the peptides 'melanin-concentrating hormone' (MCH) or hypocretins (Hcrt) sit predominantly in lateral hypothalamic regions, but are also observed medially in the dorsomedial capsule of the ventromedial nucleus (VMH) and in the dorsomedial nucleus (Peyron et al., 1998; Swanson et al., 2005). The MCH neuron population is not homogeneous, and at least two subpopulations were described on the basis of their time of birth, projection patterns, expression of CART and NK3 receptor (Brischoux et al., 2001, 2002; Cvetkovic et al., 2004). Neurons of the first subpopulation (A-MCH neuronal type) are born around the 11th day of embryonic life (E11) and project in the spinal cord; neurons of the second subpopulation (B-MCH type) are generated around E13, project throughout the cortical mantle, express CART and NK3 and are often more medially located in the perifornical region and the rostromedial zona incerta. In Brischoux et al. (2002), we had noticed that very medially located MCH neurons (in the capsule of the VMH or in the posterior part of the periventricular nucleus – generated between E14 and E16) do not belong to the A- or B-MCH neuronal types but may correspond to another MCH phenotype. By contrast, the Hcrt neurons form a more homogeneous cell population, and are generated in one sharp peak at E12 (Amiot et al., 2005). They are also often localized more medially than the A-MCH neurons in the perifornical region and the capsule of the VMH in the rat. As the capsule of the VMH is often considered as a cell poor zone, the detection of several peptides suggests that they might be co-expressed in the same cell bodies. Thus, the aims of this study was to better understand the anatomy of the RFRP system with regard to other peptidergic systems observed in this region: our specific objectives was first to provide a detailed map of the RFRP neuron distribution in the rat hypothalamus, verify a putative co-expression of the RFRP mRNA with MCH and Hcrt. Then, the period of birth of the neurons producing the prepro-RFRP mRNA was determined and compared to the already published data concerning the genesis of MCH and Hcrt neurons (Brischoux et al., 2001, 2002; Amiot et al., 2005).

2. Materials and methods

All manipulations and experiments were performed in accordance with the European Union Legislation and with the recommendations of our institution. Adult rats Long-Evans (19 male and 6 female rats, weighing between 250 and 350 g, Charles River Laboratories, L'Arbresle, France) were maintained on a 12-h light/dark cycle, with standard rodent chow and water *ad libitum*.

2.1. Tissue preparation

Animals were deeply anesthetized with an intraperitoneal injection of 7% chloral hydrate (1 ml per 200 g animal weight, Prolabo). Animals were perfused between 9 and 11 AM through the ascending aorta with 0.9% NaCl followed by a 1% paraformaldehyde (PFA) fixative solution in 0.1 M phosphate buffer. Brains were post-fixed for 12 h in the same fixative at 4 °C, immersed overnight at 4 °C in a 15% sucrose solution and frozen on dry ice. Serial sections of the frozen brains were performed in the frontal plane (10 μm) using a cryostat microtome, and stored at −40 °C on gelatinized slides until treatment.

2.2. Immunohistochemical analysis (IHC)

Sections were rinsed in phosphate-buffered saline (PBS) containing 0.3% Triton X-100, and then incubated overnight at room temperature with the primary antibody. After rinsing in PBS + 0.3% Triton X-100, labeling was revealed by incubation of the sections with secondary antibodies conjugated to Fluoprobes-488 (1:400, Molecular Probes-Interchim) or Cyanine-3 (1:400, Jackson ImmunoResearch-Interchim) for 1 h at room temperature. All antisera (-AS), a goat anti-human Hcrt (1:500, Santa-Cruz Inc.), a rabbit anti-salmon MCH (1:200, Risold et al., 1992; multiple citations since), were diluted in PBS containing 10% lactoproteins, 1% bovine serum albumin, 0.1% Triton X-100 and 0.01% sodium azide.

2.3. 5-bromo-2′-desoxyuridine (BrdU) injections

As described previously (Brischoux et al., 2001, 2002; Amiot et al., 2005), 6 pregnant females (between E11 and E16) received an intraperitoneal injection of BrdU prepared according to the method described par Markakis and Swanson (1997) (150 mg BrdU per kg animal weight, dissolved in 0.07 M NaOH solution warmed at 65 °C). Detection of the BrdU was done in the brains of the two months old male progeny (a total of 14 male rats: 3 per stages from E12 to E15 and 2 at E16). After an acid hydrolysis in 1 N HCl solution for 5 min at 65 °C, sections were rinsed in PBS solution for 5 min twice and in a PBS solution containing 0.3% Triton X100 for 30 min at room temperature. Incubation with mouse anti-BrdU antibody was performed overnight at room temperature. Labeling was revealed using donkey anti-mouse antibody conjugated to cyanine-3.

2.4. In situ hybridization (ISH)

A digoxygenin (DIG-)-labeled riboprobe has been synthesized. RNA was obtained from adult rat