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THE SATIETY MOLECULE NESFATIN-1 IS CO-EXPRESSED WITH MELANIN CONCENTRATING HORMONE IN TUBERAL HYPOTHALAMIC NEURONS OF THE RAT

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Abstract—Overlapped in the tuberal hypothalamic area (THA), melanin-concentrating hormone (MCH) and hypocretin (Hcrt) neurons contribute to the integrated regulation of food intake, energy regulation and sleep. Recently, physiological role in appetite suppression has been defined for a novel hypothalamic molecule, nesfatin-1. Acute i.c.v. infusion of nesfatin-1 (nesf-1) promotes anorexia whereas chronic treatment reduces body weight in rats. This satiety molecule is expressed in neurons from areas prominently involved in appetite regulation including THA. We therefore sought functionally relevant to determine whether nesf-1 might be a reliable signaling marker for a new cell contingent within THA, in addition to MCH and Hcrt neurons. Thus, we completed a detailed topographical mapping of neurons immunostained for nesf-1 (nesf-1+) together with cell quantification in each discrete nucleus from THA in the rat. We further combined the immunodetection of nesf-1 with that of MCH or Hcrt to assess possible co-expression. More than three quarters of the nesf-1+ neurons were encountered in nuclei from the lateral half of THA. By double immunofluorescent staining, we showed that all neurons immunoreactive for melanin concentrating hormone (MCH+) neurons depicted nesf-1 immunoreactivity and ~80% of the nesf-1+ neurons were labeled for MCH. Maximal co-expression rates were observed in the lateral THA containing ~86% of the double-labeled neurons plotted in THA. The present data suggest that nesf-1 co-expressed in MCH neurons may play a complex role not only in food intake regulation but also in other essential integrative brain functions involving MCH signaling, ranging from autonomic regulation, stress, mood, cognition to sleep.

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Abbreviations: CART, cocaine-amphetamine related transcript; DAB, 3,3′-diaminobenzidine-4 HCl; Hcrt, hypocretins/orexins; LHA, lateral hypothalamic area; L-THA, lateral part of the tuberal hypothalamic area; MCH, melanin concentrating hormone; MCH+, neurons immunoreactive for melanin concentrating hormone; M-THA, medial part of the tuberal hypothalamic area; nesf-1+, neurons immunoreactive for nesf-1; PB, phosphate buffer; PBST, phosphate buffer containing 0.9% NaCl and 0.3% Triton X-100; PBST-Az, phosphate buffer containing 0.9% NaCl and 0.3% Triton X-100 and 0.1% sodium azide; PeF, perifornical area; PS, paradoxical sleep; PVN, paraventricular nucleus; Subl, subincertal nucleus; THA, tuberal hypothalamic area; ZI, zona incerta.

Key words: MCH, orexins, hypocretins, sleep, food intake regulation, hypothalamus.

A growing body of data involves the tuberal hypothalamic area (THA) from the posterior hypothalamus as a key center for the sleep/waking regulation (Saper et al., 2001; Luppi et al., 2006). Two major cell contingents that greatly overlap within the THA (Bittencourt et al., 1992; Broberger et al., 1998; Peyron et al., 1998; Bayer et al., 2002; Swanson et al., 2005) seem to have an antagonist role, the hypocretin-containing (Hcrt) and melanin concentrating hormone-containing (MCH) neurons. On one side, evidence supports the critical engagement of the Hcrt-containing neurons for the promotion of arousal and in human narcolepsy linked to a depletion of Hcrt neurons (review in Peyron et al., 2000; Sakurai, 2007). On the other side, the MCH-containing neurons may play a role in sleep, including paradoxical sleep (PS) (Verret et al., 2003; Goutagny et al., 2004, 2005; Modirrousta et al., 2005; Hanriot et al., 2007). A large majority (~60%) of the MCH-containing neurons from the THA indeed expressed the c-Fos transcription factor (used as an index of activity) during the recovery period consecutive to a selective PS deprivation in rats, suggesting their selective activation during this sleep state. In contrast, the neighboring Hcrt-containing neurons remained unlabeled for c-Fos (Verret et al., 2003). An intriguing functional result from these studies was that half of these c-Fos-labeled neurons remained negative for MCH or Hcrt. This observation sheds light that 1) half of the hypothalamic neurons contributing to sleep are still neurochemically undefined and 2) signaling molecules from THA remain to be discovered.

In that context, a new hypothalamic bioactive molecule called nesfatin-1 (nesf-1), corresponding to the N terminal peptide fragment processed from the NEFA/nucleobindin2 protein (NUCB2; residue 1–82) has been recently isolated and its satiety properties characterized (Oh et al., 2006). Briefly, acute i.c.v. administration of nesf-1 decreases food intake in a dose-dependent manner whereas chronic treatment reduces body weight. At the anatomical level, the largest populations of neurons immunoreactive for nesf-1 (nesf-1+) were found in hypothalamic areas known to have an important role in anorexia such as the arcuate, paraventricular (PVN) and supraoptic nuclei (Oh et al., 2006; Kohno et al., 2008). Surprisingly, nesf-1+ neurons in discrete nuclei of THA were also briefly reported. Initially described as a “hunger center,” the lesion of THA results in severe anorexia and weight loss in perfect agreement with the orexigenic properties endowed by both MCH and Hcrt

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(see recent reviews Pissios et al., 2006; Coll et al., 2007; Sakurai, 2007). Regarding the large range of other integrative roles from endocrine and autonomic responses to energy homeostasis, cognition or vigilance involving THA neurons, we though functionally relevant to determine by using immunohistochemical approaches whether nesf-1 might be a reliable signaling marker for a new hypothalamic cell group. Our initial goal was thus to have a detailed mapping completed with quantification of nesf-1-secreting neurons and to characterize their topographical distribution through THA nuclei forming the "MCH and Hcrt" area (Bittencourt et al., 1992; Broberger et al., 1998; Peyron et al., 1998; Bayer et al., 2002; Swanson et al., 2005). For this purpose, we combined dual nesf-1/MCH and nesf-1/Hcrt immunohistochemical and immunofluorescent staining to compare the distribution of nesf-1+ neurons with that of neurons immunoreactive for melanin concentrating hormone (MCH+) or Hcrt+ neurons. During this experimental process, an expression of nesf-1 in some MCH-immunoreactive neurons from THA has been reported (Brailoiu et al., 2007). As a consequence, we strengthened our analysis by quantifying the range of MCH and nesf-1 co-expression while considering the discrete THA nuclei encountering for MCH-immunoreactive neurons. Part of the present results has been previously presented in an abstract form (Fort et al., 2007).

EXPERIMENTAL PROCEDURES

Histological procedures

Male Sprague–Dawley rats (200–250 g, IFFA Credo, L'Arbresle, France; $n=6$), anesthetized with a lethal dose of Nembutal (60 mg/kg), were perfused transcardially with a Ringer's lactate solution containing 0.1% heparin followed by 500 ml of an ice cold 4% paraformaldehyde fixative solution in 0.1 M phosphate buffer (PB; pH 7.4). Brains were rapidly removed to be immersed in a cryoprotecting solution of 30% sucrose in PB for at least 48 h at 4 °C. Hypothalamic blocks were frozen with CO_2 gas, coronal sections were cut on a freezing microtome at a thickness of 25 μm collected in phosphate buffer containing 0.9% NaCl, 0.3% Triton X-100 (PBST) and 0.3% hydrogen peroxide (H_2O_2). Free-floating sections were rinsed and stored in phosphate buffer containing 0.9% NaCl and 0.3% Triton X-100 with 0.1% sodium azide (PBST-Az) at 4 °C until processing.

Immunohistochemical procedures

Sections were processed for different single and dual histochemical (a, b, b′, c and c′) and fluorescent (d and e) immunostaining, as reported in the Table 1.

For dual immunohistochemical staining (b, b′, c and c′), classical protocols of sequential incubations were used. The first step consisted in the immunodetection of either MCH, Hcrt or nesf-1. Sections were thus incubated in 1) a primary antiserum either to MCH raised in chicken, to Hcrt-A raised in goat or to nesf-1 raised in rabbit (Ab24, Oh et al., 2006; Kohno et al., 2008) diluted in PBST-Az for 72h at 4 °C (see Table 1 for chemical suppliers); 2) a secondary anti-chicken, anti-goat or anti-rabbit biotinylated IgG in PBST for 90 min at room temperature under gentle stirring; 3) avidin–biotin–HRP complex (ABC; 1/1000; Elite Kit, Vector Laboratories, Burlingame, CA, USA) in PBST for 90 min at room temperature under gentle stirring; and 4) 0.05 M Tris–HCl buffer (pH 7.6) containing 0.025% 3,3′-diaminobenzidine-4 HCl (DAB; Sigma, Saint Quentin Fallavier, France) and 0.003% H_2O_2 and 0.6% nickel ammonium sulfate for 20 min. After stopping the reaction by extensive rinses, the staining appeared as a dense black cytoplasmic coloration of immunoreactive neurons. In a second step, these pretreated sections were submitted to the same sequential incubations for the immunodetection of the appropriate marker (nesf-1, MCH or Hcrt). At the end of this protocol, sections were immersed in the same Tris–HCl solution without nickel ammonium sulfate, the reaction product appearing thus colored in orange–brown.

For double immunofluorescent staining (d and e, Table 1), sections were incubated with mixtures of appropriate primary

Table 1. Combination and sequential processing of primary and secondary antibodies used for dual immunohistochemical and immunofluorescent staining of nesf-1, MCH or Hcrt

Experiment	Marker	Primary antibody	Secondary biotinylated IgG	Technique—coloration
(a)	nesf	Rabbit 1/40,000 Ab24 (gift Dr. Mori)	Donkey anti-rabbit 1/1000 Ref 611-7602, Rockland	DAB-Ni—black
(b)	nesf	Rabbit 1/40,000	Donkey anti-rabbit 1/1000	DAB-Ni—black
	MCH	Chicken 1/30,000 Ref AB 5857, Chemicon	Goat anti-chicken 1/1000 Ref BA-9010, Vector Labs	DAB—brown
(b′)	MCH	Chicken 1/90,000	Goat anti-chicken 1/1000	DAB-Ni—black
	nesf	Rabbit 1/20,000	Donkey anti-rabbit 1/1000	DAB—brown
(c)	nesf	Rabbit 1/40,000	Donkey anti-rabbit 1/1000	DAB-Ni—black
	Hcrt	Goat 1/20,000 Ref sc-8070, Santa Cruz	Horse anti-goat 1/1000 Ref BA-9050, Vector Labs	DAB—brown
(c′)	Hcrt	Goat 1/50,000	Horse anti-goat 1/1000	DAB-Ni—black
	nesf	Rabbit 1/20,000	Donkey anti-rabbit 1/1000	DAB—brown
(d)	nesf	Rabbit 1/7500	Donkey anti-rabbit 1/500 Ref A21206, Molecular Probes	Alexa 488—green
	MCH	Chicken 1/10,000	Goat anti-chicken 1/500 Ref A11040, Molecular Probes	Alexa 546—yellow
(e)	nesf	Rabbit 1/7500	Donkey anti-rabbit 1/500	Alexa 488—green
	Hcrt	Goat 1/1000	Donkey anti-goat 1/500 Ref A11056, Molecular Probes	Alexa 546—yellow

Suppliers: Rockland Immunochemicals, Inc., Philadelphia, PA, USA; Chemicon International, Inc., Temecula, CA, USA; Vector Laboratories, Burlingame, CA, USA; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; Molecular Probes, Invitrogen, Carlsbad, CA, USA.