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Do enteric neurons make hypocretin? ★

Christian R. Baumann^{a,c,*}, Erika L. Clark^a, Nigel P. Pedersen^{a,d}, Jonathan L. Hecht^b, and Thomas E. Scammell^a

^aDepartment of Neurology, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, Boston, USA ^bDepartment of Pathology, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, Boston, USA ^cDepartment of Neurology, University Hospital, Zurich, Switzerland ^dPain Management Research Institute and Medical Program, University of Sydney, Sydney, Australia

Abstract

Hypocretins (orexins) are wake-promoting neuropeptides produced by hypothalamic neurons. These hypocretin-producing cells are lost in people with narcolepsy, possibly due to an autoimmune attack. Prior studies described hypocretin neurons in the enteric nervous system, and these cells could be an additional target of an autoimmune process. We sought to determine whether enteric hypocretin neurons are lost in narcoleptic subjects. Even though we tried several methods (including whole mounts, sectioned tissue, pre-treatment of mice with colchicine, and the use of various primary antisera), we could not identify hypocretin-producing cells in enteric nervous tissue collected from mice or normal human subjects. These results raise doubts about whether enteric neurons produce hypocretin.

Keywords

Orexin; Hypocretin; Immunohistochemistry; Enteric nervous system

1. Introduction

Hypocretins (orexins) are excitatory neuropeptides produced by neurons in the lateral and posterior hypothalamus. Prior reports have described hypocretin-like immunoreactivity in the enteric nervous system and in endocrine cells in the stomach, intestine, and pancreas [1–6]. One of these studies also used reverse transcriptase polymerase chain reaction (RT-PCR) to identify mRNA coding for hypocretin and its receptors [1]. Expression of hypocretin in the gut, however, remains controversial because immunostaining and RT-PCR can produce false positive results.

Narcolepsy with cataplexy is caused by a selective loss of the hypocretin-producing neurons in the hypothalamus [7–9], but the cause of this cell loss is unknown. Because narcolepsy is linked to specific HLA alleles, an autoimmune etiology has been considered, but little direct evidence supports this hypothesis [10]. Because the brain is relatively insulated from the immune system, an autoimmune attack might initially target hypocretin-producing neurons in other sites such as the enteric nervous system. Therefore, we sought to identify hypocretin-producing neurons in the gut of normal and narcoleptic mice and human subjects.

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* Corresponding author. E-mail address: cbaumann@bidmc.harvard.edu (C.R. Baumann).

2. Methods

2.1. Mouse tissue

We immunostained brain, stomach, duodenum, jejunum, and colon from hypocretin ligand knockout mice and wild type littermates. All tissues were from male, 15 week old mice that had been backcrossed to C57BL/6J for 8 generations (the same strain was used by Kirchgeßner et al., 1999) [1]. For positive controls, we immunostained hypothalamic sections from wild type mice for hypocretin-1. For negative controls, we immunostained hypothalamic sections from hypocretin knockout mice and omitted the primary antibodies when staining wild type tissue. All experiments were approved by the Institutional Animal Care and Use Committees of Beth Israel Deaconess Medical Center and Harvard Medical School.

2.2. Human tissue

Tissue from subjects with no clinical evidence of neurologic disease was provided by the Pathology Department of Beth Israel Deaconess Medical Center. Stomach, duodenum, ileum, rectum, and pancreas specimens were removed during surgeries and fixed in 10% formalin for 1 week. Studies of human tissues were approved by the Committee on Clinical Investigations of Beth Israel Deaconess Medical Center.

2.3. Whole mount technique

18 h after intraperitoneal treatment with colchicine (5 mg/kg) and food deprivation (in a first series, we did not treat mice with colchicine), mice were euthanized under ketamine/xylazine anesthesia, and enteric tissue was immediately removed and placed in sterile Krebs solution (in mmol: NaCl 117, KCl 5, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25, NaH₂PO₄ 1.2, glucose 10, bubbled with 95% O₂ and 5% CO₂, pH 7.4). After opening along the mesenteric border, the tissue was pinned in a Sylgardlined Petri dish, and mucosa and submucosa were carefully removed. Thereafter, the tissue was fixed for 1 h in 4% formalin in phosphate buffer at pH 7.4, then rinsed in phosphate-buffered saline (PBS), and stored in 0.05% PBS–sodium azide.

2.4. Sectioning

Mice were perfused transcardially with 150 ml of 0.9% saline followed by the same volume of 10% buffered formalin phosphate. After overnight equilibration in 20% sucrose, human and mouse tissue was cut on a cryostat in 20 µm sections. Brains were cut coronally, and gut tissue longitudinally. Sections were then mounted on gelatin-coated slides.

2.5. Single-label Immunohistochemistry for hypocretin and ChAT

After treatment with 0.3% hydrogen peroxide (1 h, in PBS with 0.5% Triton X-100) and blocking in 3% normal horse serum (2 h, in PBS–sodium azide containing Triton X-100), murine and human sections were incubated overnight in goat anti-hypocretin-1 (orexin-A) antiserum (1:2500; Santa Cruz Biotechnology, CA, USA, Lot# A2604, catalogue# sc-8070) or in goat anti-hypocretin-2 (orexin-B) antiserum (1:10,000; Santa Cruz, CA, USA, Lot# E1404, cat# sc-8071), followed after 6 PBS washes by biotinylated donkey anti-goat secondary antiserum at 1:500 (Jackson ImmunoResearch Laboratories, PA, USA, Lot# 71193, cat# 705-065-147). Higher concentrations of the primary antibody (1:200, 1:1000) and 48 h incubation did not improve sensitivity of staining, and a lower concentration (1:7500) reduced sensitivity. The tissue was then reacted for 1 h with ABC (1:500, Vector Laboratories, CA, USA), and diaminobenzidine (DAB, Vector Laboratories, CA, USA) with 1% hydrogen peroxide were used to produce a brown reaction product. Slides were examined with a Zeiss Axioplan 2 microscope.

This immunostaining clearly labeled hypocretin in mouse and human brain but not the gut, so we repeated the staining using antiserum from several prior reports of hypocretin in the gut (affinity-purified, polyclonal, rabbit anti-hypocretin-1 antiserum, Alpha Diagnostic International, TX, USA, Lot# 305960A, cat# OXA-11A, at 1:500, 1:2000, 1:10,000) [1–6], and using hypocretin-1 antiserum which was used in multiple previous publications [9,11,12] (a kind gift from Dr. Takeshi Sakurai).

Adjacent gut sections were immunostained with goat anti-ChAT antiserum (1:500, Millipore, MA, USA, Lot# 0608037072, cat# AB144P) and with biotinylated donkey anti-goat secondary antiserum (Jackson Laboratories, PA, USA, Lot# 71193, cat# 705-065-1147).

2.6. Double immunohistochemistry for hypocretin and ChAT

After incubation in 0.3% hydrogen peroxide and in 3% normal horse serum, sections were incubated overnight in rabbit anti-hypocretin-1 (orexin-A) antiserum (1:2000, Alpha Diagnostic International, TX, USA, Lot# 305960A, cat# OXA-11A), followed by biotinylated donkey anti-rabbit IgG (Jackson Laboratories, PA, USA, Lot# 74820, cat# 711-065-152, at 1:500), and Alexa 488 conjugated streptavidin (Invitrogen, CA, USA, Lot# 53729A, cat# S11223 at 1:1000). Sections were then incubated overnight in goat anti-ChAT antiserum (1:500; Millipore, MA, USA, Lot# 0608037072), and then in Cy3-conjugated donkey anti-goat IgG (Jackson Laboratories, PA, USA, Lot# 58839) at 1:500. Slides were examined with a Zeiss LSM 5 Pascal laser scanning confocal microscope.

3. Results

We did not find any definite hypocretin-immunoreactive (-IR) cells in sections of human or mouse stomach, duodenum, ileum, or rectum. Hypocretin immunoreactivity was also absent in flat-mounted preparations of mouse stomach, duodenum and ileum. This lack of hypocretin immunoreactivity was not due to technical problems as all three primary antisera clearly labeled numerous hypocretin-IR neurons in mouse and human perifornical and lateral hypothalami. The hypocretin immunoreactivity appeared specific at our working concentrations as each antiserum produced no labeling in hypocretin knockout mice. This lack of hypocretin-IR in gut tissue was not due to technical problems as ChAT-IR neurons were abundant in the myenteric and submucosal plexi of mouse and human stomach, duodenum and ileum (Fig. 1).

4. Discussion

Previous studies using RT-PCR and immunohistochemistry reported hypocretin-producing cells in gut tissue of different species. However, even though we replicated the immunostaining techniques of these studies [1–6] and tried other methods, we could not identify hypocretin-IR cells in murine or human enteric tissue. The cause of this discrepancy remains unclear. The prior reports may have been mistaken due to non-specific immunostaining or false positive results in RT-PCR. Unfortunately, our attempts to explore this question with the corresponding authors and other contributors to the reports on hypocretin in the gut remained unsuccessful.

The presence of hypocretin cells in the gut could have significant clinical implications for understanding narcolepsy. A loss of hypocretin-producing enteric neurons in people with narcolepsy could support an autoimmune etiology of the disorder. Furthermore, as enteric neurons are more accessible, loss of these neurons could enable earlier diagnosis and a better understanding of the pathology of narcolepsy.

Though we used several methods, we could not replicate the finding of hypocretin-IR cells in mouse or human gut tissue. Of course, we cannot be certain that the gut truly lacks hypocretin-

producing neurons, and we hope that these results will be verified by further work, e.g. by immunoprecipitation.

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References

1. Kirchgeßner AL, Liu M. Orexin synthesis and response in the gut. *Neuron* 1999;24:941–51. [PubMed: 10624957]
2. de Miguel MJ, Burrell MA. Immunocytochemical detection of orexin A in endocrine cells of the developing mouse gut. *J Histochem Cytochem* 2002;50:63–69. [PubMed: 11748295]
3. Naslund E, Ehrstrom M, Ma J, Hellstrom PM, Kirchgeßner AL. Localization and effects of orexin on fasting motility in the rat duodenum. *Am J Physiol Gastrointest Liver Physiol* 2002;282:G470–9. [PubMed: 11841997]
4. Ouedraogo R, Naslund E, Kirchgeßner AL. Glucose regulates the release of orexin-a from the endocrine pancreas. *Diabetes* 2003;52:111–7. [PubMed: 12502500]
5. Ehrstrom M, Naslund E, Levin F, Kaur R, Kirchgeßner AL, Theodorsson E, Hellstrom PM. Pharmacokinetic profile of orexin A and effects on plasma insulin and glucagon in the rat. *Regul Pept* 2004;119:209–12. [PubMed: 15120482]
6. Ehrstrom M, Gustafsson T, Finn A, Kirchgeßner A, Gryback P, Jacobsson H, Hellstrom PM, Naslund E. Inhibitory effect of exogenous orexin a on gastric emptying, plasma leptin, and the distribution of orexin and orexin receptors in the gut and pancreas in man. *J Clin Endocrinol Metab* 2005;90:2370–7. [PubMed: 15671114]
7. Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D, Albin R, Li R, Hungs M, Pedrazzoli M, Padigaru M, Kucherlapati M, Fan J, Maki R, Lammers GJ, Bouras C, Kucherlapati R, Nishino S, Mignot E. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat Med* 2000;(6):991–997. [PubMed: 10973318]
8. Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM. Reduced number of hypocretin neurons in human narcolepsy. *Neuron* 2000;27:469–74. [PubMed: 11055430]
9. Crocker A, Espana RA, Papadopoulou M, Saper CB, Faraco J, Sakurai T, Honda M, Mignot E, Scammell TE. Concomitant loss of dynorphin, NARP, and orexin in narcolepsy. *Neurology* 2005;65:1184–8. [PubMed: 16247044]
10. Scammell TE. The frustrating and mostly fruitless search for an autoimmune cause of narcolepsy. *Sleep* 2006;29:601–2. [PubMed: 16774147]
11. Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kangawa K, Sakurai T, Yanagisawa M, Nakazato M. Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. *Proc Natl Acad Sci U S A* 1999;96:748–53. [PubMed: 9892705]
12. Kunii K, Yamanaka A, Nambu T, Matsuzaki I, Goto K, Sakurai T. Orexins/hypocretins regulate drinking behaviour. *Brain Res* 1999;842:256–61. [PubMed: 10526122]

Fig. 1. Immunolabeling of enteric neurons. A. ChAT-IR neurons labeled with DAB in a whole mount of murine jejunum. B. Double immunohistochemistry labels ChAT-IR neurons in the myenteric plexus of murine gastric corpus (red, Cy3 label), but hypocretin-IR neurons (green, Alexa 488) are absent. Scale bars=150 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)