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Concomitant loss of dynorphin, NARP, and orexin in narcolepsy

Amanda Crocker, BS¹, Rodrigo A. España, PhD¹, Maria Papadopoulou, BS¹, Clifford B. Saper, MD, PhD¹, Juliette Faraco, PhD², Takeshi Sakurai, MD, PhD³, Makoto Honda, MD, PhD², Emmanuel Mignot, MD, PhD², and Thomas E. Scammell, MD¹

¹ Dept. of Neurology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, 617-667-0833

² Dept. of Psychiatry, Stanford University Center for Narcolepsy, Palo Alto, CA, 650-725-4913

³ Dept. of Pharmacology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan, +81-298-53-3276

Abstract

Background—Narcolepsy with cataplexy is associated with a loss of orexin/hypocretin. It is speculated that an autoimmune process kills the orexin-producing neurons, but these cells may survive yet fail to produce orexin.

Objective—To examine whether other markers of the orexin neurons are lost in narcolepsy with cataplexy.

Methods—We used immunohistochemistry and in situ hybridization to examine the expression of orexin, neuronal activity-regulated pentraxin (NARP), and prodynorphin in hypothalami from five control and two narcoleptic individuals.

Results—In the control hypothalami, at least 80% of the orexin-producing neurons also contained prodynorphin mRNA and NARP. In the patients with narcolepsy, the number of cells producing these markers was reduced to about 5–10% of normal.

Conclusions—Narcolepsy with cataplexy is likely caused by a loss of the orexin-producing neurons. In addition, loss of dynorphin and NARP may contribute to the symptoms of narcolepsy.

Introduction

Narcolepsy with cataplexy is associated with a loss of the hypothalamic neuropeptides orexin-A and -B (also known as hypocretin-1 and -2)^{1–3}. An unknown process markedly reduces the expression of orexin mRNA and immunoreactivity while sparing production of melanin-concentrating hormone in nearby cells^{1, 2}. Together with the known HLA association⁴, these observations suggest that an autoimmune or neurodegenerative process may selectively kill the orexin neurons. However, it is possible that the orexin neurons survive, yet fail to produce orexin.

The orexin neurons can also be identified by the presence of other signaling molecules. In rodents, these cells produce the endogenous opiate dynorphin⁵, and these two neuropeptides may act synergistically to increase the activity of wake-promoting brain regions⁶. The orexin neurons also contain glutamate⁷ and neuronal activity-regulated pentraxin (NARP)⁸, a secreted protein that regulates AMPA receptor clustering.

To critically test whether orexin neurons are lost in narcolepsy, we first examined whether dynorphin and NARP are produced in orexin neurons of normal human hypothalami. Using multiple techniques and markers, we then measured the expression of these peptides in the hypothalami of individuals with narcolepsy because a loss of these peptides would provide evidence for a loss of the orexin neurons, rather than a selective defect in orexin expression.

Methods

Human brain tissue

Brains from five control subjects with no clinical evidence of neurologic disease were provided by the Pathology Department of Beth Israel Deaconess Medical Center. Brains from two patients with narcolepsy with cataplexy were provided by Stanford University: Subject 321 developed narcolepsy at age 13 with daytime sleepiness and cataplexy. He had an average sleep latency of 2.2 minutes and 3 sleep onset REM periods on his Multiple Sleep Latency Test. He was treated with 50 mg/day of methamphetamine and died of a viral cardiomyopathy at age 49. Subject 325 had daytime sleepiness treated with 20 mg/day methylphenidate, cataplexy triggered by laughter and improved by 10 mg clomipramine, hypnagogic hallucinations, and sleep paralysis. These symptoms were present for at least 20 years before his death from cancer at age 72. Both narcoleptic subjects were HLA DQB1*0602 positive. The BIDMC and Stanford Committees on Clinical Investigations approved the research protocols.

All brains were removed within 24 hours of death and fixed in 10% formalin for one week. After equilibration in PBS with 20% sucrose, 10 % formalin, and 0.1% diethyl pyrocarbonate (DEPC) for 2–3 days, blocks were sectioned coronally at 40 μ m on a freezing microtome into a 1:24 series. Sections were then stored at -20°C in a cryoprotectant solution (a mixture of 25% glycerol, 35% ethylene glycol, and 40% DEPC PBS).

Immunohistochemistry for orexin combined with in situ hybridization histochemistry (ISHH) for prodynorphin

In brief, free-floating sections were reacted for 30 minutes with 3% hydrogen peroxide and incubated overnight in rabbit antiserum with affinity for orexin-A and -B (1:5,000)⁹. Sections were then incubated for 1 hr in biotinylated donkey anti-rabbit secondary antiserum (1:500; Jackson ImmunoResearch), and reacted for 1 hr with avidin/biotin complex (ABC Kit; Vector Labs). Tissue was reacted with 3,3'-diaminobenzidine (DAB) and 0.1% hydrogen peroxide to produce a light brown reaction product. All immunostaining solutions were treated with DEPC to neutralize endogenous RNA-degrading enzymes.

Using previously described ISHH techniques⁵, sections were then hybridized with ³⁵S-labeled riboprobes. A pCR4-TOPO plasmid containing bases 543–1734 of the human prodynorphin sequence (subcloned from a plasmid kindly provided by Y. Hurd, Karolinska Institute, Stockholm) was linearized with Pst 1 and transcribed with T7 polymerase to produce an antisense riboprobe. To make sense riboprobe, the plasmid was linearized using Not 1 and transcribed with T3 polymerase. After riboprobe hybridization, slides were exposed to photographic emulsion for 5 to 20 days. Emulsion silver grains at more than 3 times the background density were considered indicative of prodynorphin labeling.

To maximize sensitivity, adjacent hypothalamic sections from the narcoleptic subjects were single-labeled for orexin or prodynorphin. For simplicity, these results are plotted onto single sections in Figure 2A.

Double label immunohistochemistry for NARP and orexin-B

Sections mounted on slides were dried overnight and then boiled for 18 minutes in antigen retrieval buffer (CMX603; Cell Marque Corp.). After treatment with 3% hydrogen peroxide and blocking in 3% normal horse serum, sections were incubated overnight in rabbit anti-NARP antiserum (1:3,000; a kind gift from I. Reti, Johns Hopkins Medical Institute) and then in biotinylated donkey anti-rabbit secondary antiserum at 1:500. The tissue was reacted with ABC and DAB to produce a light brown reaction product. Sections were then incubated in goat anti-orexin-B antiserum (1:10,000; Santa Cruz Biotechnology) for 36 hours. After 3 hours of incubation with biotinylated donkey anti-goat secondary antiserum (1:500; Jackson Immunoresearch), sections were treated with ABC and SG Blue (Vector Labs) to produce a blue reaction product.

Adjacent sections were also immunostained for glial fibrillary acidic protein (GFAP) (data not shown), but in all brains, the density of GFAP-IR cells was so high near the third ventricle that no quantitative analysis could be performed.

Histologic controls

Both orexin antisera clearly labeled lateral hypothalamic neurons in sections from wild type mice but not in sections from orexin knockout mice (data not shown)¹⁰. The orexin and NARP antisera have been previously characterized^{8, 9}. The sense riboprobe produced no specific labeling.

Cell Counts and Analysis

Nuclear boundaries were defined on an adjacent series stained with thionin. All cell counts and mapping were done at 200× using a Neurolucida system on a Zeiss Axiophot microscope. Labeled neurons were counted in the entire hypothalamic section, and averaged across the three sections from the center of the orexin field. Cells in the paraventricular nucleus were counted separately because these cells contain prodynorphin and NARP but lie outside the orexin field.

Results

Co-localization of orexin with prodynorphin mRNA and NARP protein

Sections from five control individuals were immunostained for orexin followed by ISHH for prodynorphin mRNA. The immunostaining clearly labeled cell bodies and proximal dendrites, and ISHH with the prodynorphin antisense probe produced dense clusters of silver grains over neuronal cell bodies with little background signal.

Orexin immunoreactive (-IR) neurons were distributed in a pattern similar to that previously reported^{1, 2, 11}. Most orexin-IR neurons were found in the posterior and lateral hypothalamic areas, but the anterior, dorsomedial, and ventromedial hypothalamic nuclei also contained a few scattered cells (Figure 1). Sections from the center of the orexin field contained an average of 244 orexin-IR neurons (Table 1).

These same regions contained dense labeling for prodynorphin mRNA, and most of this signal co-localized with orexin. On average, 80% of orexin-IR neurons also contained prodynorphin mRNA, and the best-preserved tissue showed 94% co-localization. Neurons in the paraventricular nucleus contained only prodynorphin mRNA with no orexin immunoreactivity.

Sections from control individuals were also immunolabeled for NARP and orexin-B. The number and distribution of orexin-B-IR neurons were indistinguishable from that labeled by the other orexin antiserum. NARP immunoreactivity was present in 91% of the orexin-B-

containing neurons, and NARP also was expressed in the paraventricular nucleus, with a few scattered cells in the ventromedial nucleus.

These observations demonstrate that prodynorphin mRNA and NARP are expressed in the great majority of orexin neurons. With the exception of the paraventricular nucleus, these labels are not present in other nearby cells and thus serve as useful, specific markers of the orexin neurons.

Loss of orexin, prodynorphin, and NARP in narcolepsy

To maximize sensitivity, we first used single label immunostaining and ISHH to examine the expression of orexin and prodynorphin in the brains from two individuals with well-documented narcolepsy with cataplexy. Compared to the control subjects, case 321 had only 6% of the normal number of orexin-IR neurons and 11% of the normal number of prodynorphin neurons (Figure 2). As seen in normal brains, prodynorphin mRNA-containing cells were common in the paraventricular nucleus (15 cells/section vs. 14 in the controls) and abundant in the medial temporal lobe and striatum, suggesting that this loss of prodynorphin is limited to the orexin neurons. Case 325 showed a similar reduction, with 4% of the usual number of orexin-IR neurons, but the quality of this tissue was inadequate for prodynorphin ISHH.

Sections from these narcoleptic subjects were then double-labeled for NARP and orexin-B. The number of orexin-B-IR neurons was reduced to 4–5% of normal, closely matching the number seen with the other orexin antiserum. The number of NARP-IR neurons in the orexin field was reduced to only 5–7% of normal. In the few remaining orexin neurons, double labeling was similar to that in the normal hypothalami with most (68–74%) of the orexin-B-labeled neurons containing NARP. The number of NARP-IR cells in the paraventricular nucleus was normal (118 cells/section vs. 103 in the controls), thus serving as a useful anatomic control.

Discussion

Our observations demonstrate that most orexin neurons in the human hypothalamus produce prodynorphin mRNA and NARP protein. Moreover, in patients with narcolepsy, the number of neurons expressing these gene products is reduced in proportion to the loss of orexin neurons. These observations suggest that narcolepsy with cataplexy is caused by a loss of the orexin neurons themselves, rather than a failure to produce orexin.

Several lines of evidence now support this hypothesis. Disrupted orexin signaling is sufficient to produce the major symptoms of narcolepsy in several animal models^{10, 12–14}. In mice lacking the orexin neurons¹³, the orexin field completely lacks prodynorphin mRNA⁵ and NARP (R.E.; data not shown), further supporting the co-localization of these markers. We now show that within the orexin field of people with narcolepsy, the number of neurons containing prodynorphin mRNA, orexin, or NARP is reduced to 5–10% of normal. This cell loss is probably limited to the orexin neurons because we detected no loss of NARP or prodynorphin outside the orexin field. Although the orexin neurons might fail to produce multiple peptides, these observations make selective neurodegeneration or an autoimmune attack on the orexin neurons much more likely.

Three methodologic limitations warrant comment. First, we examined brains from only two individuals with narcolepsy, but our observations are consistent with a prior report showing a marked reduction in the number of orexin-IR neurons². Our two patients have not been previously reported, and thus our neuropathologic observations contribute significantly to the six cases already published^{1, 2, 15}. Second, our narcoleptic subjects were treated at some time with amphetamines, and methamphetamine can transiently increase prodynorphin mRNA in the striatum and hypothalamus of rats^{16–18}. However, ectopic expression of dynorphin is

unlikely to have occurred as the number of hypothalamic neurons producing prodynorphin and orexin were similarly reduced. Third, mRNA may have degraded in some brains with longer post mortem intervals. We detected prodynorphin mRNA in 80% of orexin neurons on average, but the brains with the shortest intervals had > 90% co-localization, suggesting that most or perhaps all orexin neurons contain dynorphin. Prodynorphin mRNA also may be expressed at low levels in a few other regions of the human hypothalamus¹⁹, and the more limited distribution of labeling in our study was probably also caused by a post mortem loss of mRNA.

Loss of dynorphin and NARP may contribute to the symptoms of narcolepsy. Though selective disruption of the orexin gene is sufficient to produce sleepiness and cataplexy^{10, 14}, NARP may enable the orexin neurons to promote wakefulness by enhancing glutamate signaling^{7, 20}. In addition, dynorphin and other products of the prodynorphin gene bind to opiate receptors, and opiates can improve the subjective sleepiness and cataplexy of narcolepsy^{21–23}. Most importantly, mice simply lacking orexin have sleepiness and cataplexy, but mice lacking the orexin neurons have obesity as well^{13, 24}, suggesting that loss of dynorphin or other signaling molecules contributes to the mild obesity of human narcolepsy^{25, 26}. In the near future, narcolepsy may be treated with orexin agonists, but restoration of dynorphin and NARP signaling may also be necessary to correct all the symptoms of narcolepsy.

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Figure 1.

Prodynorphin and NARP co-localize with orexin. A) In coronal hypothalamic sections from a control subject, most neurons containing orexin-IR also produce prodynorphin mRNA (black circles) and are mainly located in the lateral and posterior hypothalamic areas (LHA and PHA) above and around the fornix (fx). A few neurons apparently containing only orexin (white circles) or prodynorphin (grey circles) are scattered within this region, and a separate group of neurons producing only prodynorphin mRNA lie in the paraventricular nucleus of the hypothalamus (PVH). B) Neurons double-labeled for orexin-B and NARP (black circles) have a similar distribution. This region also contains a few neurons apparently containing only orexin-B (white circles) or only NARP (grey circles). The PVH contains many cells producing only NARP. Other abbreviations: 3V, third ventricle; opt, optic tract; LT, lateral tuberal nucleus; VMH, ventromedial hypothalamic nucleus; DMH, dorsomedial hypothalamic nucleus; TM, tuberomammillary nucleus; SO, supraoptic nucleus; st, stria terminalis; IGP, internal globus pallidus; MMC, mammillary nucleus, magnocellular part; SUM, supramammillary nucleus; cp, cerebral peduncle.

Figure 2.

Loss of prodynorphin and NARP in narcolepsy. A) Sections from a subject with narcolepsy show a marked reduction in the number of neurons labeled for orexin (white circles). Within the orexin field, the number of neurons expressing prodynorphin mRNA (grey circles) is also reduced, but the paraventricular nucleus still contains a moderate number of prodynorphin-labeled cells. B) This individual also has a substantial loss of neurons double-labeled for orexin-B and NARP (black circles) within the orexin neuron field. This region also contains a few cells apparently containing only orexin-B (white circles) or only NARP (grey circles), but the PVH still contains a large number of neurons producing NARP alone.

Table 1

Prodynorphin, orexin, and NARP cell counts in the orexin neuron field

Subject Number	Age/sex	Prodynorphin mRNA- containing neurons/section	Orexin- IR neurons/ section	% of orexin-IR neurons containing prodynorphin	NARP- IR neurons/ section	Orexin-B- IR neurons/ section	% of orexin-B- IR neurons containing NARP
Control subjects							
302	59 male	321	327	94.1%	328	320	94.2%
304	67 male	182	196	87.8%	222	192	86.2%
326	49 male	137	243	54.4%	255	251	90.9%
327	34 female	170	222	73.5%	227	209	93.2%
329	72 female	232	234	90.2%	350	336	91.1%
Mean ± SEM		208 ± 32	244 ± 22	80.0 ± 7.2%	276 ± 26	262 ± 29	91.1 ± 1.4%
Narcolepsy subjects							
321	49 male	22 (10.6%)	15 (6.1%)	ND	21 (7.6%)	14 (5.3%)	73.60%
325	72 male	ND	9 (3.7%)	ND	14 (5.1%)	11 (4.2%)	67.6%