Orexins (also called hypocretins) are peptide neurotransmitters expressed in neurons of the lateral hypothalamic area (LHA). Mice lacking the orexin peptides develop narcolepsy-like symptoms, whereas mice with a selective loss of the orexin neurons develop hypophagia and severe obesity in addition to the narcolepsy phenotype. These different phenotypes suggest that orexin neurons may contain neurotransmitters besides orexin that regulate feeding and energy balance. Dynorphin neurons are common in the LHA, and dynorphin has been shown to influence feeding; hence, we studied whether dynorphin and orexin are colocalized. In rats, double-label in situ hybridization revealed that nearly all (94%) neurons expressing prepro-orexin mRNA also expressed prodynorphin mRNA. The converse was also true: 96% of neurons in the LHA containing prodynorphin mRNA also expressed prepro-orexin mRNA.

Double-label immunohistochemistry confirmed that orexin-A and dynorphin-A peptides were highly colocalized in the LHA. Wild-type mice and orexin knock-out mice showed abundant prodynorphin mRNA-expressing neurons in the LHA, but orexin/ataxin-3 mice with a selective loss of the orexin neurons completely lacked prodynorphin mRNA in this area, further confirming that within the LHA, dynorphin expression is restricted to the orexin neurons. These findings suggest that dynorphin-A may play an important role in the function of the orexin neurons.

Key words: orexin; hypocretin; dynorphin; narcolepsy; obesity; feeding

The neuropeptides orexin-A and orexin-B (also called hypocretin-1 and -2) are expressed in neurons of the lateral hypothalamic area (LHA), with a few orexin cells extending into the dorsomedial hypothalamic nucleus (DMH). Orexins may play a major role in regulating arousal, and lack of the orexin peptides or receptors is associated with symptoms of narcolepsy in mice (Chemelli et al., 1999), dogs (Lin et al., 1999), and humans (Nishino et al., 2000; Peyron et al., 2000; Than nickal et al., 2000). Orexin may not be the only functionally important neurotransmitter in these neurons: transgenic mice in which the orexin neurons are selectively destroyed develop not only narcolepsy, but also hypophagia and marked obesity (Hara et al., 2001), deficits only mildly evident in mice lacking the orexin peptides.

Although LHA neurons express a variety of neurotransmitters, few have been identified within the orexin neurons. Orexin does not colocalize with melanin-concentrating hormone, cocaine and amphetamine-related transcript, or nitric oxide synthase (Peyron et al., 1998; Cutler et al., 2001; Elias et al., 2001). The neuropeptide galanin is found in some orexin neurons, but many orexin neurons lack galanin (Hakansson et al., 1999). Orexin terminals innervating the tuberomammillary nucleus contain glutamate (F. Torrealba and C. B. Saper, personal communication), but orexin terminals in other areas have not been examined. Finally, dynorphin is expressed in many LHA neurons and has been implicated in the regulation of feeding (Gosnell et al., 1986), but its colocalization with orexin has not been directly examined.

To determine if orexin neurons contain dynorphin, we performed double-label in situ hybridization for prepro-orexin and prodynorphin mRNA. To confirm colocalization of the respective neuropeptides, we also performed double-label immunohistochemistry for orexin-A and dynorphin-A. Finally, we examined the colocalization of orexin and dynorphin in wild-type mice, in orexin knock-out mice, and in orexin/ataxin-3 mice that have a selective loss of orexin neurons.
MATERIALS AND METHODS

Animals and tissue. All work was approved by the Animal Care and Use Committees of Harvard Medical School and University of Texas Southwestern Medical Center. Experiments used Sprague-Dawley rats, 275–300 gm (Harlan, Indianapolis, IN), C57BL/6 (Harlan), orexin knock-out mice, orexin/ataxin-3 transgenic mice, or the wild-type littermates of these mice. Orexin knock-outs and littermates were 16 weeks old, whereas orexin/ataxin-3 mice and littermates were 38–42 weeks old. Orexin knock-out mice and wild-type littermates were genetically similar to those previously described (50% C57BL/6, 50% 129SvEv) (Chemelli et al., 1999). The orexin/ataxin-3 mice contain a transgene in which the human orexin promoter drives expression of a CAG-elongated form of ataxin-3 protein that results in the gradual and selective loss of orexin neurons by early adulthood (Hara et al., 2001). By 12 weeks of age, no orexin peptide or mRNA can be detected in the brains of these mice. The background strain of the orexin/ataxin-3 mice was 75% C57BL6 and 25% DBA1.

Animals were transcardially perfused with 4% paraformaldehyde, and brains were removed and equilibrated overnight in PBS with 20% sucrose in diethylpyrocarbonate-treated water. Brains were sectioned on a freezing microtome into five series of 30 μm sections (three series for mice). Rats used for double-label immunohistochemistry received the axonal transport blocker colchicine (1.5% in 120 mM NaCl; Schemel et al., 1999). The orexin/ataxin-3 mice contain a transgene in which the human orexin promoter drives expression of a CAG-elongated form of ataxin-3 protein that results in the gradual and selective loss of orexin neurons by early adulthood (Hara et al., 2001). By 12 weeks of age, no orexin peptide or mRNA can be detected in the brains of these mice. The background strain of the orexin/ataxin-3 mice was 75% C57BL6 and 25% DBA1.

Riboprobes and hybridization buffer for in situ hybridization. psP6 plasmid containing bases 270–1988 of the rat preproorexin cDNA sequence (Civelli et al., 1985) (a kind gift from A. Watts, University of Southern California) was linearized with EcoRI and transcribed with SP6 polymerase to produce antisense mRNA riboprobes for use on rat tissue. psP6 plasmid containing the same insert in the opposite direction was used to produce sense riboprobes by linearizing with PstI and transcribing with SP6 polymerase. The prodynorphin riboprobe used for in situ hybridization on mouse tissue was constructed from a 446 bp fragment of an expressed sequence tag (GenBank accession number AA066590) containing bases 293–738 of the prodynorphin coding region (Josefsen et al., 1998). This fragment was amplified using the polymerase chain reaction (left primer, TCCGATGAGTTCAGGAT; right primer, CCGAACTCTCTTGGGGTAT) and inserted into the pcR4 TOPO plasmid (Invitrogen, San Diego, CA). This plasmid was linearized with NotI or PstI and transcribed with T3 or T7 polymerase to produce antisense or sense riboprobes, respectively.

The prepro-orexin riboprobe was generated from a Bluescript II SK(+) plasmid containing bases 97–384 of the rat prepro-orexin cDNA sequence as previously described (Sakurai et al., 1998). Across this fragment, the rat and mouse cDNA sequences are 98% homologous [Basic Local Alignment Search Tool (BLAST), National Institutes of Health], making this probe suitable for hybridization in both species. This plasmid was linearized with BamHI and transcribed with T7 to produce antisense probe.

All riboprobes were described using nucleotides labeled with either 35S or digoxigenin and were diluted in hybridization buffer as previously described (Simmons et al., 1989; Kerner et al., 1998). Radiolabeled riboprobes were diluted to give 3 × 106 counts per 125 μl, whereas digoxigenin-labeled probes were diluted 1:100 in hybridization buffer.

Double-label in situ hybridization. Double-label in situ hybridization procedures were adapted from previously described methods (Simmons et al., 1989; Kerner et al., 1998; Elias et al., 2001). Slight modifications included replacement of the proteinase K pretreatment with microwave pretreatment of tissue for 10 min on high heat in citrate buffer, pH 6.0, as previously described (Marcus et al., 2001). Sections were incubated in hybridization buffer containing both orexin and dynorphin riboprobes for 12–16 hr at 56°C, followed by overnight incubation in alkaline-phosphatase-conjugated sheep anti-digoxigenin antibodies (1:5000 dilution; Roche Products, Hertfordshire, UK). Blue–purple reaction product was visualized by incubation for 4–8 hr in nitroblue tetrazolium chloride (Roche) (Kerner et al., 1998). Slides were coated with a thin layer of parlodion (Sigma) to prevent chemical interactions between the alkaline phosphatase reaction product and photographic emulsion–developer. Slides were dipped in NTB2 photographic emulsion (International Bio-technologies, New Haven, CT), stored in the dark for 4 d, and developed with D-19 developer (Eastman Kodak, Rochester, NY) for 2 min. No specific labeling was seen when the antisense riboprobes were omitted or replaced with sense probes.

RESULTS

Dynorphin and orexin colocalization in the rat

Hypothalamic sections from four rat brains were processed for double-label in situ hybridization for prepro-orexin and prodynorphin. Almost all neurons expressing prepro-orexin mRNA (94 ± 2% SEM) also expressed prodynorphin mRNA (Fig. 1A–E). In the LHA and DMH (excluding the pars compacta), the converse was also true: almost all prodynorphin mRNA-expressing neurons (96 ± 1%) also expressed prepro-orexin mRNA. This high degree of colocalization was maintained throughout the entire extent of the orexin cell population. Many neurons expressing prodynorphin were seen in adjacent hypothalamic areas, such as the supraoptic nucleus, the paraventricular hypothalamic nucleus and ending at the premammillary area. These sections included the entire orexin field, with no orexin neurons occurring outside of these sections. The LHA was defined broadly to include all hypothalamic regions lateral to the DMH and ventromedial hypothalamic nuclei, with the exception of the supraopticus nucleus. We also counted orexin and dynorphin cells within the DMH, except for the pars compacta of the DMH, which contains many dynorphin-expressing neurons that are morphologically and cytoarchitecturally distinct from dynorphin neurons in the LHA. Nuclear landmarks were clearly identifiable under dark-field illumination.

Double-labeled immunoreactive cells were counted under fluorescent episcopic illumination. To avoid false detection of double-labeled neurons caused by antibody cross-reactivity, we only counted neurons in which immunoreactivities for both dynorphin (red immunofluorescence) and orexin (green immunofluorescence) were relatively intense. In particular, neurons showing strong dynorphin immunoreactivity, but comparatively weak orexin immunoreactivity (which was stained second, and therefore most likely to be artifactual) were conservatively interpreted as single-labeled dynorphin-immunoreactive neurons. No double-labeled neurons were evident when either primary antisera was omitted.

To confirm whether dynorphin and orexin peptides were colocalized, three brains from colchicine-treated rats were processed for double-label immunohistochemistry. The distributions of orexin-A and dynorphin-A-immunoreactive neurons were almost identical to the mRNA distributions seen with in situ hybridization. Nearly all orexin-immunoreactive neurons (94 ± 2%) were dynorphin-immunoreactive (Fig. 2). Conversely, within the LHA and DMH (excluding the pars compacta of the DMH) almost all dynorphin-A-immunoreactive neurons (97 ± 1%) were
We found that nearly all orexin neurons contain dynorphin at both the mRNA and protein levels in rats, and that in the LHA, dynorphin is only expressed in the orexin neurons. Wild-type mice showed a similarly high degree of colocalization of dynorphin and prepro-orexin mRNA. By comparison, orexin knock-out mice lacked prepro-orexin mRNA but still had abundant prodynorphin mRNA-expressing neurons in the LHA, whereas orexin/ataxin-3 mice lacked both prepro-orexin and prodynorphin mRNA in the LHA.

**DISCUSSION**

We minimized several common sources of artifactual double-labeling. To avoid cross-hybridization between riboprobes, we used riboprobes of at least several hundred bases in length that have no significant similarities to each other or to other known rat or mouse genes (verified using BLAST). To limit chemical interactions between the alkaline phosphatase immunohistochemical reaction product and overlying photographic emulsion, we coated the tissue with a thin layer of parlodion before applying the emulsion. The effectiveness of these procedures was shown by the presence of some single-labeled neurons of both types in all double-label in situ hybridizations using wild-type animals and by the lack of double-labeling when either riboprobe was omitted. Finally, the use of orexin-A and dynorphin-A primary antisera that were both made in rabbits raised the possibility of artifactual double-labeling caused by antibody cross-reactivity. However, histochemical controls and stringent counting criteria eliminated artifactual double-labeling, as seen in trials in which one primary antibody was omitted.

The current findings corroborate previous observations showing that dynorphin immunoreactivity in the LHA colocalizes with immunoreactivity for a particular antisera raised against prolactin (Griffond et al., 1993), although prolactin immunoreactivity in the LHA is not detected by monoclonal antibodies to prolactin (Harlan and Scammell, 1991). The prolactin antisera used by Griffond et al. (1993) was later shown to recognize a fragment of the prepro-orexin peptide (Risold et al., 1999), indirectly suggesting that dynorphin in LHA neurons might colocalize with orexin. However, the polyclonal antisera used by Griffond et al. (1993) might have recognized additional antigens as well. By using double-label in situ hybridization and transgenic animals, we avoided problems with antigen cross-reactivity.

**Functional implications**

Although orexin is produced mainly within neurons of the LHA, dynorphin neurons are found in many other hypothalamic regions, as well as in the cortex, striatum, and brainstem. Dynorphin probably mediates many functions in the brain, but within the normal C57BL/6 mice, prodynorphin mRNA was present in nearly all prepro-orexin-containing neurons, whereas nearby regions such as the supraoptic nucleus and pars compacta of the DMH contained many prodynorphin-expressing neurons that never expressed prepro-orexin mRNA. In three orexin knock-out mice, labeling for prepro-orexin was completely absent, but the distribution and number of prodynorphin-containing neurons in the LHA was the same as seen in their wild-type littermates (Fig. 3). In three orexin/ataxin-3 mice, both prepro-orexin and prodynorphin labeling were completely absent from the LHA, whereas the number of prodynorphin-containing cells in adjacent regions was similar to that seen in their wild-type littermates.
orexin neurons, dynorphin may play a role that is coordinated with that of orexin. Dynorphin levels in the hypothalamus increase markedly at night, when rats are mostly awake (Przewlocki et al., 1983). Similarly, Fos expression increases in orexin neurons at night (Scammell et al., 2000; Estabrooke et al., 2001), and CSF levels of orexin are also increased at night (Fujiki et al., 2001). Food deprivation increases prepro-orexin mRNA (Cai et al., 1999), whereas combined deprivation of food and water increases hypothalamic dynorphin levels (Przewlocki et al., 1983). Dynorphin fibers are found in most brain regions containing orexin fibers, including the monoaminergic nuclei such as the tuberomammillary nucleus, raphe nuclei, and locus coeruleus (Fallon and Leslie, 1986; Peyron et al., 1998; van den Pol, 1999), suggesting that dynorphin may be released at similar places and times as orexin.

Orexin knock-out mice have a modest reduction in food intake and a mild tendency toward obesity (Chemelli et al., 2001; Willie et al., 2001). Orexin/ataxin-3 mice have more severe obesity and hypophagia than orexin knock-out mice (Hara et al., 2001). These different phenotypes may be attributable to the different testing environments and background strains of the mice or because the orexin/ataxin-3 mice are normal at birth and may have fewer compensatory adaptations. However, another intriguing possibility is that the more severe abnormalities of the orexin/ataxin-3 mice are attributable to loss of other neurotransmitters in the orexin neurons that regulate feeding and metabolic activity. Consistent with this hypothesis, feeding is promoted by injections of dynorphin-A or dynorphin agonists either systemically or into hypothalamic regions where orexin terminals are abundant (Morley and Levine, 1981; Gosnell et al., 1986). The effects of dynorphin on body weight and metabolism are unknown; prodynorphin knock-out mice have been reported (Sharifi et al., 2001), but have not been tested for changes in feeding, metabolic activity, or energy balance.

Orexin and dynorphin may also have related effects on sleep–wake regulation. Injections of orexin-A into the locus coeruleus increase wakefulness and decrease REM sleep (Bourgin et al., 2000). Although dynorphin is mainly a κ-opioid agonist, it also binds to the μ-opioid receptor, and pontine injections of a μ receptor agonist also decrease REM sleep (Cronin et al., 1995).

Despite their coordinated actions, orexin and dynorphin may have paradoxically opposing electrophysiological effects. Orexin excites neurons in the locus coeruleus (Horvath et al., 1999; Ivanov and Aston-Jones, 2000), whereas dynorphin inhibits these neurons via the κ-opioid receptor (Beacham et al., 2000). Orexin increases intracellular calcium levels in dopaminergic neurons (Nakamura et al., 2000), presumably a sign of neuronal activation, whereas dynorphin inhibits firing of cultured dopaminergic neurons (Ronken et al., 1993). Orexin excites neurons in the dorsal raphe nucleus (Brown et al., 2001) and the tuberomammillary nucleus (Eriksson et al., 2000), but the effects of dynorphin on these neurons are unknown.

The perspective of our experiments and discussion has focused on dynorphin-A, but the prodynorphin gene also produces dynorphin-B (also called rimorphin), leumorphin, α neo-endorphin and β neo-endorphin (Suda et al., 1983; James et al., 1984). These peptides are also κ agonists and may augment the actions of dynorphin-A.

Our findings may have implications for understanding narcolepsy in humans. Many narcoleptics with cataplexy have extremely low concentrations of orexin-A in their CSF (Nishino et al., 2000), and postmortem studies show a loss of orexin mRNA and peptide in the hypothalamus as well (Peyron et al., 2000;
Thannickal et al., 2000). However, it is unknown whether orexin neurons are absent from the brains of narcoleptics or whether the neurons are simply failing to produce orexin mRNA and peptides. If orexin and dynorphin are colocalized in normal human brains, then the presence or absence of dynorphin could establish whether the orexin neurons, or just orexin expression, is lost in human narcolepsy.

The nearly complete colocalization of dynorphin with orexin in the LHA suggests that dynorphin may play an important role in the regulation of rapid eye movement sleep through activation of the locus coeruleus. Dynorphin in the LHA is abundant in wild-type and orexin knock-out mice but completely absent in the orexin/ataxin-3 mice. Scale bar, 500 μm.

Figure 3. Dark-field photomicrographs showing silver grains corresponding to prodynorphin hybridization in wild-type mice (A), orexin knock-out mice (B), and orexin/ataxin-3 transgenic mice (C), which have a selective loss of orexin neurons. Prodynorphin expression in the LHA is abundant in wild-type and orexin knock-out mice but completely absent in the orexin/ataxin-3 mice. Scale bar, 500 μm.

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The nearly complete colocalization of dynorphin with orexin in the LHA suggests that dynorphin may play an important role in the function of the orexin neurons. Orexin neurons may regulate sleep–wake behavior, feeding, and metabolic activity, and dynorphin may influence some of these behaviors. Dynorphin in the orexin neurons may also play other roles that have not yet been explored. Further research, including the development of transgenic mice in which the prodynorphin gene is disrupted only in the orexin neurons, is needed to help elucidate the role of dynorphin within the orexin cells.

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