Orexin/Hypocretin Excites the Histaminergic Neurons of the Tuberomammillary Nucleus

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The hypothalamic orexin (hypocretin) neuropeptides are associated with the regulation of sleep and feeding, and disturbances in orexinergic neurotransmission lead to a narcoleptic phenotype. Histamine has also been shown to play a role in the regulation of sleep and feeding. Therefore, we studied the relationship between the orexin and histamine systems of the CNS using electrophysiology, immunocytochemistry, and the reverse transcriptase (RT)-PCR method.

Both orexin-A and orexin-B depolarized the histaminergic tuberomammillary neurons and increased their firing rate via an action on postsynaptic receptors. The depolarization was associated with a small decrease in input resistance and was likely caused by activation of both the electrogenic Na^+/Ca^{2+} exchanger and a Ca^{2+} current. In a single-cell RT-PCR study using primers for the two orexin receptors, we found that most tuberomammillary neurons express both receptors and that the expression of the orexin-2 receptor is stronger than that of the orexin-1 receptor. Immunocytochemical studies show that the histamine and orexin neurons are often located very close to each other. The contacts between these two types of neurons seem to be reciprocal, because the orexin neurons are heavily innervated by histaminergic axons. These results suggest a functional connection between the two populations of hypothalamic neurons and that they may cooperate in the regulation of rapid-eye-movement sleep and feeding.

Key words: orexin; orexin receptors; histamine; tuberomammillary; electrophysiology; PCR

The tuberomammillary (TM) neurons in the posterior hypothalamus send out varicose axons that innervate most parts of the CNS and release histamine (HA) (Panula et al., 1984). The activity of the TM neurons is strongly associated with behavioral state, and they fire tonically in a regular pattern during waking, little during slow-wave sleep, and not at all during rapid-eye-movement (REM) sleep (Vanni-Mercier et al., 1984). The TM neurons are most likely inhibited during sleep by the prominent GABAergic and galaninergic inputs they receive from the sleep-active neurons in the ventrolateral preoptic area, and both GABA and galanin inhibit the TM neurons (Schonrock et al., 1991; Yang and Hatton, 1997; Sherin et al., 1998; Stevens et al., 1999). The physiological functions in which the HAergic system has been implicated include the regulation of waking and feeding behaviors (Onodera et al., 1994; Brown et al., 2001b). Several pharmacological studies have shown that HA influences the ability to sustain waking. Thus, treatment with an inhibitor of HA synthesis leads to increased slow-wave sleep and REM sleep in rats and increased slow-wave sleep in cats (Lin et al., 1988; Itowi et al., 1991). The effect on waking seems to be mediated by the H_1 receptor, because H_1 agonists decrease all phases of sleep, whereas H_1 antagonists increase sleep (Monti et al., 1986, 1994; Lin et al., 1988). Histamine is also involved in the control of feeding, with food intake being depressed by activation of H_1 receptors, whereas either treatment with H_1 antagonists or inhibition of HA synthesis increases feeding in rats (Mercer et al., 1996; Sakata et al., 1997).

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The orexin/hypocretin peptides are produced by neurons within and around the lateral and posterior hypothalamus (de Lecea et al., 1998; Sakurai et al., 1998). Central administration of orexin-A increases arousal (Hagan et al., 1999), whereas a disrupted orexin system leads to narcolepsy. Dogs with a mutated orexin-2 receptor (OR_2) and orexin knock-out mice are both narcoleptic (Chemelli et al., 1999; Lin et al., 1999), whereas human narcolepsy is associated with an almost complete loss of orexin neurons and a lack of orexin-A in the CSF (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000). In addition to its arousing action, orexin-A stimulates feeding (Sakurai, 1999; Rodgers et al., 2000), although the effect is weak and may be a secondary effect of an increased metabolic rate (Lubkin and Stricker-Krongrad, 1998; Edwards et al., 1999).

The wake-promoting modafinil induces Fos expression in the TM nucleus and orexin neurons of the rat (Scammell et al., 2000), which suggests a functional connection between these two neuronal populations. Furthermore, the TM neurons are densely innervated by orexin-containing fibers (Peyron et al., 1998; Chemelli et al., 1999), and the orexin peptides have been implicated in physiological roles similar to those of HA. To investigate the interrelationship between these two systems further, we have studied the orexinergic inputs to the TM nucleus using intracellular recordings and single-cell PCRs from TM neurons as well as immunocytochemical methods.

MATERIALS AND METHODS

Electrophysiology. Experiments were performed on posterior hypothalamic slices from 3- to 4-week-old male Wistar rats. After decapitation, the brain was placed in an ice-cold solution made up of (in mM): 208 sucrose, 26 $NaHCO_3$, 10 glucose, 2.1 $MgCl_2$, 1.8 KCl, 1.5 $CaCl_2$, and 1.2 KH_2PO_4. The solution was bubbled to a pH of 7.4 with a mixture of 95% O_2 and 5% CO_2. Slices were cut at a thickness of 300–400 μm as described previously (Stevens and Haas, 1996) and transferred to artificial CSF (ACSF), which had the same composition as above except that

the sucrose was replaced with 125 mM NaCl. The slices were held in ACSF at room temperature (22–24°C) for at least 1 hr before starting the recording. The slices were then transferred to a thermostat-controlled recording chamber, in which they were submerged in ACSF with a flow rate of 1.8–2.0 ml/min^{-1} and a temperature of 32–33°C. Intracellular recordings were obtained using sharp glass microelectrodes filled with K^+ acetate (4 M) with resistances of 90–130 MΩ. Data acquisition was done with an Axoclamp-2A amplifier and a Digidata 1200 interface board (Axon Instruments, Foster City, CA). After additional amplification, the signal was fed to a chart recorder and to a PC running Clampex 7 software (Axon Instruments).

All chemicals were from Merck (Darmstadt, Germany) except orexin-A and orexin-B (Bachem, Heidelberg, Germany), *N*-methyl-D-glucamine (NMDG) and $NiCl_2$ (Sigma, Steinheim, Germany), tetrodotoxin (TTX; Alomone Laboratories, Jerusalem, Israel), and KB-R7943 mesylate (Tocris, Bristol, UK). All tested compounds were applied by bath.

The recordings were made from the lateral part of the TM nucleus, where the density of HAergic neurons is very high (Panula et al., 1984; Staines et al., 1987). Haas and Reiner (1988) have described the electrophysiological features of immunocytochemically identified HAergic neurons; they have also shown that this type of neuron predominates in the TM nucleus.

We used the following electrophysiological criteria to identify TM neurons. They should exhibit a regular, spontaneous firing rate (typically 2–8 Hz) and an absence of burst firing. Furthermore, they should have a resting membrane potential of approximately −50 mV, a broad action potential with a Ca^{2+} shoulder on the downstroke, and a long after-hyperpolarization. Finally, an inward current should be activated during a large hyperpolarizing step and a transient outward K^+ current should be activated after the step. Curve fitting was performed according to the following equation: $Y = Y_{min} + (Y_{max} - Y_{min})/(1 + 10^{((LogEC50 - Log\ [orexin]) \times Hill\ slope)})$ with Prism 3.0 (GraphPad Software, San Diego, CA). *Y* indicates depolarization in millivolts. Data were tested for significance with Student's two-tailed *t* test. All values are given as means ± SEM.

Cellular RNA harvest and RT-PCR. For preparation of isolated cells, the lateral part of the TM nucleus was dissected from the slice and incubated with papain (Sigma) in crude form (0.3–0.5 mg/ml) for 40 min at 37°C. Thereafter, the tissue was placed in a bath solution with the following composition (in mM): 150 NaCl, 3.7 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, and 10 HEPES, pH 7.4. Cells were separated by gentle pipetting. Neurons visually selected on an inverted microscope were digitally photographed and approached with a patch electrode and a gigaohm seal was obtained. After establishing the whole-cell configuration with the patched neuron, its cytoplasm was sucked into the electrode. This was done under visual control to ensure that only the cytoplasm and not the entire neuron entered the electrode. The electrodes were fabricated from thick-walled borosilicate glass tubes and had resistances of 2–5 MΩ after filling with solution (in mM: 140 CsCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, and 10 HEPES/CsOH), sterilizing by autoclave, and adjusting to a pH of 7.2 The cells were voltage-clamped by an EPC-9 amplifier (HEKA Electronics, Lambrecht, Germany) at the potential −55 mV. Cell identification was verified by reverse transcriptase (RT)-PCR analysis of histidine decarboxylase (HDC) expression. The protocols of the RT reaction and PCR amplification were similar to those described previously (Vorobjev et al., 2000). The primers designed to recognize HDC cDNA have been described previously (Sergeeva et al., 2001); they were flanking a region with three introns, with the genomic DNA fragment size amounting to 6031 bp, versus 457 bp for the cDNA size. Sequencing of the amplification products, which was done on an automatic sequencer (model 377; Applied Biosystems International, Weiterstadt, Germany), revealed the identity as the known rat HDC-cDNA sequence (GenBank accession number M29591). The thin-walled PCR tubes contained a mixture of first-strand cDNA template (2–5 μl), 10× PCR buffer (5 μl), a 10 pM concentration each of sense and antisense primer, and a 200 μM concentration each of deoxyNTP (dNTP) and 2.5 U of *Taq* polymerase. The final reaction volume was adjusted to 50 μl with nuclease-free water (Promega, Madison, WI). The Mg^{2+} concentration was 1.5 mM for all reactions. The *Taq* enzyme, PCR buffer, Mg^{2+} solution, and four dNTPs were all purchased from Qiagen (Erkrath, Germany). All oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany) and amplification was performed on a thermal cycler (GenAmp 9600; Perkin-Elmer, Weiterstadt, Germany). A two-round amplification strategy was used in each protocol. In each round 35 cycles of the following thermal programs were used: denaturation at 94°C for 48 sec, annealing at 53°C for 1 min, and extension at 72°C for 90 sec. For the second amplification round, 1 μl of the product from the first amplification was used as a template. The following primers were used for the PCR analysis of orexin receptor expression: in the first round of amplification the degenerated primer "dg up" [5′-CTGGC(AT)GATGTGCT(GT)GTGAC-3′] was taken either with OR_1 cDNA-specific lower 1 primer (5′-AACAGCAGAGGGTGGCAGAT-3′) or with OR_2 cDNA-specific lower 1 primer (5′-TGGCTGTGCTCTTGAACATC-3′). For the second round of cDNA amplification, the primers for the OR_1 were upper 2 (5′-TGTTAGTGGACATCACCGAATC-3′) and lower 2 (5′-TGAAGCTGAGAGTCAGCACTG-3′); for the OR_2, the lower 2 primer (5′-GGCAATGCAGCTCAATGTAA-3′) was used in combination with the degenerated primer dg up. Results of amplification were analyzed by agarose gel (1.5%) electrophoresis and staining with ethidium bromide gels. All products of the second round of amplification were purified (PCR purification kit from Qiagen) in water and subjected to sequencing in both directions.

Immunocytochemistry. The tissue was fixed according to the method of Panula et al. (1984), with the modification described by Eriksson et al. (1998). Slices of 2–3 mm thickness were fixed at 4°C for 12 hr in a fixative composed of 4% 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide and 0.2% *N*-hydroxysuccinimide (Sigma) in 0.1 M phosphate buffer, pH 7.4, cryosectioned at 34 μm thickness, and mounted on gelatin-coated slides. All antibody incubations and washes were done in PBS with 0.25% Triton X-100, and all antibody solutions contained 2% normal serum from the animal species used to produce the secondary antibody. To stain the TM neurons and their processes, we used a rabbit anti-HA serum (#19C; a gift from P. Panula, Helsinki University, Helsinki, Finland) that is highly specific for HA (Panula et al., 1990), which was diluted 1:2000–5000. An affinity-purified goat antiserum against orexin-A (Santa Cruz Biotechnology, Heidelberg, Germany) was used at dilutions of 1:500–2000. Both primary antisera were applied to the sections for 12–16 hr at 4°C, and the following steps were performed at room temperature. For fluorescence stainings the immunoreactivity was revealed by incubation with Texas Red-labeled donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) and Alexa Fluor 488-labeled donkey anti-goat IgG (1:500; Molecular Probes, Eugene, OR) for 90 min. For fluorescence double stainings, both primary antisera were applied together, and this was followed by incubation in a mixture of the two secondary fluorochrome-labeled antisera. The peroxidase double stainings were done in sequence. After the incubation with anti-orexin-A serum, the slides were incubated with a biotinylated rabbit–anti-goat serum (1:300; Vector Laboratories, Burlingame, CA) for 2 hr and then with an avidin-biotin complex (1:500; Vectastain elite; Vector) for 2 hr. The immunoreactivity was then visualized by a 6–12 min incubation in a solution of 0.03% 3,3′-diaminobenzidine tetrahydrochloride, 0.015% H_2O_2, and 0.1–0.2% $NiCl_2$ in Tris-HCl, pH 7.3, which yielded a black reaction product. The same procedure was then repeated with the anti-HA serum and a secondary biotinylated swine–anti-rabbit serum (1:200; Dako, Hamburg, Germany), and finally the color development was done without $NiCl_2$ to stain the HAergic elements brown.

Preincubation of the orexin-A antiserum with whole orexin-A peptide blocked all immunoreactivity. By replacing the primary antisera with 1% normal serum, we could exclude the possibility of the secondary antibodies binding directly to the tissue. The double-staining experiments were confirmed with single stainings to exclude artifacts attributable to species cross-reactions or incomplete wavelength selectivity of the fluorescence filters. The experiments were performed in accordance with the Animal Protection Law of the Federal Republic of Germany. All efforts were made to minimize animal suffering or discomfort and to reduce the number of animals used.

RESULTS

Electrophysiology

Stable recordings were obtained from 57 neurons that all had the characteristic electrophysiological features of TM neurons (Haas and Reiner, 1988). They exhibited spontaneous firing at 4.1 ± 0.5 Hz ($n = 34$) and had an average input resistance of 191.0 ± 6.6 MΩ ($n = 31$). Under tetrodotoxin, their resting membrane potential was −50.1 ± 0.6 mV ($n = 27$). Neurons more depolarized than −45 mV were discarded.

Bath application of orexin-A or orexin-B consistently increased

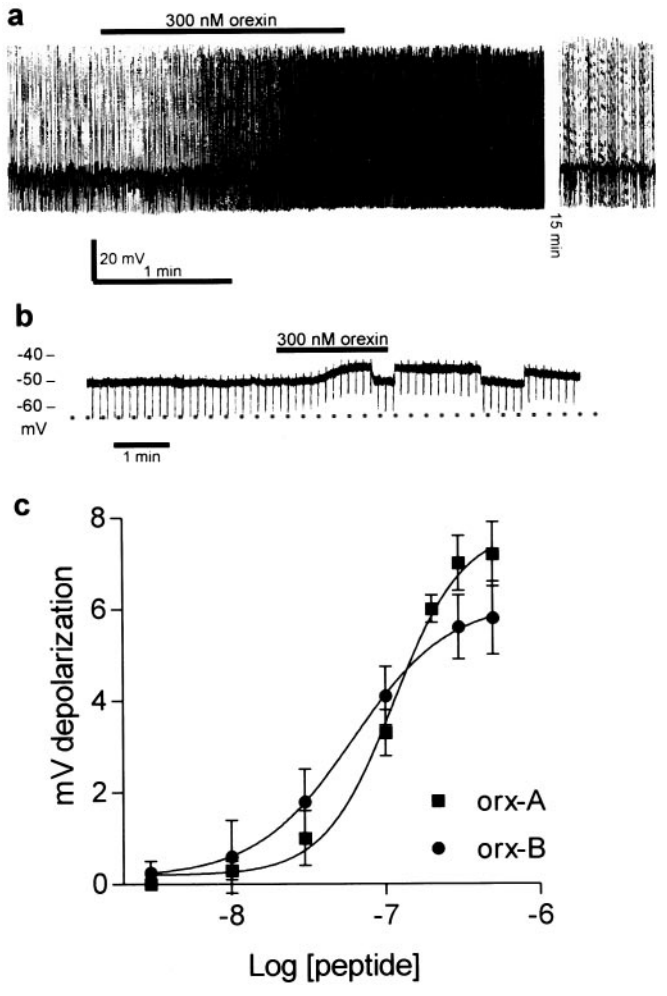

Figure 1. Intracellular recordings from TM neurons in hypothalamic slices. The *bar* indicates the presence of orexin in the recording chamber. In *A*, 300 nM orexin-A increases the firing rate of the neuron; this effect was reversed after a washout period. The neuron in *B* was recorded in the presence of tetrodotoxin, which prevents firing and causes synaptical isolation. Orexin-A strongly depolarizes the neuron, indicating a postsynaptic action. Hyperpolarizing current pulses were used to study changes in the input resistance of the neuron. When the membrane potential is manually retuned to the resting value, a small decrease in the input resistance is seen. In *C*, the dose dependence of the depolarization is shown. The data were obtained under tetrodotoxin and demonstrate the maximal postsynaptic effect of different doses of orexin-A (*orx-A*) and orexin-B (*orx-B*). Each data point represents 3–10 recordings.

the spontaneous firing rate of the TM neurons, and this effect reversed completely 15–30 min after termination of treatment (Fig. 1*A*). In five closely monitored cells, the firing increased by 73 ± 31% in the presence of 300 nM orexin-A. After a washout period of ≥1 hr, repeated applications of the peptide indicated no signs of desensitization. In the presence of tetrodotoxin, which prevents firing and causes synaptic isolation, a slight reduction of the input resistance to 92.2 ± 1.8% (range, 88–100%; $n = 6$) of the control value was observed (Fig. 1*B*), and both peptides at 3–500 nM depolarized the neurons with a rather steep dose dependency (Fig. 1*C*). Orexin-B at 300 nM depolarized the neurons by 5.3 ± 0.5 mV ($n = 4$) compared with 7.0 ± 0.5 mV ($n = 10$) for 300 nM orexin-A, but there was no significant difference between the magnitude of depolarization caused by the two peptides at any concentration tested. In the rest of the experiments the applied dose was 300 nM peptide for 2 min.

The following experiments, designed to elucidate the mechanism of the depolarization by orexin-A, are summarized in Figure 2*A*. The possible involvement of a K^+ current in the depolarization was examined by increasing the external K^+ concentration from 3 to 18 mM. This should lead to an estimated shift in the reversal potential for K^+ from −100 to −55 mV. The neurons were then held at −50 mV by current injection. Under these conditions, where the driving force for a K^+ current would be very low, orexin-A still caused a depolarization of 6.2 ± 0.6 mV ($n = 3$). Therefore, we could exclude the possibility of a K^+ current as a major contributor to the depolarization. To further investigate the ionic selectivity of the orexin-induced effect, we replaced 125 mM NaCl in the ACSF with 125 mM NMDG-Cl. This abolished the depolarization by orexin-A in two of three neurons, suggesting a dependence on external Na^+. Therefore, we tested the effects of blocking the Na^+/Ca^{2+} exchanger (NCX). The selective blocker of the NCX, KB-R7943 (Iwamoto et al., 1996) at 80 μM, strongly suppressed the depolarization, but a residual 1–3 mV depolarization remained in four of five cells (Fig. 2*B*). The onset of this depolarization was delayed by 1–2 min and it also developed much more slowly compared with the controls. It has been shown that Ni^{2+} in millimolar concentrations blocks the NCX (Kimura et al., 1987), and at this concentration it should also block Ca^{2+} channels (Stevens and Haas, 1996). We found that 3 mM Ni^{2+} applied for 2–5 min abolished the depolarization in four of five cells (Fig. 2*B*) and that the stronger blocking efficiency of Ni^{2+} compared with KB-R7943 was statistically significant (0.4 ± 0.5 mV and 2.0 ± 0.7 mV depolarization, respectively; $p < 0.05$). Finally, we studied the effects of KB-R7943 and Ni^{2+} on the depolarization by orexin-B. Ni^{2+} strongly attenuated the depolarization, whereas KB-R7943 had an intermediate effect (Fig. 2*C*). Similar to the results with orexin-A, there was a significant difference between the two treatments (0.5 ± 0.3 mV vs 2.7 ± 0.6 mV depolarization; $p < 0.05$) Thus, these experiments indicate that the mechanisms of action on TM neurons are similar for both peptides.

PCR

Acutely dissociated HDC-positive neurons had somata that were 16–30 μm in diameter with polygonal to round shapes and several dendrites. Some neurons from the lateral TM region that were collected for the single-cell PCR study turned out to be HDC-negative; these presumed interneurons or neurons from regions adjacent to the TM were always smaller (8–15 μM) and were not investigated further. In the amplifications with orexin receptor-specific primers, the obtained amplimers had the expected sizes of 108 bp and 156 bp for OR_1 and OR_2, respectively. Genomic DNA amplification products with our primers would have the expected sizes 605 bp for OR_1 and 653 bp for OR_2, but these products were never seen on the stained gels. The obtained sequences corresponded to the known cDNAs for rat OR_1 (GenBank accession number AF041244) and OR_2 (GenBank accession number AF041246). The majority of the HDC-positive neurons (9 of 12) expressed both types of orexin receptors; one cell expressed only OR_1, 1 cell expressed only OR_2, and in 1 cell neither receptor was expressed (Fig. 3). Although we did not do a detailed quantification, we observed that the signal for OR_2 was generally strong, whereas the OR_1 signal was more variable in strength and usually considerably weaker.

Immunocytochemistry

The double stainings revealed that immunoreactive orexin (orexin-IR) neurons and immunoreactive HA (HA-IR) neurons

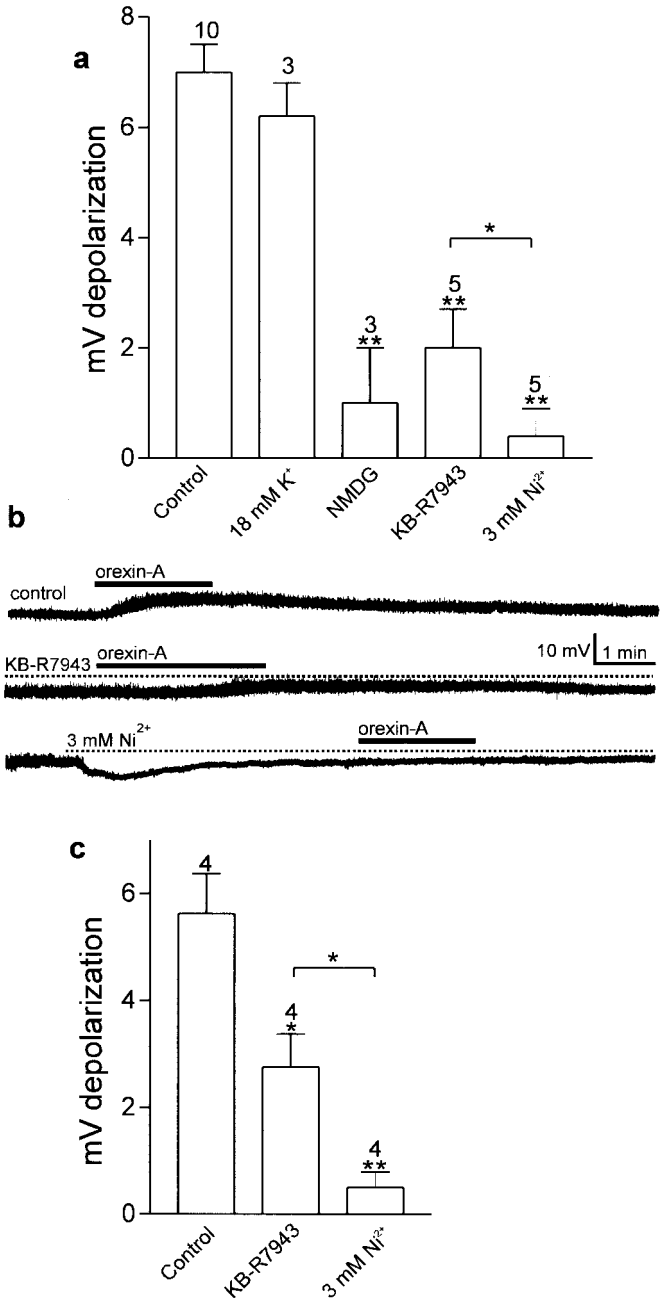

Figure 2. Characterization of the mechanism of depolarization. In *A*, the effects of different treatments on the depolarization induced by 300 nM orexin-A are summarized. Increasing the external K^+ concentration had no significant effect on the response. Replacing most of the external Na^+ with $NMDG^+$ resulted in efficient blocking, indicating dependency on external Na^+. The selective NCX blocker KB-R7943 (80 μM) also strongly suppressed the depolarization. Treatment with 3 mM $NiCl_2$ blocked the depolarization almost completely. In the *top tracing* in *B*, the voltage recording shows a representative depolarization of a TM neuron by 300 nM orexin-A. In the *middle tracing* the slice has been preincubated with the NCX blocker KB-R7943 (80 μM). The depolarization is smaller, delayed by almost 2 min, and develops more slowly compared with the control. In the *bottom tracing* the effect of 3 mM $NiCl_2$ is shown. Ni^{2+} causes a transient hyperpolarization by itself and completely inhibits the effect of 300 nM orexin-A. In *C*, the effects on the orexin-B-induced depolarization by 3 mM $NiCl_2$ and 80 μM KB-R7943 are demonstrated. Treatment with KB-R7943 attenuates the depolarization, and Ni^{2+} has a very strong inhibitory effect. *Numbers* above *bars* indicate *n*. $^{**}p < 0.001$; $^{*}p < 0.05$.

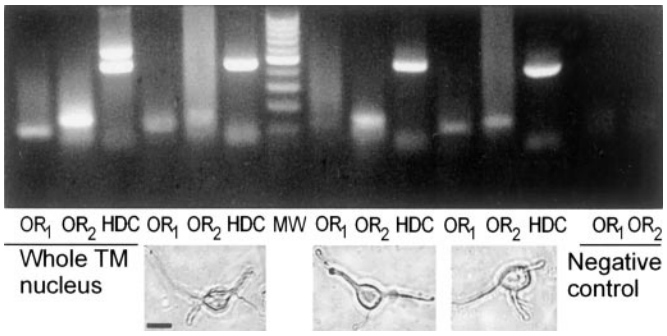

Figure 3. Representative results from the single-cell PCR study. Dissociated neurons were tested for the expression of HDC to confirm that they were histamine-producing and then studied with primers for both orexin receptors (OR_1 and OR_2). We found that most HAergic neurons expressed both orexin receptors. The results of an amplification of mRNA from whole tissue from the TM region are shown, as well as captured video images of three dissociated TM neurons, placed under their three corresponding electrophoresis lanes in the figure. All three neurons express OR_2, whereas the signal for OR_1 is weak or absent in the middle neuron. Scale bar, 20 μm. *MW*, Molecular weight markers [100-bp step DNA ladder (Promega) with the 500-bp band present at trifold intensity].

are partially colocalized to the same hypothalamic regions. In the rostral portion of the medial TM, occasional orexin-IR neurons were seen among the HA-IR neurons (Fig. 4*A*), whereas orexin-IR somata were never seen in the lateral TM. From the medial TM an area containing scattered HA-IR neurons extends out in a lateral direction to where the orexin-IR neurons are diffusely distributed in the lateral hypothalamus (Fig. 4*B*). There is an overlap between these regions where the two populations of neurons sometimes are located very close to each other (Fig. 4*C,D*). We did not observe colocalization of immunoreactivity for HA and orexin to the same neurons.

We also observed that the orexin-IR neurons were heavily innervated by HA-IR axons that often appeared to terminate on their somata. The HA-IR axons throughout the CNS normally have a characteristic varicose appearance, but at points at which they were in close contact with the orexin-IR somata, they often flattened out and bifurcated in a manner resembling synaptic specializations (Fig. 5).

DISCUSSION

The orexins have been shown to have excitatory actions or to increase the intracellular Ca^{2+} concentration in all tested brain regions, such as the arcuate, dorsal raphe, locus ceruleus, and lateral and medial hypothalamic nuclei and can also increase transmitter release (van den Pol et al., 1998; Horvath et al., 1999; Rauch et al., 2000; Brown et al., 2001a). Here we show a strong excitation of TM neurons by orexins and that the TM neurons express both orexin receptors. Furthermore, there seems to be a close and reciprocal anatomical connection between HA neurons and orexin neurons. All this suggests a complex interplay between the two neuronal populations.

The single-cell RT-PCR study revealed the expression of both OR_1 and OR_2 in most of the TM neurons, with a stronger expression of OR_2. In a recent article, the expression of OR_1 and OR_2 in the rat brain was studied with *in situ* hybridization (Marcus et al., 2001). These authors also describe a very high expression of OR_2 in the TM nucleus, but they did not see any hybridization signal for OR_1. The most likely explanation for the discrepancy between the two studies with regard to OR_1 is the

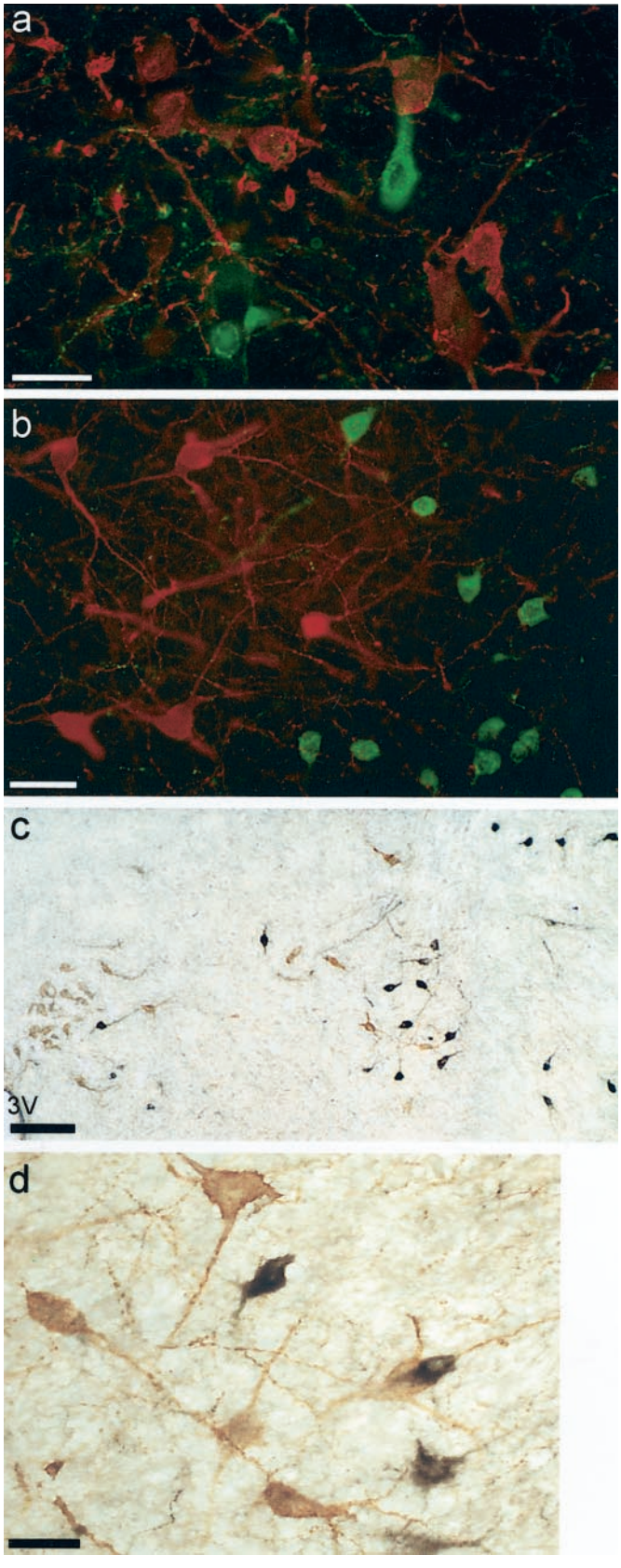

Figure 4. Anatomical interactions between the HA and orexin systems. The lateral direction is to the *right*. *A*, In the rostral parts of the medial TM nucleus, a high density of orexin-IR axons and also scattered somata (*green*) are seen among HA-IR neurons and dendrites (*red*). *B* shows

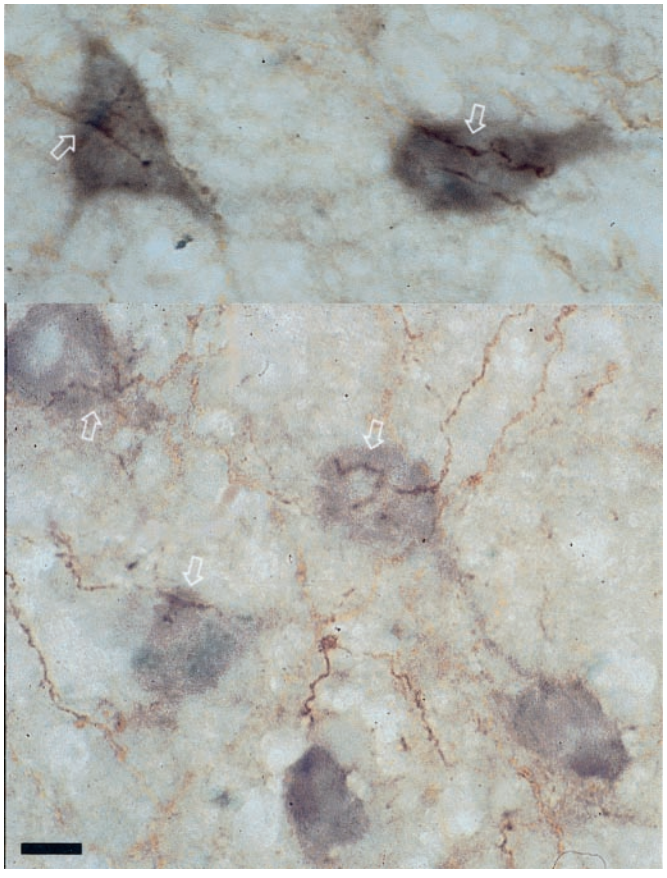

Figure 5. The orexin neurons (*gray*) receive a heavy innervation by HAergic axons (*brown*). The axons make close contacts with the neurons and often appear to flatten out and terminate on their somata (*arrows*) in a manner that resembles synaptic specializations. Scale bar, 10 μm.

different sensitivities of the detection methods used. The PCR method can detect very low levels of mRNA and can be expected to be more sensitive than *in situ* hybridization. Marcus et al. (2001) describe the OR_1 expression in many of the raphe neurons as low to moderate, and we have also studied the expression of OR_1 in isolated raphe neurons and found that the expression there was ~15-fold higher compared with the TM neurons. Another possible explanation might be the age difference between the rats used in the two studies. Because slice preparation is more successful if the animals are young, we used 3- to 4-week-old rats, but adult rats were used in the *in situ* study; it has been shown recently that the level of OR_1 mRNA in the rat hypothalamus decreases during maturation to adulthood (van den Pol et al., 2001). The presence of the OR_1 protein in TM neurons is also supported by a recent immunocytochemical study (Hervieu et al., 2001).

Orexin-B primarily activates OR_2, whereas orexin-A activates both orexin receptors, and both orexin receptors are excitatory

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HAergic neurons in the lateral portion of the medial TM nucleus extending to a region containing orexin-IR neurons. *C*, An overview showing the distribution of HA-IR (*brown*) and orexin-IR (*black*) neurons in an area extending from the third ventricle (*3V*) to parts of the perifornical area. There is a clear overlap between the distributions of these two types of neurons in regions lateral to the medial TM nucleus. *D*, Detail of an area corresponding to the central portion of *C*. Scale bars: *A, B, D*, 30 μm; *C*, 100 μm.

(Sakurai et al., 1998). Because the difference in potency between orexin-A and orexin-B was not significant, it appears that OR_2 is the physiologically more important orexin receptor in the TM neurons, which is in keeping with the expression studies. There was a tendency toward a stronger effect by orexin-A compared with orexin-B, which might indicate an additional involvement of OR_1, but to study this further we would need selective receptor blockers, especially for OR_2, which are not available at present. The blocking experiments with Ni^{2+} and KB-R7943 suggest that both peptides act through the same mechanisms, and therefore we can conclude that both orexin peptides excite the TM neurons primarily, or exclusively, via an action on OR_2.

We show here that the excitation of TM neurons occurs primarily via an activation of an NCX that causes depolarization. The NCX is electrogenic, with an exchange ratio of 3 Na^+ in for every Ca^{2+} that is pumped out, and is expressed throughout the brain (Kimura et al., 1987; Quednau et al., 1997). There are only a few other reports of this novel mechanism of increasing neuronal excitability. Recently, we found that serotonin depolarizes the TM neurons via 5-HT_{2C}-receptor-mediated activation of the NCX (Eriksson et al., 2001). Other authors have described how depolarization of neurons in the basolateral amygdala and ventromedial hypothalamus via class I metabotropic glutamate receptors, as well as H_1-receptor-mediated depolarization of neurons in the supraoptic nucleus, occur via an activation of the NCX (Smith and Armstrong, 1996; Lee and Boden, 1997; Keele et al., 2000). The metabotropic glutamate class I, 5-HT_{2C}, and H_1 receptors are all coupled to phospholipase C, and this is also true for OR_1 and OR_2 (Smart et al., 1999). The activation of NCX is therefore most likely an effect secondary to a surge in the intracellular Ca^{2+} concentration, because the activated receptors are coupled to inositol 1,4,5-triphosphate production. This Ca^{2+} is most likely released from intracellular stores, because no obvious Ca^{2+}-channel component or change in membrane conductance has been seen in association with the NCX activation in the previous studies (Smith and Armstrong, 1996; Keele et al., 2000; Eriksson et al., 2001).

In the present study, the increased conductance indicates activation of a transmembrane current in addition to the activation of NCX. It has been shown recently in an expression system that the primary response to OR_1 activation is a novel type of Ca^{2+} current that has not been described for any other receptor (Lund et al., 2000). At present, the only known compound that blocks this current is Ni^{2+} (Kukkonen and Åkerman, 2001). An activation of both the NCX and a Ca^{2+} channel in the TM neurons is supported by the fact that the selective NCX blocker KB-R7943 had a strong but not complete blocking effect, whereas Ni^{2+}, which should block both the NCX and Ca^{2+} channels, was significantly more efficient. This stronger effect of Ni^{2+} was probably not attributable to an insufficient concentration of KB-R7943, because the same concentration completely blocked the 5-HT-induced depolarization in TM neurons, which is mediated solely by NCX activation without any Ca^{2+}-channel component (Eriksson et al., 2001). It also appears that the activation of NCX by orexin is not secondary to the Ni^{2+}-sensitive residual effect, because this depolarizing mechanism developed considerably more slowly than the NCX effect. The slow Ni^{2+}-sensitive, presumed Ca^{2+}-channel component was also very variable in strength. The experiments with both NMDG and KB-R7943 would block the NCX without affecting a Ca^{2+} current, which means that out of a total of eight neurons, the depolarization was abolished in three, whereas the remaining five exhibited a 1–3 mV residual depolarization. In recent articles from our group and others, it has been shown that orexin acts in an excitatory manner by decreasing K^+ conductances in locus ceruleus and dorsal raphe neurons (Ivanov and Aston-Jones, 2000; Brown et al., 2001a). In this study, the depolarization was largely unaffected by manipulation of the external K^+ concentration, the membrane conductance was increased rather than decreased, and Ni^{2+} abolished the depolarization, none of which would fit with the involvement of a K^+ channel.

It has been shown recently that the orexin neurons express Fos in a manner that is positively correlated with wakefulness and negatively correlated with sleep (Estabrooke et al., 2001). In this article the authors also describe non-orexin-IR neurons in the perifornical area that have the same circadian pattern of Fos expression, and it is quite likely that these neurons correspond to the HA-IR neurons shown in Figure 4*D* in this study. The anti-narcolepsy drug modafinil, which has been shown recently to be an inhibitor of the dopamine transporter (Wisor et al., 2001), selectively activates these two neuronal populations in the rat (Scammell et al., 2000), although this effect was not seen in the TM nucleus of the cat (Lin et al., 1996). The orexin and TM neurons innervate each other, so it is an open question whether the two groups are activated directly or whether one is the primary target for modafinil and then activates the other group.

Histamine is believed to be released in a predominantly nonsynaptic manner and although these neurons can form synapses, it is uncommon (Diewald et al., 1997). Here we note that the HA-IR axons form structures resembling synaptic specializations terminating on the orexin-IR neurons. Electron microscopic studies will be needed to confirm this, but even if they are not real synapses it is obvious that the orexin neurons receive a very prominent innervation from the TM neurons, and this suggests that the orexin neurons are an important target for the HAergic system. Although HA often has excitatory postsynaptic effects, *in vivo* or *in vitro* recordings from the orexin neurons would be crucial for a deeper understanding of their regulation by HA and other compounds.

The TM and orexin neurons have both been implicated in the regulation of REM sleep and feeding. In this study we demonstrate a close anatomical connection between these neurons and a strong excitation of TM neurons by orexin. Together, these data indicate a functional connection between these two classes of neurons in the regulation of sleep and also suggest that their interplay may be complex.

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