Hypocretin1/OrexinA Axon Targeting of Laterodorsal Tegmental Nucleus Neurons Projecting to the Rat Medial Prefrontal Cortex

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Introduction

Wakefulness is characterized by cerebral cortex activation. The prefrontal cortex (PFC) is involved in emotional behavior, attention, memory, and perception; it comprises 3 major regions: orbital, medial, and lateral (Fuster 2001). The medial prefrontal cortex (mPFC), which is strongly activated during arousal and wakefulness, receives abundant input from the laterodorsal tegmental nucleus (LDT) (Heidbreder and Groenewegen 2003). This nucleus is associated with cortical activation during wakefulness and rapid eye movement (REM) sleep states. LDT contains 3 known neuronal populations with discharge rates that vary over the sleep–wakefulness cycle; the first is active during wakefulness and REM sleep, the second discharges only during REM sleep (el Mansari et al. 1989), and the last is active only during wakefulness (Kayama et al. 1992).

LDT contains many cholinergic neurons (Shute and Lewis 1967; Kimura and Maeda 1982; Mesulam et al. 1983) that project densely to the frontal cortex, especially to the prelimbic sector (PL) of the mPFC (Divac et al. 1978; Satoh and Fibiger 1986; Cornwall et al. 1990). Moreover, the LDT neurons that are active during both wakefulness and REM sleep are presumably cholinergic (el Mansari et al. 1989).

LDT also contains neurons with other neuromodulators such as substance P (Vincent et al. 1983), γ-aminobutyric acid (GABA), and glutamate (Wang and Morales 2009), which are intermingled with neurons containing acetylcholine (Ach), although they are at large independent populations (Wang and Morales 2009). Cholinergic neurons are ubiquitously distributed within the LDT, while noncholinergic neurons have different preferential locations (Satoh and Fibiger 1986). Thus, Wang and Morales (2009) have described that neurons containing GABA or glutamate are segregated in the rat LDT, with glutamate neurons mainly located rostrally and GABA neurons preferentially located caudally. Moreover, electrophysiological studies have shown that all these neurons stimulated cortical activation (Boucetta and Jones 2009), whereby not only cholinergic LDT neurons influence cortical circuits, as generally is presumed by disregarding LDT neurons containing other transmitters than Ach. Therefore, the elucidation of the distribution and detailed synaptic organization of LDT neurons reaching mPFC is a pending critical issue for the understanding of PFC modulation by brain stem reticular neurons.

LDT receives inputs from hypocretinergic/orexinergic neurons (Peyron et al. 1998). Hypocretin/orexin (Hcrt/Ox) neurons are exclusively located in the hypothalamus (de Lecea et al. 1998; Sakurai et al. 1998) and discharge maximally in wakefulness, decrease their activity in slow-wave sleep, and cease firing during REM sleep (Lee et al. 2005; Milejkovskiy et al. 2005). They synthesize 2 peptides, Hcrt1/Ox and Hcrt2/Ox, which bind Hcrt/Ox-R1 and Hcrt/Ox-R2. Hcrt/Ox-R1 is more abundant than Hcrt/Ox-R2 in both LDT and PFC (Greco and Shiromani 2001; Marcus et al. 2001) and binds preferentially to Hcrt/Ox-A, whose localization in LDT is studied here. Dysfunction of the Hcrt/Ox system is linked to narcolepsy, characterized by excessive daytime sleepiness, REM sleep alteration, and sleep fragmentation (Peyron et al. 2000; Thanizzas et al. 2000; Iyino et al. 2001; del Cid-Pellitero and Garzón 2007).

Hcrt/Ox application in vitro showed that Hcrt1/Ox activates LDT neurons more strongly than Hcrt2/Ox. Most cholinergic and noncholinergic LDT neurons were directly excited by application of Hcrt1/Ox in mouse brain stem slices (Burlet et al. 2002). Furthermore, Hcrt1/Ox LDT injections increased wakefulness and decreased REM sleep in non-anesthetized freely moving cats (Xi et al. 2001).

Based on the previously described rationale and on the absence of ultrastructural descriptions of Hcrt/Ox localization...
in LDT, we aim to determine (1) the distribution of LDT cholinergic and noncholinergic neurons projecting to mPFC, (2) the ultrastructural location of Hcrt1/OxA in the LDT, (3) the cellular relationships of Hcrt1/OxA-containing axons with LDT neurons projecting to PL, and (4) whether Hcrt1/OxA-containing axons target identified cholinergic LDT neurons. The obtained results will shed light in the knowledge of LDT inputs to a cortical region involved in associative function and attention and should improve our understanding of the anatomical bases supporting the wake-enhancing and cortical activation actions of Hcrt1/OxA in the LDT.

**Materials and Methods**

**FG Injections**

Eighteen male adult Sprague-Dawley rats weighing 250–300 g were used in this study. All animal procedures were done in accordance to European Community Council Directive (86/609/EEC) and were approved by the Ethical Committee for the use of laboratory animals of the Universidad Autónoma de Madrid.

The animals were anesthetized with a cocktail of ketamine (55 mg/kg intramuscularly [i.m.]), xylazine (15 mg/kg i.m.), and atropine (0.2 mg/kg i.m.). To label LDT neurons projecting to mPFC, the retrograde tracer Fluorogold (FG; Fluorochrome) was microinjected into the prelimbic mPFC, extending in some cases to medial orbital sector (MO) or circular sector (Cg1), using previously described methods (Gazón and Pickel 2004). Glass micropipettes were pulled to obtain 10–15 μm diameter tips and were filled with a 1.5% solution of FG in isotonic saline. The micropipettes were stereotaxically placed unilaterally in the mPFC (2.7 mm anterior to bregma, 0.4 mm lateral from midline, and 3.7 mm ventral from skull dorsal surface), as determined from the rat brain atlas of Paxinos and Watson (1998). FG was injected iontophoretically using continuous current (positive 5 μA, on 8 sec/off 8 sec) for 10–15 min. The micropipettes were left in place from 5 min before to 5 min after the injection to avoid spread of the solution. The skin incision was closed with a surgical thread, and topical lidocaine was applied. The rats were returned to the animal colony and housed in individual cages. They were allowed unlimited access to water and food and were maintained with a 12:12 h light:dark cycle for 6 days.

**Tissue Preparation**

Rats were perfused 6 days after FG injections, a time that has been shown to be optimal for retrograde tracing of mesopontine neurons after mPFC injections (van Bockstaele et al. 1996). The rats were anesthetized with sodium pentobarbital (33 mg/kg intraperitoneal), and their brains were fixed by aortic arch perfusion with heparin in saline (1000 U/ml) and 0.2% glutaraldehyde in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed from the cranium, and coronal sections containing the mPFC and the mesopontine region were obtained. They were postfixed in 1% sodium borohydride in 0.1 M PB to inactivate free aldehydes and 15 min in 10% methanol and 10% hydrogen peroxide in 0.1 M PB to inactivate endogenous peroxidase. After that, the sections were processed for immunohistochemical labeling as described below. After the immunolabeling procedures, sections for light microscopy observation were rinsed in 0.03 M PB and mounted on gelatine-coated glass slides, dried, dehydrated in a series of ascending concentrations of ethanol, defatted in xylene, and coverslipped with DPX mounting medium (Sigma-Aldrich).

**Single Immunohistochemical Detection of FG**

A few sections of the FG-injected rats containing either the mPFC or the mesopontine region were processed immunohistochemically for light microscope observation of the injection site and retrograde tracing, respectively. They were first rinsed in 0.1 M PB and incubated for 30 min in 0.5% bovine serum albumin (BSA; Sigma-Aldrich) in 0.1 M PB to minimize nonspecific background staining. They were then incubated overnight in rabbit anti-FG antibody (1:4000, AB155, Chemicon), 0.1% BSA, and 0.25% Triton X-100 in 0.1 M PB protected from light at room temperature. After this, the sections were incubated in biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:400, AP132B, Chemicon) and in 0.1% BSA in 0.1 M PB for 30 min, rinsed in 0.1 M PB, and incubated for 1 h in avidin-biotin-peroxidase complex (1:100, ABC, Vector Lab) (Hsu et al. 1981). Peroxidase was visualized as a brown precipitate by incubation of the tissue in 0.022% 3,3′-diaminobenzidine (DAB) and 0.003% hydrogen peroxide in 0.1 M PB at 4 °C for 6–10 min.

**Dual-Immunofluorescence Detection of FG and ChAT**

In the 18 rats with FG injections, one of the tissue series was processed for immunofluorescence detection of FG and ChAT. All procedures were done protecting the tissue from light. After extensive rinsing in 0.1 M phosphate-buffered saline, pH 7.4 (PBS), the sections were incubated in citrate buffer, pH 6.0, at 90 °C for 10 min. Then the sections were incubated in 10% donkey serum, 1% BSA, and 1% Triton X-100 in 0.1 M PBS for 2 h at room temperature. Afterward, the sections were incubated in rabbit anti-FG (1:1500, AB153, Chemicon) and goat anti-ChAT (1:1000, AB144P, Chemicon), 3% donkey serum (Sigma-Aldrich), 0.3% BSA, and 0.3% Triton X-100 in 0.1 M PBS at 4 °C for 48 h. Next, the sections were rinsed in 0.1 M PBS and incubated in fluorescent secondary antisera (donkey anti-rabbit IgG 488 nm [1:100, A21206, Alexa Fluor, Invitrogen] and donkey anti-goat IgG 546 nm [1:100, A11056, Alexa Fluor, Invitrogen]) in 0.1 M PBS at 4 °C for 2 h.

**Single Immunohistochemical Detection of Hcrt1/OxA**

Four additional naïve male Sprague-Dawley adult rats weighing 250–300 g were used in these experiments. The Hcrt1/OxA antisemur used was raised in goat against a 19-residue peptide fragment located at the C-terminus of human Hcrt1/OxA (amino acids 48–66 of the Hcrt/Ox precursor identical to the corresponding rat/mouse sequence). This antisemur has a highly specific distribution in multiple brain regions (Lee et al. 2005; Wang et al. 2005). Specificity of the anti-Hcrt1/OxA antisemur has been previously tested in western blot and immunohistoch- emistry tests; the antisemur detects the processed active Hcrt1/OxA peptide of rat origin, but it does not recognize or cross-react with Hcrt2/OxB (Guan et al. 2002). Moreover, the specificity of the Hcrt1/OxA labeling in the present study is also supported by the results of negative control experiments and species selectivity testing of the secondary antisemur.

Tissue sections were incubated in 10% donkey serum in 0.1 M PB for 3 h. They were then placed sequentially in (1) goat anti-Hcrt1/OxA antisemur (1:2000, C-19: sc-8070, Santa Cruz Biotechnology Inc.), 2% donkey serum, and 0.25% Triton X-100 in 0.1 M PB at 4 °C for 48 h; (2) biotinylated donkey anti-goat IgG (1:400, AP180B, Chemicon) and 2% donkey serum in 0.1 M PB for 1 h; and (3) lastly, ABC (1:100) in 0.1 M PB for 1 h. Hcrt1/OxA was visualized as an intense black precipitate using a modification of the glucose oxidase-DAB-nickel method (Shu et al. 1998).

**Dual-Immunohistochemical Detection of Hcrt1/OxA and FG**

Tissue sections from the LDT from the 18 FG-injected rats containing either the mPFC or the mesopontine region were processed dual detection for Hcrt1/OxA and FG. Nonspecific tissue epitopes were blocked by incubation in 10% donkey serum in 0.1 M PB for 3 h before incubating the sections in goat anti-Hcrt1/OxA (1:2000), 0.03% Triton X-100, and 2% donkey serum in 0.1 M PB at 4 °C for 48 h. Afterward, the sections were incubated in biotinylated donkey anti-goat

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IgG (1:500) in 0.1 M PB for 2 h and in ABC (1:100) in 0.1 M PB for 1 h. Hcrt1/Oxα detection was completed by using the glucose oxidase-DAB-nickel method. After abundant rinsing in 0.1 M acetate buffer, pH 6.0, and in 0.1 M PB, the sections were sequentially incubated in (1) rabbit anti-FG antisera (1:1000) and 2% donkey serum in 0.1 M PB at room temperature overnight, (2) unconjugated donkey anti-rabbit IgG (1:50, AP182, Chemicon) and 2% donkey serum in 0.1 M PB for 30 min, and then (3) rabbit peroxidase anti-peroxidase (1,500, PAP18, Chemicon) in 0.1 M PB for 2 h. The peroxidase reaction product was visualized by incubating the tissue in 0.02% DAB and 0.035% hydrogen peroxide in 0.1 M PB for 10 min. The light brown immunoperoxidase reaction product identifying FG-labeled neurons was easily distinguishable from the nickel-intensified black reaction product that marked Hcrt1/Oxα fibers.

**Data Analysis**

The tissue sections processed for single and dual immunohistochemistry were studied with a Nikon Eclipse E600 microscope. Selected representative photographs of the different immunolabelings (FG, ChAT, and Hcrt1/Oxα) were taken with a digital camera (digital camera DMX1200, Nikon) adapted to the microscope and appropriate computer software (Nikon Camera Software DMX1200 ACT-1).

Adjacent Nissl sections were used to delimit FG deposits in mPFC and to draw the retrograde-labeled neurons within LDT (bregma -8.3 to -9.3 mm). Outlines of sections and major brain structures were drawn by overlapping FG-immunolabeled sections and the adjacent Nissl-stained sections using a magnifying projector (Ernst leitz GMBH Wetzler; objective Milaron 1:2.5/90 mm); these drawings were scanned (EPSON Expression 1600), and FG-labeled neurons were plotted on drawings of coronal brain stem sections using high magnification (×20–40).

Immunofluorescence sections were examined by biomapping using a confocal microscope (Leica TCS SP2 Spectral microscope; Leica Microsystems); LDT area was studied in 1-μm thick optical sections obtained through the depth of the tissue with a ×20 multi-immersion objective. FG single-labeled cells and FG/ChAT dual-labeled cells were counted (Leica Confocal software Lite) throughout the LDT of the 18 FG-injected rats. The immunolabeled images were assembled and labeled with text in Canvas (Canvas X, ACD Systems International Inc.) to obtain the composite figures depicting FG and/or ChAT localization.

The ultrastructural analysis was carried out in 12 vibratome sections processed for Hcrt1/Oxα and FG in 6 rats and 8 vibratome sections processed for Hcrt1/Oxα and ChAT in 4 rats. Two ultrathin sections, respectively, cut from opposite sides of the vibratome sections were obtained and completely examined in each animal from resin blocks including an LDT section. All immunoreactive processes were counted in electron micrographs at ×50 000 magnification. The countings were obtained from an area of 852 ± 79.79 μm² in the Hcrt1/Oxα + FG experiment and an area of 389 ± 19.19 μm² in the Hcrt1/Oxα + ChAT experiment; the minimal areas examined in each animal, respectively, were 50 350.33 and 32 010.5 μm².

The classification of identified cellular elements was based on standard descriptions (Peters et al. 1991). Axons were resectioned by their small caliber and their lack of ribosomes. The maximum diameter along the short axonal axis was measured (ImageJ 1.40 software, National Institutes of Health) in Hcrt1/Oxα-immunoreactive profiles as a criterion for estimating the real diameter of the profile regardless of the plane of section. The diameter is directly proportional to the cross-sectional surface of the profile, especially in rather cylindrical structures such as axons. We also measured the circularity (=perimeter²/area) in all the Hcrt1/Oxα-containing axons. This parameter indicates how close the diameter of a profile is to a perfect circle. Thus, circularity of 1.0 would indicate that the axon profile is quite close to its transverse plane, while progressively lower circularities are found in axons that are longitudinally or nontransversely sectioned.

When sectioned transversely, axons had smooth contours and were often grouped in bundles. Longitudinally cut axons usually displayed varicosities with vesicles. Axon boutons were identified by the presence of numerous synaptic vesicles and were ≥0.25 μm in diameter. Varicosities (boutons en passant) ranged 0.250–0.699 μm in diameter, and axon terminals were ≥0.7 μm, as measured both in random ×100 micrographs in the light microscope images (ImageJ 1.40 software) and also in obviously longitudinal sectioned varicose axons in electron microscope images. These criteria were used to classify an axonal bouton as a varicosity or a terminal. Interrucentive axons of unmethylated axons were ≥0.25 μm wide and might or might not contain some vesicles. Dendrites usually contained abundant endoplasmic reticulum and were distinguished from unmethylated axons by their larger diameter and/or prevalence of uniformly distributed microtubules. Neuronal somata were recognized by the presence of a nucleus, Golgi apparatus, and rough endoplasmic reticulum. Both dendrites and somata were often found postsynaptic to axonal boutons. Synaptic contacts were classified as symmetric or asymmetric based on the characteristics of their pre- and postsynaptic densities (Gray 1959). Zones of closely spaced parallel plasma membranes that lacked discernible synaptic densities but were otherwise not separated by glial processes were defined as appositions or nonsynaptic contacts. All Hcrt1/Oxα-immunoreactive axons within each ultrathin section were photographed at ×50 000 with a digital camera coupled to the...
electron microscope and saved in TIFF format. The tissue was examined to determine (1) the relative frequency with which Hcrt1/OxA immunoreactivity was located within axon boutons, unmyelinated axons, or myelinated axons; (2) Hcrt1/OxA-immunolabeling area density (number of Hcrt1/OxA-immunolabeled profiles per analyzed surface unit) for each animal and ultrathin section; (3) the number and type of morphologically recognizable synapses or appositions established by Hcrt1/OxA axons with FG-labeled, ChAT-labeled, or unlabeled profiles; and (4) measurements of area, perimeter, circularity, and mean diameter of each Hcrt1/OxA-labeled profile (ImageJ 1.40 software). Chi-square tests and analysis of variance (ANOVA) tests followed by post hoc Fisher’s protected least significant difference (PLSD) were used for statistical comparison. Statistical analyses were carried out with the aid of Statview software (V 5.0, SAS Institute). Canvas X software was used to build and label the composite illustrations.

Results

Single Immunocytochemical Detection of FG

FG deposit was restricted to the prelimbic sector (PL) of the mPFC in 10 animals (Fig. 1A). In another 4 animals, the FG injections were centered in PL but also extended to the Cg1, and in the remaining 4 animals, the deposit included PL and also comprised portions of the MO. The FG deposit spanned the whole depth of the mPFC (n = 16); in one animal (PL), the FG deposit was mainly in superficial layers (layers I-IV), and in another animal (Cg1-PL), the FG injection was confined to layers I-V.

In all the animals (n = 18), FG-labeled neurons were observed in the LDT (Fig. 1B,C), although they were also detected abundantly in other brain stem structures, such as the locus coeruleus and dorsal raphe nucleus. Some FG-immunoreactive neurons were found in the pedunculopontine tegmental nucleus, lateral parabrachial nucleus, caudal linear nucleus, and median and pontine raphe nuclei. The morphology of FG neurons was mainly multipolar; the peroxidase reaction product completely filled the LDT cell bodies but also the high-order dendritic branches (Fig. 1D). FG-labeled neurons were characterized by long primary dendrites. Sometimes, especially in the side contralateral to the injection site, FG neurons were faintly stained (Fig. 1E).

FG-labeled neurons were observed throughout the entire rostrocaudal axis of the LDT, and most of them were localized in the LDT region included in the central gray (428 neurons). In all the animals, FG neurons were mainly located in the ipsilateral side (n = 18, 75.05 ± 3.60%). The distribution of FG neurons in both sides did not vary significantly between the 3 injection groups (PL, Cg1-PL, and PL-MO; F_{2,30} = 0.08, P = 0.92; Table 1).

Dual-Immunofluorescent Detection of FG and ChAT

The distribution and morphology of FG-labeled neurons were very similar on the immunohistochemistry and the immunofluorescence studies (x^2 = 0.03, P = 0.87). These neurons were located more frequently in the side ipsilateral to the FG deposit (n = 18, 75.81 ± 2.73%) in the 3 injection groups (PL: F_{1,18} = 103.53, P = 0.0001; Cg1-PL: F_{1,6} = 24.34, P = 0.003; PL-MO: F_{1,6} = 34.81, P = 0.001; Table 1). One animal (PL; R24) did not have FG-labeled neurons in the side contralateral to the injection site.

Around half of FG-immunofluorescent neurons were also positive for ChAT (n = 18, 52.05 ± 3.72%; Fig. 2). Statistically significant differences between the percentage of FG+/ChAT− and FG+/ChAT+ labeled neurons were not observed (F_{1,24} = 0.61, P = 0.44). However, when these 2 populations of neurons were analyzed in each injection group (PL, Cg1-PL, and PL-MO), ANOVA showed that FG+/ChAT+ neuron percentages were significantly higher than the percentages of FG+/ChAT− neurons in PL injections and significantly lower than FG+/ChAT− neurons in Cg1-PL (Table 2). In the side ipsilateral to the injection site, the proportion of FG+/ChAT+ neurons was higher than that of FG+/ChAT−-immunoreactive neurons (n = 18, F_{1,34} = 5.63, P = 0.02). This higher proportion of FG+/ChAT+ versus FG+/ChAT− neurons was also observed in the group of animals with FG injections restricted to PL (n = 10, F_{1,18} = 7.36, P = 0.0002). Therefore, most LDT neurons projecting to the ipsilateral mPFC and especially to the PL sector were cholinergic.

Single Immunocytochemical Detection of Hcrt1/OxA

Hcrt1/OxA-containing axons were thin, had many varicosities, and were observed throughout the entire LDT. Hcrt1/OxA immunoreactivity was moderate, so that it was quite easy to distinguish the trail of the axons and their varicosities (Fig. 1G,H). The diameter of the Hcrt1/OxA-immunoreactive varicosities was 0.355 ± 0.006 μm (mean ± standard error [SE]), as measured with ImageJ software at x100 magnification, whereas intervaricose Hcrt1/OxA-immunoreactive segments were always less than 0.236 μm wide. Axon terminals (bouton terminaux) were 0.968 ± 0.030 μm (mean ± SE).

Dual-Immunocytochemical Detection of Hcrt1/OxA and FG

Hcrt1/OxA-immunolabeled varicosities were sometimes observed very close to FG-labeled neurons in the same focal plane (Fig. 1F–H), suggesting the probable existence of cellular contacts between FG-immunoreactive neurons and Hcrt1/OxA-labeled axons in the LDT. This was confirmed in the ultrastructural study undertaken in PL animals (n = 6). FG reaction product was observed in lysosomes, multivesicular bodies, and cytoplasm of dendrites and somata. In contrast, the immunoperoxidase-DAB-nickel reaction product labeling for Hcrt1/OxA was exclusively detected in axonal profiles, in most cases filling the entire profile, and was much more electrodense than the FG immunoprecipitate (Fig. 3A–E). All Hcrt1/OxA-immunolabeled axonal boutons (varicosities and axon terminals) contained dense-cored vesicles (dcv) and large dense-cored vesicles (ldcv); sometimes these vesicles were also detected in unmyelinated axons (Fig. 3F). Hcrt1/OxA immunoreactivity was sometimes observed in specific portions of the cytoplasm, dcv, and ldcv (Fig. 3F). All Hcrt1/OxA axon terminals and many Hcrt1/OxA varicosities also contained small clear vesicles (Fig. 3A,F). ANOVA tests showed no significant variations in Hcrt1/OxA-immunoreactive profile area density (number of labeled profiles per square micrometer of analyzed surface) between animals (1.018 × 10^3 ± 0.177 × 10^3 μm^2, F_{5,42} = 1.08, P = 0.38) or ultrathin sections (0.999 × 10^3 ± 0.246 × 10^3 μm^2, F_{1,36} = 0.49, P = 0.90), assuring a low interindividual variability of the sample. Hcrt1/OxA immunoprecipitate was mainly located in unmyelinated axons (diameter ≤ 0.235 μm, n = 1277, 71.29 ± 3.98%; Fig. 3C–E) and varicosities (diameter 0.236–0.699 μm, n = 370, 24.44 ± 3.33%; Fig. 3A,B,F); it was less frequently observed in axon terminals (diameter ≥ 0.700 μm, n = 68, 4.23 ± 1.13%; Fig. 3A). There were no significant differences in the Hcrt1/OxA distribution...
between a sample including all the Hcrt1/OxA-immunoreactive profiles and a sample that contained only the profiles with circularity > 0.7 ($\chi^2 = 0.33, P = 0.95$), indicating that our criterion to measure axonal diameter was a good assessment of the real diameter of Hcrt1/OxA axons.

Hcrt1/OxA immunoreactivity was mainly observed in unmyelinated axons sectioned on a transverse plane ($79.26 \pm 2.01\%$; Fig. 3C,D), which often formed part of bundles of unmyelinated axons. Hcrt1/OxA unmyelinated axons were also detected in the longitudinal or nontransverse plane ($20.74 \pm 2.01\%$; Fig. 3B,E) and sometimes contained cv and ldcv. ANOVA indicated no significant variations between animals in the preferential transverse sectioning of Hcrt1/OxA axons ($F_{5,12} = 0.42, P = 0.83$). The preferential location of Hcrt1/OxA in transversally cut axons grouped in bundles and the few cellular contacts they make within the LDT suggest that these Hcrt1/OxA unmyelinated axons would mostly be fibers of passage traversing the LDT toward other brain regions. Hcrt1/OxA varicosities were also mainly observed in the transverse plane ($95.26 \pm 1.01\%$) in all the animals ($F_{5,12} = 0.09, P = 0.99$). Most of the Hcrt1/OxA axonal boutons were varicosity type (diameter $0.236-0.699 \mu m, 85.86 \pm 2.52\%$), but there were also terminal-type boutons (diameter $> 0.700 \mu m; 14.14 \pm 2.52\%$); there were no statistical differences between animals in the distribution of the Hcrt1/OxA-containing boutons ($F_{5,12} = 0.58, P = 0.71$).

Table 1

<table>
<thead>
<tr>
<th>Method</th>
<th>Side</th>
<th>PL (%)</th>
<th>PL-MD (%)</th>
<th>Cg1-PL (%)</th>
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<td>IH</td>
<td>Ipsilateral</td>
<td>74.75 ± 2.78</td>
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<td>Contralateral</td>
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<td>IF</td>
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<td>76.68 ± 3.71</td>
<td>76.79 ± 6.42</td>
<td>72.62 ± 6.48</td>
</tr>
<tr>
<td></td>
<td>Contralateral</td>
<td>23.32 ± 3.71</td>
<td>23.21 ± 6.42</td>
<td>27.38 ± 6.48</td>
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Note: Cg1 ($n = 4$); MO ($n = 4$); PL ($n = 10$).
Cellular Contacts

Some Hcrt1/OxA-immunoreactive profiles established cellular contacts with dendrites or somata in the LDT (16.99 ± 1.52%; Table 3). Most of the cellular contacts made by Hcrt1/OxA boutons were appositions with LDT dendrites (n = 140; Table 3, Fig. 4D), most of which were unlabeled (78.57%; Table 3) although some of them were FG-labeled dendrites (21.43%; Table 3). All the synapses were morphologically asymmetric (excitatory type, n = 53; Table 3) and were formed exclusively between Hcrt1/OxA boutons and either unlabeled (86.79%; Table 3, Fig. 4A,B) or FG-labeled (13.21%; Table 3, Fig. 4C) LDT dendrites. The incidence of cellular contacts made by Hcrt1/OxA-containing boutons was associated to the morphological type of axonal bouton; thus, Hcrt1/OxA axon terminals made contacts more frequently than Hcrt1/OxA varicosities (χ²=28.35, P < 0.0001; Fig. 5), indicating the association between bouton morphology type (varicosity or terminal) and the presence of cellular contact (apposition or synapse). Moreover, the percentage of synapses from the total contacts made by Hcrt1/OxA boutons indicated that Hcrt1/OxA axon terminals (50.00%) established more synapses than Hcrt1/OxA varicosities (19.58%), which was confirmed with chi-square test (χ²=17.21, P < 0.0001). The mean diameter of the dendrites that established synapses with Hcrt1/OxA boutons was around 0.8 μm; the ANOVA test showed significant variations in the percentage of axonal boutons (F²,14 = 5.27, P = 0.02) making synapses with dendrites having a diameter narrower than 0.5 μm and those making synapses with dendrites having a diameter wider than 0.5 μm. Furthermore, some dendrites that formed synapses with Hcrt1/OxA boutons also received synapses from other unlabeled terminals or made appositions with FG-labeled dendrites.

Hcrt1/OxA unmyelinated axons established appositions with LDT profiles (84/1277; Table 3), but synapses were not observed. These appositions were made with either unlabeled (3.93 ± 0.97%) dendrites or FG-labeled dendrites (1.24 ± 0.48%). Appositions with FG-labeled dendrites were not

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Table 2

<table>
<thead>
<tr>
<th>mPFC sector</th>
<th>FG*/ChAT− (%)</th>
<th>FG*/ChAT+ (%)</th>
<th>ANOVA</th>
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<tr>
<td>PL</td>
<td>41.71 ± 4.32</td>
<td>58.29* ± 4.32</td>
<td>F₁,₁₈ = 7.36, *P = 0.01</td>
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<td>Cg1-PL</td>
<td>59.32 ± 3.03</td>
<td>40.68* ± 3.03</td>
<td>F₁,₁₈ = 18.95, *P = 0.005</td>
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<tr>
<td>MO-PL</td>
<td>52.17 ± 11.09</td>
<td>47.83 ± 11.09</td>
<td>F₁,₁₈ = 0.08, P = 0.79</td>
</tr>
</tbody>
</table>

Note: Cg1 (n = 4); MO (n = 4); PL (n = 10).
*Fisher’s P < 0.05.
detected in one animal. Some unmyelinated axons and varicosities containing Hcrt1/OxA were close (<0.7 μm) to blood vessels or the vascular glia limitans (4.47 ± 0.70%; Fig. 4E,F), and about half of these axons were varicosities (2.47 ± 1.22%).

**Dual-Immunocytochemical Detection of Hcrt1/OxA and ChAT**

ChAT-immunoreactive neurons were clustered within the boundaries of the LDT, but they were also noticeable in surrounding areas of the central gray or tegmentum (data not shown). The density, distribution, and morphological types of Hcrt1/OxA-immunoreactive profiles in this experiment were completely comparable to those observed in the FG-injected animals. Within the LDT, Hcrt1/OxA-labeled axons made frequent contacts with cholinergic neurons. Thus, 50 Hcrt1/OxA-immunoreactive boutons (varicosities or terminals) contacted with dendrites immunolabeled for ChAT out of a total of 100 contacts established by Hcrt1/OxA boutons in the LDT tissue processed for dual Hcrt1/OxA and ChAT labeling. Although many of the Hcrt1/OxA boutons were without discernible synaptic specializations in the examined plane of section (Fig. 4G), we did observe 7 synaptic junctions onto LDT cholinergic dendrites (Fig. 4H); these synapses represented one-third of all the synapses (n = 21) made by Hcrt1/OxA-immunolabeled boutons in the tissue processed for dual Hcrt1/OxA and ChAT labeling. The remaining synapses were onto dendrites that were not labeled with ChAT-immunogold in the observed orientation.

**Discussion**

FG was chosen to detect LDT neurons projecting to mPFC (PL) because of its high sensitivity for labeling distal dendrites (fourth or fifth branching). In addition, immunoperoxidase detection of FG provides a higher sensitivity for identifying potential synaptic contacts in comparison to other visualization methods such as immunogold (van Bockstaele et al. 1994). We intentionally used a moderately intense DAB immunoprecipitation method to visualize FG so as to not obscure synaptic contacts but also to be able to distinguish FG and Hcrt1/OxA reaction products readily. FG has previously been shown to trace retrograde projections in detail; however, we cannot exclude the possibility that some axon terminals did not uptake the FG; and therefore, the FG-labeled neurons may have been underestimated. Estimation of synaptic contacts in

<table>
<thead>
<tr>
<th>Hcrt1/OxA axonal profile type</th>
<th>Appositional contacts</th>
<th>Synapses (asymmetric)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmyelinated axons (n = 1277)</td>
<td>FG 21</td>
<td>Unlabeled 63</td>
<td>FG 0</td>
</tr>
<tr>
<td>Varicosities (n = 370)</td>
<td>26</td>
<td>89</td>
<td>3</td>
</tr>
<tr>
<td>Axon terminals (n = 68)</td>
<td>4</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>173</td>
<td>7</td>
</tr>
</tbody>
</table>

Note: The contacts are classified regarding their morphological type (apposition or synapse) and the immunolabeling within the LDT dendrites (FG or unlabeled).
dual-immunolabeled tissue at the electron microscope level is highly dependent on antisera penetration, which was enhanced in our study by using free-thawing and incubation in a Triton X-100 solution. Moreover, only ultrathin sections near the tissue–Epon interface were analyzed to ensure optimal detection of the labelings (Hcrt1/OxA, FG, and ChAT).

The antisera used here are highly specific for the peptides against which they were raised as shown in previous studies (Basheer et al. 2002; Guan et al. 2002; Garzón and Pickel 2004; del Cid-Pellitero and Garzón 2011). Negative control experiments for all antisera used here consisted of omission of the primary antisera from the incubation solution; they yielded absence of immunolabeling. In the immunofluorescent sections, we studied all confocal sections (1 μm thickness) to avoid the overlapping of fluorescent signals. We also obtained microphotographs of control sections to check that the observed immunofluorescence was not an autofluorescent signal from the tissue.

The Hcrt1/OxA antiserum does not cross-react with Hcrt2/OxB antisera (Guan et al. 2002). We observed Hcrt1/OxA immunoprecipitate exclusively in axonal profiles, as has been described in other brain structures (Torrealba et al. 2003; Figure 4. Cellular contacts formed by Hcrt1/OxA-containing axons in the LDT. (A) Hcrt1/OxA varicosity (Hcrt1/OxA-V) makes an asymmetric excitatory-type synapse (curved arrow) with an unlabeled dendrite (Den). (B) Unlabeled dendrite receiving 2 asymmetric synapses (curved arrows) from an Hcrt1/OxA-immunoreactive terminal (Hcrt1/OxA-T). An FG-labeled dendrite (FG-den) showing DAB immunoprecipitate (black arrow) is seen in the nearby neuropil. (C) An Hcrt1/OxA-T establishes an asymmetric synapse (curved arrow) with a longitudinally sectioned FG-den, identified by the presence of DAB immunoprecipitate (black arrow). (D) An Hcrt1/OxA-T showing Hcrt1/OxA labeling somewhat restricted to portions of its cytoplasm forms an appositional contact with an unlabeled dendrite (Den) that receives convergent asymmetric synaptic input (curved arrow) from an unlabeled terminal (Ter). (E) Hcrt1/OxA-V makes an appositional contact with an endothelial cell (e) of a blood vessel (bv). Notice the DAB–nickel immunoprecipitate (Hcrt1/OxA) evenly distributed in the cytoplasm and Idcv of the varicosity. (F) Hcrt1/OxA unmyelinated axon (Hcrt1/OxA-Ax) in the proximity of an LDT bv. (G) An Hcrt1/OxA-T makes a nonsynaptic contact with an LDT dendrite containing multiple ChAT-immunogold particles (straight white arrows). Hcrt1/OxA-T contains small clear vesicles (ssv) near the appositional membrane and dcv that are immunoreactive to Hcrt1/OxA more distanced from the contact surface. (H) A dendrite (ChAT-den₁) containing conspicuous ChAT-immunogold labeling (straight white arrows) contacts an Hcrt1/OxA-T that also establishes a thick asymmetric synapse (curved white arrow) onto another ChAT-immunogold–labeled dendrite (ChAT-den₂). Scale bars: 0.2 μm.)
Prefrontal Cortex, Hypocretin, and Laterodorsal Tegmental Nucleus · Cid-Pellitero and Garzón

Prefrontal Cortex, Hypocretin, and Laterodorsal Tegmental Nucleus (Jones 2009; Reinoso-Sua´rez et al. 2011). The involvement of Steriade, Datta, et al. 1990; Kayama et al. 1992; Boucetta and Jones 2009). Both cholinergic and noncholinergic REM sleep is widely acknowledged (el Mansari et al. 1989; Boucetta and Morales 2009). Both cholinergic and noncholinergic nucleus (PPT), in cortical activation during wakefulness and REM sleep that has been described in rats (Kayama et al. 1992; Boucetta and Jones 2009) and in cats (Steriade, Datta, et al. 1990).

**LDT and mPFC**

Multipolar LDT neurons with extensive dendritic branches had been reported to project to the mPFC (Divac et al. 1978; Vincent et al. 1983; Satoh and Fibiger 1986; Cornwall et al. 1990). We have confirmed that the PL sector of the mPFC is a key target of LDT fibers, as had been described previously using anterograde and retrograde tracers (Satoh and Fibiger 1986; Cornwall et al. 1990).

The LDT contains the Ch6 cholinergic cell group of the brain (Armstrong et al. 1983; Mesulam et al. 1983). Moreover, the Ch6 group is the brain stem cholinergic cell group that most strongly projects to the mPFC (Satoh and Fibiger 1986). Other neurotransmitters or neuromodulators, such as GABA, glutamate, or substance P, are also found in LDT neurons (Vincent et al. 1983; Wang and Morales 2009), although the degree of coexistence with Ach is controversial. Recent experiments indicate that Ach, glutamate, and GABA are mostly found in independent subpopulations of LDT neurons (Wang and Morales 2009). Both cholinergic and noncholinergic (GABAergic and putatively glutamatergic) LDT neurons increase their discharge rate during cortical activation in rats (Boucetta and Jones 2009). Thus, our FG-labeled neurons devoid of ChAT may actually be GABA and/or glutamate LDT neurons.

The prominent role of LDT, as well as pedunculopontine nucleus (PPT), in cortical activation during wakefulness and REM sleep is widely acknowledged (el Mansari et al. 1989; Steriade, Datta, et al. 1990; Kayama et al. 1992; Boucetta and Jones 2009; Reinoso-Suárez et al. 2011). The involvement of these nuclei in REM and waking, however, is not exactly alike. The recent observation of increased expression of early transcription factors during REM sleep only in cholinergic neurons of the PPT, but not in those of the LDT (Datta et al. 2009), as well as previous reports of a differential role in the genesis of phasic REM events (Steriade, Pare, et al. 1990; Shouse and Siegel 1992) indicate the existence of heterogeneity between LDT and PPT in their roles on sleep-waking state modulation. The LDT cholinergic neurons that project to the mPFC could belong to the neuron population involved in cortical activation that is active in wakefulness and/or REM sleep that has been described in rats (Kayama et al. 1992; Boucetta and Jones 2009) and in cats (Steriade, Datta, et al. 1990).

**LDT and Hcrt1/OxA**

The observation of moderate Hcrt1/OxA immunoreactivity in LDT is consistent with previous reports of preproHcrt/preproOx location in the rat brain (Peyron et al. 1998). Hcrt1/OxA and Hcrt2/OxB increase the discharge rate of both cholinergic and noncholinergic LDT neurons in extracellularly recorded mice brain stem slices (Burlet et al. 2002). Hcrt1/OxA infusions in the mouse LDT showed that Hcrt1/OxA increases glutamate release from LDT afferents and generates an inward current that produces depolarization in vitro (Burlet et al. 2002). But in addition to activation of nonselective cation channels generating depolarization, Hcrt/Ox independently activates in LDT neurons a protein kinase C-dependent calcium transient enhancement through L-type calcium channels; this suggests that Hcrt1/OxA may also be active in the regulation of long-term homeostatic or trophic processes (Kohlmieier et al. 2004, 2008).

In our animals, Hcrt1/OxA was detected in the cytoplasm and granular vesicles (dcv and ldcv), as has been reported in other brain structures, such as the posterolateral hypothalamus (de Lecea et al. 1998), tuberomammillary nucleus (Torrealba et al. 2003), and ventral tegmental area (Balcita-Pedicino and Sesack 2007). Hcrt1/OxA axons only made asymmetric synapses with LDT profiles, suggesting an excitatory action on the postsynaptic neurons, some of which project to the PL. Actually, Hcrt1/OxA colocalizes with glutamate (Torrealba et al. 2003) but not with GABA (Balcita-Pedicino and Sesack 2007) in other regions. Although detection of Hcrt1/OxA targeting of LDT neurons projecting to PL is somewhat infrequent, it is likely that some of the unlabeled dendrites observed here belong to FG-containing neurons from a different sectional plane.

The intensity of the Hcrt1/OxA presence in dcv and ldcv in the LDT confirms and extends studies on Hcrt1/OxA location in vesicles of the tuberomammillary nucleus (Torrealba et al. 2003) and ventral tegmental area (Balcita-Pedicino and Sesack 2007) using colloidal gold immunolabeling. The fact that the Hcrt1/OxA axons only establish asymmetric synapses, the scarcity of these synapses and the location of Hcrt1/OxA in dcv and ldcv far from the synapses suggest that the excitatory actions of Hcrt1/OxA may be due to synaptic transmission or extrasynaptic volume transmission. Neuropeptides are typically stored in dcv and ldcv (Thureson-Klein 1983; Zhu et al. 1986; Peddie et al. 2008). Diffusion of neuropeptides through the extracellular space to active specific receptors at distant locations from releasing sites is characteristic of volume transmission (Fuxe et al. 2007). Therefore, Hcrt1/OxA could excite the postsynaptic neuron and maintain the action of other coexistent neurotransmitters, such as glutamate (Torrealba et al. 2003).

Hcrt1/OxA injections in the LDT of anesthetized cats significantly enhance wakefulness and suppress REM sleep (Xi et al. 2001). The appositional and synaptic contacts...
between Hcrt1/OxA axons and LDT neurons that project to the PL suggest that the LDT neurons could be involved in the cortical activation characteristic of wakefulness. In addition, LDT cholinergic neurons project to dorsolateral and ventral regions of pontine tegmentum, strongly involved in sleep-wakefulness cycle regulation in rats (Semba 1993) and cats (Rodrigo-Angulo et al. 2005). The Hcrt1/OxA innervations of these LDT neurons modulate REM sleep-suppressive effects of Hcrt1/OxA in the pontine tegmentum (Moreno-Balandrán et al. 2008). Moreover, Hcrt1/OxA infusion in the LDT increased the excitatory postsynaptic potentials in neurons of the oral pontine tegmentum (Xi et al. 2003).

Hcrt1/OxA excites cholinergic and noncholinergic LDT neurons (Burlet et al. 2002), which receive abundant asymmetric excitatory-type inputs (Garzón and Pickel 2000; Pickel et al. 2002). In the present study, we provide for the first time anatomical evidence for specific Hcrt1/OxA axon synaptic targeting of LDT cholinergic neurons, which supports previously reported hypocretinergic activation of those cholinergic neurons on electrophysiological grounds (Burlet et al. 2002). Actually, the present observation that one-third of the synapses made by Hcrt1/OxA axons in the LDT were onto ChAT-immunolabeled dendrites strongly suggests a prominent targeting of cholinergic neurons, since ChAT labeling is somewhat underestimated due to the pre-embedding immunogold technique limitations (Chan et al. 1990). Hypocretinergic activation of cholinergic LDT neurons with ascending projections to mPFC sustains electroencephalographic activation and arousal, as discussed above. Moreover, recent experiments in mice null for both Hcrt/Ox-R1 and Hcrt/Ox-R2 indicate that hypocretin signaling disruption in the LDT results in cholinergic LDT neuron upregulation, and cholinergic dysregulation could underlie the cataplexy accompanying narcolepsy in humans and some animal models (Kalogiannis et al. 2010). An additional intervening reason for cataplexy is presumably the interference with cholinergic autoregulation in the LDT (Leonard and Llinás 1994; Garzón and Pickel 2000). However, our results indicate that Hcrt1/OxA also contacts noncholinergic LDT neurons, in agreement with reported direct hypocretinergic excitation of noncholinergic LDT neurons (Burlet et al. 2002).

The synaptic incidence between Hcrt1/OxA-immunolabeled axons and LDT profiles (FG and non-FG) is probably underestimated because we analyzed nonserial ultrathin sections and the synaptic specializations occupy a small proportion of the neuron membrane surface (Peters et al. 1991). LDT is known to innervate sectors of mPFC other than PL, such as infralimbic-orbitomedial or anterior cingulate cortex (Satoh and Fibiger 1986; Cornwall et al. 1990). Therefore, Hcrt1/OxA targeting of LDT neurons projecting to the mPFC cortex is likely to be significantly higher than that reported here, since our ultrastructural study of dual Hcrt1/OxA + FG labeling included only the animals with FG injections limited solely to the PL cortex.

Neuropeptide Y, a well-known peptide involved in microvascular functions (Abounader and Hamel 1997), is contained in hypothalamic neurons targeted by Hcrt/Ox axons (Horvath et al. 1999). In addition, Hcrt/Ox-R1 activation attenuates neurogenic vasodilation of dural vessels (Holland et al. 2005), and Hcrt/Ox has also shown vasomotor effects through activation of nitricergic or GABAergic neurotransmission in the solitary tract (Shih and Chung 2007). Our observation of Hcrt1/OxA axons close to LDT blood vessels further suggests a probable role for the Hcrt/Ox system in vasomotor control.

Conclusion

In this study, we have shown for the first time that Hcrt1/OxA-containing axons appose and make excitatory-type synapses with LDT neurons that project to the PL sector of the mPFC and that more than a half of these neurons are cholinergic. Hcrt1/OxA axons show noteworthy but not exclusive targeting of LDT neurons containing Ach. We suggest that disruption of Hcrt/Ox actions in LDT might play a part in the excessive daytime sleepiness present in narcoleptics. Our findings support the contribution of Hcrt1/OxA actions on LDT cholinergic and noncholinergic neurons to cortical activation during wakefulness and REM, as well as the known roles of these neurons in sleep-wakefulness transitions (Carter et al. 2009) and maintenance of arousal characteristic of attentional functions of mPFC (Gill et al. 2000; Kozak et al. 2006).

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Notes

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