Integrative Biosciences 9: 145-152, 2005

Integrative
Biosciences
http://www.ibiosci.or.kr

Hypothalamic Orexin-A Projections to Midline Thalamic Nuclei in the Rat

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Abstract: A retrograde tracer, WGA-apo-HRP-gold, was injected into midline thalamic nuclei and subsequently orexin-A immunostaining was performed on the tuberal region of the hypothalamus in order to investigate orexinergic projections to the midline thalamus. Injection site was targeted within one specific region, i.e., paraventricular, centromedian, rhomboid, reuniens, or intermediodorsal nucleus, but it proved to be either one or a combination of these thalamic nuclei. The distribution of WG/orexin-double-labeled neurons exhibited a general pattern in that the majority of labeled cells were observed within the ventral portion of the lateral hypothalamus as well as the perifornical nucleus (PeF). A small number of double-labeled cells were also observed at the dorsomedial nucleus, the area dorsal to the PeF, dorsal portion of the lateral hypothalamus, and the posterior hypothalamus. These orexin-immunoreactive neurons might have wake-related influences over a variety of functions related with midline thalamic nuclei, which include autonomic control, associative cortical functions, and limbic regulation.

Key words: Orexin-A immunocytochemistry, WGA-apo-HRP-gold retrograde tracing, midline thalamic nuclei, hypothalamus

The neuropeptide hypocretin (orexin), recently found in perifornical sites in the tuberal region of the hypothalamus, has been known to play a crucial role in the maintenance of arousal state of the animal (de Lecea et al., 1998; Chemelli et al., 1999; Hagan et al., 1999; Horvath et al., 1999; Brown et al., 2001). The state of arousal promoted by orexin is closely associated with regulation of feeding and drinking, increased locomotor activity, enhanced sympathetic and cardiovascular activity, and energy homeostasis (Sakurai, 1998; Taheri et al., 2002; Baumann and Bassetti, 2005). The highest densities of orexin-positive axonal fibers have been found in the locus coeruleus (LC) and the dorsal raphe (DR), the laterodorsal tegmental nucleus, and the pedunculopontine tegmental nucleus of the rat and the cat, further suggesting an important role for this peptide in wake-related behaviors (Peyron et al., 1998b; Mintz, 2001; Zhang et al., 2004).

Physiological studies reported that orexin provides excitatory influence on noradrenergic LC, serotonergic DR, and histaminergic tuberomammillary nucleus and causes wakeful state of the animal (Horvath et al., 1999; Inzunza, 2000; Brown et al., 2001; Eriksson et al., 2001, 2004). There has been, however, no retrograde labeling study to examine differential distribution of these orexinergic projection neurons within the tuberal region of the hypothalamus. Only recently, an anatomical study was performed, which suggested that LC-projecting neurons were located primarily within the dorsal half of the orexin cell group, whereas arousal-related, basal forebrain-projecting neurons were distributed within the medial half (Espana et al., 2005). Some of the distribution of orexinergic, LC- or basal forebrain-projecting neurons coincide with those of orexinergic, DR-projecting neurons (Lee et al., 2005c).

Our preliminary results indicated that orexin-immunoreactive axon terminals were extremely dense within the midline thalamic nuclei and that the density of fiber distribution was even higher than that at the monoaminergic nuclei. The most distinct midline cell group is the paraventricular nucleus located below the dorsal ventricular wall (Paxinos and Watson, 1998). Several additional cell groups can be also recognized in the periventricular gray matter, such as centromedian, reuniens, rhomboid, and intermediodorsal nuclei. These midline thalamic nuclei are classified as diffuse projection nuclei and are part of a system believed to govern the level of arousal of the brain (Jones, 1985; Ohye, 1990). Thus, the present study was performed to examine hypothalamic orexinergic projections to various regions of the midline thalamic nuclei. A retrograde tracer, WGA-apo-HRP-gold (WG), was employed to make a

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discrete injection within each thalamic nucleus and orexin immunostaining was performed for the hypothalamus in order to examine the distribution of WG/orexin-double-labeled cells in the hypothalamus.

MATERIALS AND METHODS

Twenty-three Sprague-Dawley rats of both sexes ranging in weight from 300 to 350 g were used in the present study. Prior to surgery, each rat was anesthetized with an intraperitoneal injection of chloral hydrate (3.6% in distilled water, 1 ml/100 g body weight).

Injection sites at midline thalamic nuclei

A circular-shaped piece of bone with a radius of 2 mm, centered at specific point along the midline, was removed from the dorsal surface of the skull and the superior sagittal sinus was ligated with surgical sutures rostrally and caudally. Angiovasectomy was performed between suture points in order to expose the cerebral fissure at midline. Based on the atlas of Paxinos and Watson (1998), anterior, middle, and posterior paraventricular thalamic nuclei were located between 1.6 and 3.6 mm caudal to the bregma, thus extended 2.0 mm rostro-caudally. Because of diffusion of the tracer from the center of injection, each nucleus was approached for sites 2.0, 2.5, and 3.0 mm caudal to bregma at the depth of 4.5, 5.0, and 5.5 mm from the dural surface. Rhomboid/reuniens nuclei were approached at the depth of 6.0 mm from the dural surface in the middle paraventricular thalamic level.

WGA-apo-HRP-gold (WG) injection

The WG was synthesized using inactivated WGA-HRP (Sigma, L-0390) and 20 nm (Sigma, G1652) colloidal gold, as described in Basbaum and Menetrey (1987). The injection apparatus consisted of a glass micropipette (tip diameter, 10-15 μm) hydraulically linked to a 2.0 μl Hamilton syringe. Approximately 1.0 μl of tracer was drawn into the pipette and subsequently a small amount of oil was also backfilled into the tip of the pipette right before injection, so that tissue along the pipette track would not be exposed to the tracer. We injected 0.02 μl of WG nine times (a total of 0.18 μl) at 3-min intervals into a single site within the midline thalamus and took out the pipette 30 min after the first injection trial.

Perfusion-fixation and silver enhancement reaction

After a survival period of 48-72 h following tracer injections, the animals were anesthetized with an intraperitoneal injection of chloral hydrate (3.6% in distilled water, 1 ml/100 g body weight) and perfused using 150 ml of saline followed by 600 ml of fixative containing 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS, pH 7.4). The perfusion-fixation was completed with 100 ml of PBS containing 10% sucrose. The brain was then removed and stored in 30% sucrose solution in PBS overnight. A series of 40 μm sections were prepared using a cryostat. Following rinses with distilled water, the WG was detected using a commercial silver intensification kit (Sigma, SE-100) as described in Llewellyn-Smith et al. (1992).

Hypocretin (orexin) immunocytochemistry

The prepro-orexin gene in the tuberal region of the rat hypothalamus encodes a precursor (prepro-orexin) with 130 amino acids. Rat hypocretin-1 (orexin-A) is a 39-amino acid peptide (residues 28-66), whereas hypocretin-2 (orexin-B) consists of 29-amino acids (residues 69-97) (Gautvik et al., 1996; de Lecea et al., 1998). We performed immunostaining for the hypothalamus using rabbit antibodies for both orexin-A (Chemicon, AB3704) and orexin-B (Chemicon, AB3100). Orexin-B immunostaining did not yield results despite repeated trials, thus in the present report we describe only the results obtained with anti-orexin-A.

Tissue sections through the hypothalamus were processed for orexin immunostaining using the peroxidase-anti-peroxidase (PAP) method of Sternberger (1986). Sections were washed in 0.1 M tris-buffered saline (TBS, pH 7.4) and incubated in 0.5% H_2O_2 in TBS for 20 min to inhibit endogenous peroxidase activity. After rinses, sections were incubated in 10% normal goat serum (Vector, S-1000) for 1 h to block non-specific binding. After a brief rinse, free-floating sections were incubated in 1 : 1,000 diluted rabbit anti-orexin-A (Chemicon, AB3704) for 48 h (4°C). Sections were incubated in 1 : 100 dilution of goat anti-rabbit immunoglobulin (Chemicon, AP132). After rinses, sections were incubated in rabbit PAP (Chemicon, PAP18) diluted 1 : 400. After rinses with 0.1 M Tris buffer (TB, pH 7.4), sections were reacted with 3,3'-diaminobenzidine (DAB)-H_2O_2 (Vector, SK-4100) for 1-2 min (4°C).

For the immunocytochemical procedures, positive tissue controls were performed using sections from various orexin-immunoreactive brain regions. Negative control experiments included omission of the primary antibody and substitution of an equivalent dilution of normal serum, or reaction of a series of sections with increasing dilutions of the primary antibody until all staining was lost. Similarly, omission of the secondary antibody or the PAP solution was also performed to indicate whether either the reagents or the procedures would give rise to non-specific staining.

Microscopic observations

The sections were mounted on gelatin-coated glass slides and dried overnight. They were then dehydrated using a graded series of ethanol, treated with xylene, and coverslipped

with Permount (Fisher, SP15-500). Sections were observed and pictures were taken using Nikon E600 microscopic and photographic system.

Results

For 23 rats used in the present study, the retrograde tracer WG was injected into midline thalamic nuclei and subsequently orexin-A immunostaining was performed on hypothalamic sections in order to investigate orexinergic projections to the midline thalamus. Based on the atlas of Paxinos and Watson (1998), midline thalamus was located between 1.6 and 3.6 mm caudal to the bregma, including paraventricular, centromedian, rhomboid, reuniens, and intermediodorsal nuclei. The original experimental scheme was to inject the tracer at each midline thalamic nucleus. An injection site, however, often involved more than one thalamic nucleus. Five representative examples of injection sites are shown in Fig. 1, i.e., the anterior paraventricular (A, R56), anterior paraventricular/centromedian (B, R65), rhomboid/reuniens (C, R72), posterior paraventricular (D, R58), and posterior paraventricular/intermediodorsal (E, R74) thalamic nuclei. The medio-lateral dimension of WG injection sites was in the range of 150-300 μm.

The distribution of WG/orexin-double-labeled neurons within various hypothalamic nuclei was examined for six representative injection cases (Table 1, R56, R65, R61, R63, R72, R58, and R74). Injection at anterior paraventricular nucleus alone (R56) or one in combination with the centromedian nucleus (R65) produced the retrograde

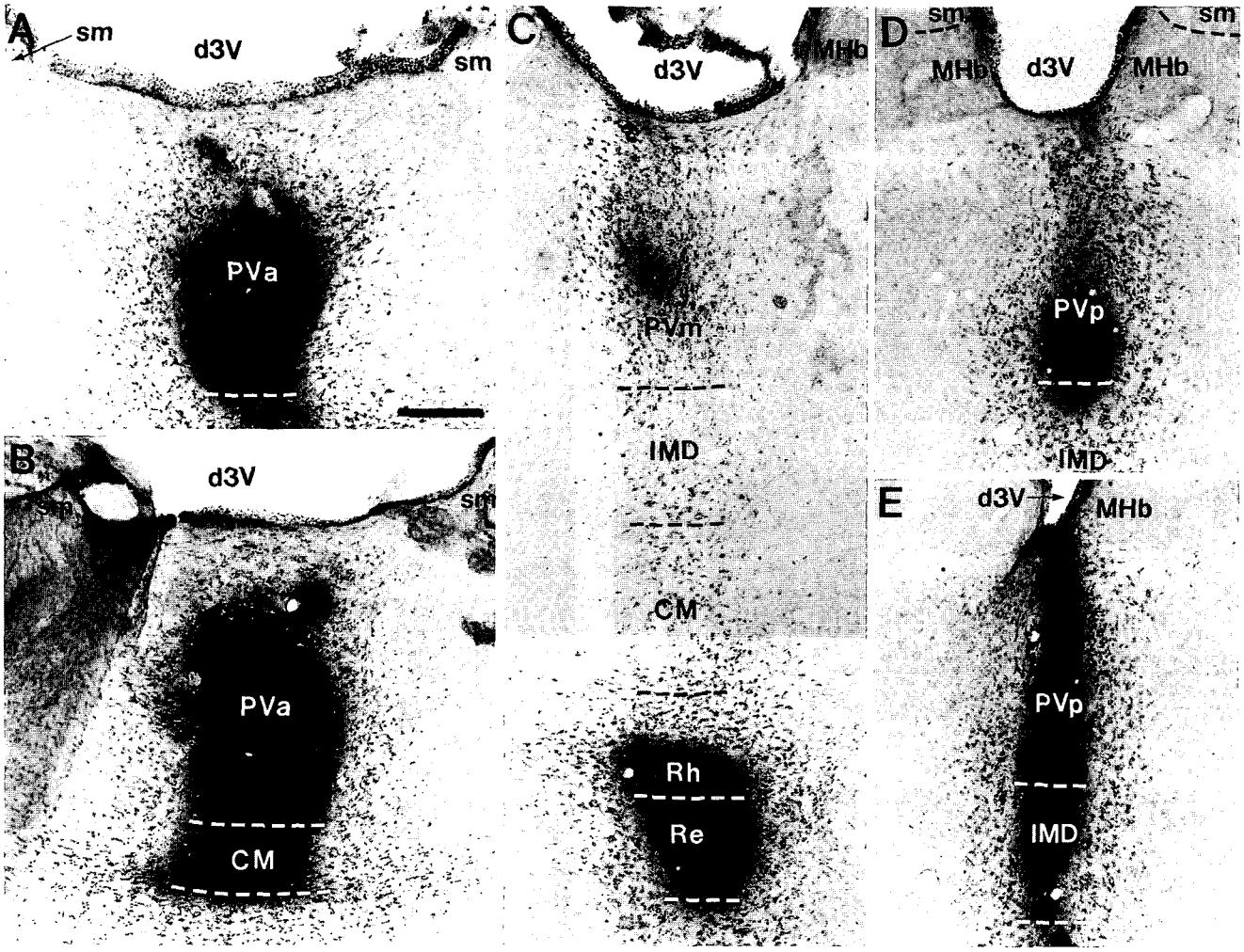

Fig. 1. Injections of WGA-apo-HRP-gold (WG) at midline thalamus produced an injection site involving one or a combination of midline thalamic nuclei. Representative injection sites were shown, including the anterior paraventricular (A, R56), anterior paraventricular/centromedian (B, R65), rhomboid/reuniens (C, R72), posterior paraventricular (D, R58), and posterior paraventricular/intermediodorsal (E, R74) thalamic nuclei. The transition from rostral (A and B), intermediate (C) to caudal (D and E) thalamic levels could be identified by the narrowing of the dorsal third ventricle (d3V), the approximation of left and right stria medullaris (sm), and the appearance of the medial habenular nucleus (MHb) on either side of d3V. CM, centromedian thalamic nucleus; IMD, intermediodorsal nucleus; PVa, anterior paraventricular thalamic nucleus; PVm, middle PV; PVp, posterior PV; Re, reuniens nucleus; Rh, rhomboid nucleus. Scale bar = 200 μm.

Table 1. The distribution of WG/orexin-double-labeled neurons within various hypothalamic nuclei (Hypo) following WG injections into various, midline thalamic nuclei

Hypo.	Inj. cases/Inj. sites						
	R56/PVa	R65/PVa+CM	R61/PVm	R63/PVm+CM	R72/Rh+Re	R58/PVp	R74/PVp+IMD
DM	4	5	0	1	0	0	0
PeF	6	9	8	7	5	6	8
dPeF	1	2	3	4	3	2	3
dLH	2	3	3	1	4	3	4
vLH	11	17	14	12	13	12	15
PH	0	0	0	0	0	1	2

CM, centromedian thalamic n.; dLH, dorsal portion of lateral hypothalamus; DM, dorsomedial hypothalamic n.; dPeF, the area dorsal to the PeF; IMD, intermediodorsal thalamic n.; PeF, perifornical nucleus; PH, posterior hypothalamus; Pva, anterior paraventricular thalamic nucleus; PVm, middle PV; PVp, posterior PV; Re, reuniens thalamic n.; Rh, rhomboid thalamic n.; vLH, ventral portion of lateral hypothalamus; WG, WGA-apo-HRP-gold.

labeling mainly at the ventral portion of the lateral hypothalamus as well as the perifornical nucleus (PeF). Retrograde labeling at dorsomedial hypothalamic nucleus was also prominent. A small number of double-labeled cells were also observed in the area dorsal to the PeF and the dorsal portion of the lateral hypothalamus, but none in the posterior hypothalamus (Table 1, R56 and R65). Representative examples of WG-single- or WG/orexin-double-labeled neurons were depicted from case R56 (Fig. 2). Labeled cells were observed at dorsomedial (Figs. 2A and B) and perifornical (Figs. 2C-E) hypothalamic nuclei. WG-labeled cells contained dense population of black granules within the cytoplasm (Figs. 2B, D, and E). The size of WG-single-labeled cells was sometimes small (Fig. 2B, straight and solid arrow), but the majority of WG/orexin-double- (Figs. 2B and D, open arrows with asterisks) or WG-single-labeled (Fig. 2E) cells were in the range of 15-25 μm and the morphology was fusiform (Figs. 2B and D) to multipolar (Fig. 2E).

Following injection at middle paraventricular nucleus (Table 1, R61) or one in combination with the centromedian nucleus (Table 1, R63), the majority of WG/orexin-double-labeled cells were observed at the ventral portion of the lateral hypothalamus as well as the perifornical nucleus. A

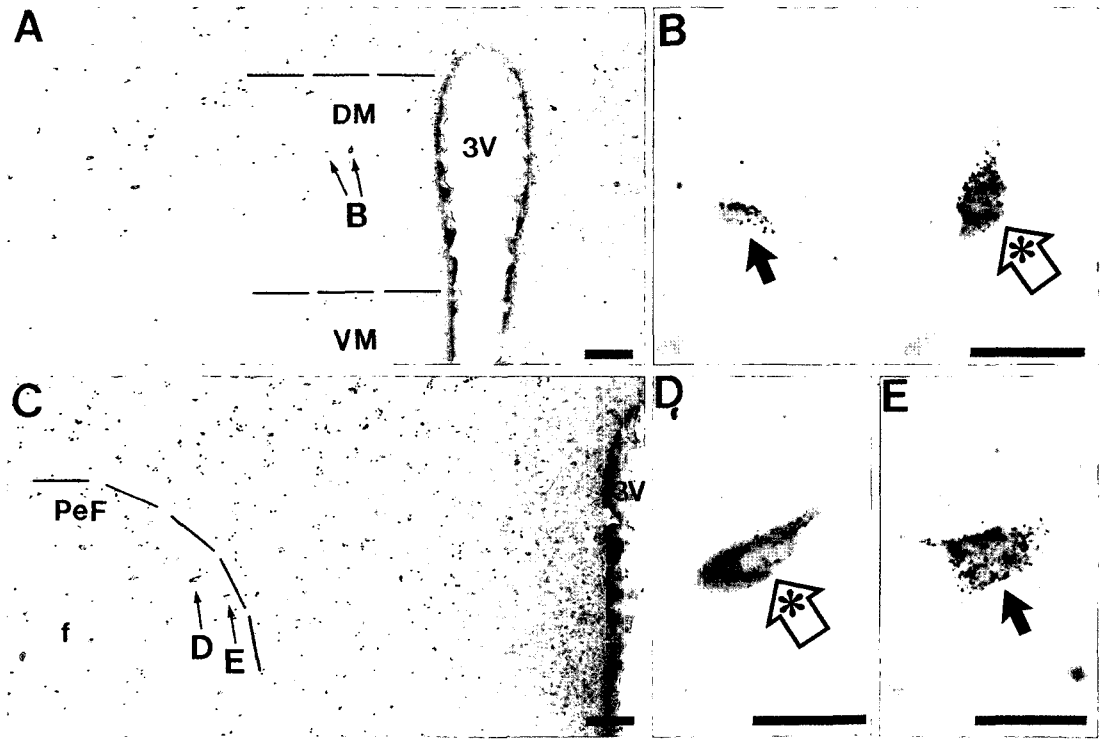

Fig. 2. Following WG injection at anterior paraventricular nucleus (R56), labeled cells were observed at dorsomedial (A and B) and perifornical (C-E) hypothalamic nuclei. Panels B and D/E are higher magnification views of WG-single- (straight and solid arrow) and WG/orexin-double-labeled (open arrows with asterisks) neurons at A and C, respectively. DM, dorsomedial hypothalamic nucleus; f, fornix; PeF, perifornical nucleus; VM, ventromedial hypothalamic n.; 3V, the third ventricle. Scale bars = 100 μm (A, C), and 25 μm (B, D, E).

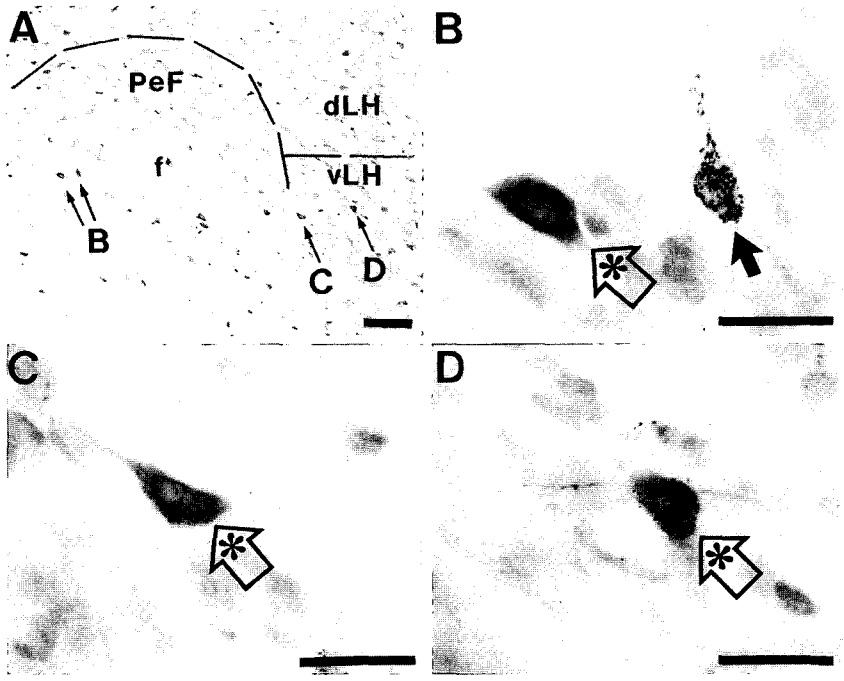

Fig. 3. Following WG injection at rhomboid/reuniens thalamic nuclei (R72), a number of labeled neurons were observed at perifornical (PeF) as well as the ventral portion of the lateral hypothalamus (vLH) in a transverse section (A). Panels B-D represent higher magnification views of WG-single-labeled (B, straight and solid arrow) as well as WG/orexin-double-labeled (B-D, open arrows with asterisks) at panel A. dLH, dorsal portion of the lateral hypothalamus; f, fornix. Scale bars = 100 μm (A) and 25 μm (B-D).

small number of labeled cells were observed in the area dorsal to the PeF and the dorsal portion of the lateral hypothalamus, whereas those at the dorsomedial hypothalamus were minimal.

An injection was made at rhomboid/reuniens thalamic nuclei (Table 1, R72). The majority of WG/orexin-double-labeled cells were observed at the ventral portion of the lateral hypothalamus. Labeling was also prominent at the PeF, the area dorsal to the PeF, and dorsal portion of the lateral hypothalamus. No double-labeled cells were observed in the dorsomedial and posterior hypothalamus. Representative examples of labeled neurons are shown in Fig. 3. Double-labeled cells were located at perifornical (Figs. 3A and B) as well as the ventral portion of the lateral hypothalamus (Figs. 3A, C, and D). The morphology of labeled cells was fusiform (Figs. 3B and C) or multipolar (Fig. 3D) and the diameter was in the range of 15-25 μm.

Injection at posterior paraventricular nucleus alone (Table 1, R58) or one in combination with intermediodorsal nucleus (Table 1, R74) produced labeling at the ventral portion of the lateral hypothalamus as well as the perifornical nucleus. Labeled cells were also observed at the area dorsal to the PeF, the dorsal portion of the lateral hypothalamus, and the posterior hypothalamus (Table 1, R58 and R74). No double-labeled cells were observed at the dorsomedial hypothalamus. Representative examples of WG-single-, orexin-single-, or WG/orexin-double-labeled neurons following WG injection at posterior paraventricular nucleus (R58) were shown in Fig. 4. Labeled neurons were observed at rostral (Figs. 4A and B) and caudal (Figs. 4C-F) levels of the ventral portion of the lateral hypothalamus. The morphology of labeled neurons was fusiform, oval-shaped, or multipolar (Figs. 4B, D, E, and F) and the diameter was either small (Figs. 4B and D, 10-15 μm) or large (Figs. 4E and F, 15-25 μm).

Discussion

The goal of the present study was to investigate differential orexinergic projections to midline thalamic nuclei. The retrograde tracer WGA-apo-HRP-gold was used, because it produces a smaller, well-circumscribed injection site (medio-lateral dimension of 150-300 μm) compared with HRP or cholera-toxin B subunit (Basbaum and Menetry, 1987; Peyron et al., 1998a). Precautions were taken to minimize leakage of the tracer along the injection pathway from the dural surface. First, a small amount of oil was backfilled into the tip of the pipette after it had been filled with tracer so that tissue along the pipette track would not be exposed to the tracer. Second, pipette tip diameters were kept small (10-15 μm) so as to minimize unwanted release. Finally, we injected tracer substances in nine increments (0.02 μl each) at 3-min intervals in a total of 0.18 μl, since retrograde labeling with WG is most successful when tracer

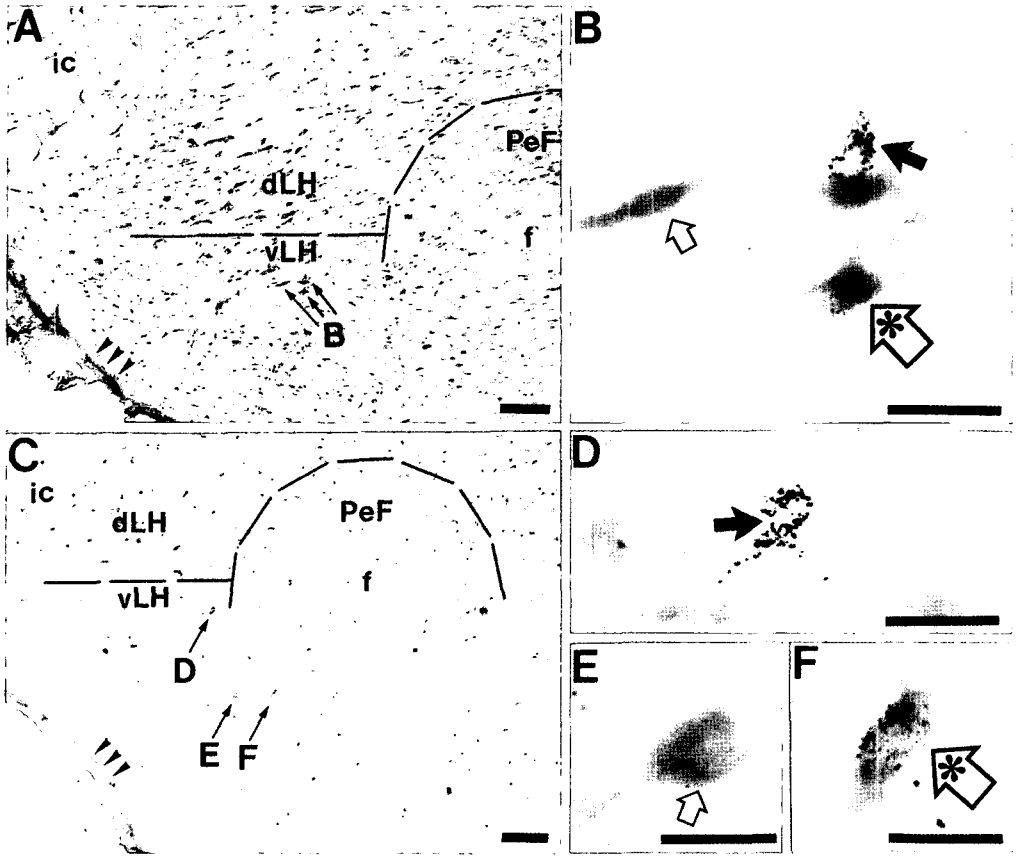

Fig. 4. Following WG injection at posterior paraventricular nucleus (R58), labeled neurons were observed at rostral (A and B) and caudal (C-F) levels of the ventral portion of the lateral hypothalamus (vLH). Examples of WG-single- (B and D, straight and solid arrows), orexin-single- (B and E, open arrows), and WG/orexin-double-labeled (B and F, open arrows with asterisks) neurons were shown at higher magnification views. Arrowheads in A and C represent the pial surface. dLH, dorsal portion of the lateral hypothalamus; f, fornix; ic, internal capsule; PeF, perifornical nucleus. Scale bars = 100 μm (A, C) and 25 μm (B, D, E, F).

substances are injected slowly over extended periods.

Previous studies reported the existence of orexin-immunoreactive cells and axon terminals in various regions of the brain. They were done, however, not in combination with anterograde or retrograde tract tracing to identify the input/output relationships of labeled cells (Peyron et al., 1998b; Mintz, 2001; Zhang et al., 2004; Sakurai et al., 2005). They reported that more than 50% of the orexin-containing cells are located in the perifornical sites at the tuberal level of the hypothalamus. Based on the atlas of Paxinos and Watson (1998), the perifornical sites they specified include the perifornical nucleus, the area dorsal to the PeF, the lateral hypothalamus, and lateral portions of the dorsomedial and posterior hypothalamus. The distribution of orexin-immunostained cells in the present study coincides with what they reported (Table 1).

We report in the present study that the ventral portion of the lateral hypothalamus as well as the perifornical nucleus provides the major orexinergic projection to midline thalamic nuclei (Table 1). A previous study indicated that the medial half of the orexin cell group provides inputs to arousal-related basal forebrain structures such as medial septal area, medial preoptic area, and substantia innominata, whereas the dorsal half sends inputs to the LC (Espana et al., 2005). Some of the distribution of orexinergic, LC- or basal forebrain-projecting neurons coincide with those of orexinergic, DR-projecting neurons (Lee et al., 2005c). Thus it seems that each hypothalamic cell group sends orexinergic projections to more than one arousal-related targets including LC, DR, basal forebrain regions, and the midline thalamus. In fact, Espana et al. (2005) reported that a large proportion of orexin neurons provides collateral axonal distribution to the LC and the basal forebrain structures. A recent study in our laboratory also reported that a large number of neurons within the lateral hypothalamus as well as perifornical region sends collateral axonal fibers to the LC and the DR (Lee et al., 2005a). Since such axon collaterals might provide simultaneous, possibly more efficient, way of influencing various arousal centers, it will be interesting to examine in future studies whether orexinergic, midline thalamus-projecting neurons send axon collaterals to LC, DR, or arousal-related basal

forebrain structures.

In addition to orexin terminals, the midline thalamic nuclei receive profuse monoaminergic innervation that derives mainly from the noradrenergic neurons of the LC and from the serotonergic neurons of the DR nucleus (Hökfelt et al., 1984; Lavoie and Parent, 1991). The monoaminergic nuclei themselves have reciprocal connections and influence one another during various sleep/wakefulness states (Lakoski et al., 1983; Ericson et al., 1989; Iwase et al., 1993; Barbara et al., 2002; Kim et al., 2004; Lee et al., 2005b). Brown et al., (2002) reported that DR serotonergic neurons receive convergent excitatory inputs from multiple arousal system such as orexinergic hypothalamus, histaminergic tuberomammillary nucleus, and noradrenergic LC. Colocalization of orexin A and glutamate immunoreactivity was also observed in axon terminals located at the tuberomammillary nucleus of the rat (Torrealba et al., 2003). Thus, it is also one of the major issues for future studies to examine at the ultrastructural level how hypothalamic orexinergic, DR-serotonergic, and LC-noradrenergic axonal fibers interact within midline thalamus and to examine the possibility of the existence of more than one neurotransmitters within axon terminals.

On the other hand, the midline thalamic nuclei send efferent projections to various autonomic and limbic-related structures (Ohye, 1990; Date et al., 1999). Efferent fibers from the paraventricular nucleus, the central nuclear complex, and the nucleus reuniens, project to the amygdaloid nuclear complex as well as ventral parts of the striatum (Mehler, 1971; Berense and Groenewegen, 1990). Some of the midline thalamic nuclei project to the anterior cingulated cortex (Vogt et al., 1979). In addition, fine myelinated and unmyelinated fibers coursing in the periventricular gray matter are thought to relate these nuclei to the hypothalamus (Jones, 1985; Ohye, 1990). Thus, it would be also interesting to examine input/output relations between the orexinergic terminals and midline thalamic neurons projecting to the amygdala, striatum, anterior cigulated cortex, and the hypothalamus. The present and future studies might clarify how these orexin-immunoreactive neurons have wake-related influences over a variety of functions related with midline thalamic nuclei, which include autonomic control, associative cortical functions, and limbic regulation.

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[Received , 2005; accepted , 2005]