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Orexin fibers in the cholinergic mesopontine tegmentum of the rat

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Abstract

Orexins, which are recently discovered neuropeptides, are though to play a critical roll in the regulation of sleep-wake state. Neurons producing orexin are preferentially located in the lateral hypothalamus, while orexin fibers are widely distributed in the central nervous system. The cholinergic neurons in the laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) nuclei are known to be involved in the regulation of sleep-wake state. Present study was therefore undertaken to reveal an orexin innervation to the cholinergic mesopontine tegmental neuron by a double immunohistochemistry for orexin-A and choline acetyltransferase (ChAT). Orexin immunoreactive fibers with varicosities were seen throughout LDT and PPT nuclei. Although the orexin fibers were often distributed in the interstices among ChAT immunoreactive cells, some of fibers appeared on ChAT immunoreactive cells. Furthermore, the regional differences in the density of orexin fibers were observed in the LDT and PPT nuclei.

Key words: orexin, choline acetyltransferase, laterodorsal tegmental nucleus, pedunculopontine tegmental nucleus, immunohistochemistry

The orexin-A and -B, also called hypocretin-1 and -2, are recently identified neuropeputides (de Lecea et al., 1998), which derived from the same precursor (Sakurai et al., 1998), synthesized preferentially on the neurons in the lateral hypothalamic area (Broberger et al., 1998). On the ground of the location of orexin neurons, they have been thought to regulate the appetite (Sakurai et al., 1998). However, the remarkable evidences indicating the contribution of orexin to the sleep/arousal system of the brain are accumulating (Hagan et al., 1999; Estabrooke et al., 2001). The dogs suffering the narcolepsy, which is a disabling sleep disorder, comprise the mutation in the orexin receptor gene (Lin et al., 1999). The mice knocked out the orexin gene show the malfunction of sleep-wake cycle (Chemelli et al., 1999). Moreover, the human brains of narcoleptic patients show a large amount of degeneration of orexin neurons in the lateral hypothalamus (Thannickal et al., 2000). In spite of the restricted location of orexin neurons in the brain, their fibers are widely distributed throughout

the brain (Peyron et al., 1998; Cutler et al., 1999; Nambu et al., 1999; van den Pol, 1999; Date et al., 2000), involving the locus coeruleus, basal forebrain, dorsal raphe, tuberomammillary, laterodorsal tegmental (LDT), and pedunculopontine tegmental (PPT) nuclei (Yanagihara, 2001), where are compromised in the regulation of the sleep-wake cycles (Sinton and McCarley, 2000). It has been reported that the injection of orexin-A into the LDT induced the change of wake-sleep cycles in the cat (Xi et al., 2001). This paper showed the orexin innervation to the cholinergic neurons in the LDT and PPT nuclei by a double immunohistochemistry for orexin-A and choline acetyltransferase (ChAT) that is the enzyme synthesizes the acetylcholine.

Adult female Sprague-Dawley rats (200-350 g: CLEA) were used. The rat was anesthetized with intraperitoneal injection of sodium pentobarbital (100mg/Kg) and perfused cardially with 50 ml of saline followed by 200 ml of 4% paraformaldehyde buffered fixative solution. The brain was removed from the skull, postfixed in the same fixative solution for 4 hours, and then stored in 30% sucrose buffer solution at 4℃ for a week. The brainstem through the mesopontine tegmentum was sliced coronally at 30 μm thickness by the freezing microtome, and sections were collected in the phosphate buffered saline as 4 sets of serial sections. A set of the sections was used for a double immunohistochemistry for orexin-A and ChAT. The sections were soaked in 5% normal donkey serum in phosphate buffered saline containing 3% Triton X-100 (PBS-T) for 30 minutes, and then transferred in the cocktail of anti-bodies of orexin-A (Santa Cruz Biotechnology) at a dilution of 1/2000 and choline acetyltransferase (Chemicon) at a dilution of 1/500 in PBS-T containing 1.5% normal donkey serum for overnight at 4℃. The section were rinsed with PBS three times for 5 minutes each, and then soaked in HRP conjugated anti-rabbit IgG (Chemicon) at a dilution of 1/500 in PBS-T containing 1.5% normal donkey serum for 2 hours at room temperature. After three times of rinses, the sections were immersed in 0.05M Tris-HCl buffer, pH 7.6, containing 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide to produce brown precipitations on immunoreactive components. After sufficient rinses with PBS, the sections were soaked in biotinylated anti-goat IgG at a dilution of 1/500 in PBS-T containing 1.5% normal donkey serum for 30 minutes, rinsed with PBS, and then incubated in the ABC solution (Vector) for 30 minutes. The sections were rinsed with PBS and then developed with the DAB-hydrogen peroxide solution containing 0.04% nickel chloride to make black staining on immunoreactive structures. After several rinsing, the sections were mounted on glass slides coated with MSA (Matunami), air-dried, and coverslipped with Enteran (Merck).

ChAT immunoreactive neurons were detected by diffuse brown staining of the cytoplasm and proximal dendrites. Orexin immunoreactive fibers were found as black fine lines, which could be easily distinguished from brown products of ChAT immunoreactivity. Both LDT and PPT nuclei contained a large number of ChAT immunoreactive cells. Orexin fibers were detected throughout the mesopontine brainstem observed in this experiment. Dens accumulation of orexin fibers was seen in the periaqueductal gray, including the locus coeruleus, dorsal raphe, and LDT nuclei. In the LDT and PPT nuclei, orexin immunoreactive fibers were

distributed throughout the nuclei and most of fibers possessed varicosities. Some varicose fibers were appeared on the ChAT immunoreactive cells, although the varicose fibers were often distributed in the interstices among ChAT immunoreactive cells (Fig. 1). The regional differences in the density of orexin fibers

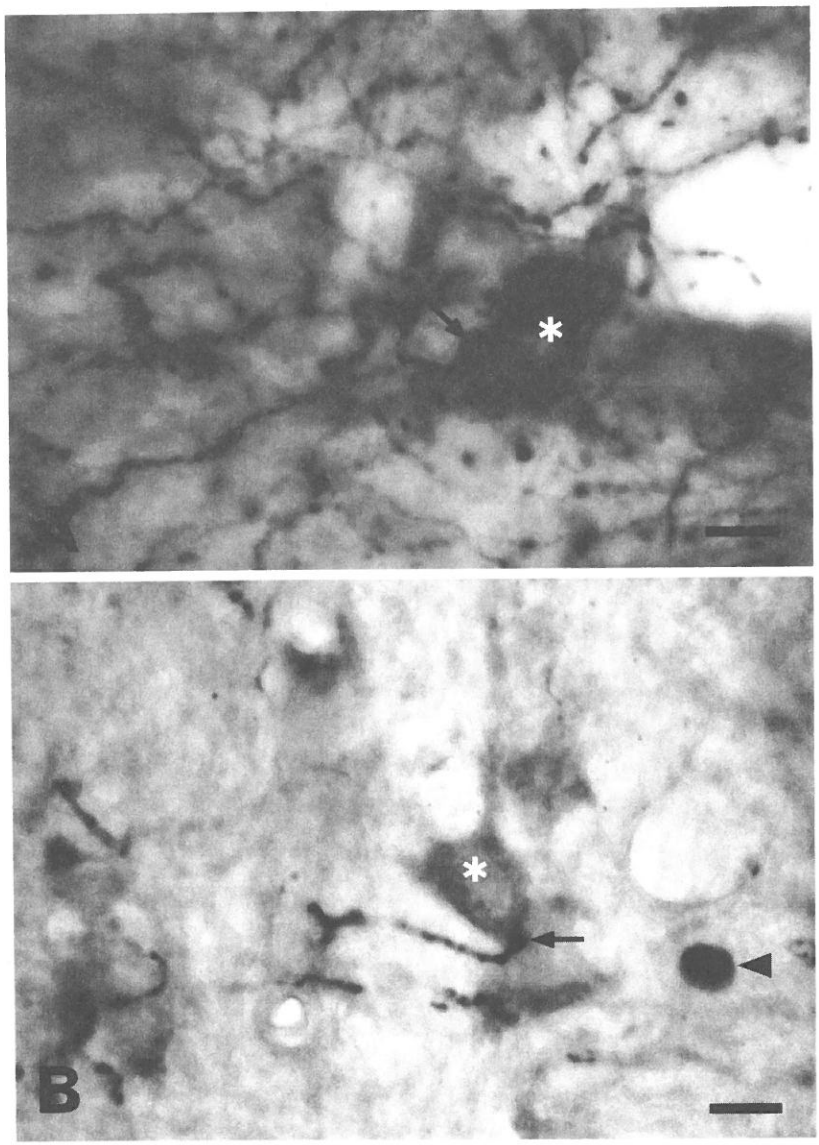

Fig. 1. Photomicrographs of coronal sections showing orexin-choline acetyltransferase (ChAT) interaction in the mesopontine tegmentum. A, A rich amount of dark black orexin-A immunoreactive fibers with varicosities appear in the laterodorsal tegmental nucleus. Some of fibers (solid black arrow) contact with brown ChAT immunoreactive cells (white asterisk). B, Orexin-A immunoreactive fibers (solid black arrow) lie in close apposition to ChAT immunoreactive cells (white asterisk) in the pedunculopontine tegmental nucleus. A solid arrowhead indicates a blood cell. Scale bars = 10 μ m.

were observed in the LDT and PPT nuclei. Most densely populated fibers were seen in the dorsal part of rostral LDT and in the lateral part of caudal LDT. The remaining part of the LDT also contained abundant fibers. Meanwhile the PPT nucleus contained medium to small amount of fibers.

Orexin-A binds to both orexin-1 and orexin-2 receptors (Sakurai et al., 1998). Both types of receptors were detected on LDT and PPT neurons (Trivedi et al., 1998; Hervieu et al., 2001; Marcus et al., 2001), but the chemical properties of these neurons were not speculated. The present observation that orexin fibers contact with cholinergic cells provides a morphological basis of the orexin action to the cholinergic cells in the LDT and PPT.

The noradrenergic cells in the locus coeruleus were abundantly received orexin fibers not only in the dendritic area but also on the cell bodies (Horvath et al., 1999). On the other hand, in the LDT/PPT nuclei the orexin fibers with varicosities were often distributed in the interstices among ChAT immunoreactive cells. The neurons in the LDT and PPT nuclei have wide dendritic area, where maximum path length of dendrite is around 350 μm, and possess dendritic varicosities that increased in distal dendritic segments (Surkis et al., 1996). Moreover, the synapses are mainly detected on the dendrites of cholinergic cells in the PPT nucleus (Steininger et al., 1997), and the majority of synapses make contacts with non-cholinergic axons (Garzon and Pickel, 2000). Due to the technical limitations, distal dendrites of ChAT immunoreactive cells were not visible in this experiment. It is likely, therefore, that the orexin fibers with varicosities present in the space among cholinergic cells may contact with dendrites of them in the LDT and PPT nuclei.

The cholinergic cells in the LDT and PPT nuclei are received various inputs (Rye, 1997), such as the noradrenergic, serotonergic, GABAergic, and cholinergic inputs, providing inhibitory effects on the target cells(Koyama and Kayama, 1993; Leonard and Llinas, 1994; Sakai and Koyama, 1996). However, the majority of dendritic synapses on the cholinergic cells reveal an asymmetric type (Steininger et al., 1997), indicating the excitatory synapses. It has been reported that the orexin provides an excitatory activity on the neuronal cells (de Lecea et al., 1998). Although the histaminergic (Khateb et al., 1990) and glutamatergic (Inglis and Semba, 1996) inputs, which may be excitatory, are also detected on the cholinergic cells in the LDT and PPT nuclei, a dens distribution of the orexin varicose fibers in LDT and partly in PPT nucleus is suggesting considerable contributions of orexin to the cholinergic cells.

The orexin fibers were unevenly distributed in the LDT and PPT nuclei. It has been reported that glutamate receptors are detected predominantly in PPT and less in LDT nucleus (Inglis and Semba, 1996). Conversely, orexin fibers are densely populated in the LDT and lesser in the PPT. It seems that the orexinergic and glutamatergic synapses on the cholinergic neurons tend to be segregated each other within the LDT and PPT nuclei.

Orexin has an essential roll in sleep/arousal system by projecting to various neurons of the brain. The cholinergic LDT and PPT neurons are known to act on wake and REM-sleep states (Steriade and McCarley,

1990). The orexinergic projection to the cholinergic mesopontine tegmental neurons presented in this experiment is likely involved in the regulation of sleep-wake state.

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