学 位 論 文 の 要 旨

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学 位 論 文 名　Glutamatergic Lateral Parabrachial Neurons Innervate Orexin-containing Hypothalamic Neurons in the Rat

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論 文 内 容 の 要 旨

INTRODUCTION

Orexin (ORX), which is specifically localized in neurons within the hypothalamus, is involved in food intake and sleep-wakefulness regulation. The parabrachial nucleus (PBN) receives gustatory and general visceral inputs from the nucleus of the solitary tract as well as nociceptive inputs from medullary and spinal lamina I neurons, and relays these inputs to the forebrain including the hypothalamus. The PBN-hypothalamic projection originates primarily from the lateral PBN. Judging from the above, it seems quite probable that ORX-containing hypothalamic neurons are under the direct influence of the lateral PBN in the control of feeding and/or in the regulation of sleep. However, the question whether or not ORX-containing hypothalamic neurons receive monosynaptic inputs from the lateral PBN remains unanswered.

Most of the PBN neurons express vesicular glutamate transporter 2 (VGLUT2) mRNA that is a marker for glutamatergic neurons, whereas small numbers of them express glutamic acid decarboxylase (GAD) 67 mRNA that is a marker for GABAergic neurons. As far as we know, there have been no studies to examine whether the lateral PBN-hypothalamic projection is glutamatergic or GABAergic.

In the present study, we first provide definitive evidence for the existence of a monosynaptic pathway from the lateral PBN to ORX-containing hypothalamic neurons by using a combined anterograde tracing with biotinylated dextranamine (BDA) and immunohistochemistry for ORX, and then examine whether hypothalamus-projecting lateral PBN neurons express VGLUT2 mRNA or GAD67 mRNA by using a combined retrograde tracing with cholera toxin B subunit (CTb) and *in situ* hybridization technique.

MATERIALS AND METHODS

The experiments were carried out on male Wister rats. All surgical procedures were performed under general anesthesia with intraperitoneal injection of chloral hydrate (350 g/kg).

In the combined anterograde tracing and immunohistochemistry experiments, injections of BDA into the lateral PBN were made stereotaxically by iontophoresis in 11 rats. After 7-10 days of survival, the rats were perfused transcardially with a solution composed of 4% paraformaldehyde (PA) and 0.2% glutaraldehyde in 0.1 M phosphate buffer (PB). The brains were postfix and saturated with 20% sucrose in 0.1 M PB. Subsequently, the brains were sectioned 40 μm thick on a vibrating microtome. BDA-labeled axons were visualized with avidin-biotin peroxidase complex (ABC) and stained dark blue by using diaminobenzidine (DAB) and nickel ammonium sulfate as a chromogen. ORX neurons were detected immunohistochemically and stained brown by using DAB as a chromogen. In the electron microscopic experiments, BDA-labeled axons were detected with ABC, and then silver-gold intensification of DAB reaction product of BDA was performed. ORX neurons were detected as mentioned above. The specimens were cut out from the hypothalamus, postfixed with osmium tetroxide, stained with uranyl acetate, dehydrated, cleared in propylene oxide, and then embedded flat in Epon. Subsequently, serial ultrathin sections were cut on an ultramicrotome, stained with lead citrate, and then examined under an electron microscope.

In the combined retrograde tracing and *in situ* hybridization experiments, CTb injections were made into the ORX field dorsal to the fornix by iontophoresis in 9 rats. After 7–10 days survival, the rats were perfused with a solution composed of 4% PA and 1% picric acid in 0.1 M PB. The brains were postfixed and cut into frontal sections of 30 μm thickness on a freezing microtome. The sections containing PBN were incubated in the hybridization buffer containing antisense digoxigenin (DIG)-labeled VGLUT2 riboprobe (0.5 μg/μl) or GAD67 riboprobe (0.5 μg/μl) for 16–48 h at 50 °C. The sections were washed in standard saline citrate (SSC) with 50% formamide, treated with RNase, washed in SSC with 50% formamide, incubated overnight in the Tris-buffer saline containing sheep anti-DIG antibody conjugated to peroxidase, and then reacted with tyramide-conjugated Cy3. After *in situ* hybridization, sections were processed to detect CTb immunohistochemically; the sections were treated with goat anti-CTb, biotinylated rabbit anti-goat IgG, and then Alexa488-conjugated streptavidin. The sections containing hypothalamus were incubated in goat anti-CTb and rabbit anti-ORX-A. Subsequently, the sections were incubated in Cy3-conjugated anti-goat IgG and Alexa488-conjugated anti-rabbit IgG. Finally, the sections were observed not only under an epifluorescent microscope but also under a confocal laser scanning microscope.

RESULTS AND DISCUSION

In the rats injected with BDA into the lateral PBN, the sections were immunostained for

ORX after a visualization of BDA. In the tuberal hypothalamus, moderate to dense plexuses of BDA-labeled fibers and terminals were found in the dorsomedial nucleus (DMH), and adjacent areas dorsal and lateral to the nucleus, as well as in the dorsal perifornical area (PF) and dorsal lateral hypothalamus (LH). Some labeled fibers were distributed in the ventromedial nucleus and ventral LH. A few labeled fibers were seen in other hypothalamic regions, such as the anterior hypothalamic area and arcuate nucleus. ORX-ir neurons were distributed predominantly in the PF and dorsal LH, and additionally in the DMH across the tuberal hypothalamus. The prominent overlapping distribution of BDA-labeled fibers and ORX-ir neurons was found in the lateralmost part of the DMH and adjacent dorsal PF just dorsal to the fornix; this overlapping field was referred to as "suprafornical area" in the present study. In this area, bouton-like varicosities labeled with BDA were often closely apposed to cell bodies and dendrites of the ORX-ir neurons. When the "suprafornical area" was examined under the electron microscope, BDA-labeled terminals were densely packed with the electron-dense DAB reaction product and silver-gold grains filling up the entire space between the synaptic vesicles and the mitochondria, whereas ORX-ir neuronal somata and dendrites contained scattered irregular patches of the electron-dense reaction deposits. When we examined the targets of 198 BDA-labeled axon terminals forming synapses with the "suprafornical area" neurons in 5 rats, approximately 34% (n=68) of them were found to make synapses with ORX-ir neurons, as well as 130 terminals formed synapses with non-ORX-ir neurons. Almost all these synapses were of asymmetrical type, which are usually thought to be excitatory.

VGLUT2 mRNA-positive neurons were distributed in all of the PBN subnuclei with differences in labeling intensity among the subnuclei, whereas GAD67 mRNA-positive neurons are present in the PBN with differences in the number among the subnuclei. In the rats injected with CTb into the "suprafornical area", CTb-labeled neurons were distributed predominantly in the lateral PBN and additionally in the medial PBN, bilaterally with an ipsilateral predominance. When the confocal images were analyzed, almost all the CTb-labeled PBN neurons were positive for VGLUT2 mRNA; 1041 (93%) out of 1120 lateral PBN neurons labeled with CTb expressed VGLUT2 mRNA. On the other hand, there were only a few CTb-labeled PBN neurons that were positive for GAD67 mRNA; 18 (2%) out of 964 lateral PBN neurons labeled with CTb expressed GAD67 mRNA. Together with the above-mentioned morphological features of the synapses, the results suggest that the lateral PBN axons in the "suprafornical area" are generally excitatory and glutamatergic.

CONCLUSION

The present study demonstrated that there exists a monosynaptic pathway from the lateral PBN to ORX-containing hypothalamic neurons in the "suprafornical area" and that "suprafornical area"-projecting PBN neurons express VGLUT2 mRNA, suggesting that the ORX-containing neurons is under the excitatory influence of the glutamatergic PBN neurons.