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Research Report

Glutamatergic lateral parabrachial neurons innervate orexin-containing hypothalamic neurons in the rat

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ABSTRACT

We performed this study to understand the anatomical substrates of parabrachial nucleus (PBN) modulation of orexin (ORX)-containing neurons in the hypothalamus. After biotinylated dextranamine (BDA) injection into the lateral PBN and immunostaining of ORX-containing neurons in the rat, the prominent overlap of the distribution field of the BDA-labeled fibers and that of the ORX-immunoreactive (ir) neurons was found in the lateralmost part of the dorsomedial nucleus and adjacent dorsal perifornical area (this overlapping field was referred to as "supraformical area" in the present study), and the labeled axon terminals made asymmetrical synaptic contacts with somata and dendrites of the ORX-ir neurons. We further revealed that almost all the "supraformical area"-projecting lateral PBN neurons were positive for vesicular glutamate transporter 2 mRNA and very few of them were positive for glutamic acid decarboxylase 67 mRNA. The present data suggest that ORX-containing neurons in the "supraformical area" may be under the excitatory influence of the glutamatergic lateral PBN neurons probably for the regulation of arousal and waking.

1. Introduction

Orexin (ORX), also called hypocretin, is recently identified neuropeptide, which is specifically localized in neurons within the hypothalamus, including the perifornical area (PF), lateral hypothalamus (LH) and dorsomedial nucleus (DMH) (de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998; Nambu et al., 1999; Modirrousta et al., 2005; Henny and Jones, 2006; Hahn, 2010). ORX-containing neurons receive projection fibers from multiple forebrain and brainstem cell

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Abbreviations: 3V, 3rd ventricle; 4n, trochlear nerve or its root; AHP, posterior part of the anterior hypothalamic area; Arc, arcuate hypothalamic nucleus; cl, central parabrachial subnucleus; cr, crescent parabrachial subnucleus; CnF, cuneiform nucleus; dl, dorsal parabrachial subnucleus; DLL, dorsal nucleus of the lateral lemniscus; DMH, dorsomedial hypothalamic nucleus; exl, external lateral parabrachial subnucleus; exm, external medial parabrachial subnucleus; f, fornix; ic, internal capsule; il, internal lateral parabrachial subnucleus; KF, Kölliker-Fuse nucleus; m, medial parabrachial subnucleus; mcp, middle cerebellar peduncle; me5, mesencephalic trigeminal tract; Mo5, motor trigeminal nucleus; mt, mammillothalamic tract; opt, optic tract; PH, posterior hypothalamic area; Pr5, principal sensory trigeminal nucleus; Re, reuniens thalamic nucleus; scp, superior cerebellar peduncle; sl, superior lateral parabrachial subnucleus; STh, subthalamic nucleus; Sub, submedius thalamic nucleus; vl, ventral lateral parabrachial subnucleus; VM, ventromedial thalamic nucleus; VMH, ventromedial hypothalamic nucleus; vsct, ventral spinocerebellar tract; ZI, zona incerta

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groups (Sakurai et al., 2005; Yoshida et al., 2006), and send their axons to many brain regions and spinal cord (Peyron et al., 1998; Nambu et al., 1999; van den Pol, 1999). ORX was originally found to regulate the food intake (Sakurai et al., 1998; Sutcliffe and de Lecea, 2000), and shortly thereafter the ORX system was revealed to be involved in sleep-wakefulness regulation and narcolepsy (reviewed in Taheri et al., 2002). On the other hand, the parabrachial nucleus (PBN), which is composed of neurons surrounding the superior cerebellar peduncle along its course through the dorsolateral pons, has been initially divided into medial and lateral parts; the medial PBN receives projection fibers from the rostral, gustatory portion of the nucleus of the solitary tract (Norgren, 1978), while the lateral PBN receives those from the more caudal portion of the nucleus of the solitary tract where general visceral afferents terminate (Herbert et al., 1990). Furthermore, the PBN has been known to receive nociceptive inputs from medullary (trigeminal) and spinal lamina I neurons (Cechetto et al., 1985; Slugg and Light, 1994). The PBN constitutes main relay for these inputs to the forebrain including the hypothalamus, amygdala and bed nucleus of the stria terminalis (Saper and Loewy, 1980; Fulwiler and Saper, 1984; Krukoff et al., 1993; Alden et al., 1994; Bester et al., 1997; Karimnamazi and Travers, 1998; Bester et al., 1999). The PBN-hypothalamic projection has been shown to originate primarily from the lateral PBN (Saper and Loewy, 1980; Fulwiler and Saper, 1984; Krukoff et al., 1993; Bester et al., 1997). Judging from the above, it seems quite probable that the terminal field of the lateral PBN fibers is overlapped with the distribution field of ORX-containing neurons in the hypothalamus, and therefore 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.

As for the neurotransmitter of the PBN neurons, our previous study (Yokota et al., 2007) indicated that 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. Furthermore, PBN neurons projecting to the rostral ventral respiratory group and phrenic nucleus (Yokota et al., 2004, 2007), as well as to the rostral ventrolateral medulla (Len and Chan, 1999) and dorsal raphe nucleus (Lee et al., 2003) have been revealed to be glutamatergic, while PBN neurons projecting to the nucleus of the solitary tract are GABAergic (Len and Chan, 2001). As far as we know, however, 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.

2. Results

2.1. *Overlapping distribution of lateral PBN fibers and ORX-ir neurons*

2.1.1. *Light microscopic observation*

In 5 out of 11 operated rats received a successful injection of BDA into the lateral PBN (Figs. 1A and B), the sections including the hypothalamus were immunostained for ORX. In these rats, anterogradely labeled axons were observed bilaterally with a clear-cut ipsilateral predominance in the hypothalamus. Fig. 2 shows a series of camera lucida drawings that illustrate the distribution of anterograde labeling and ORX-immunoreactive (ir) neurons after a relatively large injection of BDA into the lateral PBN (Fig. 1A). In the tuberal hypothalamus, moderate to dense plexuses of BDA-labeled fibers and terminals were found in the DMH (Figs. 2B–F), and adjacent areas dorsal (Figs. 2B–F) and lateral (Figs. 2C and D) to the nucleus, as well as in the dorsal PF and dorsal LH (Figs. 2A–F). Some labeled fibers with bouton-like varicosities 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. When the sites of BDA injection centered on the central lateral subnucleus and its immediate vicinities (Fig. 1B), the distribution pattern of labeled fibers in the hypothalamus was almost similar to that in the aforementioned case, although the anterograde labeling was less dense (Fig. 3). In these rats, moderate to dense plexuses of labeled fibers were found in the DMH (Figs. 3C–E) and adjacent areas lateral (Fig. 3C) and dorsomedial (Fig. 3E) to the nucleus. Some labeled fibers with bouton-like varicosities were distributed around the fornix as well as in the LH. Light anterograde labeling was seen in the ventromedial and arcuate nuclei. On the other hand, ORX-ir neurons were distributed predominantly in the PF and dorsal LH, and additionally in the DMH across the tuberal hypothalamus (Figs. 2 and 3), in accordance with previous studies (de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998; Nambu et al., 1999; Modirrousta et al., 2005; Henny and Jones, 2006; Hahn, 2010). In both cases, 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 (Figs. 1C, 2C, D and 3C); 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 (Figs. 1D and E). A light overlap of the fibers and neurons with small numbers of their contacts was also seen in the dorsal LH.

2.1.2. *Electron microscopic observation*

When the “suprafornical area”, where the prominent overlapping distribution of BDA-labeled axon terminals and ORX-ir neurons had been found, was examined under the electron microscope, BDA-labeled axon terminals were densely packed with the electron-dense diaminobentizine (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