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Cholecystokinin and substance P immunoreactive projections to the paraventricular thalamic nucleus in the rat

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Abstract

Cholecystokinin (CCK) and substance P (SP) are thought to play an important role in a variety of stress responses. Both CCK- and SP-positive fibers innervating the thalamus are found principally in the midline nuclei, including the paraventricular thalamic nucleus (PVT), which has strong reciprocal connections with the medial prefrontal cortex. In the present study, we determined the source of the CCK- and SP-immunoreactive fibers to the PVT, employing combination of retrograde neuronal tracing and immunohistochemistry in the rat. The PVT-projecting neurons showing CCK immunoreactivity were detected in the dorsomedial nucleus of the hypothalamus, and ventral mesencephalic periaqueductal gray, including the Edinger-Westphal nucleus and the dorsal raphe nucleus. Sources of SP afferents to the PVT were detected in the Edinger-Westphal nucleus, the mesopontine tegmentum and the medullary raphe nucleus. CCK- and SP-immunoreactive fibers may exert modulatory influence on the prefrontal cortical activity via the PVT and regulate behavioral components of stress-adaptation responses.

Keywords: Midline thalamus; Cholecystokinin; Substance P; Retrograde tracing; Immunohistochemistry; Dorsomedial hypothalamic nucleus; Edinger-Westphal nucleus; Stress

1. Introduction

The densities of neuropeptide-immunoreactive cells and fibers in the thalamus have been reported as being relatively low with a few notable exceptions. Among these exceptions, the midline region of the thalamus, whose functions are not well understood, is heavily innervated by various peptidergic fibers, e.g. substance P (SP), cholecystokinin (CCK), a corticotrophin-releasing factor, neuropeptide Y, and somatostatin (Bentivoglio et al., 1991). The paraventricular thalamic nucleus (PVT), the most dorsal component of the thalamic midline, is known to be strongly activated following a variety of stressors and thus might be suggested to play a role in stress-related information targeted for viscerolimbic areas in the brain.

Among neuropeptides innervating the PVT, SP and CCK are of particular interest, due to the roles ascribed to these peptides as neuromodulators in the integrated hypothalamic stress response, mediating stress–neuroendocrine interaction (Siegel et al., 1987). SP controls vomiting and various behavioral, neurochemical and cardiovascular responses to stress, besides its best-known role as a pain neurotransmitter. For example, recent clinical trials have confirmed the efficacy of NK1 receptor (i.e., SP receptor) antagonists to alleviate depression and emesis (Rupniak and Kramer, 1999). CCK has been shown to be involved in numerous physiological functions such as feeding behavior, central respiratory control and cardiovascular tonus, vigilance states, memory processes, nociception, and emotional and motivational responses (Fink et al., 1998; Noble and Roques, 1999) and has been associated with several neuropsychiatric diseases such as schizophrenia as well as anxiety and panic attacks (Bourin et al., 1998; Bradwejn et al., 1995).

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Although it seems important to identify the origins of the SP-ergic and CCK-ergic innervation to the PVT in the contexts mentioned above, only limited researchers have investigated this issue so far. For example, Bhatnager et al. (2000) identified the origins of CCK innervations mainly in brainstem areas, including the lateral parabrachial, periaqueductal gray and dorsal raphe. However, previous findings of altered CCK levels in various hypothalamic regions during acute stress exposure suggested the importance of hypothalamic CCK, related to the negative feedback actions of glucocorticoids (Siegel et al., 1987). Intracerebroventricular administration of CCK produced a wide spectrum of endocrine effects, likely via a hypothalamic site of action (Vijayan et al., 1979). The origin of SP innervation of the PVT has not been so far documented. The aim of the present study was therefore to determine the detailed sites of origin of CCK- and SP-projection fibers to the PVT by immunohistochemistry for CCK and SP combined with retrograde tract tracing. A preliminary report of this study was presented in abstract form (Otake and Nakamura, 2003).

2. Materials and methods

2.1. Animals and tissue preparation

Sprague–Dawley rats (male; 200–250 g; Nippon Bio-Supp. Center, Tokyo, Japan) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). All efforts were made to minimize the number of animals used and their suffering. Injections of a 1% solution of cholera toxin B-subunit (CT-b, Research Biochemicals International, Natick, MA, USA) diluted with 0.2 M sodium phosphate buffer (PB, pH 7.5) were delivered from glass micropipettes in the dorsal midline thalamus using coordinates from the atlas of Paxinos and Watson (Paxinos and Watson, 1998). Iontophoretic ejections were made using positive pulses (7 μA, 7 s on/off) from a Midgard precision current source (Stoelting, Wood Dale, IL, USA) for 15–20 min. Three to four days later, these rats were re-anesthetized and colchicine (150 μg/10 μl saline) was slowly injected unilaterally into the lateral ventricle to build up transmitter concentration in cell bodies, and then, after 1 more day, the animals were re-anesthetized and perfused transcardially with 200 ml heparinized saline followed by 500 ml of Zamboni's fixative.

The surgical procedures and pre- and post-operative care of the animals were approved by the Tokyo Medical and Dental University Animal Care Committee and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication 80-23, revised 1996).

The brains were removed and immersed overnight at 4 °C in 0.1 M PB containing 10% sucrose. They were sectioned in the transverse plane at 40-μm on a freezing microtome and each 1-in-4 series was immunostained by a double or triple labeling method as follows. All histological procedures were carried out at room temperature.

2.2. Double immunohistochemistry for CT-b and CCK/CT-b and SP

Two 1-in-4 series were processed for CT-b and SP or CCK reactivity. The sections were immersed for 1 h in PB containing 1% bovine serum albumin (Wako, Osaka, Japan) and incubated overnight in a mixture of goat anti-CT-b antiserum (1:20,000; List Biological, Inc., Campbell, CA, USA) and rabbit anti-SP (1:200; Peninsula Labs, Belmont, CA, USA) or rabbit anti-CCK antibody (1:200; Chemicon, Temecula, CA, USA). The sections were rinsed in PB and then incubated for 2 h in biotinylated horse anti-goat IgG (1:1000; Vector, Burlingame, CA, USA). After additional rinsing, sections were immersed for 2 h in a mixture of avidin-TRITC (1:250; Vector) and FITC-conjugated swine anti-rabbit IgG (1:20; DakoCytomation, Glostrup, Denmark).

Sections were mounted in a glycerol-mounting medium containing 0.1% *p*-phenylenediamine (Sigma, Saint-Louis, MO, USA), examined in a Nikon fluorescent microscope (Eclipse E600) equipped with appropriate filter combinations, and photographed by using a cooled CCD camera (Keyence VB 6000/6010, Osaka, Japan) attached to a microscope. The distribution of single- and multi-labeled neurons was mapped onto drawings prepared from digital photomontage of the total area of the representative sections.

2.3. Triple immunohistochemistry for CT-b, SP and serotonin

Since many SP-containing neurons also contained serotonin in the raphe nuclei in cat (Arvidsson et al., 1994; Lovick and Hunt, 1983) as well as in human (Baker et al., 1991), coexistence of serotonin with SP and CT-b immunoreactivity was analyzed by use of the triple-labeling technique. A 1-in-4 series through the medulla was first processed for SP and serotonin using the same procedure. In brief, the sections were incubated overnight in a mixture of rat anti-serotonin (1:100; Chemicon) and rabbit anti-SP antiserum (1:200; Peninsula). The sections were placed for 2 h in biotinylated goat anti-rat antibody (1:1000; Vector), and then for 2 h in a mixture of avidin-TRITC (1:250; Vector) and FITC-conjugated swine anti-rabbit IgG (1:20; DakoCytomation). Sections were mounted in a glycerol-mounting medium. After examining and photographing the relevant areas, the sections were then immunostained for CT-b. To eliminate potential cross-reaction between avidin/biotin system reagents, the sections first went through a blocking procedure (Ferri et al., 1999); the sections were incubated in the free avidin solution (Avidin/Biotin Blocking Kit, #SP-2001, Vector) for 40 min, in 10% formaldehyde for 40 min, and then in a free biotin solution