Neuroscience 164 (2009) 1180–1190

ULTRASTRUCTURAL CHARACTERIZATION OF RELATIONSHIP BETWEEN SEROTONERGIC AND GABAERGIC STRUCTURES IN THE VENTRAL PART OF THE ORAL PONTINE RETICULAR NUCLEUS

C. DE LA ROZA* AND F. REINOSO-SUÁREZ

Departamento de Anatomía, Histología y Neurociencia, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo, 28029 Madrid, Spain

Abstract—The ventral part of the oral pontine reticular nucleus (vRPO) is involved in the generation and maintenance of rapid eye movement (REM) sleep. Both GABAergic and serotonergic neurotransmission have been implicated in the control of the sleep–wakefulness cycle. Nevertheless, the synaptic organization of serotonergic terminals in the vRPO has not yet been characterized. We performed an electron microscope study of serotonin-immunoreactive (5-HT-IR) terminals using immunoperoxidase or immunogold–silver methods. In a second set of experiments, combining GABA immunoperoxidase and 5-HT immunogold–silver techniques, we examined inputs from GABA-immunoreactive (GABA-IR) terminals to serotonergic neurons. 5-HT-IR terminals were located primarily on dendrites and occasionally on somata of unlabeled and 5-HT-IR neurons. The majority of the synapses formed by 5-HT-IR terminals were of the symmetrical type, making contacts primarily with unlabeled dendritic profiles. Moreover, 5-HT-IR terminals contacted unlabeled axon terminals that formed asymmetric synapses on dendrites. Double immunolabeling experiments showed 5-HT-IR and GABA-IR afferents, in apposition to each other, making synapses with the same dendrites. Finally, GABA-IR terminals innervated 5-HT-IR and GABA-IR dendrites. Our findings indicate that serotonin would modulate the neuronal activity through inhibitory or excitatory influences, although the action of serotonin on the vRPO would predominantly be inhibitory. Moreover, the present results suggest that the serotonin modulation of vRPO neurons might involve indirect connections. In addition, GABA might contribute to the induction and maintenance of REM sleep by inhibiting serotonergic and GABAergic neurons in the vRPO.

Key words: double immunocytochemistry, pontine reticular formation, synapse, REM sleep, serotonin, monoamines.

It has been demonstrated, by Reinoso-Suárez et al. (1994, 2001), that the ventral part of the oral pontine reticular nucleus (vRPO) is a nodal link for the generation and maintenance of rapid eye movement (REM) sleep. In fact, this is the site in the cat brainstem in which small volume microinjections of low doses of the cholinergic agonist carbachol produce enduring periods of REM sleep with a short latency (Garzón et al., 1997, 1998; Reinoso-Suárez et al., 2001). The cat vRPO, or its equivalent in the rat, is the most effective site for the induction or inhibition of REM sleep after carbachol or another neurotransmitter injections (Iwakiri et al., 1993; Bier and McCarley, 1994; Garzón et al., 1996; Kshatri et al., 1998; Horner and Kubin, 1999; Kohlmeier et al., 2002; Moreno-Balandrán et al., 2008) or after injections of $GABA_A$ receptor agonists or antagonists (Xi et al., 1999; Manquillo, 2000; Sanford et al., 2003).

In vitro experiments in the rat have demonstrated that GABA and serotonin evoke hyperpolarization in vRPO neurons (Núñez et al., 1998). Light (Mugnaini and Oertel, 1985; Ford et al., 1995; De la Roza and Reinoso-Suárez, 2006) and electron microscope studies (De la Roza and Reinoso-Suárez, 2003, 2006; De la Roza et al., 2004) have described GABA-immunoreactive (GABA-IR) neurons and/or terminals in the vRPO. By using a combination of the physical dissector, electron microscopy and the post-embedding immunogold techniques, we have demonstrated that 30% of all vRPO synaptic terminals are immunoreactive to GABA and that they form symmetric (inhibitory) synapses on vRPO somata and the different parts of the dendritic tree, including distal regions (De la Roza and Reinoso-Suárez, 2006). Although some of these GABA-IR terminals could arise from local neurons, most of the axon terminals probably originate from structures that are connected with the vRPO and that contain GABA-IR neurons. Recent anatomical studies in our laboratory indicate that some GABA-IR neurons in the reticular thalamic nucleus and hypothalamic areas project to the vRPO (De la Roza et al., 2004; Rodrigo-Angulo et al., 2008). Our results indicate that posterior lateral hypothalamus, a crucial structure for maintaining normal wakefulness, would modulate vRPO neurons through a GABAergic pathway as well as by other inhibitory or excitatory pathways (De la Roza et al., 2004).

It is accepted that mesopontine tegmentum serotonin-immunoreactive (5-HT-IR) neurons contribute to the suppression of REM sleep (Hobson et al., 1975; Portas and Mccarley, 1994; Portas et al., 1996; Horner et al., 1997). Studies on the distribution of 5-HT-IR-containing neurons performed in the brainstem of rats (Steinbusch, 1981) and cats (Wiklund et al., 1981; Jacobs et al., 1984) have revealed that although most of these neurons are located in the raphe nuclei, many others are found throughout the mesopontine tegmentum. Rodrigo-Angulo et al. (2000) reported that almost half of the total serotonin-positive neurons located in the mesopontine tegmentum are situated in

*Corresponding author. Tel: +34-914975354; fax: +34-914975338.
E-mail address: carmen.roza@uam.es (C. De la Roza).

Abbreviations: BSA, bovine serum albumine; DAB, diaminobenzidine; EM, electron microscope; NGS, normal goat serum; PB, phosphate buffer; PBS, PB saline solution; REM sleep, rapid eye movement sleep; TBS, Tris–buffered saline; vRPO, ventral part of the oral pontine reticular nucleus.

0306-4522/09 $ - see front matter © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2009.08.050

mesopontine tegmentum structures other than raphe nuclei, some of them located in the vRPO. Anatomical experiments using neural tracing combined with immunohistochemistry have demonstrated that, in the cat, the main source of serotonin to the vRPO arises from these non-raphe mesopontine tegmentum structures (Rodrigo-Angulo et al., 2000). These neurons may be the origin of the dense network of 5-HT-IR fibers and varicosities observed in the vRPO (Kobayashi et al., 1994; Rodrigo-Angulo et al., 2000) that would indicate that the serotonin modulation of REM sleep could be the result of activation in non-raphe mesopontine serotonin neurons.

Elucidating the neuronal mechanisms mediating the role of serotonin in the vRPO requires a clear understanding of its synaptic microcircuitry. However, to the best of our knowledge, the synaptic organization of serotonergic terminals in the vRPO has not yet been characterized. To address this subject, we performed an electron microscopic analysis of 5-HT-IR profiles in this structure. Furthermore, we investigated if the GABAergic afferents contact serotonergic neurons in the vRPO as occurs in other brain areas associated with the sleep–wake cycle (Gervasoni et al., 2000; Fiske et al., 2006). Preliminary results have been published in abstract form De la Roza and Reinoso-Suarez, 2001.

EXPERIMENTAL PROCEDURES

Tissue preparation

The experiments were performed on five adult cats (between 2.5 and 3 kg) following the European Community Council Directive guidelines (86/609/EEC) and were approved by the Animal Care and Use Committee of the Facultad de Medicina, Universidad Autónoma de Madrid. All efforts were made to limit the number of animals used and to minimize suffering. Animals were deeply anesthetized with Nembutal (35 mg/kg, i.p.) and, when they reached a deep anesthetic state, were perfused through the aorta with 0.9% saline followed by 3.8% acrolein (Polysciences, Warrington, PA, USA) in a solution of 2% paraformaldehyde (Sigma, St. Louis, MO, USA) dissolved in phosphate buffer 0.1 M pH=7.4 (PB), followed by 2% paraformaldehyde dissolved in PB. Brains were then removed from the skulls, dissected and postfixed for 30 min in 2% paraformaldehyde. Blocks containing the vRPO were serially sectioned at 50 μm on a vibratome in the frontal plane. Vibratome (Ted Pella Inc., Redding, CA, USA) sections were collected in two series; one series was Nissl-stained to delineate architectonic borders and its adjacent series was treated for single immunocytochemical detection of 5-HT or GABA or for double immunolabeling of 5-HT and GABA.

Immunocytochemistry

Vibratome sections were immersed, for cryoprotection, in a graded series of sucrose solutions (10% and 20% in 0.05 M PB, pH 7.4) and left in 25% sucrose and 10% glycerol in 0.05 M PB. After this, in order to increase the penetration of immunoreagents, they were quickly frozen in liquid nitrogen and thawed in 0.1 M PB at room temperature. After repeated washing, sections were treated with 1% sodium borohydride ($NaBH_4$; Sigma, St. Louis, MO, USA) in 0.1 M PB for 30 min to reduce free aldehyde groups and enhance immunostaining and then repeatedly washed in 0.1 M PB solutions until no bubbles emerged from the tissue.

Single immunolabeling for 5-HT. Free-floating sections were processed by immunoperoxidase or immunogold–silver techniques to detect serotonin with methods of different resolution and sensitivity. Briefly, in both cases, vibratome sections were rinsed in 0.1 M Tris–buffered saline (TBS, pH 7.6, 0.9% saline, Sigma, St. Louis, MO, USA) and then incubated for 60 min in 10% normal goat serum (NGS) in 0.1 M TBS. Sections were then placed for 42 h at 4 °C in a solution containing rabbit anti-5-HT (ImmunoStar, cat. n° 20080, Hudson, WI, USA) diluted (1:4000 or 1:5000) in 1% NGS-TBS This antibody is a widely used antiserum that was raised in rabbit against serotonin coupled to bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) with paraformaldehyde. Preadsorption studies of the antiserum with 25 μg/ml of serotonin/BSA, conducted by the manufacturer, abolished all tissue staining. Preadsorption of the antiserum with 5-hydroxyindole-3-acetic acid, 5-hydroxytryptophan and dopamine (at 5–25 μg/ml concentrations) did not attenuate staining.

For immunoperoxidase labeling, the sections were rinsed in 0.1 M TBS several times, incubated in goat anti-rabbit Ig G conjugated to biotin (1:400 in 0.1 M TBS; Vector Laboratories, Burlingame, CA, USA) for 1 h and then in avidin-biotin-peroxidase complex (1:100 in 0.1 M TBS; Vectastain Elit Kit, Vector Laboratories, Burlingame, CA, USA) for 30 min. Peroxidase reaction was carried out with 0.022% 3,3′-diaminobenzidine (Sigma, St. Louis, MO, USA) and 0.003% hydrogen peroxide in 0.1 M TBS. Sections were rinsed in 0.1 M TBS between incubations. For immunogold–silver localization of serotonin, sections were rinsed in 0.01 M phosphate-buffered saline (PBS, pH=7.4, 0.9% saline) and transferred to a blocking solution containing 0.8% BSA and 0.1% gelatin in 0.01 M PBS for 10 min. The sections were then incubated in a secondary goat anti-rabbit IgG coupled to 1 nm gold particles (1:50 dilution in the blocking solution, Amersham Corporation, Arlington Heights, IL, USA) for 2 h. Sections were rinsed several times in 0.01 M PBS and post-fixed for 10 min in 2% glutaraldehyde in 0.01 M PBS to ensure adherence of the bound colloidal gold. The diameter of immunogold–silver particles was increased with a silver enhancement kit (IntenS-EM kit, Amersham Corporation, Arlington Heights, IL, USA) for 5–7 min. The specificity of the 5-HT antibody was verified by omission of this antiserum in the immunocytochemical protocol.

Single immunolabeling for GABA. Free-floating sections were processed by immunoperoxidase technique, as described above, to detect GABA. A monoclonal anti-GABA raised in mouse (1:7000; mAb 3A12, generously provided by Dr. C. Matute, Universidad del País Vasco, Vizcaya, Spain) and a biotinylated horse antimouse Ig G (1:400; Vector Laboratories, Burlingame, CA, USA) was used in this immunoreaction. The production and characterization of the monoclonal anti-GABA has been described in detail elsewhere (Matute and Streit, 1986); this antibody is widely used in immunocytochemistry to locate GABA in different brain areas and species (Domenici et al., 1988; Ginestal and Matute, 1993; Echevarría et al., 1997; Pérez-Cerdá et al., 1999).

Double immunolabeling for 5-HT and GABA. Immunoperoxidase detection of GABA and immunogold-silver labeling of 5-HT were performed according to the method described by Chan et al. (1990). Vibratome sections were incubated for 42 h at 4 °C in a solution of 0.1 M TBS containing a mixture of rabbit anti-5-HT antibody (1:4000 or 1:5000; Immunostar, cat. n° 2080) and the monoclonal anti-GABA described above (1:7000; mAb 3A12). Following incubation in primary antibodies, sections were washed in 0.1 M TBS and incubated in biotinylated horse antimouse Ig G (1:400; Vector Laboratories, Burlingame, CA, USA) for 30 min. Subsequently, GABA-immunoreactivity was revealed by incubation in avidin-biotin-peroxidase complex and a peroxidase reaction, as described above. Sections were then rinsed in 0.01 M PBS, blocked for 10 min in 0.8% BSA and 0.1% gelatin in 0.01 M PBS and processed for immunogold–silver labeling of serotonin, as previously described. Control sections were incubated in parallel, following the same procedure, except that one of the primary