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Immunohistochemical localization of orexins (hypocretins) in the brain of reptiles and its relation to monoaminergic systems

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ABSTRACT

With the aim of gaining more insight into the evolution of the orexinergic systems in the brain of vertebrates we have conducted a comparative analysis of the distribution of orexin-immunoreactive cell bodies and fibers in two reptiles, the lizard *Gekko gecko* and the turtle *Pseudemys scripta elegans*. In both species most immunoreactive neurons were found in the periventricular hypothalamic nucleus and in the infundibular hypothalamus. Only in the gecko, orexinergic cell bodies were present in the dorsolateral hypothalamic nucleus and the periventricular preoptic nucleus. Fiber labeling was observed in all main brain subdivisions but was more abundant in regions such as the septum, preoptic area, suprachiasmatic nucleus, lateral hypothalamic area and median eminence. Less conspicuous was the innervation of the olfactory bulbs, pallial regions, habenula, dorsomedial and dorsolateral thalamic nuclei, torus semicircularis and spinal cord. Double immunohistofluorescence techniques were applied for the simultaneous detection of the orexinergic systems and the catecholaminergic or serotoninergic systems in the brain of reptiles. Actual colocalization of orexins and catecholamines or serotonin in the same neurons was not observed. However, orexinergic innervation was found in dopaminergic, noradrenergic and serotoninergic cell groups, such as the substantia nigra and ventral tegmental area in the midbrain tegmentum, the locus coeruleus, the nucleus of the solitary tract and the raphe nuclei.

The comparison of the distribution of orexin-immunoreactive neurons and fibers found in reptiles with those reported for other vertebrates reveals a strong resemblance but also notable variations. In addition, the relation between the orexinergic and monoaminergic systems observed in the brain of reptiles seems to be a shared feature among vertebrates.

1. Introduction

The orexins, also known as hypocretins, are neuropeptides first found in the human brain as regulators of feeding behavior (Sakurai et al., 1998). Two orexins, A and B (or hypocretins 1 and 2, respectively) were described to derive from different proteolitic postraductional processing of the same precursor, prepro-orexin, whose gene in rat is expressed exclusively in the brain (de Lecea et al., 1998; Sakurai et al., 1998). Immunohistochemical studies in mammals have shown that the neurons producing orexins are localized in particular positions of the hypothalamus and perifornical region and elaborate networks of immunoreactive

Abbreviations: Acc, nucleus accumbens; ADVR, anterior dorsal ventricular ridge; Alh, lateral hypothalamic area; Am, amygdaloid complex; BST, bed nucleus of the stria terminalis; Cb, cerebellum; cc, central canal; Cxd, dorsal cortex; Cxl, lateral cortex; Cxm, medial cortex; dh, dorsal horn of spinal cord; DF, dorsal funiculus; Dl, dorsolateral thalamic nucleus; Dlh, dorsolateral hypothalamic nucleus; Dm, dorsomedial thalamic nucleus; epl, external plexiform layer; flm, fasciculus longitudinalis medialis; Gc, griseum centrale; GP, globus pallidus; Hbl, lateral habenula; Hbm, medial habenula; igl, internal granular layer; inf, infundibulum; Ip, interpeduncular nucleus; ir, infundibular recess; Is, isthmic nucleus; LA, lateral amygdala; lfb, lateral forebrain bundle; Lc, locus coeruleus; LDT, Laterodorsal tegmental nucleus; LF, lateral funiculus; LM, pretectal lentiform mesencephalic nucleus; MA, medial amygdala; Ma, mamillar region; me, median eminence; MP, medial posterior thalamic nucleus; Mri, medial nucleus of the infundibular recess; Mt, medial thalamic nucleus; NdB, nucleus of the diagonal band of Broca; Nolfa, anterior olfactory nucleus; Nsl, lateral septal nucleus; Nsm, medial septal nucleus; Nsol, nucleus of the solitary tract; ob, olfactory bulb; oc, optic chiasm; Pb, parabrachial nucleus; pc, posterior commissure; Pd, posterodorsal nucleus; PDVR, posterior dorsal ventricular ridge; Ph, periventricular hypothalamic nucleus; POp, periventricular preoptic nucleus; PV, paraventricular nucleus; Ra, raphe nuclei; Rai, inferior raphe nucleus; Ras, superior raphe nucleus; RA8, reptilian A8 nucleus; RF, reticular formation; Ri, inferior reticular nucleus; Rm, median reticular nucleus; Rot, nucleus rotundus; Rs, superior reticular nucleus; SC, suprachiasmatic nucleus; Sn, substantia nigra; SNc, substantia nigra, pars compacta; SNr, substantia nigra, pars reticulata; So, supraoptic nucleus; sol, solitary tract; Str, striatum; tect, mesencephalic tectum; Tegm, mesencephalic tegmentum; to, optic tract; Tor, torus semicircularis; v, ventricle; Vds, nucleus descendens nervi trigemini; Ves, nucleus vestibularis superior; VF, ventral funiculus; vh, ventral horn of spinal cord; Vpr, nucleus sensorius principalis nervi trigemini; VTA, ventral tegmental area; IIIv, third ventricle; Xm, nucleus motorius nervi vagi; XII, nucleus nervi hypoglossi.

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fibers originated from these neurons are widely distributed in almost all main brain regions (Broberger et al., 1998; Peyron et al., 1998; Cutler et al., 1999; Nambu et al., 1999; Dube et al., 2000; McGranaghan and Piggins, 2001; Mintz et al., 2001; Zhang et al., 2001, 2004; Nixon and Smale, 2007). Moreover, by means of double immunohistofluorescence it was corroborated that both orexins colocalize in the same neuronal hypothalamic cells and their projections (Zhang and Luo, 2002; Zhang et al., 2004). However, the two orexin receptors 1 and 2 bind the orexins with different affinity and showed distinct patterns of distribution in the brain (Sakurai et al., 1998; Kilduff and de Lecea, 2001), raising the possibility that there may be differences in the functional roles for orexins A and B (Nixon and Smale, 2007). In agreement with their widespread distribution in the brain multiple functions for orexins have been demonstrated, such as the control of feeding and energy homeostasis (Lubkin and Stricker-Krongrad, 1998; Sakurai et al., 1998; Wolf, 1998; Dube et al., 1999; Haynes et al., 1999; Sweet et al., 1999; Volkoff et al., 1999, 2005; Yamanaka et al., 1999; Karteris et al., 2005; Volkoff, 2006; Carter et al., 2009) or the regulation of sleep–wake cycle and related pathologies like narcolepsy (Chemelli et al., 1999; Hagan et al., 1999; Lin et al., 1999; Beuckmann and Yanagisawa, 2002; Kukkonen et al., 2002; Baumann and Bassetti, 2005; Matsuki and Sakurai, 2008; Takahashi et al., 2008). In addition, orexins also regulate the release of adenohypophyseal hormones (Pu et al., 1998; Malendowicz et al., 1999; Mitsuma et al., 1999; Tamura et al., 1999; Russell et al., 2000; Kohsaka et al., 2001; Seoane et al., 2004; Barb and Matteri, 2005; Martynska et al., 2006), the integrated control of autonomic function (Shirasaka et al., 2002; Ferguson and Samson, 2003; Berthoud et al., 2005; Yasuda et al., 2005) and the stimulation of gastrointestinal functions (Okumura and Takakusaki, 2008).

Most investigations about the precise localization of orexins in the brain have been conducted in rodent species (Peyron et al., 1998; Cutler et al., 1999; Nambu et al., 1999; McGranaghan and Piggins, 2001; Mintz et al., 2001; Nixon and Smale, 2007). However, due to the conserved molecular structure of orexins across vertebrates (Sakurai et al., 1998; Shibahara et al., 1999; Alvarez and Sutcliffe, 2002; Ohkubo et al., 2002), several studies have used antibodies against mammalian orexins to localize orexin-immunoreactive (orexin-ir) cell bodies and fibers in representatives of fish (Kaslin et al., 2004; Huesa et al., 2005; Amiya et al., 2007), amphibians (Shibahara et al., 1999; Galas et al., 2001; Singletary et al., 2005; Suzuki et al., 2008; López et al., 2009) and birds (Ohkubo et al., 2002; Phillips-Singh et al., 2003; Singletary et al., 2006). The results of these studies suggested a highly conserved organization of orexinergic systems in the brain of vertebrates. However, only in a few cases, direct cross-species comparison of the distribution of cell bodies and fibers containing orexins have been made and have shown peculiar differences both among mammals (Nixon and Smale, 2007) and amphibians (López et al., 2009).

Surprisingly, there is no information concerning the specific localization of orexins in the brain of reptiles. To our knowledge, only a brief report has pointed out the presence of orexin-ir cells along the third ventricle within the periventricular nucleus in the green anole lizard (Farrell et al., 2003). Considering the crucial position of reptiles in a phylogenetic perspective and the previously observed differences between lizards and turtles for other neurochemical markers (Smeets et al., 2001, 2003, 2006; Muñoz et al., 2008), the present study of the distribution of orexin immunoreactivity in the brain of the turtle *Pseudemys scripta elegans* and the lizard *Gekko gecko* has been carried out for a better understanding of primitive and derived traits of this system in reptiles and, more generally, vertebrates. An additional goal of our study was to establish the relationship between the orexin-ir structures and the aminergic cells and fibers in the reptilian brain. Data in mammals, amphibians and zebrafish are available about the different degree of colocalization and/or codistribution of orexin-ir cells and fibers with catecholaminergic elements in the mesencephalic tegmentum, locus coeruleus and caudal rhombencephalon and with serotoninergic cells in the raphe nuclei (Peyron et al., 1998; Baldo et al., 2003; Wang et al., 2003; Kaslin et al., 2004; Zhang et al., 2004; Balcita-Pedicino and Sesack, 2007). Therefore in our study, immunohistochemistry for the detection of orexins was systematically combined with the detection of tyrosine hydroxylase (TH, the first and rate-limiting enzyme for catecholamine synthesis) and serotonin.

2. Materials and methods

For the present study, a total of twelve red-eared turtles, *Pseudemys scripta elegans*, and seven lizards *Gekko gecko* were used. The animals were purchased from an authorized commercial supplier and were housed in an air-conditioned room with controlled temperature (25 °C) and natural light conditions. The original research reported herein was performed according to the regulations and laws established by European Union (86/609/EEC) and Spain (Royal Decree 1201/2005) for care and handling of animals in research.

The turtles were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (50–100 mg/kg, Normon Labs, Madrid, Spain). Corneal and pedal withdrawal reflexes disappeared within 10–15 min and the heart was exposed when ventral plastron were removed by two lateral incisions. In turn, the lizards were deeply anesthetized by inhalation of diethyl ether (Panreac, Barcelona, Spain).

All animals were perfused transcardially with physiological saline followed by 200 ml of cold 4% paraformaldehyde in a 0.1 M phosphate buffer (PB, pH 7.4). Two animals of each species received an intraperitoneal injection of colchicine (20 mg for each 100 g of animal weight) dissolved in saline, one-day prior the perfusion. The brain and the upper spinal cord were removed and kept in the same fixative for 2–3 h. Subsequently, they were immersed in a solution of 30% sucrose in PB for 4–6 h at 4 °C until they sank, embedded in a solution of 20% gelatin with 30% sucrose in PB, and stored for 6 h in a 3.7% formaldehyde solution at 4 °C. The brains were cut on a freezing microtome at 40 μm in the frontal plane and sections were collected in PB.

2.1. Orexin immunohistochemistry

The free-floating sections were rinsed twice in PB, treated with 1% H_2O_2 in PB for 20 min to reduce endogenous peroxidase activity, rinsed again three times in PB and processed by the peroxidase antiperoxidase (PAP) method (Sternberger, 1979). This included a first incubation of the sections in a goat anti-orexin-A (Santa Cruz Biotechnology, Santa Cruz, CA, USA; code sc-8070) or goat anti-orexin-B serum (Santa Cruz Biotechnology; code sc-8071), diluted 1:500 in PB containing 0.5% Triton X-100, 15% normal rabbit serum, and 2% bovine serum albumin (BSA), for 48 h at 4 °C. Subsequently, the sections were rinsed three times in PB for 10 min and incubated for 60 min at room temperature in rabbit anti-goat serum (Chemicon, Temecula, CA) diluted 1:50. After rinsing again three times for 10 min, the sections were incubated for 90 min in goat PAP complex (diluted 1:500; Chemicon). Secondary antiserum and PAP complex were diluted in PB containing 0.5% Triton X-100, 15% NRS and 2% BSA. Finally, the sections were rinsed three times for 10 min in PB and subsequently stained in 0.5 mg/ml 3,3′-diaminobenzidine (DAB; Vector SK4100) intensified with nickel (Adams, 1981), with 0.01% H_2O_2 in PB for 5–10 min. The series of sections were mounted on glass slides (mounting medium: 0.25% gelatin) in 0.1 M Tris–HCl buffer (pH 7.6) and, after dehydration, coverslipped with Entellan (Merck, Darmstadt, Germany). Some sections were counterstained with cresyl violet to facilitate analysis of the results.

The specificity of the immunohistochemical reaction was corroborated with controls that included: (1) staining of some selected sections with preimmune goat serum; (2) controls in which either the primary antibody, secondary antibody or the PAP complex was omitted; (3) homologous and heterologous preabsorptions of the primary antibody with synthetic blocking peptides for orexin-A or orexin-B (both of Santa Cruz Biotechnology; code sc-8070P and sc-8071P respectively; 0.1, 1.0 or 10 μM). In all these negative controls, the immunostaining was eliminated, even when the goat anti-orexin-A or goat anti-orexin-B was preabsorbed with the synthetic blocking peptides at low concentration (0.1 μM).

2.2. Double orexin and TH or serotonin (5-HT) immunohistochemistry

A procedure based on immunohistofluorescence was used as follows: (1) first incubation for 72 h at 4 °C in a mixture of goat anti-orexin-A or goat anti-orexin-B (diluted 1:500) and mouse anti-TH (diluted 1:1000; Immunostar, USA; code P22941) or rabbit anti-5-HT (diluted 1:1000; Immunostar, USA; code 20080); (2) second incubation for 90 min at room temperature in a mixture of secondary antisera: donkey anti-goat Alexa 594 (red fluorescence; diluted 1:300; Molecular Probes, Denmark) and chicken anti-mouse Alexa 488 (green fluorescence; diluted