Journal of Chemical Neuroanatomy 45 (2012) 36–44

Contents lists available at SciVerse ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Mapping of CGRP in the alpaca diencephalon

R. Coveñas [a,*], M.L. Sánchez [a], A. Mangas [a], L.E. Medina [a], L.A. Aguilar [b], Z. Díaz-Cabiale [c], J.A. Narváez [c]

[a] Institute of Neurosciences of Castilla y León (INCYL), Laboratory of Neuroanatomy of the Peptidergic Systems, Salamanca, Spain
[b] Cayetano Heredia Peruvian University, School of Medicine "Alberto Hurtado", Lima, Peru
[c] University of Málaga, School of Medicine, Department of Physiology and Pharmacology, Málaga, Spain

ARTICLE INFO

Article history:
Received 16 March 2012
Received in revised form 19 July 2012
Accepted 25 July 2012
Available online 31 July 2012

Keywords:
Calcitonin gene-related peptide
Hypothalamus
Thalamus
Camelids
Immunohistochemistry
Lama pacos

ABSTRACT

We report the distribution of immunoreactive cell bodies and fibers containing calcitonin gene-related peptide in the alpaca diencephalon. This study was carried out in alpacas that lived from birth to death at 0 m above sea level. Immunoreactive fibers were widely distributed throughout the thalamus and hypothalamus. A moderate density of these fibers was found in the zona incerta, the central medial, subparafascicular, reuniens and rhomboid thalamic nuclei, in the preoptic, anterior, lateral and dorsal hypothalamic areas, around the fornix, in the posterior, ventromedial and paraventricular hypothalamic nuclei and in the lateral mammillary nucleus. Cell bodies were only found in the hypothalamus: a high density in the paraventricular and supraoptic hypothalamic nuclei and a low density in the anterior, lateral and dorsal hypothalamic areas, around the fornix, and in the posterior and ventromedial hypothalamic nuclei. The widespread distribution of calcitonin gene-related peptide in the alpaca diencephalon suggests that it is involved in many physiological actions that must be investigated in-depth in the future, since alpacas lives from 0 m above sea level to altitudes of up to 5000 m altitude and hence the involvement of neuropeptides in special and unique regulatory physiological mechanisms could be suggested.

1. Introduction

In South America camelid research has been focused in general on their reproductive mechanisms (Bravo et al., 1996; Correa et al., 1997; Ratto et al., 1997, 2005, 2006) due to the economic importance of their wool, although recently the distribution of three neuropeptides belonging to different families of peptides (leucine-enkephalin, calcitonin gene-related peptide (CGRP), somatostatin-28 (1–12)) and tyrosine hydroxylase has been reported in the alpaca central nervous system (brainstem and diencephalon) (de Souza et al., 2007, 2008; Coveñas et al., 2011; Marcos et al., 2011). In this sense, the distribution of CGRP has been reported in the alpaca brainstem (de Souza et al., 2008) and the colocalization of this neuropeptide with tyrosine hydroxylase has been also studied in the same central nervous system region of this ungulate (Marcos et al., 2011). Comparing the distribution of immunoreactive cell bodies containing the above-mentioned neuropeptides, it should be remarked that both the distribution and number of cell bodies containing CGRP are considerably higher than those observed for perikarya containing leucine-enkephalin or somatostatin-28 (1–12) (de Souza et al., 2007, 2008; Coveñas et al., 2011). In all cases, these studies were carried out in control animals; that is, in alpacas not treated with colchicine. Thus, the aim of this study was to increase our knowledge of the chemical

Abbreviations: III, third ventricle; AD, anterodorsal thalamic nucleus; AHy, anterior hypothalamic area; AM, anteromedial thalamic nucleus; Arc, arcuate nucleus; AV, anteroventral thalamic nucleus; ci, capsula interna; CL, centrolateral thalamic nucleus; CM, central medial thalamic nucleus; cp, cerebral peduncle; DA, dorsal hypothalamic area; Fx, fornix; LD, laterodorsal thalamic nucleus; LG, lateral geniculate nucleus; LH, lateral hypothalamic area; LHb, lateral habenular nucleus; LM, lateral mammillary nucleus; LP, lateroposterior thalamic nucleus; MD, mediodorsal thalamic nucleus; ME, median eminence; MHb, medial habenular nucleus; MM, medial mammillary nucleus; mt, mammillothalamic tract; opt, optic tract; ox, optic chiasm; PA, preoptic area; PC, paracentral thalamic nucleus; PH, posterior hypothalamic nucleus; PV, paraventricular thalamic nucleus; PVH, paraventricular hypothalamic nucleus; Re, reuniens thalamic nucleus; Rh, rhomboid thalamic nucleus; Rt, reticular thalamic nucleus; s, stria medullaris; SCh, suprachiasmatic nucleus; SO, supraoptic hypothalamic nucleus; SPF, subparafascicular thalamic nucleus; STh, subthalamic nucleus; VA, ventroanterior thalamic nucleus; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VMH, ventromedial hypothalamic nucleus; VPL, ventroposterior thalamic nucleus, lateral part; VPM, ventroposterior thalamic nucleus, medial part; ZI, zona incerta.

* Corresponding author at: University of Salamanca, Institute of Neurosciences of Castilla y León (INCYL), Laboratory 14, c/Pintor Fernando Gallego, 1, 37007 Salamanca, Spain. Tel.: +34 923294400x1856; fax: +34 923294549.
E-mail address: covenas@usal.es (R. Coveñas).

0891-0618/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchemneu.2012.07.004

neuroanatomy of the CGRP system in the alpaca brain, and in this sense here we report the distribution of CGRP-immunoreactive fibers and cell bodies in the alpaca thalamus and hypothalamus according to an immunohistochemical technique. The distribution found is compared with that described for CGRP in the mammalian diencephalon (Skofitsch and Jacobowitz, 1985a,b; see Palkovits, 1988; Coveñas et al., 2001, 2002) and with the distribution of somatostatin-28 (1–12) recently described in the alpaca thalamus and hypothalamus (Coveñas et al., 2011).

The diencephalon is an important region of the central nervous system and is involved in food intake, olfactory, neuroendocrine, vestibular, somatosensorial, stress, thermoregulation, blood pressure, visual, auditive, taste, immunoregulatory, drinking, reproductive and nociceptive mechanisms (see Swaab, 1997; Coveñas et al., 2001, 2002 for a review). The neuropeptide CGRP, composed of 37 amino acids, has also been implicated in many physiological actions (e.g., hyperthermia, vasodilatation, locomotor activity, social behavior and regulation of astrocytes) (see de Souza et al., 2008) and hence knowledge of the distribution of neuropeptides (e.g., CGRP) in the alpaca brain will serve in the future to better understand, for example, the involvement of these substances in reproductive and social behavior, as well as at different altitudes, since this camelid lives from sea level to heights of more than 5000 m, suggesting the possible existence of special and unique regulatory mechanisms.

2. Materials and methods

2.1. Animals

We used seven male adult alpacas (*Lama pacos*) (70–80 kg) obtained from the Cayetano Heredia Peruvian University (Faculty of Veterinary Medicine and Animal Sciences, Lima, Peru). As previously reported (Coveñas et al., 2011), the animals were maintained at 0 m (sea level) from birth to death under standard conditions of light (lights on at 06:00 h and off at 20:00 h) and temperature (26° C) and had free access to food and water. The experimental design, protocols, and procedures of this work were performed under the principles of laboratory animal care and under the guidelines of the ethics and legal recommendations of Peruvian and Spanish legislation. This work was also approved by the research commission of the Cayetano Heredia Peruvian University (Lima, Peru).

2.2. Tissue preparation, immunocytochemistry and specificity of the antisera

The perfusion of the animals, the tissue preparation and the histological and immunocytochemical procedures used in this work have been published previously (see de Souza et al., 2008; Coveñas et al., 2011), as well as the characteristics of the polyclonal CGRP antiserum (obtained at the laboratory of Professor Gérard Tramu, University of Bordeaux I, France) and the specificity of the immunostaning observed (see de Souza et al., 2008). In brief, the anti-CGRP was raised in rabbits against immunogens prepared by coupling the whole synthetic CGRP peptide to human serum albumin with glutaraldehyde. Before the immunohistochemical application, the antiserum was preabsorbed with the carrier protein and the coupling agent in order to prevent non-specific immunoreactivity. Histological controls were carried out to determine the specificity of the immunostaining (preabsorption of anti-CGRP with synthetic CGRP; omission of the first antibody; preabsorption of anti-CGRP with other related peptides such as amylin and calcitonin) (see de Souza et al., 2008) (Fig. 2B). In order to avoid possible interference by endogenous peroxidases, free-floating sections were treated with distilled water containing NH_3 (20%), NaOH (1%) and H_2O_2 (30%) before carrying out the immunocytochemical procedure (Guntern et al., 1989). In all cases, the results found confirmed the specificity of the antisera used in this study.

2.3. Mapping

Mapping was carried out following the frontal planes of the alpaca diencephalon that appear in a recently published article addressing the distribution of somatostatin-28 (1–12) in the diencephalon of the alpaca (see Coveñas et al., 2011). Moreover, the brain atlas of *Lama glama* (available from the Mammalian Brain Collections of the University of WI, Madison, USA) was used. Contiguous sections to that reacted for CGRP were stained for Nissl substance with cresyl violet in order to locate the diencephalic nuclei. For the nomenclature of the diencephalic nuclei, we used that published previously in the alpaca diencephalon (see Coveñas et al., 2011).

As previously reported (de Souza et al., 2007, 2008; Coveñas et al., 2011), the density of the immunoreactive cell bodies and fibers was established: cell bodies (high density: >20 cell bodies/region/section; moderate: 10–20 cell bodies/region/section; low: <10 cell bodies/region/section) and fibers (high, moderate, low and single). This involved viewing the sections under illumination by light at constant magnification with reference to photographs of a defined series of densities for immunoreactive fibers (high, moderate, low) established previously (de Souza et al., 2007, 2008; Coveñas et al., 2011). Moreover, as previously published, immunoreactive fibers were considered short (<90 μm), medium (90–120 μm) or long in length (>120 μm) and cell bodies were considered small (diameter below 15 μm); medium-sized (15–25 μm), and large (above 25 μm) (see Coveñas et al., 2011). The size of the cell body was measured when the nucleus was visible.

Finally, photomicrographs were obtained with an Olympus DP50 digital camera attached to a Kyowa Unilux 12 microscope. To improve the visualization of the results, only the brightness and contrast of the images were adjusted, with no any further manipulation of the photographs. Adobe Photograph 6.0 software was used to view the images and adjust their brightness and contrast.

3. Results

Fig. 1 and Table 1 show the distribution and density of immunoreactive fibers and cell bodies containing CGRP in the alpaca diencephalon. In the seven animals used in this study both the distribution and density of the immunoreactive structures observed in this zone were fairly similar. Thus, in 33 nuclei/regions (18 thalamic/epithalamic, 14 hypothalamic and the subthalamic nucleus) of the alpaca diencephalon we found CGRP-immunoreactive fibers (in general, thin, short/medium in length, non-branched, and with varicosities) (Figs. 2A, 3E, 4F and 5C, D). A moderate density of such fibers was observed in 14 diencephalic nuclei/regions (5 thalamic and 9 hypothalamic) and a low density was found in 19 of them (13 thalamic/epithalamic, 5 hypothalamic and the subthalamic nucleus) (see Table 1). CGRP-immunoreactive perikarya (a high or low density) were only found in the

Table 1
Density of CGRP-immunoreactive fibers and cell bodies in the alpaca diencephalon.

Nuclei	CGRP	
	CB	F
AHy	+	++
AM	–	+
Arc	–	+
CL	–	+
CM	–	++
DA	+	++
Around Fx	+	++
LD	–	+
LH	+	++
LHb	–	+
LM	–	++
LP	–	+
MD	–	+
ME	–	+
MHb	–	+
MM	–	+
PA	–	++
PC	–	+
PH	+	++
PV	–	+
PVH	+++	++
Re	–	++
Rh	–	++
SCh	–	+
SO	+++	+
SPF	–	++
STh	–	+
VL	–	+
VM	–	+
VMH	+	++
VPL	–	+
VPM	–	+
ZI	–	++

CB, cell bodies (+++: high density; +: low density). F, fibers (++: moderate density; +: low density). For nomenclature of the nuclei, see list of abbreviations.