Distribution of CGRP in the Minipig Brainstem

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KEY WORDS calcitonin gene-related peptide; mesencephalon; pons; medulla; immunocytochemistry

ABSTRACT For the first time, an in-depth study has been made of the distribution of fibers and cell bodies containing calcitonin gene-related peptide (CGRP) in the minipig brainstem using an indirect immunoperoxidase technique. The animals studied were not treated with colchicine. Cell bodies containing CGRP were found in 20 nuclei/regions of the brainstem. These perikarya were located in somatomotor, brachiomotor and raphae nuclei, nucleus ambiguus, substantia nigra, nucleus reticularis tegmenti pontis, nucleus prepositus hypoglossi, nuclei olivaris inferior and superior, nuclei pontis, formatio reticularis, nucleus dorsalis tegmenti of Gudden, and in the nucleus reticularis lateralis. Fourteen of the 20 brainstem nuclei showed a high density of immunoreactive cell bodies. In comparison with other species, the minipig, together with the rat, show the most widespread distribution of cell bodies containing CGRP in the mammalian brainstem. Immunoreactive fibers were also observed in the brainstem. However, in the minipig brainstem the density of these fibers is low, as in many brainstem nuclei only single immunoreactive fibers were observed. A high density of immunoreactive fibers was only observed in the pars caudalis of the nucleus tractus spinalis nervi trigemini and in the nucleus ventralis tegmenti of Gudden. According to the observed anatomical distribution of the immunoreactive structures containing CGRP, the peptide could be involved in motor, somatosensory, gustative, and autonomic mechanisms. *Microsc. Res. Tech. 77:374–384, 2014.*

INTRODUCTION

The Göttingen minipig was developed in 1961–1962 at the Institute of Animal Breeding and Genetics of the University of Göttingen (Germany). The present characteristics of the Göttingen minipig as a small, white miniature pig with good fertility and stable genetics, and easy to handle were obtained as a result of crossbreeding the Minnesota minipig with the Vietnamese potbelly pig and the German Landrace (Bollen and Ellegaard, 1997). The pig brain (gyrencephalic) is more similar to the primate brain than lissencephalic brains from small laboratory animals. The pig is affordable, it is easily handled, and its use may potentially avoid some of the ethical considerations concerning the use of primates as laboratory animals (Jelsing et al., 2006). The pig model is increasingly used in the field of neuroscience; for example, it has been increasingly used for molecular imaging studies using positron emission tomography (Sauleau et al., 2009).

Abbreviations: III, nervus oculomotorius; IV, nervus trochlearis; V, nervus trigeminus; Vm, nervus trigeminus, radix motoria; Vs, nervus trigeminus, radix sensoria; VI, nervus abducens; VII, nervus facialis; VIII, nervus vestibulo-cochlearis; VIIIc, nervus vestibulo-cochlearis, pars cochlearis; VIIIv, nervus vestibulo-cochlearis, pars vestibularis; XII, nervus hypoglossus; Am, nucleus ambiguous; AP, area postrema; AQ, aqueductus cerebri; AVT, area ventralis tegmenti; cc, central canal; CCS, commissura colliculi superior; CInf, colliculus inferior; CP, commissura posterior; CR, nucleus centralis raphae; CSi, colliculus superior, stratum intermedium; CSp, colliculus superior, stratum profundum; CSs, colliculus superior, stratum superficiale; CT, corpus trapezoideum; Cu, nucleus cuneatus; CuE, nucleus cuneatus externalis; CVP, nucleus cochlearis posteroventral; D, nucleus Darkschewitsch; DG, nucleus dorsalis tegmenti of Gudden; DMX, nucleus dorsalis motorius nervi vagus; DPCS, decussatio pedunculorum cerebellarium superior; DR, nucleus dorsalis raphae; FC, fasciculus cuneatus; FG, fasciculus gracilis; FLD, fasciculus longitudinalis dorsalis; FLM, fasciculus longitudinalis medialis; FR, formatio reticularis; G, nucleus gracilis; GC, substantia grisea centralis; ICO, commissura colliculi inferior; In, nucleus intercalates; IP, nucleus interpeduncularis; LC, locus ceruleus; LL, lemniscus lateralis; LLd, nucleus lemnisci lateralis, pars dorsalis; LM, lemniscus medialis; MV, nucleus motorius nervi trigemini; NIII, nucleus nervi oculomotorius; NIV, nucleus nervi trochlearis; NVI, nucleus nervi abducens; NVII, nucleus nervi facialis; NXII, nucleus nervi hypoglossi; NCH, nuclei cochleares; NCInf, nucleus colliculi inferior; NCS, nucleus centralis superior; NCT, nucleus corporis trapezoidei; NInt, nucleus interstitial of vestibular nerve; NMV, nucleus tractus mesencephali nervi trigemini; NO, nucleus ovalis; nP, nuclei pontis; NR, nucleus rubber; NSVc, nucleus tractus spinalis nervi trigemini, pars caudalis; NSVi, nucleus tractus spinalis nervi trigemini, pars interpolaris; NSVo, nucleus tractus spinalis nervi trigemini, pars oralis; NTS, nucleus tractus solitarius; Oli, nucleus olivaris inferior; Ols, nucleus olivaris superior; PbL, nucleus parabrachialis lateralis; PbM, nucleus parabrachialis medialis; PCI, pedunculus cerebellaris inferior; PCM, pedunculus cereberallis medialis; PCS, pedunculus cerebellaris superior; PH, nucleus prepositus hypoglossi; PrV, nucleus sensorius principalis nervi trigemini; PT, nucleus pretectalis; Py, tractus pyramidalis; RL, nucleus reticularis lateralis; Rt, fasciculus retroflexus; RTP, nucleus reticularis tegmenti pontis; SNc, substantia nigra, pars compacta; SNr, substantia nigra, pars reticulate; TS, tractus solitarius; TSV, tractus spinalis nervi trigemini; TTS, tractus tectospinalis; V3, ventriculus tertius; V4, ventriculus quartus; VesI, nucleus vestibularis inferior; VesL, nucleus vestibularis lateralis; VesM, nucleus vestibularis medialis; VesS, nucleus vestibularis superior; VG, nucleus ventralis tegmenti of Gudden; VR, nucleus ventralis raphae.

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Calcitonin gene-related peptide (CGRP) is a compound composed of 37 amino acids and originates from the calcitonin gene. The calcitonin family of peptides comprises calcitonin, amylin, CGRP, and adrenomedullin (Poyner et al., 2002). CGRP, in addition to substance P and somatostatin, is an important neuropeptide involved in the transmission of sensorial information (Ribeiro-da-Silva, 1995). CGRP is released from activated trigeminal sensory nerves, dilates intracranial blood vessels, and transmits vascular nociception (Arulmani et al., 2004). CGRP has also been implicated in other physiological actions (hyperthermia, locomotor activity, social behavior, the regulation of astrocytes, glucose metabolism, increases in the number of acetylcholine receptors at the level of the neuromuscular junction, etc) (Batten et al., 1989; de Souza et al., 2008; Gibson et al., 1988; New and Mudge, 1986; Raynaud et al., 1994). The colocalization of CGRP with substance P or with gamma-aminobutiric acid (GABA) and the presence of CGRP in intrinsic neurons of the brainstem have also been reported (Aita et al., 2008; Batten et al., 1989; Maison et al., 2003). It is also known that motor neurons containing CGRP located in the nucleus ambiguus project to the striated muscle of the larynx and pharynx, but CGRP immunoreactivity is absent from vagal preganglionic motor neurons projecting to structures in the chest and abdomen (McWilliam et al., 1989). Migraine is a neurological disorder that is associated with an increase in plasma CGRP levels (Arulmani et al., 2004) and for this reason its treatment has attracted considerable attention (Olesen et al., 2004); in fact, CGRP receptor antagonists are now emerging as a new generation of migraine drugs (Recober and Russo, 2009). Two receptors, CGRP1 and CGRP2, have been described for the CGRP (Häppölä et al., 1993; Waugh et al., 1999). These receptors show different affinities for the C-terminal fragment of human CGRP (Dennis et al., 1990).

According to immunocytochemical techniques, it is known that fibers and cell bodies containing CGRP are widely distributed through the mammalian central nervous system (Batten et al., 1989; Conti and Sternini, 1989; Coveñas et al., 2003; Marcos et al., 1999; McWilliam et al., 1989; Quartu et al., 1992; Palkovits, 1988; Skofitsch and Jacobowitz, 1985; Tashiro et al., 1991; Unger and Lange, 1991). Moreover, the quantitative distribution of CGRP has also been reported in the rat central nervous system by radioimmunoassay (Skofitsch and Jacobowitz, 1985). However, no previous information appears to be available in the literature concerning the presence of fibers and cell bodies containing neuropeptides in the minipig central nervous system following the implementation of immunocytochemical techniques, except for a recent study carried out by our group on the distribution of immunoreactive structures containing methionine-enkephalin in the minipig brainstem (Sánchez et al., 2013). Thus, the chemical neuroanatomy of neuropeptides in the minipig central nervous system has not yet been studied in-depth, a field awaiting further development.

In light of the above, the aims of this study were to increase our knowledge of the chemical neuroanatomy of CGRP in the mammalian brainstem, in particular in the minipig brainstem, and to compare the distribution of fibers and cell bodies containing CGRP found in this species with those previously described for the same peptide in the brainstem of other mammals. Another aim was to compare the distribution of CGRP-immunoreactive structures in the minipig brainstem with that located in the same region of the minipig central nervous system containing methionine-enkephalin (Sánchez et al., 2013). Knowledge about the distribution of CGRP in the minipig brainstem will serve in the future to better understand the involvement of this peptide in numerous physiological actions and for future neuroanatomical, neuropharmacological, and behavioral studies.

MATERIALS AND METHODS

Animals and Tissue Preparation

As previously reported (Sánchez et al., 2013), in this study the animals used were treated according to the resolution of the Association for Research in Vision and Ophthalmology. We used four Göttingen minipigs, two male and two female, 14 months old. The animals were kept under light–dark cycles (12/12 h). They were fed once a day while water was freely available. The minipigs were anaesthetized using 8 mg/kg Zoletil (tiletamine and zolazepam), 0.04 mg/kg atropine and 300 μg/kg Domtor (medetomidine), after which animals were deeply anaesthetized with Propofol administered through an intravenous cannula inserted into the ear (Sánchez et al., 2013). Then, the animals were euthanized with an intravenous injection of saturated KCl (Sánchez et al., 2013). As previously reported (Sánchez et al., 2013), the brains were fixed in paraformaldehyde and after fixation, the brainstem pieces were cryoprotected in sucrose in phosphate-buffered saline (PBS). They were then embedded in Tissue Tek medium (Leica). Cryosections were cut at 25 μm thickness and were stored in PBS until use.

Immunocytochemistry

Coronal sections were prepared for immunostaining with the avidin-biotin-peroxidase (ABC) technique (see Sánchez et al., 2013). Free floating sections were preincubated for 30 min in 10% normal horse serum in PBS (pH 7.2) containing 0.3% TritonX-100 and incubated overnight at 4°C in the same solution supplemented with anti-CGRP and diluted 1/3,000. The sections were then rinsed extensively in PBS (30 min) and transferred to the secondary antibody for 1 h at room temperature (biotinylated antirabbit immunogamma globulin diluted 1/200). The sections were washed in PBS (30 min) and incubated with Vectastain ABC reagent (diluted 1/100) for 1 h at room temperature. After the sections had been washed in PBS (30 min) and Tris-HCl buffer (pH 7.6, 10 min), tissue-bound peroxidase was developed with H_2O_2 using 3, 3'diaminobenzididine as chromogen. The sections were rinsed with PBS and coverslipped with PBS/glycerol (1/1).

Specificity of the Antisera

The polyclonal antibody used in this study, anti-CGRP (obtained from the laboratory of Professor Gérard Tramu, University of Bordeaux I, France), was raised in rabbits against immunogens prepared by coupling the whole synthetic CGRP to a carrier protein (human serum albumin) with glutaraldehyde, as

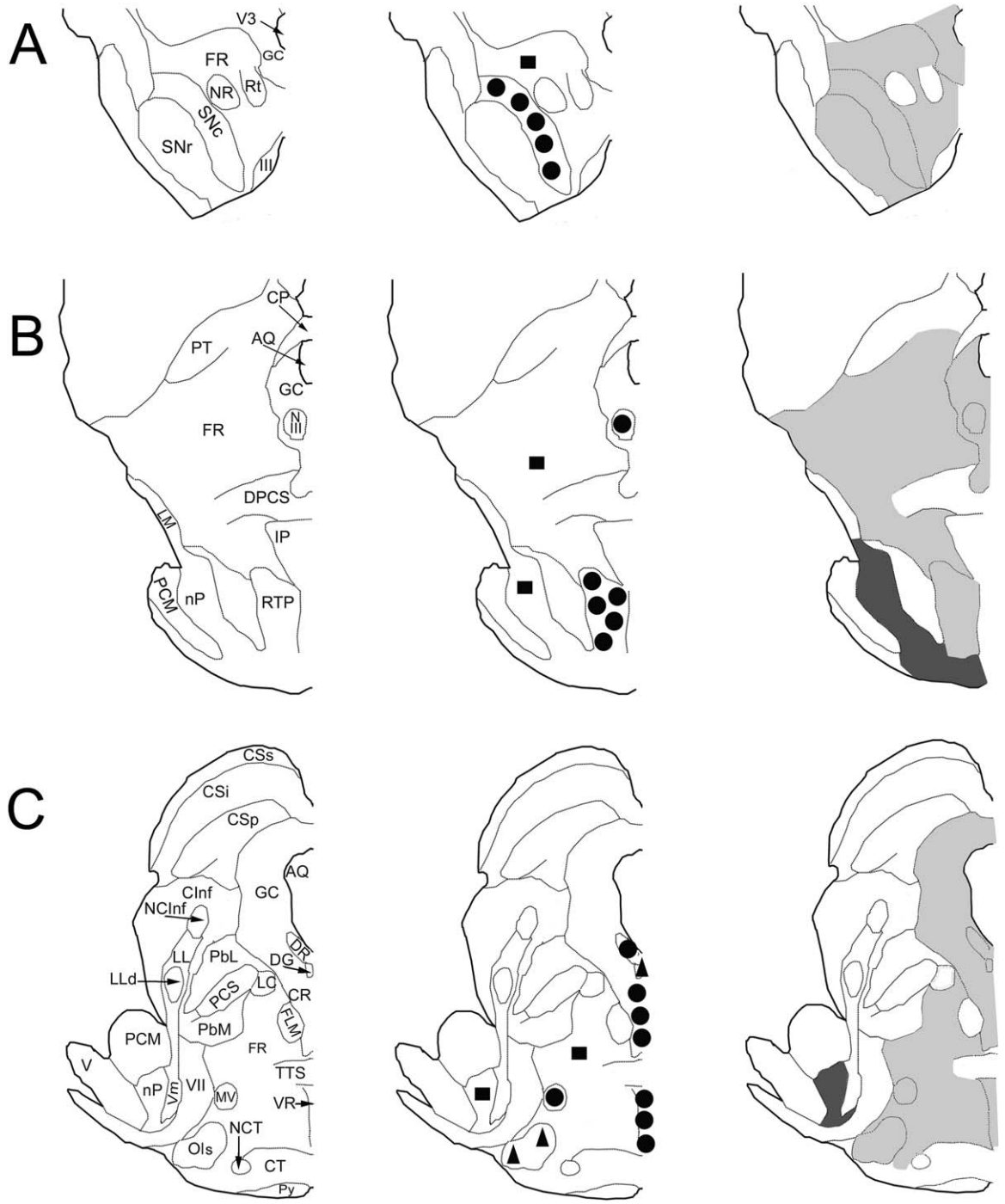

Fig. 1. Distribution of CGRP-immunoreactive fibers and perikarya in frontal planes of the minipig brainstem from rostral (**A**) to caudal (**F**) levels. Cell bodies containing CGRP are represented by closed circles (high density), triangles (moderate density), and squares (low density), whereas immunoreactive fibers are represented by slightly dark (single axons), moderately dark (low density), strongly dark (moderate density), and black (high density). For nomenclature of the structures, see list of abbreviations.

previously reported (Marcos et al., 1999). Rabbit antiserum was preabsorbed with the carrier protein and the coupling agent to prevent nonspecific immunoreactivity due to the anticarrier antibodies. This preabsorption was carried out before the immunocytochemical applications.

As previously reported (de Souza et al., 2008), histological controls were also 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). To

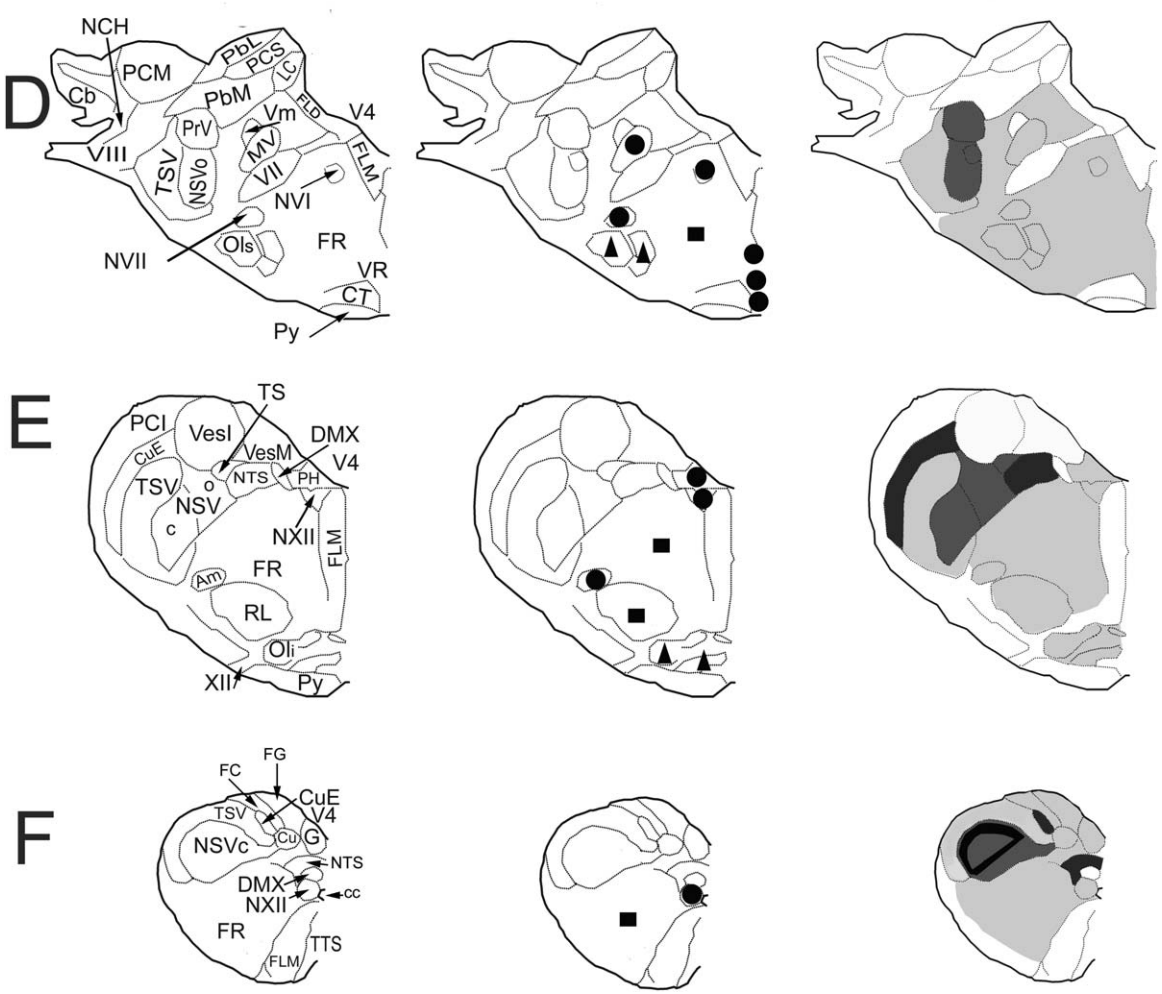

Fig. 1. Continued

avoid possible interference by endogenous peroxidase, free-floating sections were treated with distilled water containing NH_3 (20%), NaOH (1%), and H_2O_2 (30%) before carrying out the immunocytochemical procedures (Guntern et al., 1989). As previously reported (Coveñas et al., 2012; de Souza et al., 2008), in all cases the results found confirmed the specificity of the antisera used in this research.

Mapping

As previously reported (see Sánchez et al., 2013), mapping was carried out according to the frontal planes of the pig brainstem published by Félix et al. (1999). For the nomenclature, we also used this stereotaxic atlas. Moreover, a number of adjacent sections which reacted for CGRP were routinely stained for Nissl substance with cresyl violet to locate the brainstem nuclei. The density of the immunoreactive cell bodies and fibers was established: cell bodies (high density: >10 cell bodies/region/section; moderate: 5–10 cell bodies/region/section; low: <5 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, and low) established previously (Coveñas et al., 2011; de Souza et al., 2008). The 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 40 μm); medium-sized (41–50 μm), and large (above 50 μm). 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 further manipulation of the photographs. Adobe Photograph 2.0 software was used to view the images and adjust their brightness and contrast.

RESULTS

Figure 1 and Table 1 show the distribution and density of immunoreactive fibers and cell bodies containing CGRP in the minipig brainstem. In the animals studied (males and females), both the distribution and density of the immunoreactive structures (fibers and cell bodies) observed in the pig brainstem were fairly

TABLE 1. CGRP-immunoreactive fibers and cell bodies in the minipig brainstem.

N/R	F	CB	N/R	F	CB	N/R	F	CB	N/R	F	CB
III	–	–	Cu	S	–	MV	S	+++	PbM	–	–
IV	–	–	CuE	++	–	NCH	–	–	PCI	–	–
V	–	–	CVP	–	–	NCInf	–	–	PCM	–	–
Vm	–	–	D	–	–	NCS	S	+++	PCS	–	–
Vs	–	–	DG	S	++	NCT	–	–	PH	S	+++
VI	–	–	DMX	–	–	NIII	S	+++	PrV	+	–
VII	–	–	DPCS	–	–	NInt	–	–	PT	–	–
VIII	–	–	DR	S	+++	NIV	S	+++	Py	–	–
VIIIc	–	–	FC	S	–	NMV	S	–	RL	S	+
VIIIv	–	–	FG	S	–	NO	–	–	Rt	–	–
XII	–	–	FLD	–	–	nP	+	+	RTP	S	+++
Am	S	+++	FLM	–	–	NR	–	–	SNc	S	+++
AP	–	–	FR	S	+	NSVc	+++	–	SNr	S	–
AVT	–	–	G	S	–	NSVi	+	–	TS	+	–
CCS	–	–	GC	S	–	NSVo	+	–	TSV	S	–
CInf	–	–	ICO	–	–	NTS	++	–	TTS	–	–
CP	–	–	In	–	–	NVI	S	+++	VesI	–	–
CR	S	+++	IP	–	–	NVII	S	+++	VesL	–	–
CSi	–	–	LC	–	–	NXII	S	+++	VesM	–	–
CSp	–	–	LL	–	–	Oli	S	++	VesS	–	–
CSs	–	–	LLd	–	–	Ols	S	++	VG	+++	–
CT	–	–	LM	–	–	PbL	–	–	VR	S	+++

CB: cell bodies (+++: high density; ++: moderate density; and +: low density). F: fibers (+++: high density; ++: moderate density; and +: low density; S: single). –: no immunoreactivity. N: nucleus. R: region.
For nomenclature of the nuclei, see list of abbreviations.

similar. CGRP-immunoreactive structures were distributed throughout the brainstem, although this distribution was not widespread. In fact, we observed immunoreactive structures in 36 (40.9%) of the 88 minipig brainstem nuclei/tracts/regions. CGRP-immunoreactive cell bodies were found in 20 nuclei/regions (22.7%), whereas in 16 nuclei/tracts/regions (18.1%) we found only fibers containing the neuropeptide (Table 1).

Immunoreactive perikarya were >30 μm in diameter. Table 2 shows the size of the cell bodies containing CGRP: small (31–40 μm), medium (41–50 μm), and large (51–60 μm). The largest cell bodies (60–100 μm) were observed in the brainstem nuclei ambiguus, nervi abducens, nervi facialis, and prepositus hypoglossi, as well as in the formatio reticularis (Figs. 3C and 3D, 4B–4D). Most of the immunoreactive perikarya were piriform, oval, or polygonal, although in the brainstem nuclei a no given shape predominated over the others (Figs. 2C, 3E–3H, 4E and 4F, 5A). In general, CGRP-immunoreactive cell bodies showed at least two visible processes (Figs. 3E–3H). We observed 14 brainstem nuclei showing a high density of immunoreactive perikarya: four somatomotor nuclei [nervi oculomotorius (Fig. 5D), nervi trochlearis (Figs. 5A and 5C), nervi abducens (Figs. 4C and 4D), and nervi hypoglossi (Figs. 2C and 2D)]; the nucleus ambiguus (Fig. 5B); two brachiomotor nuclei [motorius nervi trigemini (Fig. 4F); and nervi facialis (Fig. 4B)]; four raphae

TABLE 2. Morphological characteristics of CGRP-immunoreactive cell bodies in the minipig brainstem.

	Most abundant			Others		
Nuclei/regions	Size	Shape	Processes	Size	Shape	Processes
Am[a]	Large	Polygonal	2–3	Large	Piriform	1
CR	Small	Oval	2	Small	Piriform	1
DG	Small	Oval	2	Small	Piriform	1
DR	Small	Oval	2	Small	Piriform	1
FR[a]	Large	Polygonal	3	Large	Oval	2
MV	Medium	Piriform	1	Medium	Polygonal	2–3
NCS	Small	Oval	2	Small	Piriform	1
NIII	Medium	Piriform	1	Medium	Polygonal	2–3
NIV	Medium	Polygonal	3	Medium	Piriform	1
nP	Small	Oval	2	Small	Polygonal	2
NVI[a]	Large	Polygonal	3	Large	Oval	2
NVII[a]	Large	Polygonal	3	Large	Oval	2
NXII	Large	Oval	1	Large	Polygonal	3
Oli	Large	Polygonal	2–3	Large	Piriform	1
Ols	Large	Polygonal	2	Large	Piriform	1–2
PH[a]	Large	Piriform	1–2	Large	Polygonal	3
RL	Medium	Piriform	1	Medium	Polygonal	3
RTP	Small	Piriform	1	Small	Oval	2
SNc	Large	Piriform	1	Large	Oval	1
VR	Small	Oval	2	Small	Piriform	1

[a]Also indicates the presence of the largest cell bodies observed (60–100 μm in diameter).
For nomenclature of the nuclei, see list of abbreviations.

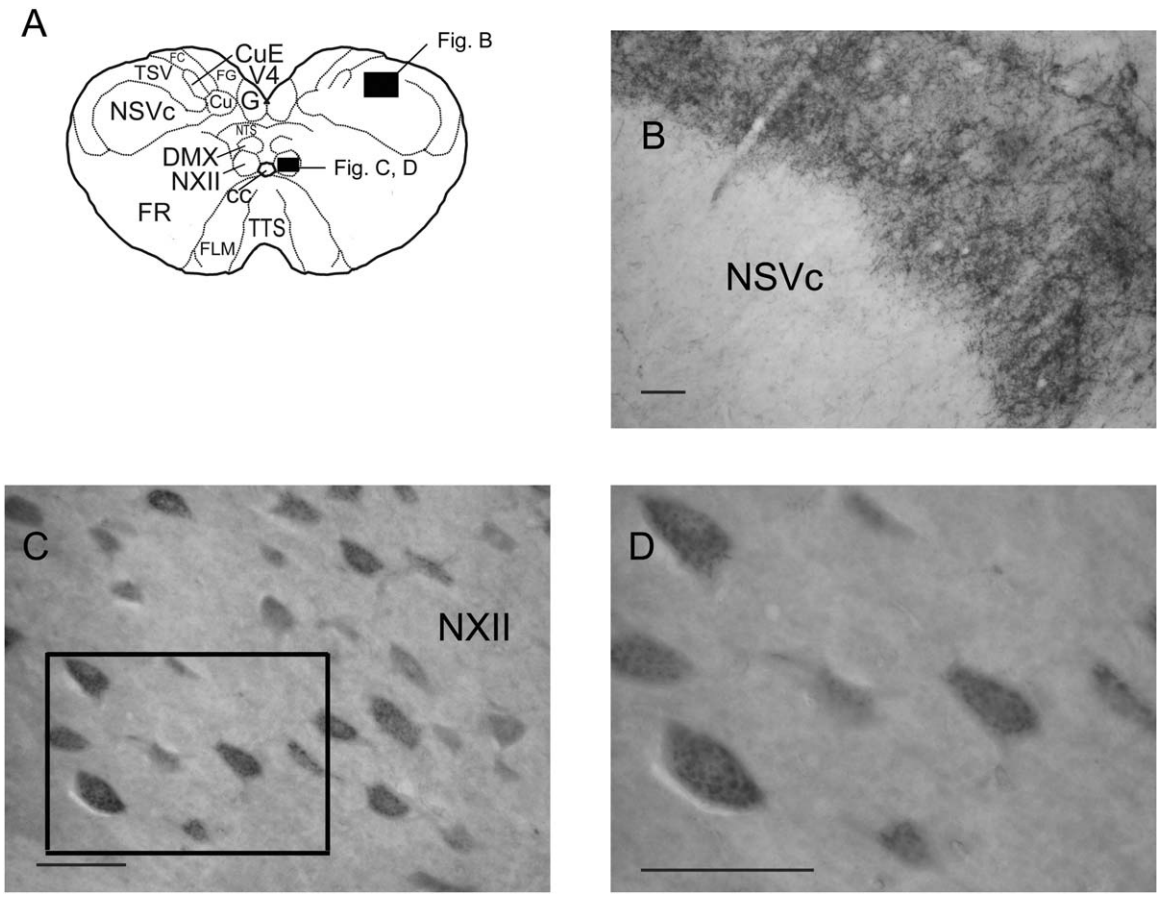

Fig. 2. Immunoreactive fibers and cell bodies containing CGRP. **A**: Frontal section of the minipig medulla oblongata (caudal region). For nomenclature of the nuclei, see list of abbreviations. The photographs shown in (B)–(D) were respectively taken from the regions delimited by the rectangles in (A) [indicated as (B)–(D)]. **B**: Low-power magnification of fibers containing CGRP located in the nucleus tractus spinalis nervi trigemini, pars centralis (NSVc). **C**: Medium-power magnification of cell bodies containing CGRP located in the nucleus nervi hypoglossi (NXII). D: High-power magnification of the region delimited by a rectangle in (C). Scale bar: 100 μm.

nuclei (centralis raphae, dorsalis raphae (Fig. 5C), centralis superior, and ventralis raphae); substantia nigra, pars compacta; nucleus reticularis tegmenti pontis; and nucleus prepositus hypoglossi (Figs. 3C and 3D). Moreover, three nuclei showed a moderate density of immunoreactive perikarya [nucleus dorsalis tegmenti of Gudden, nucleus olivaris inferior, nucleus olivaris superior (Fig. 4E)], and three nuclei showed a low density of these cell bodies (nuclei pontis, formatio reticularis, and nucleus reticularis lateralis; Table 1).

We observed immunoreactive fibers in 36 nuclei/tracts/ regions of the minipig brainstem. However, in 27 of them we only found single fibers. This means that although the distribution of CGRP-immunoreactive fibers is moderately widespread the density of fibers containing CGRP is low in the minipig brainstem. Except for nuclei/tracts/regions in which we found single fibers, we only observed a low/moderate/high density of CGRP-immunoreactive fibers in nine brainstem regions: two nuclei showed a high density (Fig. 2B), two nuclei a moderate density, and five nuclei/tracts a low density (Table 1). Some of the brainstem nuclei in which we observed a low/moderate/high density of CGRP-immunoreactive fibers are involved in somatosensory [nucleus cuneatus externalis (Fig. 3B), pars caudalis, oralis, and interpolaris of the nucleus tractus spinalis nervi trigemini (Fig. 2B)] and gustative (nucleus tractus solitarius) mechanisms, as well as in the control of autonomic function (nucleus tractus solitarius). The most abundant CGRP-immunoreactive fibers were thin, short, with varicosities, medium/long (100–200 μm) in length, and unbranched.

DISCUSSION

CGRP in the Pig

In a previous study, the presence of CGRP-immunoreactive cell bodies in the pig brainstem was reported in the nucleus nervi hypoglossi (Sienkiewicz et al., 2010). This is in agreement with our results. The study conducted by Sienkiewicz et al. (2010) was carried out in juvenile (4-months-old) female pigs not treated with colchicine. The authors focused their study only in this brainstem nucleus, in which several other neuropeptides (e.g., substance P and somatostatin), in addition to CGRP, were found. In another study, the presence of fibers and cell bodies containing CGRP or other neuropeptides was reported in the pig stellate ganglion (Häppölä et al., 1993). In this latter

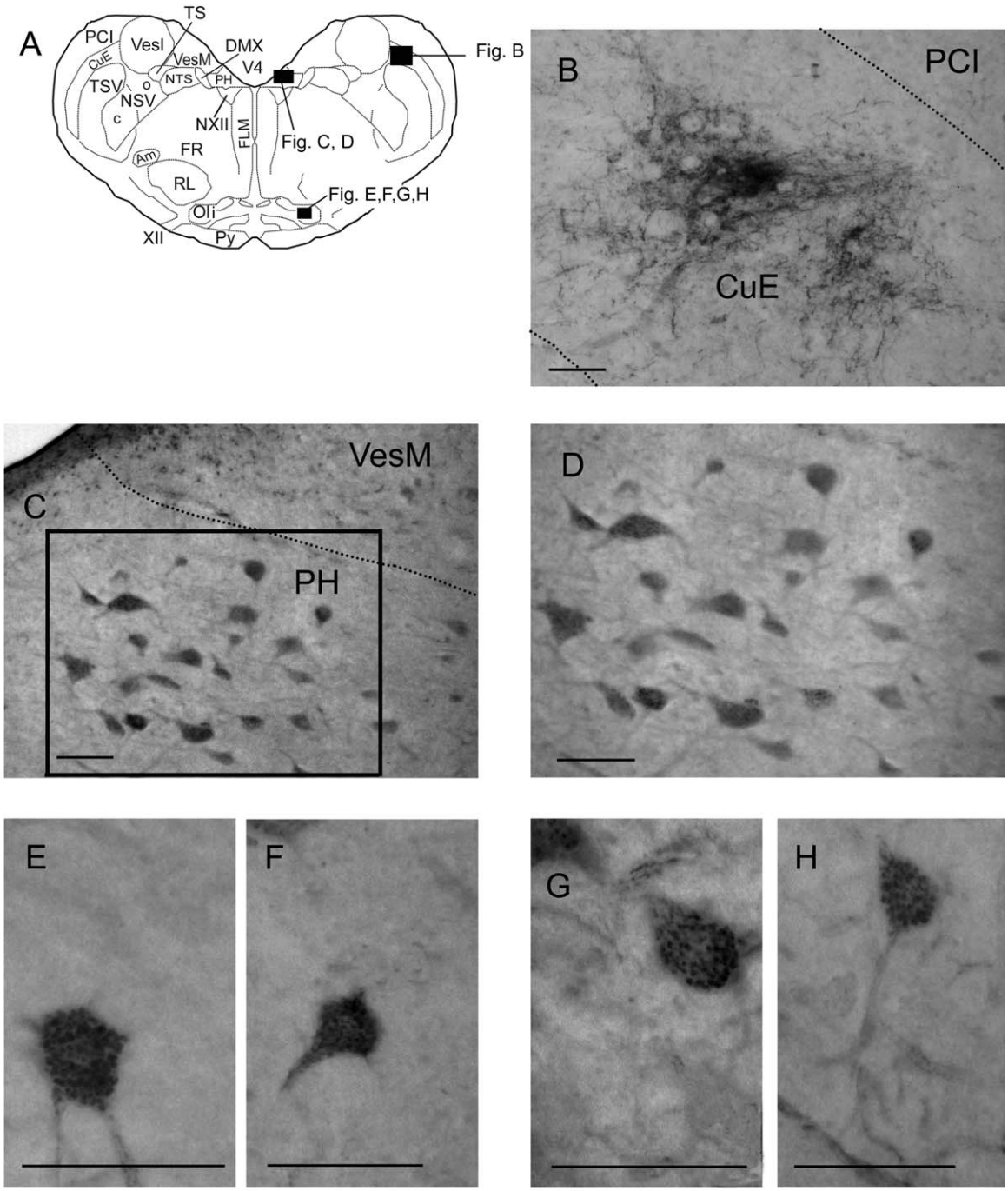

Fig. 3. Immunoreactive fibers and cell bodies containing CGRP. **A**: Frontal section of the minipig medulla oblongata (rostral region). For nomenclature of the nuclei, see list of abbreviations. The photographs shown in (B)–(H) were respectively taken from the regions delimited by the rectangles in (A) [indicated as (B)–(H)]. **B**: Low-power magnification of fibers containing CGRP located in the nucleus cuneatus externalis (CuE). **C**: Medium-power magnification of cell bodies containing CGRP located in the nucleus prepositus hypoglossi (PH). **D**: High-power magnification of the region delimited by a rectangle in (C). E–H: High-power magnification of four cell bodies located in the nucleus olivaris inferior (Oli). Scale bar: 100 μm.

study, colchicine was not administered. Accordingly, here, we described for the first time in-depth the distribution of fibers and cell bodies containing CGRP in the minipig brainstem. Here, as in the two works reported above (Häppölä et al., 1993; Sienkiewicz et al., 2010), we used animals not treated with colchicine.

Neuropeptides in the Minipig Brainstem

The distribution of methionine-enkephalin in the minipig brainstem in animals not treated with colchicine has been published recently (Sánchez et al., 2013). On comparing the distribution of both

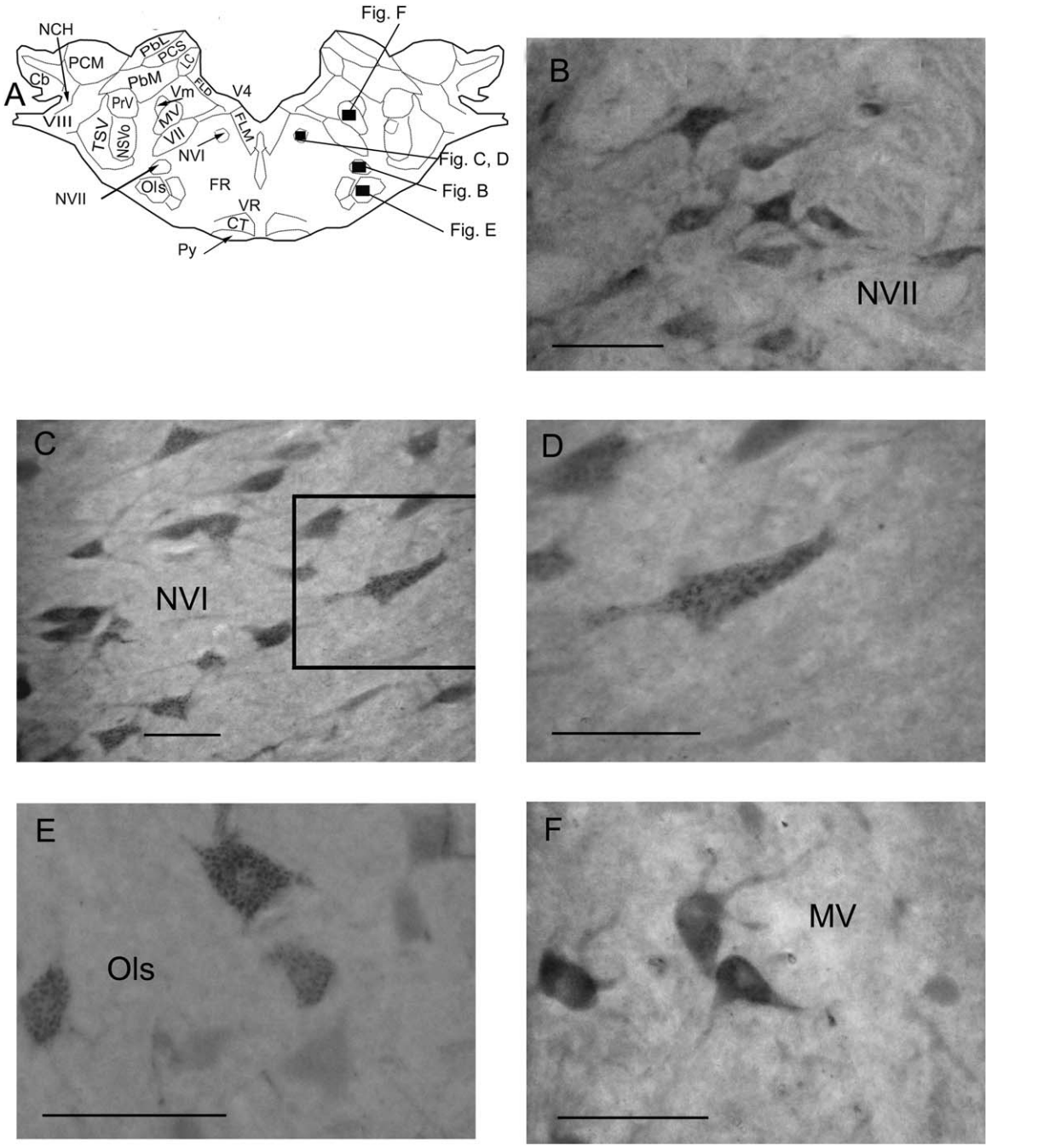

Fig. 4. Immunoreactive cell bodies containing CGRP. **A**: Frontal section of the minipig brainstem. For nomenclature of the nuclei, see list of abbreviations. The photographs shown in (B)–(F) were respectively taken from the regions delimited by the rectangles in (A) [indicated as (B)–(F)]. **B**: Medium-power magnification of cell bodies containing CGRP located in the nucleus nervi facialis (NVII). **C**: Medium-power magnification of cell bodies containing CGRP located in the nucleus nervi abducens (NVI). **D**: High-power magnification of the region delimited by the rectangle in (C). **E**: High-power magnification of cell bodies containing CGRP located in the nucleus olivaris superior (Ols). **F**: High-power magnification of cell bodies containing CGRP located in the nucleus motorius nervi trigemini (MV). Scale bar: 100 μm.

methionine-enkephalin and CGRP in the minipig brainstem, it seems that the distribution of cell bodies containing CGRP is more widespread than that shown by cell bodies containing the opioid peptide and that the distribution of fibers containing methionine-enkephalin is more widespread than that shown by the CGRP-immunoreactive fibers. Both neuropeptides have been observed in cell bodies located in four nuclei of the raphae (nucleus centralis raphae, nucleus dorsalis raphae, nucleus centralis superior, and nucleus ventralis raphae), in some motor nuclei (nucleus motorius nervi trigemini, nucleus nervi oculomotorius,

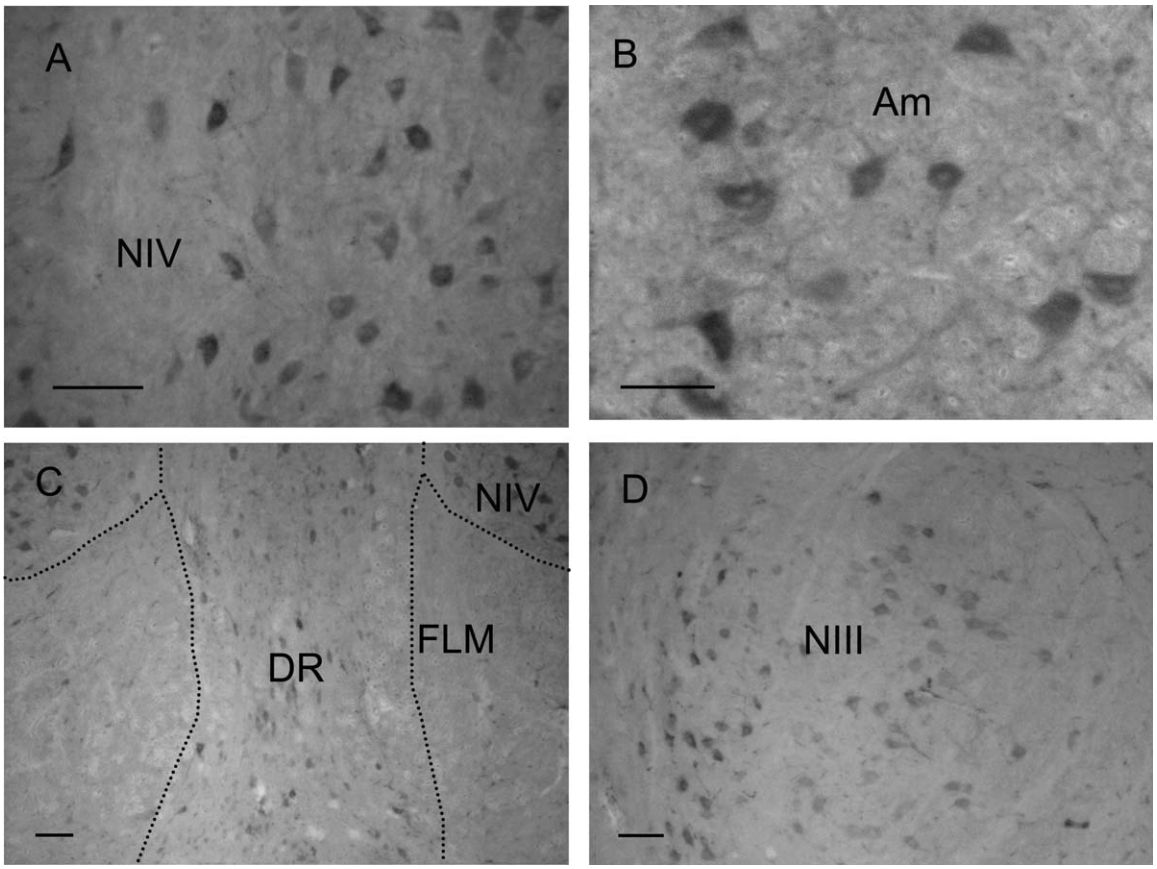

Fig. 5. Immunoreactive cell bodies containing CGRP. **A**: Medium-power magnification of the nucleus nervi trochlearis (NIV). **B**: Medium-power magnification of cell bodies located in the nucleus ambiguus (Am). **C**: Low-power magnification of cell bodies containing CGRP located in the nucleus nervi trochlearis (NIV) and in the nucleus dorsalis raphae (DR). **D**: Low-power magnification of cell bodies containing CGRP located in the nucleus nervi oculomotorius (NIII). Scale bar: 100 μm.

nucleus nervi trochlearis, nucleus nervi abducens, and nucleus nervi facialis), as well as in the formatio reticularis (mesencephalon, pons, and medulla oblongata) and in the nucleus reticularis lateralis. However, in the nuclei parabrachialis lateralis and medialis and in the nucleus ruber there are perikarya containing methionine-enkephalin, but not CGRP. By contrast, CGRP-immunoreactive cell bodies have been observed in the nucleus ambiguus, nucleus nervi hypoglossi, nucleus olivaris inferior, nucleus olivaris superior, nucleus prepositus hypoglossi, nucleus reticularis tegmenti pontis, nuclei pontis, and in the substantia nigra, pars compacta, but in these nuclei no cell bodies containing methionine-enkephalin were found. It should be noted that 11 of the 14 brainstem nuclei showing methionine-enkephalin-immunoreactive perikarya also contained CGRP-immunoreactive cell bodies. For example, perikarya containing both neuropeptides were observed in the motor (ambiguus, motorius nervi trigemini, nervi oculomotorius, nervi abducens, nervi trochlearis, nervi facialis, and nervi hypoglossi) and raphae nuclei (centralis raphae, dorsalis raphae, centralis superior, and ventralis raphae) (Sánchez et al., 2013). The morphological characteristics (e.g., size and shape) of cell bodies containing CGRP or methionine-enkephalin are quite similar and hence a possible coexistence of both neuropeptides could occur in these brainstem nuclei of the minipig.

CGRP in the Mammalian Brainstem

In the rat, the presence of cell bodies containing CGRP has been reported after the administration of colchicine, whereas in control animals (not treated with the drug) no cell bodies were observed (Skofitsch and Jacobowitz, 1985). By contrast, in other studies carried out in the same rodent, the cat or alpaca the administration of colchicine was not necessary to observe CGRP-immunoreactive cell bodies (Batten et al., 1989; de Souza et al., 2008; Kruger et al., 1988a, b). Moreover, CGRP-immunoreactive perykarya have also been observed in humans (Unger and Lange, 1991).

In general, our results are in agreement with those previously reported in the mouse (Peltier and Bishop, 1999), rat (Kruger et al., 1988a; Ma et al., 2003; Palkovits, 1988; Skofitsch and Jacobowitz, 1985), cat (Batten et al., 1989), alpaca (de Souza et al., 2008), and primates (Arvidsson et al., 1991; Unger and Lange, 1991). Thus, in both the mouse and minipig, cell bodies containing CGRP have been found in the nucleus ambiguus, nucleus motorius nervi trigemini, nucleus nervi

oculomotorius, nucleus nervi facialis, nucleus nervi hypoglossi, nucleus olivaris inferior, nucleus reticularis lateralis, and in the formatio reticularis (Peltier and Bishop, 1999). In both the rat and minipig, CGRP-immunoreactive perikarya were found in the nucleus ambiguus, nucleus motorius nervi trigemini, nucleus nervi oculomotorius, nucleus nervi trochlearis, nucleus nervi facialis, and in the nucleus nervi hypoglossi, nucleus olivaris superior, and in the nucleus reticularis lateralis (Palkovits, 1988; Skofitsch and Jacobowitz, 1985). In the cat and minipig, CGRP cell bodies were found in the nucleus ambiguus, nucleus nervi abducens, nucleus nervi facialis, nucleus nervi hypoglossi, and in the pontine formatio reticularis (Batten et al., 1989). In both the alpaca and minipig, CGRP-immunoreactive perikarya have been found in the motor nuclei nucleus motorius nervi trigemini, nucleus nervi trochlearis, nucleus nervi facialis, and nucleus nervi hypoglossi as well as in the nucleus reticularis lateralis, nucleus olivaris superior, and in the formatio reticularis (mesencephalon, pons, and medulla oblongata) (de Souza et al., 2008). In primates (monkey and human), CGRP-immunoreactive cell bodies were observed in motor nuclei (Arvidsson et al., 1991; Unger and Lange, 1991).

However, several differences merit comment. In the mouse (Peltier and Bishop, 1999), but not in the minipig, cell bodies containing CGRP have been found in the substantia grisea centralis, locus ceruleus, nucleus parabrachialis lateralis, and in the nucleus parabrachialis medialis, In the rat, cell bodies have been described in the colliculus superior, nucleus tractus solitarius, nucleus cuneatus externalis, colliculus inferior, nucleus cuneatus, nucleus gracilis, nucleus parabrachialis medialis, and in the nucleus parabrachialis lateralis (Ma et al., 2003; Palkovits, 1988; Skofitsch and Jacobowitz, 1985), but in the minipig no CGRP-immunoreactive cell bodies were observed in these nuclei. Moreover, the distribution of CGRP-immunoreactive fibers is more widespread in the rat brainstem than in the minipig brainstem. In the minipig, we did not detect CGRP-immunoreactive perikarya in the nucleus cuneatus externalis and in the pars centralis of the nucleus tractus spinalis nervi trigemini but in the cat such cell bodies were observed in these nuclei (Batten et al., 1989). In the cat, the distribution of CGRP-immunoreactive fibers located in the brainstem (Batten et al., 1989) is less widespread than that found in the minipig. In the alpaca, CGRP-immunoreactive perikarya have been observed in the colliculus superior and in the nucleus dorsalis motorius nervi vagus (de Souza et al., 2008), but in the minipig we did not observe any immunoreactive cell body in these nuclei. The distribution of CGRP-immunoreactive fibres is more widespread in the alpaca brainstem than in the minipig brainstem. In humans, the distribution of both immunoreactive cell bodies and fibers containing CGRP is quite restricted (Unger and Lange, 1991). In comparison with the distribution observed in this study, the distribution of both CGRP-immunoreactive fibers and perikarya is more widespread in the minipig than that described in humans.

Taking the above data together, it seems that the rat and the minipig show the most widespread distribution of cell bodies containing CGRP in the mammalian brainstem (in both species immunoreactive cell bodies were found in 20 nuclei), followed by the mouse (17 nuclei), the alpaca (14 nuclei), the cat (eight nuclei), and humans (five nuclei). Regarding the immunoreactive fibers containing CGRP, the alpaca showed the most widespread distribution (38 nuclei), followed by the minipig (36 nuclei), the rat (25 nuclei) and humans (four nuclei). It should be noted that in the alpaca in most of the nuclei in which CGRP-immunoreactive fibers were observed a high density of immunoreactive fibers was found, whereas in the minipig many nuclei showed single immunoreactive fibers. The observed discrepancies regarding the distribution of cell bodies and fibers containing CGRP in the mammalian brainstem could be due to technical considerations (administration of colchicine, anti-CGRP used,etc) and/or to species differences.

Possible Physiological Functions of CGRP in the Minipig Brainstem

CGRP plays an important role in nociception, hyperthermia, vasodilation, locomotor activity, social behavior, and regulation of astrocytes (Arulmani et al., 2004; de Souza et al., 2008). At present, the physiological actions of CGRP in the minipig brainstem are unknown, but the presence of CGRP-immunoreactive structures in the brainstem nuclei implies that CGRP serves different functions at these sites. Thus, the presence of a rich network of CGRP-immunoreactive fibers in the pars centralis of the nucleus tractus spinalis nervi trigemini suggests that the neuropeptide is involved in the transmission of nociceptive inputs. In fact, CGRP, substance P and somatostatin are three of the most important neuropeptides involved in nociceptive mechanisms (Lazarov, 1995; Ribeiro-da-Silva, 1995). The presence of CGRP-immunoreactive perikarya in the nucleus olivaris suggests the involvement of CGRP in auditory mechanisms. This is in agreement with the results found in the mouse in which the presence of CGRP in olivocochlear terminals in inner and outer hair cell regions has been reported (Maison et al., 2003). It has been also suggested that CGRP would exert autocrine and paracrine actions in the olivary complex (Gregg et al., 1999).

CGRP-immunoreactive cell bodies were located in all the motor nuclei of the minipig brainstem (nucleus ambiguus, nucleus nervi oculomotorius, nucleus nervi trochlearis, nucleus motorius nervi trigemini, nucleus nervi abducens, nucleus nervi facialis, and nucleus nervi hypoglossi). This means that somatic efferences and special visceral efferences arise from these neurons and suggests that CGRP could play an important role in muscular trophic mechanisms, as has been reported previously (Gibson et al., 1988). In addition to this trophic effect, CGRP is known to increase the synthesis of acetylcholine receptors at the level of the neuromuscular junction (New and Mudge, 1986). The presence of CGRP in cell bodies located in the raphae nuclei suggests that the neuropeptide could regulate the activity of spinal motor nuclei, as suggested previously (Arvidsson et al., 1991; Gualda et al., 2011).

In sum, for the first time we describe in detail the distribution of CGRP-immunoreactive fibers and cell

bodies in the minipig brainstem. This is the second study in which the distribution of a neuropeptide has been studied in the central nervous system of this mammal, as previously the distribution of fibers and cell bodies containing methionine-enkephalin has been reported in the minipig brainstem (Sánchez et al., 2013). We hope that the study and the present one will serve in the future to develop further work focused on the distribution and functions of neuropeptides in the minipig central nervous system.

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