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Distribution of methionine-enkephalin in the minipig brainstem

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ABSTRACT

We have studied the distribution of immunoreactive cell bodies and axons are containing methionine-enkephalin in the minipig brainstem. Immunoreactive axons were widely distributed, whereas the distribution of perikarya was less widespread. A high or moderate density of axons containing methionine-enkephalin were found from rostral to caudal levels in the substantia nigra, nucleus interpeduncularis, nucleus reticularis tegmenti pontis, nucleus dorsalis raphae, nucleus centralis raphae, nuclei dorsalis and ventralis tegmenti of Gudden, locus ceruleus, nucleus sensorius principalis nervi trigemini, nucleus cuneatus externalis, nucleus tractus solitarius, nuclei vestibularis inferior and medialis, nucleus ambiguus, nucleus olivaris inferior and in the nucleus tractus spinalis nervi trigemini. Immunoreactive perikarya were observed in the nuclei centralis and dorsalis raphae, nucleus motorius nervi trigemini, nucleus centralis superior, nucleus nervi facialis, nuclei parabrachialis medialis and lateralis, nucleus ventralis raphae, nucleus reticularis lateralis and in the formatio reticularis. We have also described the presence of perikarya containing methionine-enkephalin in the nuclei nervi abducens, ruber, nervi oculomotorius and nervi trochlearis. These results suggest that in the minipig the pentapeptide may be involved in many physiological functions (for example, proprioceptive and nociceptive information; motor, respiratory and cardiovascular mechanisms).

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 ambiguus; AP, area postrema; AQ, aqueductus cerebri; AVT, area ventralis tegmenti; Cb, cerebellum; 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 postero-ventral; 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 intercalatus; 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 ruber; 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 reticulata; TS, tractus solitarius; TSV, tractus spinalis nervi trigemini; TTS, tractus tectospinalis; 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; V3, ventriculus tertius; V4, ventriculus quartus.

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1. Introduction

The Göttingen minipig and the domestic pig are increasingly used as non-primate models in experimental studies of neurological diseases. In recent years these species are being used in the laboratory as experimental animal models in order to answer several scientific questions related to circulatory and metabolic processes, as well as to xenotransplantation and neurobiology (e.g., brain disorders) (Phillips et al., 1982; Imai et al., 2006; Groth, 2007; Lind et al., 2007; Sauleau et al., 2009). 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, 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 is more similar to the primate brain than lissencephalic brains from small laboratory animals. The pig is affordable, it is easy to handle and its use may potentially avoid some of the ethical considerations concerning the use of primates as laboratory animals (Jelsing et al., 2006). The development of the pig brain is also considered more similar to the human brain with respect to myelination and electrical activity (Dickerson and Dobbing, 1966; Thibault and Margulies, 1998; Fang et al., 2005). In ophthalmology the pig and minipig have been used as a novel model for glaucoma (Ruiz-Ederra et al., 2005; Vecino and Sharma, 2011; Galdos et al., 2012) showing many similarities with respect the human glaucoma. However, little is known about classical neurotransmitter and neuropeptide distributions in the pig brain.

Since the discovery of opiate receptors and enkephalins, many biochemical and immunohistochemical studies have been carried out. Thus, many works have been focused on the distribution of the pentapeptide methionine-enkephalin (Met-enk) in the mammalian central nervous system using immunocytochemical techniques. This distribution has been reported in several mammals such as the rat, cat, dog, monkey and human (Haber and Elde, 1982a, b; Ploska et al., 1982; Conrath-Verrier et al., 1983; Inagaki and Parent, 1985; Conrath et al., 1986; Coveñas et al., 1986; Merchenthaler et al., 1986; Coveñas et al., 1988; Palkovits, 1988; Pego-Reigosa et al., 2000; Marcos, 2007; Duque et al., 2011). The pentapeptide, located in axons and cell bodies, showed a widespread distribution throughout the mammalian central nervous system, suggesting that Met-enk is involved in many physiological actions. In fact, it is known that the peptide is implicated in the control of pain messages, in visual mechanisms, inhibits the release of substance P, vassopresin, dopamine and acetylcholine and influences the hypophysis (Vázquez et al., 1990; Strand, 1999; see Coveñas et al., 2007 for review). It is also known that Met-enk is derived from both the proenkephalin and proopiomelanocortin opioid precursors, as well as that Met-enk binds to delta receptors (Strand, 1999; Rozenfeld et al., 2007).

Although the distribution of Met-enk in the mammalian central nervous system is well known, there are specific mammalian species in which the data regarding such peptide are absent or fragmentary. This is the case for the minipig and the domestic pig; in the latter, the presence of Met-enk has been reported in various regions of the brain after using radioimmunoassay (Kumar et al., 1991; Yan et al., 1995; Waters et al., 1997). In the minipig brainstem, to our knowledge, there is no study on the distribution of axons and perikarya containing Met-enk. The brainstem is an important part of the central nervous system. This brain region receives somatic and visceral inputs, its neurons send motor outputs (impulses) via the cranial nerves, which innervate the head, neck, thorax and subabdominal structures and sensory organs and is implicated in sleep, vocalization, eye movement, pain, analgesia, sexual, visual, attentive, auditory, motor, respiratory and in cardiovascular mechanisms (see Coveñas et al., 2003 for review).

Thus, the aim of this study was to increase the knowledge of the chemical neuroanatomy of Met-enk in the mammalian brainstem, and in particular in the minipig brainstem and to compare the distribution of axons and perikarya containing the peptapeptide found in this species with those previously described in the brainstem of other mammals. Knowledge of the distribution of Met-enk in the minipig brainstem will serve in this species to better understand in the future the involvement of this pentapeptide in numerous physiological actions and for future neuroanatomical, neuropharmacological and behavioral studies.

2. Materials and methods

2.1. Animals

This study was carried out according to the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use of animals in research. We used five Göttingen minipigs, three males and two females 14 months old weighing 35–40 kg. Animals were kept and were fed under a 12:12 h light: dark cycle and are fed once a day with water ad libitum.

Minipigs were anaesthetized using 8 mg/kg Zoletil (tiletamine and zolazepam), 0.04 mg/kg atropine and 300 μg/kg Domtor (medetomidine); then animals were deeply anaesthetized using Propofol administered through an intravenous cannula inserted into the ear. Afterwards, animals were euthanized with an intravenous injection of saturated KCl.

2.2. Tissue preparation, immunocytochemistry and specificity of the antisera

The brains were removed from the skull, the brainstems were dissected out and cut into three pieces (the first contains the mesencephalon, the second the pons and the third the medulla oblongata). The thickness of the pieces varied from 5–9 mm. As previously reported (Ruiz-Ederra et al., 2004), brainstem pieces were fixed by immersion in 4% paraformaldehyde for 12 h at 4° C. After fixation, the brainstem pieces were cryoprotected in 30% sucrose in phosphate-buffered saline (PBS) for 12 h at 4 °C. They were then embedded in Tissue Tek medium (Leica). Cryosections were cut at 25 μm thickness and were stored in PBS until their use.

Histological sections were processed for immunostaining with the avidin–biotin-peroxidase (ABC) technique. Free-floating sections were pre-incubated for 30 min in 10% normal horse serum in PBS (pH 7.2), containing 0.3% Triton X-100 and then incubated overnight at 4° C in the same solution supplemented with anti-Met-enk antiserum (1/5000 final dilution). The sections were rinsed extensively in PBS (30 min) and transferred to the secondary antibody for 1 h at room temperature (biotinylated anti-rabbit 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 washing the sections in PBS and Tris-HCl buffer (pH 7.6), the tissue-bound peroxidase was developed with H_2O_2, using 3,3′-diaminobenzidine as chromogen. The sections were rinsed with PBS and cover-slipped with PBS/glycerol (1/1).

The immunocytochemical procedure used in this work has been published previously (see Pego-Reigosa et al., 2000), as well as the characteristics of the polyclonal Met-enk antiserum (obtained from the laboratory of Professor Gérard Tramu, University of Bordeaux I, France) and the specificity of the immunostaining observed (see Pego-Reigosa et al., 2000). The primary antiserum was raised in rabbits against immunogens prepared by coupling the synthetic Met-enk peptide to hemocyanin with glutaraldehyde and was further purified by affinity chromatography (see Pego-Reigosa et al., 2000). 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-Met-enk with synthetic Met-enk; omission of the primary antibody; preabsorption of anti-Met-enk with other related peptides such as leucine-enkephalin, methionine-enkephalin-Arg^6-Gly^7, methionine-enkephalin-Arg^6-Gly^7-Leu^8 alpha-neo-endorphin, beta-endorphin) (see Pego-Reigosa et al., 2000) (Fig. 2D). In order to avoid possible interference by endogenous peroxidases, free-floating sections were treated with a mixture of 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 pig brainstem published by Félix et al. (1999). For the nomenclature, we also followed this stereotaxic atlas. Moreover, a series of contiguous sections to that reacted for Met-enk was routinely stained for Nissl substance with cresyl violet in order to identify the brainstem nuclei.

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The density of the immunoreactive perikarya and axons 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 axons (high, moderate, low and single). This involved viewing the minipig sections under illumination by light at a constant magnification (20×) with reference to photographs (taken at the same magnification, 20×) of a defined series of densities for immunoreactive axons (high, moderate, low) established previously in the cat diencephalon after using a beta-endorphin (1–27) antibody (Coveñas et al., 1996). Moreover, immunoreactive axons were considered short (<90 μm), medium (90–120 μm) or long in length (>120 μm) and perikarya were considered small (diameter below 20 μm); medium-sized (20–40 μm), and large (above 40 μm).

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

The distribution and density of immunoreactive axons and perikarya containing methionine-enkephalin in the minipig brainstem are indicated in Fig. 1 and Table 1.

In the animals studied (males and females) both the distribution and density of the immunoreactive structures (axons and perikarya) observed in the minipig brainstem were fairly similar. Immunoreactive axons were widely distributed throughout the brainstem, whereas the distribution of cell bodies was less widespread. In general, the most abundant perikarya were oval, polygonal or round, medium or large in size, and showed 0–3 processes. In a total of 88 nuclei/tracts/regions studied of the minipig brainstem, we found Met-enk-immunoreactive axons and/or perikarya in 61 of them (69.31%), Met-enk-immunoreactive axons in 61 nuclei/regions (69.31%) and Met-enk-immunoreactive cell bodies in 14 nuclei/regions (15.90%) (Table 1). Regarding the Met-enk-immunoreactive perikarya, a high, moderate and low density was respectively observed in 7 (nucleus centralis raphae, nucleus dorsalis raphae, nucleus centralis superior, nucleus nervi trochlearis, nucleus ruber, nucleus nervi abducens, nucleus ventralis raphae,), 4 (nucleus nervi oculomotorius, nucleus nervi facialis, nucleus parabrachialis medialis, nucleus reticularis lateralis) and 3 (nucleus parabrachialis lateralis, nucleus motorius nervi trigemini, formatio reticularis) nuclei/regions of the minipig brainstem (Fig. 2B, C, E, F, Fig. 3A, B, Fig. 4B, C and Fig. 6C, D) (Table 1). A high, moderate and low density of immunoreactive axons was respectively found in 4 (nuclei ventralis and dorsalis tegmenti of Gudden, nucleus interpeduncularis, nucleus tractus spinalis nervi trigemini), 18 and 28 nuclei/regions (Fig. 4D–F, Fig. 5B–F and Fig. 6A–F) (Table 1). Finally, single axons were found in 15 nuclei/regions of the minipig brainstem (Table 1). In the formatio reticularis, we observed single axons (in the most rostral part) (Fig. 1A, B) as well as low (middle part) (Fig. 1C, D) and moderate (caudal part) (Fig. 1E, F) densities of immunoreactive axons. In transversal sections immunoreactive axons were, in general, thin, short in length, branched, and with varicosities. Long axons (300–500 μm) have been also observed in the locus ceruleus and in the nuclei cuneatus, cuneatus externalis, gracilis and nervi hypoglossi. In the minipig brainstem, inmmunoreactive axons were observed in nuclei belonging to the somatosensory (nucleus cuneatus, nucleus cuneatus externalis, pars caudalis of the nucleus tractus spinalis nervi trigemini, nucleus sensorious principalis nervi trigemini), gustatory (nucleus tractus solitarius, nucleus parabrachialis medialis), vestibular (nuclei vestibularis inferior, lateralis, medialis and superior), auditory (nucleus olivaris superior, nucleus cochlearis postero-ventral, nuclei cochleares) and visual (colliculus superior) systems, as well as in autonomic regulatory centers (nucleus tractus solitarius, nucleus dorsalis motorius nervi vagus, nucleus parabrachialis). Moreover, immunoreactive axons were also observed in motor nuclei (e.g., nucleus motorius nervi trigemini, nucleus nervi abducens, nucleus nervi facialis, nucleus nervi hypoglossi, substantia nigra). However, single or no immunoreactive axons were observed in the nucleus gracilis and lemniscus medialis (somatosensory system), lemniscus lateralis, nucleus lemnisci lateralis, colliculus inferior and nucleus corporis trapezoidei (auditory system), fasciculus longitudinalis medialis (vestibular system), nucleus pretectalis (visual system) and in the nuclei nervi oculomotorius and nervi trochlearis (motor nuclei).

Cell bodies were not found at the most caudal level of the brainstem (Fig. 1F). At caudal level (Fig. 1E), Met-enk-immunoreactive perikarya were found in 2 nuclei/regions, whereas at middle levels (Fig. 1C and D) such cell bodies were seen in 7 nuclei/regions. At rostral level (Fig. 1A and B), perikarya containing Met-enk were present in 2 nuclei/regions of the minipig brainstem.

Table 1
Density of Met-enk-immunoreactive axons and cell bodies in the minipig brainstem.

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

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

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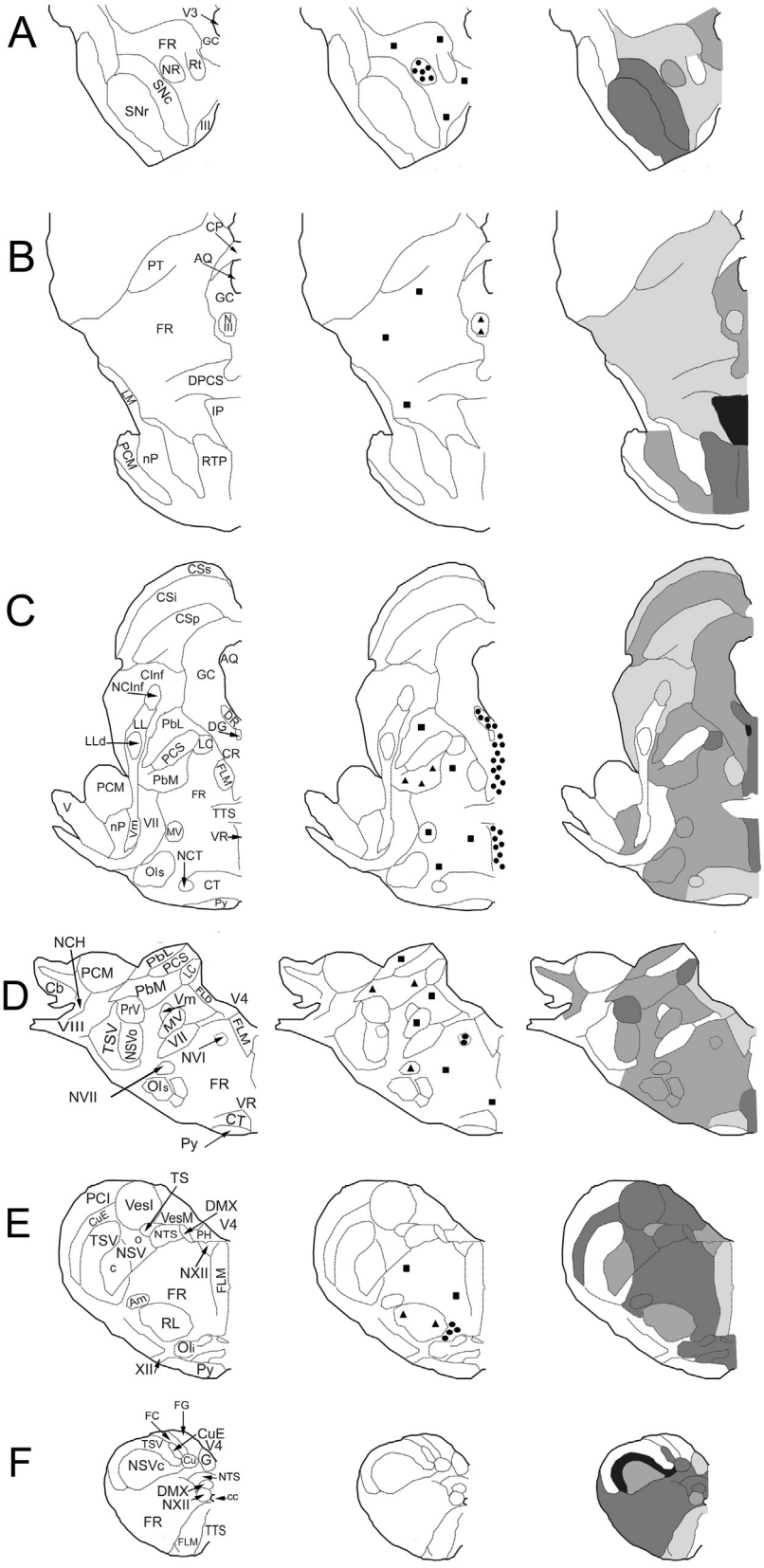

Fig. 1. Distribution of methionine-enkephalin-immunoreactive axons and perikarya in frontal planes of the minipig brainstem from rostral (A) to caudal (F) levels. Cell bodies containing methionine-enkephalin are represented by closed circles (high density), triangles (moderate density) and squares (low density), whereas immunoreactive axons 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.

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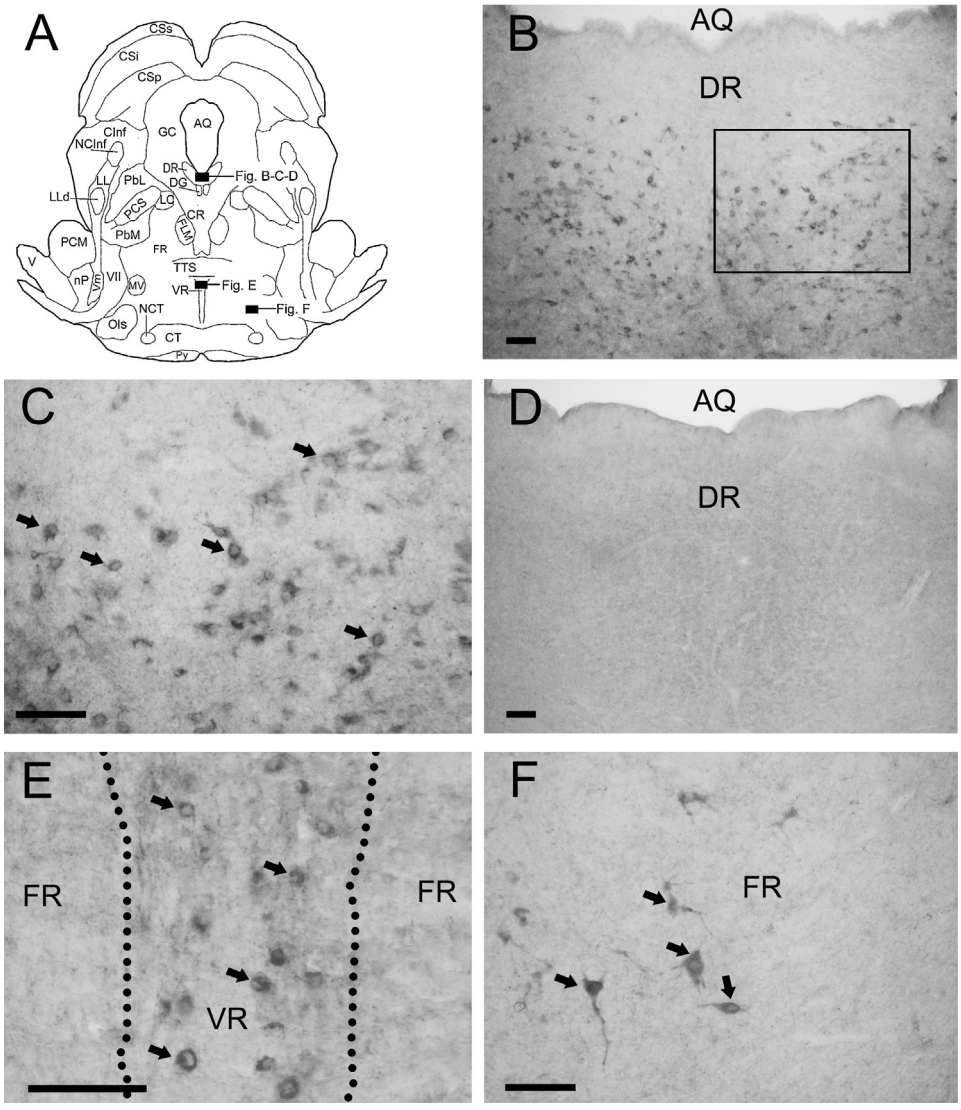

Fig. 2. Immunoreactive perikarya containing Met-enk. (A) Frontal section of the minipig mesencephalon. For nomenclature of the nuclei, see list of abbreviations. The photographs shown in B-F were respectively taken from the regions delimited by the rectangles in A (indicated as Fig. B, C, D, E and F). (B) Low magnification of perikarya containing Met-enk located in the nucleus dorsalis raphae (DR). (C) High magnification of the region delimited by a rectangle in B. Arrows indicate perikarya containing Met-enk. (D) Absence of immunoreactivity in the nucleus dorsalis raphae (DR) when the preabsorption of the primary antibody with Met-enk was carried out. (E) Perikarya (arrows) containing the neuropeptide located in the nucleus ventralis raphae (VR). (F) Immunoreactive cell bodies (arrows) in the formatio reticularis (FR). Scale bars: 100 μm.

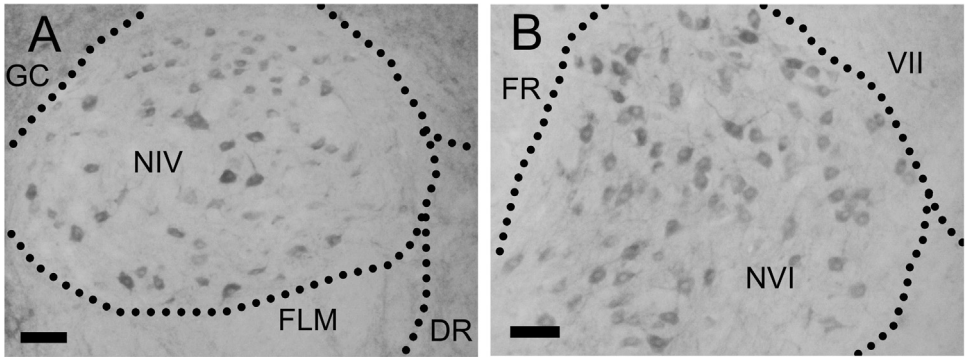

Fig. 3. Immunoreactive perikarya containing Met-enk located in the nucleus nervi trochlearis (NIV) (A) and in the nucleus nervi abducens (NVI) (B). Scale bars: 100 μm.

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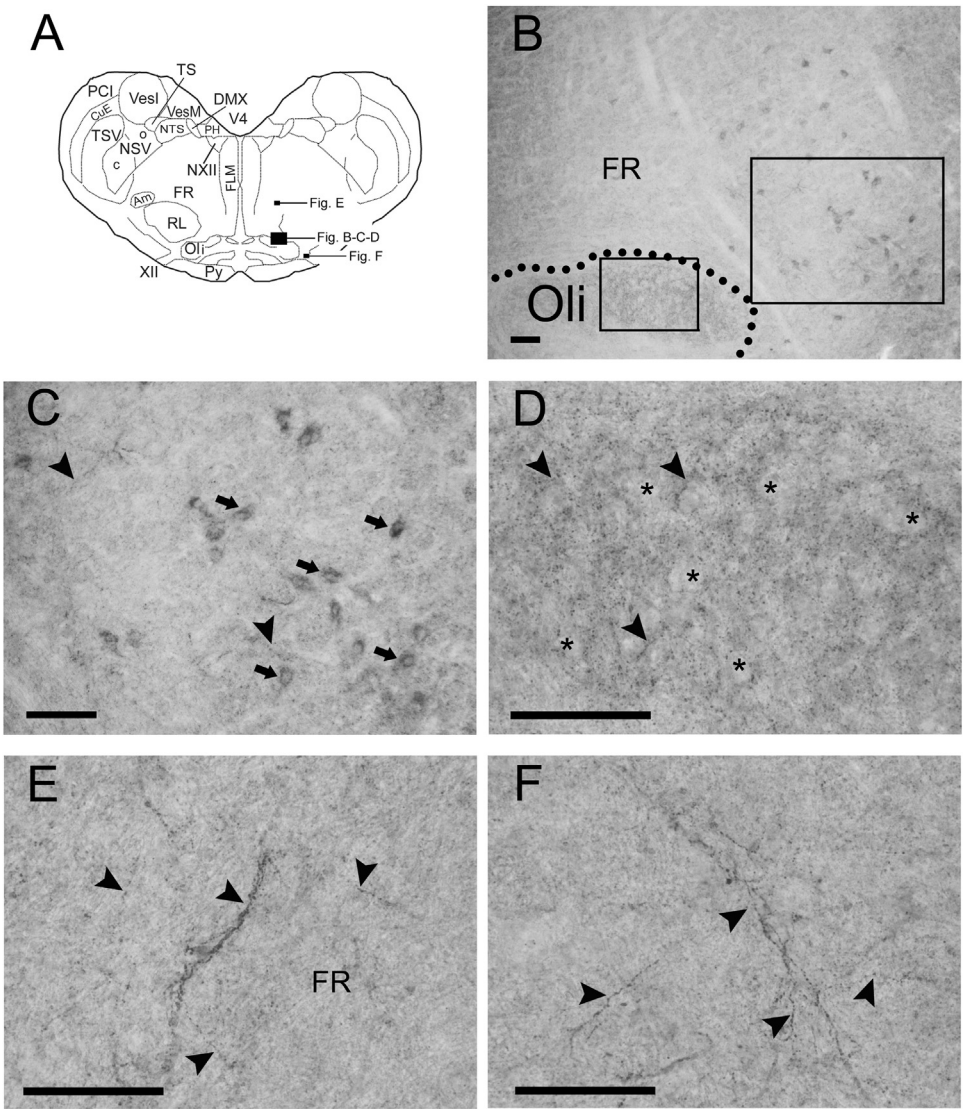

Fig. 4. Immunoreactive axons and cell bodies containing Met-enk. (A) Frontal section of the minipig medulla oblongata. For nomenclature of the nuclei, see list of abbreviations. The photographs shown in B–F were respectively taken from the regions delimited by the rectangles in A (indicated as Fig. B, C, D, E and F). (B) Low magnification of perikarya containing Met-enk located in the formatio reticularis (FR). (C) High magnification of the region delimited by the right rectangle in B. Arrows indicate perikarya containing Met-enk. (D) High magnification of the region delimited by the left rectangle in B. Arrowheads indicate immunoreactive axons located in the nucleus olivaris inferior. Asterisks indicate non-immunoreactive perikarya. (E) Immunoreactive axons (arrowheads) in the formatio reticularis (FR). (F) Axons containing Met-enk (arrowheads) located above the tractus pyramidalis. Scale bars: 100 μm.

Met-enk-immunoreactive cell bodies have been located in motor nuclei (nucleus nervi oculomotorius, nucleus nervi trochlearis, nucleus motorius nervi trigemini, nucleus nervi abducens and nucleus nervi facialis) and in raphe nuclei (nucleus centralis raphae, nucleus dorsalis raphae, nucleus ventralis raphae, nucleus centralis superior). Moreover, perikarya containing Met-enk were found in the nucleus parabrachialis (in mammals, its medial part receives gustatory inputs) and in the formatio reticularis (mesencephalon, pons and medulla oblongata, nucleus reticularis lateralis), but no cell bodies were found in sensory nuclei of the minipig brainstem. The morphological characteristics of the cell bodies containing Met-enk are shown in Table 2.

4. Discussion

This is the first report describing the distribution of axons and perikarya containing a neuropeptide in the minipig central nervous system, although some information is available regarding other strains (Yan et al., 1995; Waters et al., 1997). Histological controls were carried out in order to check the specificity of the anti-Met-enk antibody used in this study: (a) omission of the primary antibody; (b) preabsorption of anti-Met-enk with synthetic Met-enk; and (c) preabsorption of the primary antibody with other related peptides (leucine-enkephalin, beta-endorphin). Both histological and biochemical results confirmed the specificity of the anti-Met-enk antibody used in this study (see Pego-Reigosa et al., 2000). Moreover, in the dog the preabsorption of the same primary antiserum used in the present study with leucine-enkephalin did not reduce the immunoreactivity (Pego-Reigosa et al., 2000). Met-enk can be cleaved from beta-endorphin and hence the primary antiserum used here could recognize it, but this is quite improbable since, within the beta-endorphin molecule, the pentapeptide sequence is not followed by the pair of basic amino acids that is the usual signal for enzymatic processing of the polypeptide precursors (Mol et al., 1991; Young and Kemppainen, 1994; see Pego-Reigosa et al., 2000). Moreover, Met-enk sequence

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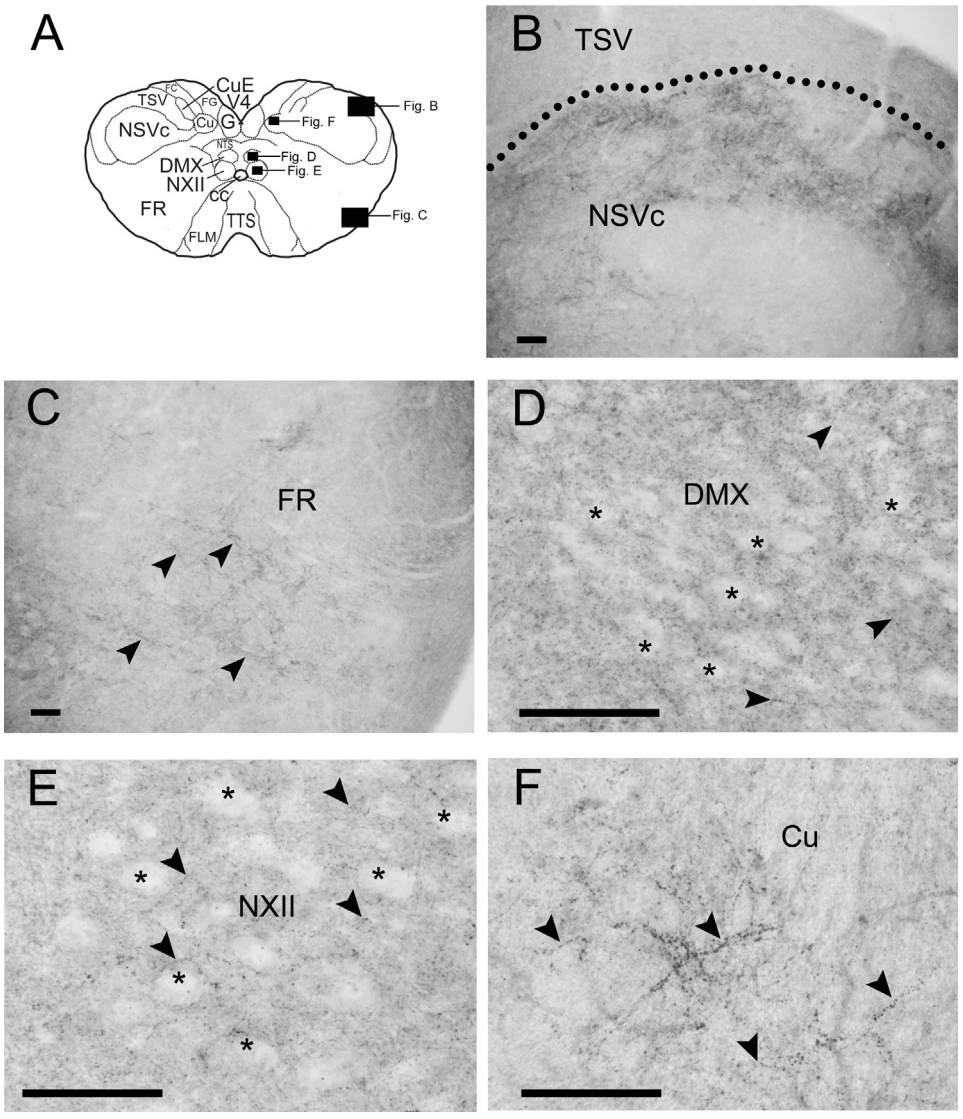

Fig. 5. Immunoreactive axons containing Met-enk. (A) Frontal section of the minipig medulla oblongata. For nomenclature of the nuclei, see list of abbreviations. The photographs shown in B-F were respectively taken from the regions delimited by the rectangles in A (indicated as Fig. B, C, D, E and F). (B) Low magnification of the pars caudalis of the nucleus tractus spinalis nervi trigemini (NSVc). (C) Immunoreactive axons (arrowheads) in the formatio reticularis (FR). (D) Axons (arrowheads) located in the nucleus dorsalis motorius nervi vagus (DMX). Asterisks indicate non-immunoreactive perikarya. (E) Immunoreactive axons (arrowheads) in the nucleus nervi hypoglossi (NXII). Asterisks indicate non-immunoreactive perikarya. (F) Axons containing Met-enk (arrowheads) located in the nucleus cuneatus (Cu). Scale bars: 100 μm.

is not included in the prodynorphin polypeptide from known species, and hence reduces to the minimum the possibility that any prodynorphin-containing neuronal population were misidentified as enkephalinergic. The presence of a moderate density of immunoreactive axons containing Met-enk in the minipig nucleus tractus solitarius is in agreement with previous studies reporting the presence of the pentapeptide in the same nucleus of Yucatan piglets (Yan et al., 1995; Waters et al., 1997), where it was reported that curtailed respiration by repeated/isolated hypoxia is unrelated to the levels of Met-enk found in the nucleus tractus solitarius (Waters et al., 1997).

It is known that the distribution of Met-enk-immunoreactive structures in the mammalian brainstem (e.g., rat, cat, dog, monkey, human) is widespread and that the distribution of immunoreactive axons is more widespread than that found for the cell bodies containing Met-enk (Hökfelt et al., 1977; Sar et al., 1978; Uhl et al., 1979; Finley et al., 1981; Haber and Elde, 1982a,b; Conrath-Verrier et al., 1983; Bouras et al., 1984; Inagaki and Parent, 1985; Merchenthaler et al., 1986; Palkovits, 1988; Pego-Reigosa et al., 2000; Coveñas et al., 2003; Marcos, 2007; Duque et al., 2011). This is in agreement with the results reported in this study. However, we have to remark that the distribution of Met-enk-immunoreactive axons in the minipig brainstem was very similar to that found in the rat (Hökfelt et al., 1977; Sar et al., 1978; Uhl et al., 1979; Finley et al., 1981; Merchenthaler et al., 1986; Palkovits, 1988), but more widespread than those previously reported in the cat (Conrath-Verrier et al., 1983; Coveñas et al., 2003; Marcos, 2007), dog (Pego-Reigosa et al., 2000), monkey (Haber and Elde, 1982a,b; Inagaki and Parent, 1985; Duque et al., 2011) and in human (Bouras et al., 1984). For example, in both rat and minipig, axons containing Met-enk have been observed in the nuclei dorsalis and ventralis tegmenti of Gudden, nuclei vestibularis, substantia nigra, locus ceruleus, nucleus interpeduncularis, nucleus ruber, nuclei olivaris inferior and superior, nucleus parabrachialis, colliculus superior, area ventralis tegmenti, substantia grisea centralis, nucleus nervi facialis, nucleus nervi hypoglossi, nucleus prepositus hypoglossi and in the nucleus reticularis lateralis. Met-enk-immunoreactive axons have been

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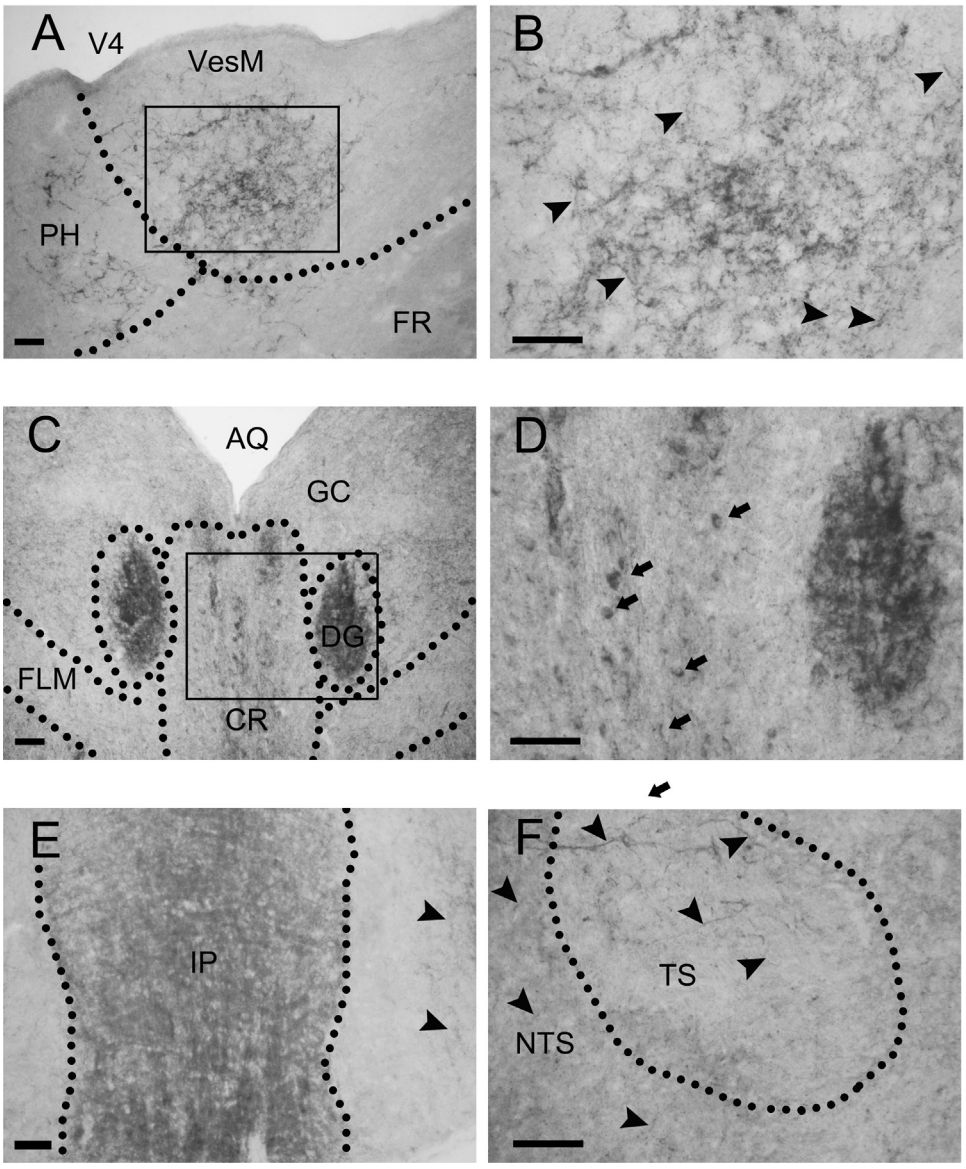

Fig. 6. (A) Immunoreactive axons in the nucleus prepositus hypoglossi (PH) and in the nucleus vestibularis medialis (VesM). (B) High magnification of the region delimited by a rectangle in A. Arrowheads indicate axons containing Met-enk. (C) Immunoreactive axons in the nucleus centralis raphae (CR), the nucleus dorsalis tegmenti of Gudden (DG) and in the substantia grisea centralis (GC). (D) High magnification of the region delimited by a rectangle in C. Arrows indicate immunoreactive cells bodies located in the nucleus centralis raphae. (E) Immunoreactive axons in the nucleus interpeduncularis (IP) and close to this nucleus (arrowheads). (F) Immunoreactive axons in the tractus solitarius (TS) and in the nucleus tractus solitarius (NTS). Arrowheads indicate immunoreactive axons. Scale bars: 100 μm.

observed in the minipig, but not in the cat for example in the following nuclei: cuneatus, dorsalis motorius nervi vagus, gracilis, pontis, ruber and nervi abducens. Regarding the dog, it should be noted that, in the present work, we have used the same anti-Met-enk antiserum and applied the same immunocytochemical method that in a previous study carried out in the dog (Pego-Reigosa et al., 2000). Thus, in the minipig but not in the dog axons containing Met-enk were found in the nuclei cuneatus, cuneatus externalis, gracilis, nervi oculomotorius, pontis, ruber, nervi abducens and olivaris superior. These differences between the minipig and the dog could be due to species differences rather than to technical differences. The distribution of Met-enk-immunoreactive axons in the minipig was more widespread than that found in the primate (monkey and human) brainstem. These axons were found in the minipig, but not in primates in the area postrema and in the nuclei ambiguus, cuneatus, corpus trapezoideum, nervi trochlearis, pontis, prepositus hypoglossi, olivaris inferior and superior. Finally, it should be noted that in minipig and rat Met-enk-immunoreactive axons have been found in the nuclei cuneatus, gracilis and pontis, but not in the cat, dog, monkey and human. Moreover, in minipig and human, immunoreactive axons containing Met-enk were found in the nucleus nervi abducens, but not in the rat, cat, dog and monkey.

On comparing our results with the distribution of Met-enk-immunoreactive perikarya in the mammalian brainstem (rat, cat, dog, primates) (Hökfelt et al., 1977; Sar et al., 1978; Uhl et al., 1979; Finley et al., 1981; Haber and Elde, 1982b; Conrath-Verrier et al., 1983; Inagaki and Parent, 1985; Merchenthaler et al., 1986; Palkovits, 1988; Pego-Reigosa et al., 2000; Coveñas et al., 2003; Marcos, 2007; Duque et al., 2011), the distribution of such cell bodies in the minipig is the most restricted. This could be due to the administration of colchicine, since in the previous research studies the rats, cats, dogs and monkeys were treated with the drug, but the minipigs were not, although species differences should not be

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Table 2
Morphological characteristics of Met-enk-immunoreactive perikarya in the minipig brainstem.

Nuclei/Regions	Most abundant			Others		
	Size	Shape	Dendrites	Size	Shape	Dendrites
Motor nuclei						
Motorius nervi trigemini	Medium 40 μm	Round	0	Medium	Polygonal	2–3
Nervi oculomotorius	Medium 35 μm	Round	0	Medium	Oval	1–2
Nervi trochlearis	Medium 40 μm	Round	0	Medium	Polygonal	3–4
Nervi abducens	Medium 40 μm	Round	0	Medium	Oval	1–2
Nervi facialis	Medium 40 μm	Oval	1–2	Medium	Round	0
Ruber	Large 55 μm	Polygonal	2–3	Medium	Oval	2
Raphe nuclei						
Centralis	Medium 25 μm	Round	0	Small/Medium	Oval	1–2
Dorsalis	Medium 30 μm	Round	0	Medium	Oval	1–2
Centralis superior	Medium 30 μm	Oval	1–2	Medium	Polygonal	3–4
Ventralis	Medium 30 μm	Oval	1	Medium	Polygonal	2–3
Formatio reticularis						
Mesencephalon	Large 50 μm	Polygonal	2–3	Medium	Oval	1–2
Pons	Large 55 μm	Polygonal	2–3	Medium	Oval	1
Medulla oblongata	Large 50 μm	Oval	1	Medium	Polygonal	2–3
Reticularis lateralis	Large 50 μm	Polygonal	2–3	Medium	Oval	1–2
Parabrachialis nuclei						
Lateralis	Medium 35 μm	Oval	1–2	Medium	Polygonal	2–3
Medialis	Medium 35 μm	Oval	1–2	Medium	Polygonal	2–3

ruled out. However, although the drug was not used, we observed Met-enk-immunoreactive perikarya in 14 nuclei/regions of the minipig brainstem.

Regarding the motor nuclei (Conrath-Verrier et al., 1983; Palkovits, 1988; Pego-Reigosa et al., 2000), the presence of Met-enk-immunoreactive cell bodies in the nuclei nervi oculomotorius, nervi trochlearis and nervi abducens has been reported in the minipig, but not in the dog, cat and rat. Perikarya containing the pentapeptide has been found in the nucleus motorius nervi trigemini in the minipig and rat, but not in dog and cat, whereas in the nucleus nervi facialis, immunoreactive cell bodies were found in the minipig and cat, but not in dog and rat. In the nucleus dorsalis motorius nervi vagus, Met-enk immunoreactive perikarya were found in the dog, but not in cat, minipig and rat. Finally, no immunoreactive cell body was found in the nucleus nervi hypoglossi in the four mentioned species. Thus, it seems that the minipig shows the most widespread distribution of cell bodies containing Met-enk in the brainstem motor nuclei. In this species, cell bodies containing the pentapeptide were found in general somatic motor neurons and in special visceral motor neurons. Cell bodies containing Met-enk located in the sensory brainstem nuclei are widely distributed in the rat and dog, and they show a more restrictive distribution in the monkey and cat, but in all the cases (Haber and Elde, 1982a; Conrath-Verrier et al., 1983; Palkovits, 1988; Pego-Reigosa et al., 2000) the distribution is more widespread than that found in the minipig (in which no immunoreactive cell body was found in the brainstem sensory nuclei).

According to the results reported in this study, some physiological implications of Met-enk can be suggested in the minipig brainstem. The neuroanatomical results reported here indicates that Met-enk, acting as a neurotransmitter and/or neuromodulator, could be involved in many physiological functions in the minipig brainstem such as in motor, respiratory and cardiovascular mechanisms and in the control/transmission of the nociceptive/propioceptive information. The presence of Met-enk-immunoreactive axons surrounding non-immunoreactive perikarya in the nucleus olivaris inferior suggests that the peptide controls those neurons which provide the climbing axon input to the cerebellum. Moreover, the presence of a high density of immunoreactive cell bodies in four raphe nuclei suggests that the pentapeptide could be involved in the control of nociceptive transmission and in circadian rhythms.

We have no data indicating whether Met-enk-immunoreactive neurons located in the minipig brainstem are local or projecting neurons, as well as the sources of the immunoreactive axons are unknown. However, according to the morphological results reported here, it seems that the perikarya containing Met-enk observed in the nuclei nervi oculomotorius, nervi trochlearis, ruber, centralis superior and nervi abducens could be projecting neurons, since in these nuclei a high/moderate density of cell bodies and a single/low density of immunoreactive axons were found. The presence of both a moderate density of perikarya and single axons containing Met-enk in the nucleus nervi oculomotorius suggests that those perikarya are projecting neurons involved in the regulation of eye movements. It is known that in other mammals the nucleus reticularis lateralis sends projections to the cerebellum; this could also occur in the minipig, since in that nucleus a moderate density of immunoreactive perikarya was found. Thus, the

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presence of the pentapeptide in cell bodies located in several cranial motor nuclei suggests that the enkephalinergic neurons generate special visceral efferents (nucleus nervi facialis) and somatic efferents (nucleus nervi trochlearis, nucleus nervi oculomotorius, nucleus nervi abducens), according to the type of muscle innervated by the neurons located in the cranial motor nuclei.

In summary, for the first time in the minipig brainstem a detailed immunohistochemical study on the distribution of a neuropeptide (Met-enk) has been carried out. Our study should be useful for future neuroanatomical, neuropharmacological and behavioral studies, since the minipig is increasingly used as non-primate models in basic experimental studies of neurological diseases.

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References

Bollen, P., Ellegaard, L., 1997. The Göttingen minipig in pharmacology and toxicology. Physical Therapy 80, 3–4.
Bouras, C., Taban, C.H., Constantinidis, J., 1984. Mapping of enkephalins in human brain. An immunohistofluorescence study on brains from patients with senile and presenile dementia. Neuroscience 12, 179–190.
Conrath, M., Coveñas, R., Romo, R., Cheramy, A., Bourgoin, S., Hamon, M., 1986. Distribution of met-enkephalin immunoreactive fibers in the thalamus of the cat. Neuroscience Letters 65, 299–303.
Conrath-Verrier, M., Dietl, M., Arluison, M., Cesselin, F., Bourgoin, S., Hamon, M., 1983. Localization of met-enkephalin-like immunoreactivity within pain-related nuclei of cervical spinal cord, brainstem and midbrain in the cat. Brain Research Bulletin 11, 587–604.
Coveñas, R., Belda, M., Marcos, P., de León, M., Narváez, J.A., Aguirre, J.A., Tramu, G., González-Barón, S., 2003. Neuropeptides in the cat brainstem. Current Topics in Peptide & Protein Research 5, 41–61.
Coveñas, R., Burgos, C., Conrath, M., 1988. Immunocytochemical study of met-enkephalin-like cell bodies in the cat hypothalamus. Neuroscience Research 5, 353–360.
Coveñas, R., de León, M., Narváez, J.A., Tramu, G., Aguirre, J.A., González-Barón, S., 1996. An immunocytochemical mapping of beta-endorphin (1-27) in the cat diencephalon. Neuropeptides 30, 261–271.
Coveñas, R., Mangas, A., Narváez, J.A., 2007. Introduction to neuropeptides. In: Coveñas, R., Mangas, A., Narváez, J.A. (Eds.), Focus on Neuropeptide Research. Transworld Research Network, Trivandrum, pp. 1–26.
Coveñas, R., Romo, R., Cheramy, A., Cesselin, F., Conrath, M., 1986. Immunocytochemical study of enkephalin-like cell bodies in the thalamus of the cat. Brain Research 377, 355–361.
Dickerson, J.W.T., Dobbing, J., 1966. Prenatal and postnatal growth and development of the central nervous system of the pig. Proceedings of the Royal Society of London Series B-Biological Sciences 166, 384–395.
Duque, E., Mangas, A., Díaz-Cabiale, Z., Narváez, J.A., Coveñas, R., 2011. Neuropeptides in the monkey brainstem. In: Williams, R.M. (Ed.), Monkeys: Biology, Behavior and Disorders. Nova Science Publishers, New York, pp. 151–166.
Fang, M., Li, J., Gong, X., Antonio, G., Lee, F., Kwong, W.H., Wai, S.M., Yew, D.T., 2005. Myelination of the pig's brain: a correlated MRI and histological study. Neurosignals 14, 102–108.
Félix, B., Léger, M.-E., Albe-Fessard, D., 1999. Stereotaxic atlas of the pig brain. Brain Research Bulletin 1–138.
Finley, J.C.W., Maderdrut, J.L., Petrusz, P., 1981. The immunocytochemical localization of enkephalin in the central nervous system of the rat. Journal of Comparative Neurology 198, 541–565.
Galdos, M., Bayón, A., Rodríguez, F.D., Micó, C., Sharma, S.C., Vecino, E., 2012. Morphology of retinal vessels in the optic disk in a Göttingen minipig experimental glaucoma model. Veterinary Ophthalmology 1, 36–46.
Groth, C.G., 2007. Prospects in xenotransplantation: a personal view. Transplantation Proceedings 39, 685–687.
Guntern, R., Vellet, P.G., Bouras, C., Constantinidis, J., 1989. An improved immunohistostaining procedure for peptides in human brain. Experientia 45, 159–161.
Haber, S., Elde, R., 1982a. The distribution of enkephalin immunoreactive fibers and terminals in the monkey central nervous system: an immunohistochemical study. Neuroscience 7, 1049–1095.
Haber, S., Elde, R., 1982b. The distribution of enkephalin immunoreactive neural cell bodies in the monkey brain: preliminary observations. Neuroscience Letters 32, 247–252.
Hökfelt, T., Elde, R., Johansson, O., Terenius, L., Stein, L., 1977. The distribution of enkephalin-immunoreactive cell bodies in the rat central nervous system. Neuroscience Letters 5, 25–31.
Imai, H., Konno, K., Nakamura, M., Shimizu, T., Kubota, C., Seki, K., Honda, F., Tomizawa, S., Tanaka, Y., Hata, H., Saito, N., 2006. A new model of focal cerebral ischemia in the miniature pig. Journal of Neurosurgery 104, 123–132.
Inagaki, S., Parent, A., 1985. Distribution of enkephalin-immunoreactive neurons in the forebrain and upper brainstem of the squirrel monkey. Brain Research 359, 267–280.
Jelsing, J., Nielsen, R., Olsen, A.K., Grand, N., Hemmingsen, R., Pakkenberg, B., 2006. The postnatal development of neocortical neurons and glial cells in the Göttingen minipig and the domestic pig brain. Journal of Experimental Biology 209, 1454–1462.
Kumar, M.S., Becker, T., Ebert, K., 1991. Distribution of substance P, GnRH, met-enkephalin in the central nervous system of the pig. Brain Research Bulletin 26, 511–514.
Lind, N.M., Moustgaard, A., Jelsing, J., Vajta, G., Cumming, P., Hansen, A.K., 2007. The use of pigs in neuroscience: modeling brain disorders. Neuroscience and Biobehavioral Reviews 31, 728–751.
Marcos, P., 2007. Neuropeptides in the cat brainstem. In: Coveñas, R., Mangas, A., Narváez, J.A. (Eds.), Focus on Neuropeptide Research. Transworld Research Network, Trivandrum, pp. 57–101.
Merchenthaler, I., Maderdrut, J.L., Altschuler, R.A., Petrusz, P., 1986. Immunocytochemical localization of proenkephalin-derived peptides in the central nervous system of the rat. Neuroscience 17, 325–348.
Mol, J.A., van Mansfeld, A.D., Kwant, M.M., van Wolferen, M., Rothuizen, J., 1991. The gene encoding proopiomelanocortin in the dog. Acta Endocrinologica 125, 77–83.
Palkovits, M., 1988. Neuropeptides in the brain. In: Martini, L., Ganong, W.F. (Eds.), Frontiers in Neuroendocrinology, vol. 10. Raven Press, New York, pp. 1–44.
Pego-Reigosa, R., Coveñas, R., Tramu, G., Pesini, P., 2000. Distribution of met-enkephalin immunoreactivity in the diencephalon and the brainstem of the dog. Journal of Chemical Neuroanatomy 19, 243–258.
Phillips, R.W., Panepinto, L.M., Spangler, R., Westmoreland, N., 1982. Yucatan miniature swine as a model for the study of human diabetes mellitus. Diabetes 31, 30–36.
Ploska, A., Taquet, H., Javoy-Agid, F., Gaspar, P., Cesselin, F., Berger, B., Hamon, M., Legrand, J.C., Agid, Y., 1982. Dopamine and methionine-enkephalin in human brain. Neuroscience Letters 33, 191–196.
Rozenfeld, R., Abul-Husn, N.S., Gomez, I., Devi, L.A., 2007. An emerging role for the delta opioid receptor in the regulation of mu opioid receptor function. The Scientific World Journal 7, 64–73.
Ruiz-Ederra, J., García, M., Hernández, M., Urcola, H., Hernández-Barbáchano, E., Araiz, J., Vecino, E., 2005. The pig eye as a novel model of glaucoma. Experimental Eye Research 81, 561–569.
Ruiz-Ederra, J., García, M., Hicks, D., Vecino, E., 2004. Comparative study of the three neurofilament subunits within pig and human retinal ganglion cells. Molecular Vision 10, 83–92.
Sar, M., Stumpf, W.E., Miller, R.J., Chang, K.-J., Cuatrecasas, P., 1978. Immunohistochemical localization of enkephalin in rat brain and spinal cord. Journal of Comparative Neurology 182, 17–38.
Sauleau, P., Lapouble, E., Val-Laillet, D., Malbert, C.H., 2009. The pig model in brain imaging and neurosurgery. Animal 3, 1138–1151.
Strand, F., 1999. Neuropeptides: Regulators of Physiological Processes. MIT Press, Cambridge, Massachusetts.
Thibault, K.L., Margulies, S.S., 1998. Age-dependent material properties of the porcine cerebrum: effect on pediatric inertial head injury criteria. Journal of Biomechanics 31, 1119–1126.
Uhl, G.R., Goodman, R.R., Kuhar, M.J., Childers, S.R., Snyder, S.H., 1979. Immunohistochemical mapping of enkephalin containing cell bodies, fibers and nerve terminals in the brain stem of the rat. Brain Research 166, 75–94.
Vázquez, J., Muñoz, M., Coveñas, R., 1990. Alterations on the distribution of methionine-enkephalin-like in the cat thalamus after electroacupuncture: an immunocytochemical study. Journal fur Hirnforschung 31, 555–561.
Vecino, E., Sharma, S.C., 2011. Glaucoma animal models. In: Rumelt, S. (Ed.), Glaucoma: Basic and Clinical Concepts. InTech, Rijeka, pp. 319–334.
Waters, K.A., Laferrière, A., Paquette, J., Goodyer, C., Moss, I.R., 1997. Curtailed respiration by repeated vs. isolated hypoxia in maturing piglets is unrelated to NTS ME or SP levels. Journal of Applied Physiology 83, 522–529.
Yan, S., Zhang, C., Laferrière, A., Moss, I.R., 1995. Met-enkephalin-like immunoreactivity in microdialysates from nucleus tractus solitarii in piglets during normoxia and hypoxia. Brain Research 687, 217–220.
Young, D.W., Kemppainen, R.J., 1994. Molecular forms of beta-endorphin in the canine pituitary gland. American Journal of Veterinary Research 55, 567–571.

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