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Distribution of Neurotensin and Somatostatin-28 (1–12) in the Minipig Brainstem

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Summary

Using an indirect immunoperoxidase technique, an in depth study has been carried out for the first time on the distribution of fibres and cell bodies containing neurotensin and somatostatin-28 (1–12) (SOM) in the minipig brainstem. The animals used were not treated with colchicine. The distribution of neurotensin- and SOM-immunoreactive fibres was seen to be quite similar and was moderate in the minipig brainstem: a close anatomical relationship between both neuropeptides was observed. The distribution of cell bodies containing neurotensin or SOM was quite different and restricted. Cell bodies containing neurotensin were found in four brainstem nuclei: nucleus centralis raphae, nucleus dorsalis raphae, in the pars centralis of the nucleus tractus spinalis nervi trigemini and in the nucleus ventralis raphae. Cell bodies containing SOM were found in six nuclei/regions of the brainstem: nucleus ambiguus, nucleus dorsalis motorius nervi vagus, formatio reticularis, nucleus parabrachialis medialis, nucleus reticularis lateralis and nucleus ventralis raphae. According to the observed anatomical distribution of the immunoreactive structures containing neurotensin or SOM, the peptides could be involved in sleep-waking, nociceptive, gustatory, motor, respiratory and autonomic mechanisms.

Introduction

The domestic pig and the Göttingen minipig are increasingly used as non-primate models in experimental studies of neurological diseases. The pig brain (gyrencephalic) is more similar to the primate brain than lissencephalic brains from small laboratory animals. The pig model is increasingly used in the field of neuroscience; for example, it has been used for molecular imaging studies using positron emission tomography (Sauleau et al., 2009). Furthermore, in recent years, the domestic pig and the Göttingen minipig have been used in the laboratory as experimental animal models to address several scientific issues 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 the 60s 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 cross-breeding the Minnesota minipig with the Vietnamese potbelly pig and the German Landrace (Bollen and Ellegaard, 1997). 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 that of the human brain with respect to myelination and electrical activity (Dickerson and Dobbing, 1966; Thibault and Margulies, 1998; Fang et al., 2005). In ophthalmology, pigs and minipigs have been used as a novel model for glaucoma (Ruiz-Ederra et al., 2004; Vecino and Sharma, 2011; Galdos et al., 2012), because in these

animals, this disease shows many similarities to glaucoma in humans. However, little is known about the distribution of classical neurotransmitters and neuropeptides in the pig/minipig central nervous system.

Regarding the neuropeptides, currently the distribution of calcitonin gene-related peptide and methionine-enkephalin has only been explored in the minipig central nervous system, in particular in the brainstem (Sánchez et al., 2013, 2014). Thus, the distribution of other neuropeptides belonging to other peptide families (e.g. neurotensin, somatostatin) in the minipig brainstem is currently unknown. Accordingly, the present work focuses on the distribution of neurotensin and somatostatin in that region (brainstem) of the minipig central nervous system. It is known that both neuropeptides are involved in analgesia, in respiratory and cardiovascular mechanisms and in the sleep-waking cycle (Yaksh et al., 1982; Reichlin, 1983; Dalsgaard et al., 1984; Moga and Gray, 1985; Spampinato et al., 1988; Carpentier et al., 1996; Jolas and Aghajanian, 1997; Zhao et al., 1998; Llona and Eugenin 2005; Mitchell et al., 2009) and that somatostatin is also present in many nuclei of the mammalian brainstem (e.g. cat) in which the presence of neurotensin has been described (de León et al., 1992). Moreover, in other mammals (e.g. rat, alpaca, human) neurotensin and somatostatin have been located in the periaqueductal grey-raphe magnus pathway (Beitz et al., 1983), nucleus tractus solitarius (Forssmann et al., 1979; Yamada et al., 1995; de Souza et al., 2014), trigeminal ganglion (del Fiacco and Quartu, 1994; Zhao et al., 1998), periaqueductal grey (Urban and Smith, 1993; Fodor et al., 1997), nucleus dorsalis motorius nervi vagus (Higgins and Schwaber, 1983; Duan and Shimizu, 1992), locus ceruleus (de León et al., 1991; Moyse et al., 1992) and in the substantia nigra (Whitford et al., 1986; Castel et al., 1992). All these data suggest a physiological interaction between both neuropeptides in the mammalian brainstem.

Neurotensin is a tridecapeptide that was originally isolated from the bovine hypothalamus (Carraway and Leeman, 1973), and it is widely distributed throughout the mammalian central nervous system (Kataoka et al., 1979; Uhl et al., 1979; Jennes et al., 1982; Triepel et al., 1984; Mai et al., 1987). The highest concentration of this neuropeptide is found in the amygdala, the lateral septum, the ventral tegmental area and the substantia nigra. Immunocytochemical and radioimmunoassay studies addressing the distribution of neurotensin in the mammalian central nervous system have been carried out in rats (Kobayashi et al., 1977; Uhl et al., 1977; Kahn et al., 1980; Jennes et al., 1982; Meister et al., 1986; Meister and Hökfelt, 1988), guinea pigs (Triepel et al., 1984), cats (de León et al., 1991), monkeys (Kataoka et al., 1979) and humans (Cooper et al., 1981; Mai et al., 1987), and the distribution of neurotensin-binding sites in the central nervous system of mammals has also been studied (Sarrieau et al., 1985; Moyse et al., 1987). The physiological functions of neurotensin have been reviewed recently (St-Gelais et al., 2006; Mustain et al., 2011; Boules et al., 2013). In the central nervous system, neurotensin elicits many biological activities that affect haemodynamic, glucoregulatory, nociceptive, neuroendocrine (it controls the release of prolactin, luteinizing hormone and thyrotrophin) and thermoregulatory processes (Bissette et al., 1976; Clineschmidt et al., 1979; Coveñas et al., 2007). In addition, the peptide induces muscle relaxation, decreases locomotor activity and food consumption, inhibits gastric acid secretion, stimulates pancreatic secretion and controls the release of dopamine, and it has also been implicated in alcoholism and inflammatory processes (Kitabgi et al., 1992; Marcos et al., 1997; Binder et al., 2001; St-Gelais et al., 2006; Coveñas et al., 2007). It is also known that neurotensin has effect on cholinergic, GABAergic and serotoninergic transmission (Wenk et al., 1989; Ferraro et al., 2001; Buhler et al., 2005).

To date, three prosomatostatin-derived peptides have been observed in the mammalian central nervous system: somatostatin-14, somatostatin-28 (the entire sequence of somatostatin-14 at its carboxyl terminal plus a double pair of basic amino acids) and somatostatin-28 (1–12) corresponding to the first 12 amino acids of somatostatin-28 (Finley et al., 1981; Reichlin, 1983; Benoit et al., 1985). In the sheep hypothalamus, it has been reported that all somatostatin-14-immunoreactive cell bodies also contain somatostatin-28 (1–12) (Scanland et al., 2003). According to the immunocytochemical and radioimmunoassay studies carried out, somatostatin is widely distributed throughout the mammalian central nervous system (Brownstein et al., 1975; Dierickx and Vandesande, 1979; Forssmann et al., 1979; Barden et al., 1981; Fuxe et al., 1984; Coveñas et al., 2011). In the mammalian central nervous system, somatostatin inhibits the release of noradrenaline and growth hormone and stimulates the release of acetylcholine and serotonin (Reichlin, 1983). Central administration of somatostatin causes behavioural changes, such as decreased sleep, excessive grooming, difficulty in breathing and hypersensitivity to tactile stimuli (Reichlin, 1983; Johansson et al., 1984; Llona and Eugenín, 2005). Moreover, somatostatin is involved in sensory processes including vestibular sensitivity, somatosensoriality and proprioception, the sleep-waking cycle and arousal, and the peptide controls several neurovegetative functions, including the regulation of cardiovascular and respiratory activities as well as gastric acid secretion (Carpentier et al., 1996).

In the light of the above, here, we were prompted (i) to study the distribution of cell bodies and fibres containing

neurotensin and somatostatin in the minipig brainstem using an immunoperoxidase technique and (ii) to compare our findings with the distribution of methionine–enkephalin and calcitonin gene-related peptide previously described in the minipig brainstem (Sánchez et al., 2013, 2014). A further aim was to compare our findings with those reported previously on the distribution of neurotensin and somatostatin in the mammalian brainstem rat, cat, alpaca, etc.). In the future, knowledge of the distribution of both neuropeptides in the minipig brain will serve to better understand the involvement of these substances in multiple physiological functions.

Materials and Methods

Animals

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 five Göttingen minipigs, three males and two females, of 14 months old. The animals were kept under a 12:12 hour light: dark cycle and were fed once a day, with water *ad libitum*.

The minipigs were anaesthetized using 8 mg/kg Zoletil (tiletamine and zolazepam), 0.04 mg/kg atropine and 300 μg/kg Domtor (medetomidine). Then, the animals were deeply anaesthetized using propofol administered through an intravenous cannula inserted into an ear vein. Following this, the animals were euthanized with an intravenous injection of saturated KCl (Sánchez et al., 2013).

Tissue preparation and immunocytochemistry

As previously reported (Sánchez et al., 2013), the brainstems were fixed 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, Nussloch, Germany). Cryosections were cut at 25 μm thickness and were stored in PBS until use (Sánchez et al., 2013).

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-neurotensin anti-serum (diluted 1/3000) or with anti-somatostatin-28 (1–12) anti-serum (diluted 1/5000). 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 coverslipped with PBS/glycerol (1/1).

Specificity of the anti-sera

The immunological characteristics of the polyclonal neurotensin and somatostatin-28 (1–12) anti-sera used here have been reported previously (Studler et al., 1988; Lenders et al., 1989; de León et al., 1992; Coveñas et al., 2011; de Souza et al., 2014). Briefly, the anti-sera were raised in rabbits against immunogens prepared by coupling the synthetic neurotensin or the synthetic 1–12 sequence of somatostatin-28 to a carrier protein (human serum albumin) with glutaraldehyde. Rabbit anti-sera were pre-absorbed with the carrier protein and the coupling agent to prevent non-specific immunoreactivity due to the anti-carrier antibodies. This pre-absorption was carried out before the immunohistochemical application. Both anti-sera were obtained at the laboratory of Professor Gérard Tramu, University of Bordeaux I (France). It has been reported that competitive interference by several partial peptide sequences of synthetic neurotensin with the binding of (^{125}I-Tyr^{3})-neurotensin to the anti-serum indicated that the anti-neurotensin anti-serum used here is highly specific for the COOH-terminal sequence of neurotensin and that no cross-reactivity with other peptides such as substance P, angiotensin I and bradykinin occurs (Studler et al., 1988).

In addition, as previously reported (de León et al., 1992; Coveñas et al., 2011; de Souza et al., 2014), histological controls were carried out to determine the specificity of the immunostaining (pre-absorption of anti-neurotensin with synthetic neurotensin, pre-absorption of anti-somatostatin-28 (1–12) with synthetic somatostatin-28 (1–12), in both cases 100 μg/ml of diluted anti-serum; omission of the primary antibody). Moreover, the pre-absorption of anti-somatostatin-28 (1–12) with an excess of synthetic somatostatin-28, somatostatin-14, neurotensin, neuropeptide Y, angiotensin II, substance P, cholecystokinin and methionine–enkephalin was carried out, as well as the pre-absorption of anti-neurotensin with an excess of synthetic substance P, angiotensin II, beta-endorphin, somatostatin-28 (1–12), vasoactive intestinal peptide and neuropeptide Y (de León et al., 1991, 1992; Coveñas et al., 2011; de Souza et al., 2014). Staining was not blocked when anti-somatostatin-28 (1–12)/anti-neurotensin was pre-absorbed with the above antigens. In addition, 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 confirmed the specificity of the anti-sera used in this study.

Mapping

As previously reported (Sánchez et al., 2013, 2014), mapping was carried out following the frontal planes of the pig brainstem atlas published by Félix et al. (1999). We also followed this stereotaxic atlas for the nomenclature. In addition, a series of sections contiguous to those reacted for neurotensin or for somatostatin-28 (1–12) were routinely stained for Nissl substance with cresyl violet to locate the brainstem nuclei.

The density of the immunoreactive cell bodies and fibres 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 fibres (high, moderate, low and single). This involved viewing the sections under illumination at constant magnification with reference to photographs of a defined series of densities for immunoreactive fibres (high, moderate and low) established previously (de Souza et al., 2008; Coveñas et al., 2011). Immunoreactive fibres were considered short (<90 μm), medium (90–120 μm) or long in length (>120 μm), and cell bodies were considered small (diameter below 20 μm); medium-sized (20–40 μm) and large (above 40 μm).

Photomicrographs were obtained with an Olympus DP50 digital camera (Tokyo, Japan) attached to a Kyowa Unilux 12 microscope (Tokyo, Japan). 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 (Adobe Systems Incorporated, San José, California, USA) was used to view the images and adjust their brightness and contrast.

Results

General considerations

The distribution and density of the immunoreactive fibres and cell bodies containing neurotensin (NT) or somatostatin-28 (1–12) (SOM) in the minipig brainstem are shown in Fig. 1 and Table 1. Table 2 shows the size and the shape of the immunoreactive perikarya. In general, in all the animals studied (males and females), the distribution and density of NT-immunoreactive fibres and cell bodies, observed in the nuclei of the minipig brainstem, were quite similar and the same was the case of the SOM-immunoreactive structures. In general, the distribution of NT- and SOM-immunoreactive fibres in the minipig brainstem was quite similar, but the distribution of immunoreactive cell bodies containing NT or SOM was very different (Table 1). NT- and SOM-immunoreactive fibres showed a moderate distribution in the minipig brainstem (fibres containing NT were found in 41 nuclei/regions/tracts of the 88 nuclei/regions/tracts of the minipig brainstem and fibres containing SOM in 40 nuclei/regions/tracts) (Table 1). The distribution of NT- and SOM-immunoreactive perikarya was quite restricted: cell bodies containing NT were only observed in four nuclei and those containing SOM only in six brainstem nuclei. NT- and SOM-immunoreactive perikarya were only observed in one brainstem nucleus, the nucleus ventralis raphae (Table 1).

NT-immunoreactive structures

Immunoreactive cell bodies and fibres containing NT were observed in 41 nuclei/regions/tracts of the minipig brainstem (Table 1), that is NT-immunoreactivity was observed in 46.5% of the minipig brainstem nuclei/regions/tracts. NT-immunoreactive perikarya were found in four nuclei (4.5%). A high density of cell bodies containing NT was found in the nuclei centralis raphae, dorsalis raphae and ventralis raphae (Figs 1 and 4c–f). In general, these perikarya were oval and medium-sized (24–26 μm) and showed 1–2 processes (Table 2). Polygonal and small (15 μm) immunoreactive neurons showing 2–3 processes and present a moderate density were observed in the pars centralis of the nucleus spinalis nervi trigemini (Table 2, Figs 1 and 2c,d).

A high density of immunoreactive fibres was only found in two brainstem nuclei: the ventral part of the pars centralis of the nucleus spinalis nervi trigemini (Figs 1 and 3e) and the nucleus ventralis tegmenti of Gudden. Moreover, in 12 brainstem nuclei/regions/tracts, we observed a moderate density of NT-immunoreactive fibres (Figs 2b,e, 3b,c and 4b); in 14, a low density (Fig. 3d,f); and in 13, single fibre (Fig. 1 and Table 1). Most of these immunoreactive fibres showed varicosities and were short. We observed the longest NT-immunoreactive fibres in the nucleus cuneatus (Fig. 2f).

SOM-immunoreactive structures

Immunoreactive structures containing SOM were found in 40 nuclei/regions/tracts (45.4% of the 88 brainstem nuclei/regions/tracts) (Table 1). Immunoreactive cell bodies were only observed in six nuclei/regions (6.8%). A moderate density was found in the nucleus dorsalis moto-

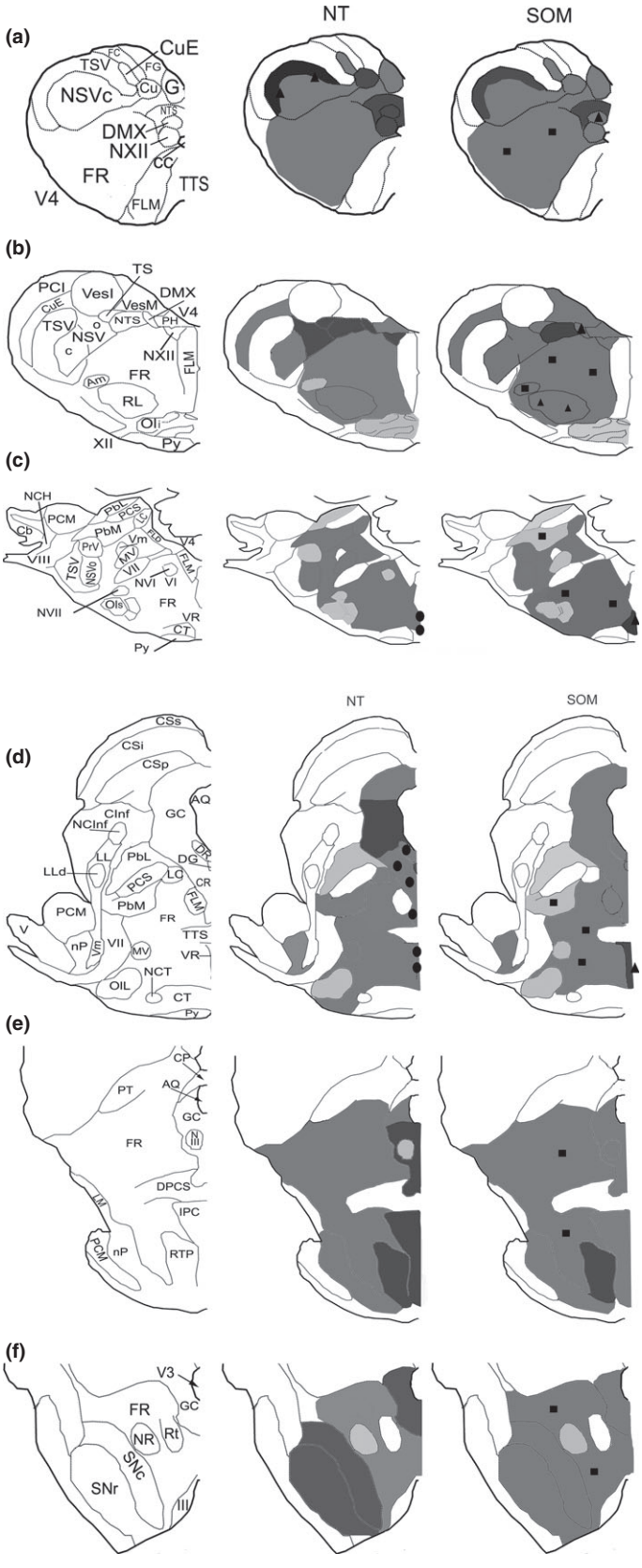

Fig. 1. Distribution of NT- and SOM-immunoreactive fibres and perikarya in frontal planes of the minipig brainstem from caudal (a) to rostral (f) levels. Cell bodies containing NT and SOM are represented by closed circles (high density), triangles (moderate density) and squares (low density), whereas immunoreactive fibres are represented by slightly dark (single axons), moderately dark (low density), strongly dark (moderate density) and black (high density) shades. The first column represents six frontal sections; the second and the third ones show the distribution and density of the immunoreactive fibres and cell bodies, respectively, containing NT and SOM. For nomenclature of the structures, see list of abbreviations. NT, neurotensin; SOM, somatostatin.

Table 1. Density of NT- and SOM-immunoreactive fibres and cell bodies in the minipig brainstem

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

CB, cell bodies (+++: high density; ++: moderate density; +: low density); F, fibres (+++: high density; ++: moderate density; +: low density; s: single); –, no immunoreactivity; N, nucleus; R, region. For nomenclature of the nuclei, see list of abbreviations: Am, nucleus ambiguus; AP, area postrema; AQ, aqueductus cerebri; AVT, area ventralis tegmenti; cc, central canal; CCS, commissura colliculi superior; CInf, inferior colliculus; CP, commisura posterior; CR, nucleus centralis raphae; CSi, superior colliculus, stratum inter-medium; CSp, superior colliculus, stratum profundum; CSs, superior colliculus, stratum superficiale; CT, corpus trapezoideum; Cu, nucleus cuneatus; CuE, nucleus cuneatus externalis; CVP, nucleus cochlearis posteroventral; D, nucleus of 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 inter-calatus; IPC, nucleus inter-peduncularis centralis; LC, locus ceruleus; LL, lemniscus lateralis; LLd, nucleus lemnisci lateralis, pars dorsalis; LM, lemniscus medialis; MV, nucleus motorius nervi trigemini; NCH, nuclei cochleares; NCInf, nucleus colliculi inferior; nCS, nucleus colliculi superior; NCS, nucleus centralis superior; NCT, nucleus corporis trapezoidei; NIII, nucleus nervi oculomotorius; NInt, nucleus interstitial of vestibular nerve; NIV, nucleus nervi trochlearis; NMV, nucleus tractus mesencephali nervi trigemini; NO, nucleus ovalis; nP, nuclei pontis; NR, nucleus ruber; NSVc nucleus tractus spinalis nervi trigemini, pars centralis; NSVi, nucleus tractus spinalis nervi trigemini, pars inter-polaris; NSVo; nucleus tractus spinalis nervi trigemini, pars oralis; NTS, nucleus tractus solitaries; NVI, nucleus nervi abducens; NVII, nucleus nervi facialis; NXII, nucleus nervi hypoglossi; 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 pre-positus hypoglossi; PrV, nucleus sensorius principalis nervi trigemini; PT, nucleus pre-tectalis; 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 solitaries; 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; 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 vestibulocochlearis; VIIIc, nervus vestibulocochlearis, pars cochlearis; VIIIv, nervus vestibulocochlearis, pars vestibularis; XII, nervus hypoglossus.

rius nervi vagus, nucleus reticularis lateralis and in the nucleus ventralis raphae (Table 1). In the three nuclei, the cell bodies containing SOM were medium-sized (28–30 μm) and showed 2–3 processes (Table 2). In general, in the case of the nucleus dorsalis motorius nervi vagus, the immunoreactive perikarya were oval and, in the other two nuclei, most of the immunoreactive cell bodies were polygonal (Table 2, Figs 1, 5c,d, 6c,d and 7e,f). In the nucleus ambiguus, a low density of large (45 μm) and oval SOM-immunoreactive cell bodies showing 2–3 processes was found (Table 2, Figs 1 and 6b), whereas oval and medium (26 μm) immunoreactive neurons showing 2–3 processes were observed in the nucleus parabrachialis medialis (Table 2, Figs 1 and 7c,d). Finally, scattered,

Table 2. Morphological characteristics of NT- and SOM-immunoreactive cell bodies in the minipig brainstem. We have included them to show better the data. No immunoreactivity was found in these nuclei.

	NT			SOM		
Nuclei/Regions	Size	Shape	Processes	Size	Shape	Processes
Am				Large 46 μm	Oval	2–3
CR	Medium 26 μm	Oval	1–2			
DMX				Medium 28 μm	Oval	2
DR	Medium 26 μm	Oval	1–2			
FR				Large 41 μm	Polygonal	2–3
NSVc	Small 15 μm	Polygonal	2–3			
PbM				Medium 26 μm	Oval	2–3
RL				Medium 38 μm	Polygonal	2–3
VR	Medium 24 μm	Oval	1–2	Medium 30 μm	Polygonal	2–3

large polygonal SOM-immunoreactive perikarya were visualized throughout the formatio reticularis (Table 2, Figs 1, 5e–h, 6e–h and 7b).

Immunoreactive fibres containing SOM were found in 40 nuclei/regions/tracts of the minipig brainstem (Fig. 1). A moderate density of SOM-immunoreactive fibres was only observed in the pars centralis of the nucleus tractus spinalis nervi trigemini, nucleus ventralis raphae, nucleus reticularis tegmenti pontis and nucleus tractus solitarius (Fig. 5b). In other brainstem nuclei/regions/tracts, we only observed a low density of immunoreactive fibres (28 nuclei/regions/tracts) or single fibre (eight nuclei/regions/tracts) (Fig. 1). In general, SOM-immunoreactive fibres showed varicosities and were short.

Discussion

Here, we report for the first time the mapping of the NT- and SOM-immunoreactive structures in the minipig brainstem. As far as we are aware, no reference addressing this topic have been published previously. This is the third study carried out by our group focused on the distribution of neuropeptides in the minipig central nervous system, as previously the distribution of both methionine–enkephalin and calcitonin gene-related peptide has also been studied in the minipig brainstem (Sánchez et al., 2013, 2014). We carried out controls to show the specificity of the immunoreactivity and, in all cases, the results confirmed the specificity of the anti-NT and anti-SOM used in this work (Studler et al., 1988; de León et al., 1991, 1992; Coveñas et al., 2011; de Souza et al., 2014).

Neuropeptides in the minipig brainstem

The mapping of methionine–enkephalin (MET) and calcitonin gene-related peptide (CGRP) in the minipig brainstem has been published recently (Sánchez et al., 2013, 2014). As in the present case, the animals were not treated with colchicine. MET-immunoreactive structures (fibres and/or cell bodies) were observed in 61 nuclei/regions of the minipig brainstem; NT-immunoreactive structures in 41 of them; SOM-immunoreactive structures in 40; and CGRP-immunoreactive structures in 36. It is important to note that CGRP-immunoreactive cell bodies were found in 20 nuclei/regions of the minipig brainstem (14 nuclei/regions showed a high density of cell bodies) and that MET-immunoreactive perikarya were found in 14 of them (seven nuclei/regions showed a high density of cell bodies) (Sánchez et al., 2013, 2014). However, SOM- and NT-immunoreactive cell bodies were, respectively, found in only six and four nuclei/regions of the minipig brainstem.

Cell bodies containing NT, SOM, MET or CGRP were observed in the nucleus ventralis raphae. However, it seems that according to the morphological characteristics of the immunoreactive perikarya (size, shape...), each neuropeptide is located in a different neuronal population (Sánchez et al., 2013, 2014). Moreover, SOM-, MET- and CGRP-immunoreactive cell bodies were located in the formatio reticularis and in the nucleus reticularis lateralis. The morphological characteristics of the cell bodies containing the above neuropeptides are similar in the case of the neurons

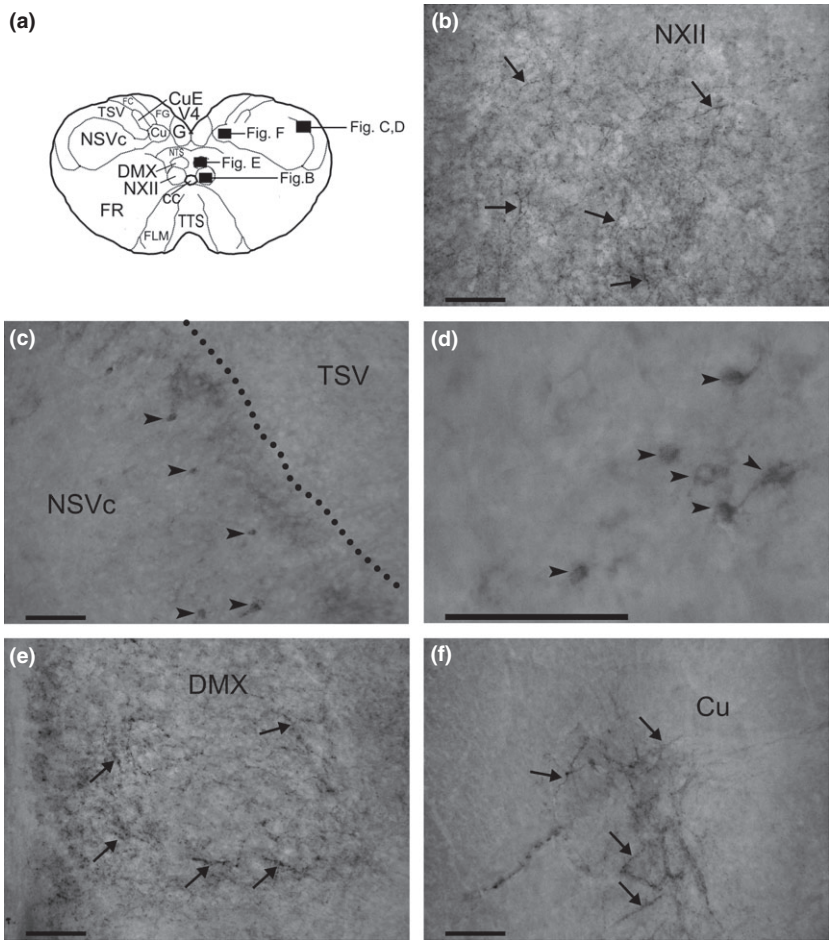

Fig. 2. Immunoreactive fibres and cell bodies containing NT. (a) Frontal section of the minipig medulla oblongata (caudal region). For nomenclature of the nuclei, see list of abbreviations. The photographs shown in b–f were, respectively, taken from the regions delimited by the rectangles in a (indicated as Fig. b–f). (b) Medium-power magnification of fibres containing NT located in the nucleus nervi hypoglossi (NXII). (c) Medium-power magnification of the nucleus tractus spinalis nervi trigemini, pars centralis (NSVc). (d). High-power magnification of the nucleus tractus spinalis nervi trigemini, pars centralis (NSVc). (e) Medium-power magnification of fibres containing NT located in the nucleus dorsalis motorius nervi vagus (DMX). (f) Medium-power magnification of fibres containing NT located in the nucleus cuneatus (Cu). Arrowheads indicate cell bodies and arrows fibres. Scale bar: 100 μm. NT, neurotensin.

located in the formatio reticularis, but not in the case of the neurons located in the nucleus reticularis lateralis. NT-, MET- and CGRP-immunoreactive perikarya have been observed in the nuclei centralis raphae and dorsalis raphae (Sánchez et al., 2013, 2014). In both nuclei, it seems that the peptidergic neurons show different morphological characteristics. In three minipig raphae nuclei (nucleus centralis raphae, nucleus dorsalis raphae and nucleus centralis superior), the presence of peptidergic neurons containing MET and CGRP (Sánchez et al., 2013, 2014), but not SOM (which was observed in the present study), has been reported. Moreover, MET and CGRP, but not NT/SOM, have been observed in motor nuclei of the minipig brainstem (e.g. nucleus motorius nervi trigemini, nucleus nervi oculomotorius, nucleus nervi trochlearis, nucleus nervi abducens and nucleus nervi facialis) (Sánchez et al., 2013, 2014). The nucleus parabrachalis medialis showed cell bodies containing MET or SOM, but in the nuclei parabrachialis lateralis and ruber, perikarya containing only MET were observed. In the nucleus ambiguus, cell bodies containing CGRP or SOM have been visualized, whereas in other nuclei such as the nervi hypoglossi, olivaris inferior, pre-positus hypoglossi, olivaris superior, reticularis tegmenti pontis and substantia nigra, pars compacta, only CGRP-immunoreactive perikarya have been observed. In the nucleus dorsalis motorius nervi vagus and in the nucleus tractus spinalis nervi trigemini, only cell bodies, respectively, containing SOM and NT were observed. Finally, it is important to note that in the motor nuclei motorius nervi trigemini, nervi oculomotorius, nervi trochlearis, nervi abducens and nervi facialis, only cell bodies containing MET or CGRP were found.

Regarding the distribution of immunoreactive fibres containing neuropeptides in the minipig brainstem, the most widespread distribution was found for the fibres containing MET (61 nuclei/regions). Fibres containing NT, SOM or CGRP showed a similar distribution (41, 40 and 36 nuclei/regions, respectively) (Sánchez et al., 2013, 2014). Here, we demonstrate a close anatomical relationship between the immunoreactive fibres containing NT and those containing SOM, as in almost all the brainstem nuclei in which we observed NT-immunoreactive fibres,

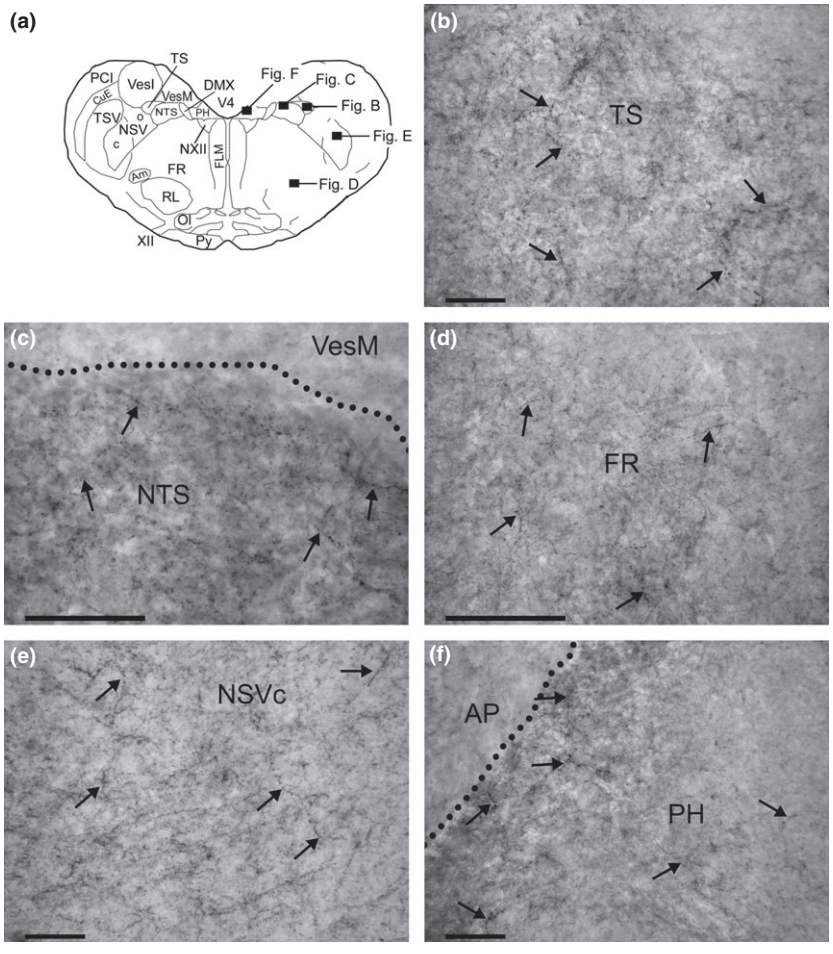

Fig. 3. Immunoreactive fibres containing NT. (a) Frontal section of the minipig medulla oblongata (rostral region). For nomenclature of the nuclei, see list of abbreviations. The photographs shown in b–f were, respectively, taken from the regions delimited by the rectangles in a (indicated as Fig. b–f). (b): Medium-power magnification of fibres containing NT located in the tractus solitarius (TS). (c) High-power magnification of fibres containing NT located in the nucleus tractus solitarius (NTS). (d) High-power magnification of immunoreactive fibres located in the formation reticularis (FR). (e) Medium-power magnification of immunoreactive fibres located in the nucleus tractus spinalis nervi trigemini, pars centralis (NSVc). (f) Medium-power magnification of fibres containing NT in the nucleus pre-positus hypoglossi (PH). Arrows indicate fibres. Scale bar: 100 μm. NT, neurotensin.

fibres containing SOM were also found. In many nuclei/regions of the minipig brainstem (31 of them), fibres containing MET, CGRP, NT and SOM were observed (e.g. nucleus ambiguus, nucleus centralis raphae, nucleus cuneatus, nucleus cuneatus externalis, nucleus dorsalis tegmenti of Gudden, nucleus dorsalis raphae, nucleus gracilis and substantia grisea centralis). This means that in many nuclei of the minipig brainstem, a possible interaction among the four above-mentioned neuropeptides occurs and that there is an elaborate modulation of functions in which these brainstem nuclei are involved.

In sum, in the minipig brainstem, MET is the most widespread neuropeptide (it was observed in 61 nuclei/regions) and the CGRP-immunoreactive cell bodies showed the most widespread distribution. The distribution of CGRP-, NT- and SOM-immunoreactive fibres was fairly similar, but the distribution of NT- and SOM-immunoreactive perikarya was quite restricted. Moreover, except in the nucleus ventralis raphae, the distribution of NT- and SOM-immunoreactive cell bodies was different. In many nuclei of the minipig brainstem, fibres containing MET, CGRP, NT and SOM were found.

Neurotensin in the mammalian brainstem

The mapping of NT-immunoreactive structures in the mammalian brainstem has been carried out in the rat, cat, alpaca, monkey and humans (Kessler et al., 1987; Mai et al., 1987; Uhl et al., 1977; Palkovits, 1988; de León et al., 1991; Coveñas et al., 2008; de Souza et al., 2014). In general, our results are in agreement with the findings describing the distribution of NT-binding sites in the rat brainstem (Kessler et al., 1987). For example, in the rodent, NT-binding sites were observed in the nucleus dorsalis motorius nervi vagus, nucleus tractus solitarius, nucleus cuneatus externalis, nucleus reticularis lateralis, nucleus reticularis tegmenti pontis, and nuclei pontis, in which we found NT-immunoreactive fibres in the minipig. Moreover, in general, the data reported from radioimmunoassay and immunocytochemical studies carried out in the rat (see Palkovits, 1988) are in agreement

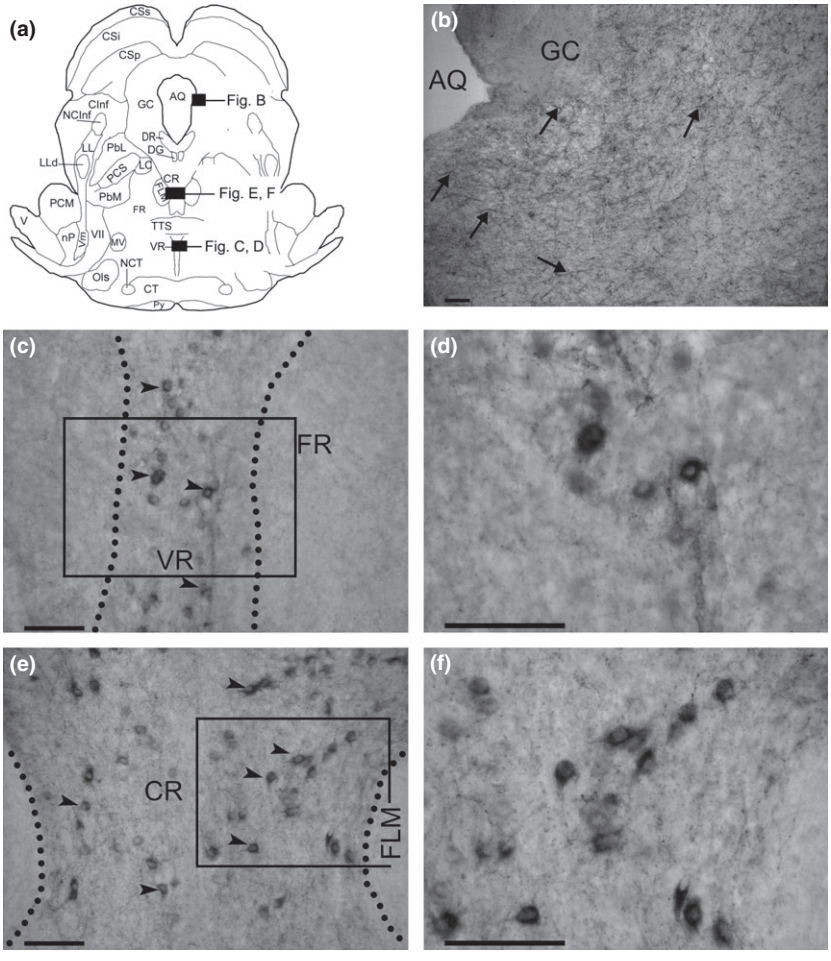

Fig. 4. Immunoreactive fibres and cell bodies containing NT. (a) Frontal section of the minipig mesencephalon. For nomenclature of the nuclei, see list of abbreviations. The photographs shown in b–f were, respectively, taken from the regions delimited by the rectangles in a (indicated as Fig. b–f). (b) Low-power magnification of fibres containing NT located in the substantia grisea centralis (GC). (c) Medium-power magnification of the nucleus ventralis raphae (VR). (d) High-power magnification of the region delimited by a rectangle in c. (e) Medium-power magnification of cell bodies containing NT in the nucleus centralis raphae (CR). (f) High-power magnification of the region delimited by a rectangle in e. Arrowheads indicate cell bodies and arrows fibres. Scale bar: 100 μm. NT, neurtensin

with those found in the minipig regarding the distribution of NT. That is, the distribution of NT-immunoreactive fibres is quite similar in the rat and minipig brainstems. In both species, fibres containing the peptide were observed, for example, in the formatio reticularis, nucleus nervi facialis, raphae nuclei, nucleus reticularis lateralis, substantia grisea centralis, substantia nigra and nucleus tractus solitarius. However, the distribution of NT-immunoreactive perikarya is more widespread in the rat (Palkovits, 1988) than in the minipig. In the rat brainstem, many nuclei showed immunoreactive cell bodies (e.g. area ventralis tegmenti, nucleus dorsalis raphae, nucleus centralis raphae, locus ceruleus, nucleus ambiguus...), which were not observed in the minipig. The widespread distribution of the NT-immunoreactive cell bodies in the rat brainstem could be due to the administration of colchicine (Palkovits, 1988), as the rodents were treated with this drug whereas the minipigs were not.

In comparison with the cat, it is important to note that in both the cat and minipig, the same methodology and the same anti-NT antibody were used, except that in the cat colchicine was administered (de León et al., 1991). In general, the distribution of NT-immunoreactive fibres is quite similar in both species. Thus, in both the cat and the minipig, fibres containing NT were found, for example, in the area postrema, nucleus centralis raphae, nucleus dorsalis motorius nervi vagus, substantia grisea centralis, formatio reticularis, locus ceruleus, nucleus tractus solitarius and nucleus olivaris inferior (de León et al., 1991). As in the case of the rat, in the feline, a more widespread distribution of NT-immunoreactive perikarya was found (e.g. area postrema, formatio reticularis, locus ceruleus and nucleus tractus solitarius) (de León et al., 1991) in comparison with the minipig. This difference is probably due to the administration of colchicine in the lateral ventricle of the cat (de León et al., 1991).

In comparison with the alpaca (de Souza et al., 2014), in general, the distribution of NT-immunoreactive fibres is quite similar to that found here in the minipig. Thus, in both species, NT-immunoreactive fibres were found,

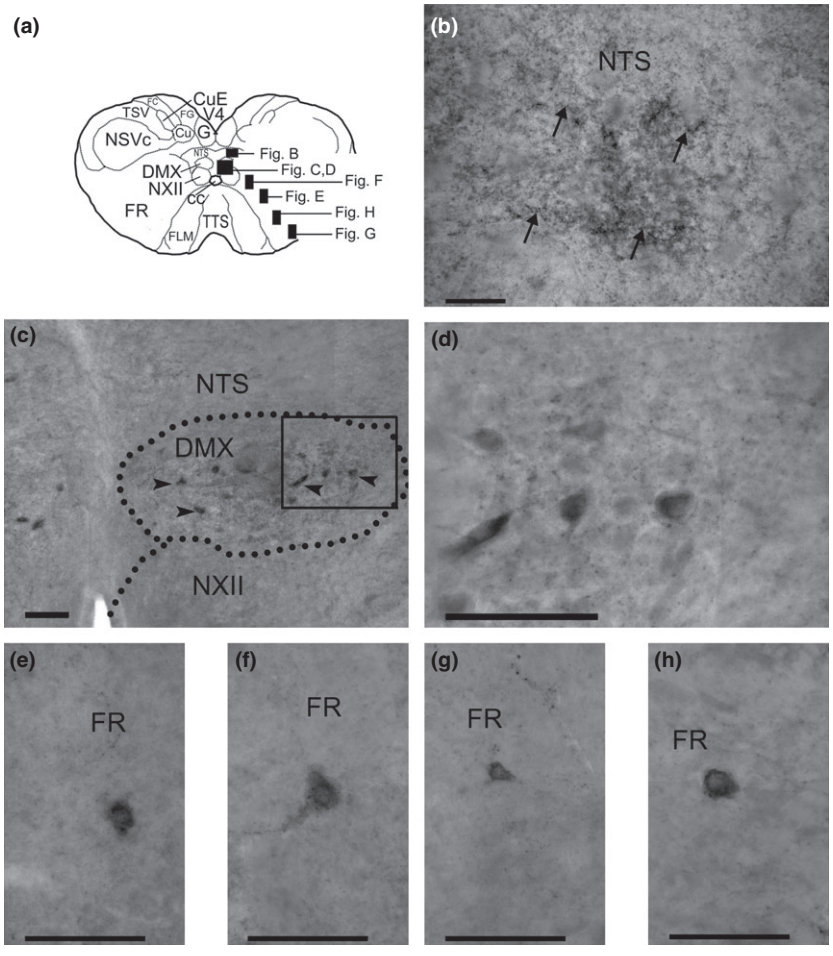

Fig. 5. Immunoreactive fibres and cell bodies containing SOM. (a) Frontal section of the minipig medulla oblongata (caudal region). For nomenclature of the nuclei, see list of abbreviations. The photographs shown in b–h were, respectively, taken from the regions delimited by the rectangles in a (indicated as Fig. b–h). (b) Medium-power magnification of fibres containing SOM located in the nucleus tractus solitarius (NTS). (c) Low-power magnification of the nucleus dorsalis motorius nervi vagus (DMX). (d) High-power magnification of the region delimited by a rectangle in c. (e–h) High-power magnification of cell bodies located in the formatio reticularis (FR). Arrowheads indicate cell bodies and arrows fibres. Scale bar: 100 μm. SOM, somatostain

for example, in the nucleus cuneatus, nucleus dorsalis motorius nervi vagus, nucleus dorsalis raphae, formatio reticularis, locus ceruleus, nucleus inter-peduncularis centralis and nucleus nervi trochlearis. The distribution of immunoreactive cell bodies containing NT in both the alpaca and minipig brainstems is quite restricted (de Souza et al., 2014). In the alpaca, six brainstem nuclei displayed NT-immunoreactive cell bodies, and in the minipig, we only observed four brainstem nuclei containing NT-immunoreactive perikarya. It should be noted that in both species, the same anti-NT and methodology (e.g. no colchicine administration) were applied (de Souza et al., 2014). However, the distribution of the NT-immunoreactive cell bodies is quite different in both species, as only the nucleus tractus spinalis nervi trigemini shows immunoreactive cell bodies in the alpaca and minipig.

As in the present case, in the monkey (Coveñas et al., 2008), we applied the same methodology (animals not treated with colchicine) and we used the same anti-NT antibody. In the brainstems of both species, the distribution of NT-immunoreactive fibres was quite similar, but, in the monkey, no immunoreactive cell bodies containing NT were found.

In humans, cell bodies containing NT have been observed in the nucleus tractus spinalis nervi trigemini, superior colliculus and substantia grisea centralis (Mai et al., 1987). In the minipig, perikarya containing NT have only been observed in the first nucleus. Immunoreactive fibres have been visualized in these three nuclei and in the nucleus dorsalis motorius nervi vagus, nucleus tractus solitarius, tractus solitarius, formatio reticularis, nucleus tractus mesencephalic nervi trigemini and substantia nigra (Mai et al., 1987). In general, these findings are in agreement with the results found in the minipig brainstem.

In sum, it seems that, in general, the distribution of the NT-immunoreactive fibres in the mammalian brainstem is quite similar (except in humans, in which it is more restricted), but the distribution of perikarya containing NT in the brainstem of rats and cats is more widespread than the distribution found in the minipig, monkey and humans. This difference could be due to technical reasons (administration of colchicine). However,

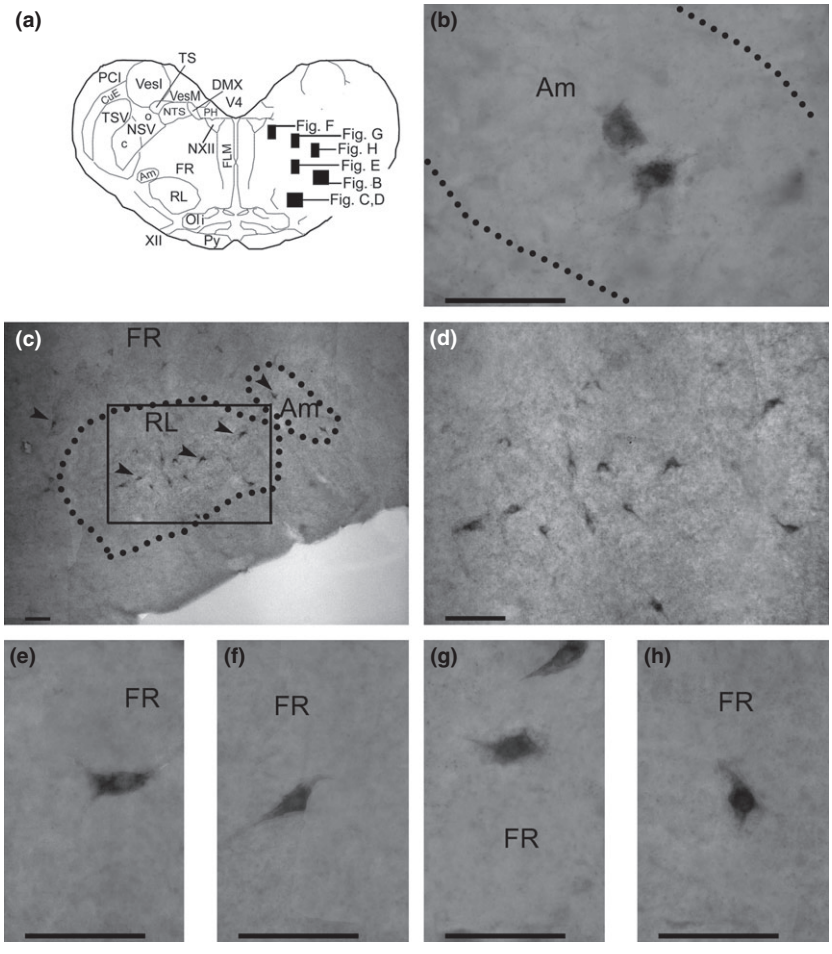

Fig. 6. Immunoreactive fibres and cell bodies containing SOM. (a) Frontal section of the minipig medulla oblongata (rostral region). For nomenclature of the nuclei, see list of abbreviations. The photographs shown in b–h were, respectively, taken from the regions delimited by the rectangles in a (indicated as Fig. b–h). (b) High-power magnification of cell bodies containing SOM located in the nucleus ambiguus (Am). (c) Low-power magnification of immunoreactive cell bodies located in the nucleus reticularis lateralis (RL) and nucleus ambiguus (Am). (d) High-power magnification of the region delimited by a rectangle in c. (e–h) High-power magnification of cell bodies located in the formatio reticularis (FR). Arrowheads indicate cell bodies. Scale bar: 100 μm. SOM, somatostatin

in the case of alpacas, it seems that the different distribution of NT-immunoreactive cell bodies observed in this camelid, in comparison with that found in the minipig would be due to species differences.

Somatostatin in the mammalian brainstem

The literature contains only a few references regarding the distribution of SOM-immunoreactive structures in the mammalian brainstem (de León et al., 1992; Pego-Reigosa et al., 2001). Applying the same methodology as here, except for the administration of colchicine, and using the same anti-SOM antibody, the distribution of SOM has been reported in the cat and dog brainstem (de León et al., 1992; Pego-Reigosa et al., 2001). In general, the distribution of SOM-immunoreactive fibres is quite similar in the cat, dog and minipig. In all three species, immunoreactive fibres have been observed, for example, in the substantia grisea centralis, nucleus dorsalis raphae, nucleus inter-peduncularis centralis, locus ceruleus, nucleus cuneatus and nucleus ambiguus. However, the distribution of cell bodies containing SOM is widely more widespread in the cat and the dog (de León et al., 1992; Pego-Reigosa et al., 2001) in comparison with the minipig. This different distribution could be due to the administration of colchicine to both the feline and the dog (de León et al., 1992; Pego-Reigosa et al., 2001). Regarding the rat, a radioimmunoassay study reported the presence of SOM in the medulla oblongata, pons and mesencephalon (Benoit et al., 1982), as we observed in the minipig.

It seems that, in general, the results found in the minipig are in agreement with the findings reported for the distribution of somatostatin-immunoreactive fibres in the mammalian brainstem (e.g. Japanese dancing mouse, rat, sheep, monkey and humans), as well as with results showing the distribution of receptors for somatostatin (Forssmann et al., 1979; Finley et al., 1981; Johansson et al., 1984; Bennett-Clarke and Joseph, 1986; Bouras et al., 1987; Spangler and Morley, 1987; Chigr et al., 1989; del Fiacco and Quartu, 1994; Carpentier et al., 1996; Fodor et al., 1997; Zhao et al., 1998; Lavezzi et al., 2004; Sienkiewicz et al., 2010). However, the distribution of somatostatin-immunoreactive cell bodies in the rat and

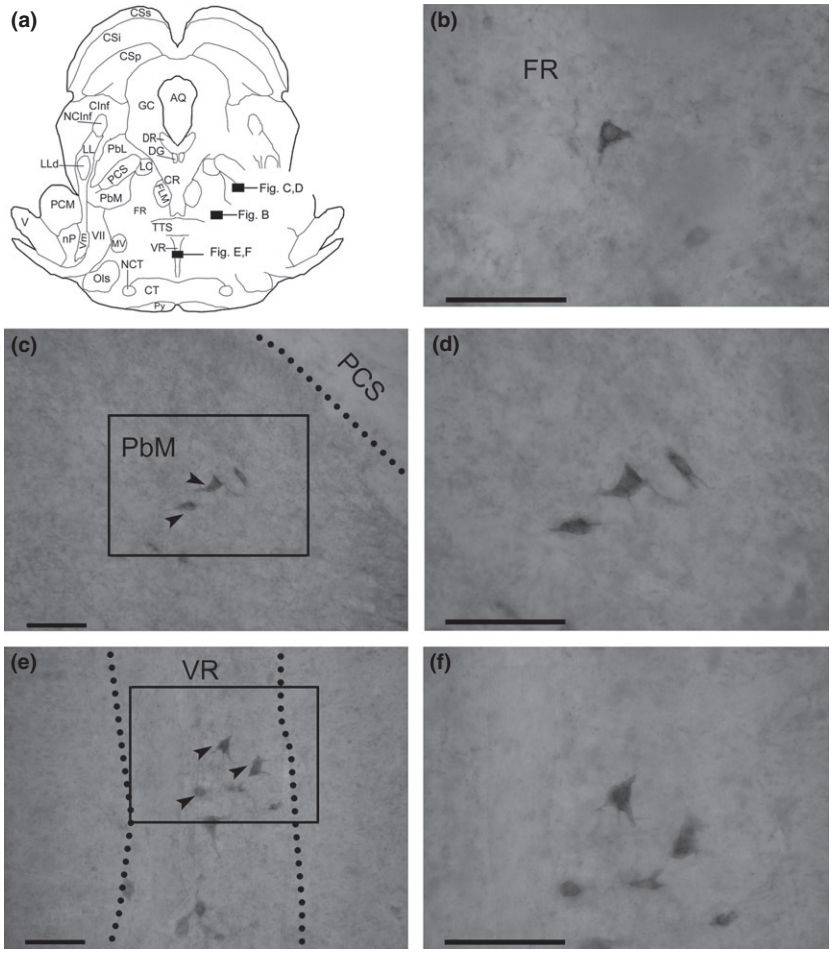

Fig. 7. Immunoreactive fibres and cell bodies containing SOM. (a) Frontal section of the minipig mesencephalon. For nomenclature of the nuclei, see list of abbreviations. The photographs shown in b–f were, respectively, taken from the regions delimited by the rectangles in a (indicated as Fig. b–f). (b) High-power magnification of a cell body containing SOM located in the formation reticularis (FR). (c) Medium-power magnification of the nucleus parabrachialis medialis (PbM). (d) High-power magnification of the region delimited by a rectangle in c. (e) Medium-power magnification of immunoreactive cells bodies located in the nucleus ventralis raphae (VR). (f) High-power magnification of the region delimited by a rectangle in e. Arrowheads indicate cell bodies. Scale bar: 100 μm. SOM, somatostatin

in humans is more widespread than the distribution of SOM-immunoreactive perikarya observed in the minipig. In the case of rats, the rodents were treated with colchicine (Johansson et al., 1984).

Possible physiological functions of neurotensin and SOM in the minipig brainstem

The distribution of NT in the minipig brainstem suggests that the neuropeptide plays many important physiological actions. It is known that NT is involved in anti-nociceptive, cardiovascular, auditive and respiratory mechanisms (Clineschmidt et al., 1979; Morin-Surun et al., 1986; de León et al., 1991; Chigr et al., 1992; Mitchell et al., 2009), that NT excites periaqueductal grey neurons (Li et al., 2001) and depolarizes neurons by post-synaptic mechanisms (Duan and Shimizu, 1992), and that NT is involved in dopamine transmission. It is also known that NT antagonizes the effects of dopamine at D_2 receptors via a NTS1/D_2 receptor–receptor interaction (Jiang et al., 1994), and the presence of NT-binding sites and acetylcholinesterase has been reported in several brainstem nuclei (e.g. nucleus dorsalis motorius nervi vagus, nucleus tractus solitarius and raphae nuclei) (Kessler et al., 1987).

The presence of NT-immunoreactive structures in the minipig nuclei centralis raphae, dorsalis raphae, ventralis raphae, substantia grisea centralis and tractus spinalis nervi trigemini suggests that the neuropeptide could be involved in controlling the sleep-waking cycle and in the regulation of the somatosensory/nociceptive information (Cape et al., 2000). Moreover, the presence of NT in the nuclei cuneatus, cuneatus externalis, gracilis, tractus solitarius, dorsalis motorius nervi vagus and parabrachialis medialis suggests the involvement of NT in sensory, gustatory and autonomic mechanisms (de León et al., 1991). The presence of scarce NT-immunoreactive fibres in the minipig motor brainstem nuclei (nervi oculomotorius, nervi trochlearis, nervi abducens and nervi facialis) should be noted. This suggests that the neuropeptide does not play an important role in these motor nuclei. However, low and moderate densities of NT-immunoreactive

fibres have been visualized in the nucleus motorius nervi trigemini and in the nucleus nervi hypoglossi, respectively. Moreover, a moderate density of NT-immunoreactive fibres has been observed in the substantia nigra, suggesting a role of the neuropeptide in motor mechanisms (Chen et al., 2006). Finally, it should be noted that no immunoreactive structure containing NT has been located in either the inferior and superior colliculi or in the vestibular and cochlear nuclei.

It is known, for example, that the peptide somatostatin is involved in sleep, nociceptive, respiratory and cardiovascular mechanisms (Beitz et al., 1983; Higgins and Schwaber, 1983; Reichlin, 1983; Moga and Gray, 1985; Carpentier et al., 1998; Lavezzi et al., 2004; Bartsch et al., 2005) and that somatostatin controls neuronal activity in the locus ceruleus (Olpe et al., 1987).

The presence of SOM in the minipig brainstem nuclei ventralis raphae, ambiguus, reticularis lateralis, dorsalis motorius nervi vagus and parabrachialis medialis suggests that the neuropeptide is involved in the sleep-waking cycle (Carpentier et al., 1996), in motor and position control, parasympathetic functions,and respiratory mechanisms (Ramírez-Jarquín et al., 2012). Moreover, according to the distribution of SOM-immunoreactive structures in the minipig brainstem, the neuropeptide could play a role in sensory/gustatory processes and in autonomic control (Spary et al., 2008). Finally, no SOM-immunoreactive structure was observed in the inferior and superior colliculi.

In sum, for the first time, in the minipig brainstem, a detailed immunohistochemical study addressing the distribution of NT and SOM has been carried out. Our study should be useful for future neuroanatomical, neuropharmacological and behavioural studies, as the minipig is increasingly used as a nonprimate model in basic experimental studies on neurological diseases.

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