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Brain afferents to the medullary dorsal reticular nucleus: a retrograde and anterograde tracing study in the rat

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Abstract

The medullary dorsal reticular nucleus (DRt) was recently shown to belong to the supraspinal pain control system; neurons within this nucleus give origin to a descending projection that increases spinal nociceptive transmission and facilitates pain perception [Almeida *et al.* (1999), *Eur. J. Neurosci.*, **11**, 110–122]. In the present study, the areas of the brain that may modulate the activity of DRt neurons were investigated by using of tract-tracing techniques. Injection of a retrograde tracer into the DRt resulted in labelling in multiple areas of the brain. In the contralateral orbital, prelimbic, infralimbic, insular, motor and somatosensory cortices labelling was prominent, but a smaller ipsilateral projection from these same areas was also detected. Strong labelling was also noted in the central amygdaloid nucleus, bed nucleus of stria terminalis and substantia innominata. Labelled diencephalic areas were mainly confined to the hypothalamus, namely its lateral and posterior areas as well as the paraventricular nucleus. In the mesencephalon, the periaqueductal grey, red nucleus and deep mesencephalic nucleus were strongly labelled, whereas, in the brainstem, the parabrachial nuclei, rostroventromedial medulla, nucleus tractus solitarius, spinal trigeminal nucleus, and the parvocellular, dorsal, lateral and ventral reticular nuclei were the most densely labelled regions. All deep cerebellar nuclei were labelled bilaterally. These data suggest that the DRt integrates information from the somatosensory, antinociceptive, autonomic, limbic, pyramidal and extrapyramidal systems while triggering its descending facilitating action upon the spinal nociceptive transmission.

Introduction

The medullary dorsal reticular nucleus (DRt; Lima, 1990; Swanson, 1998) is an area strongly involved in nociceptive processing. Anatomical studies have revealed a closed reciprocal circuit between the DRt and spinal dorsal horn. Descending DRt fibres make asymmetrical, presumably excitatory (Shepherd, 1990; Todd & Spike, 1993) synaptic contacts upon lamina I spinal cells that, in turn, project back to the nucleus (Almeida *et al*., 1993); in addition, ascending fibres originating in laminae I and IV–V neurons make asymmetrical synaptic contacts upon DRt neurons projecting back to the same spinal laminae (Almeida *et al*., 2000). These data revealed the existence of a putative reverberative excitatory loop between the DRt and spinal lamina I. The ascending spinal projection targeting the DRt conveys nociceptive information as a large percentage of lamina I neurons targeting the DRt (Almeida & Lima, 1997) and most neurons recorded in the DRt (Villanueva *et al*., 1988, 1989; Roy *et al*., 1992) are activated by noxious stimulation. Regarding the descending action of the loop, different lines of evidence have shown that the DRt has a facilitating action upon pain perception: (i) glutamate stimulation of the DRt resulted in a decrease of the tail-flick latency (Almeida *et al*., 1996); (ii) electrical or chemical lesioning of the nucleus increased the tail-flick latency and the response temperature in the tail-flick and hot plate tests, respectively (Almeida *et al*., 1996); (iii) lesioning the DRt decreased the pain-like response in both the acute and inflammatory pain phases of the formalin test (Almeida *et al*., 1999a); (iv) the attenuation in nociceptive behaviour resulting from DRt lesioning is accompanied by a decrease in noxious-evoked c-*fos* spinal expression in laminae I–II and IV–V (Almeida *et al*., 1999a); (v) glutamate stimulation of the DRt resulted in the facilitation (increase) of the postdischarge of wide-dynamic-range (WDR) spinal nociceptive neurons (Dugast *et al*., 2000), an effect that is transiently reversed by DRt administration of the local analgesic lidocaine (C.Dugast, A.Almeida & D.Lima, unpublished data); (vi) DRt glutamate-activation reduced the stimulation frequency and/or the number of stimulation pulses needed to trigger wind-up in WDR neurons (Dugast *et al*., 2001). The above data indicate that the nucleus has a pro-nociceptive action that is mediated by a descending excitatory projection impinging upon spinal nociceptive neurons (reviewed by Lima & Almeida, 2002).

A large number of studies have been dedicated to the descending nociceptive inhibitory influences triggered by the periaqueductal grey matter (PAG) –rostroventromedial medulla (RVM) –spinal cord circuit and by the noradrenergic pontomesencephalic nuclei projecting to the cord (for extensive reviews see Basbaum & Fields, 1984; Hammond, 1986; Gebhart & Randich, 1990; Jones, 1992). In contrast, the descending facilitating influences, whose existence

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TABLE 1. Density of retrogradely labelled neurons after small (rat 691) and large (rat 536) CTb injections restricted to the Drt

Location of labelled neurons	Small injection of CTb		Large injection of CTb	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Telencephalon				
Orbital cortex		++		++
Prelimbic and infralimbic cortices		++		++++
Secondary motor cortex	+	+++	+	+++
Primary motor cortex	+	++++	++	+++++
Secondary somatosensory cortex	+	++++	+	+++++
Primary somatosensory cortex	+	++++	+	++++
Dysgranular/granular insular cortices	+	++++	+++	+++++
Agranular insular cortex	+++	++++	+++	+++++
Cingulate cortex				+
Retrosplenial granular cortex		+		+
Secondary auditory cortex				++++
Primary auditory cortex				++
Perirhinal cortex		+	+	+++
Ectorhinal cortex		+		++
Parietal association cortex				+
Temporal association cortex		+		++
Lateral/medial preoptic area			+	
Bed nucleus stria terminalis	+		++++	+
Substantia innominata	++		++	
Globus pallidus, lateral	++		++	
Central amygdaloid nucleus	+++++	+	+++++	+
Diencephalon				
Paraventricular hypothalamic nucleus	+++	++	++++	+++
Parasubthalamic nucleus	+		+++	+++
Zona incerta	++	+	++	+
Posterior hypothalamic area			++	++
Lateral hypothalamic area	++++	++	+++++	+++
Parafascicular thalamic nucleus	+	+	+	
Mesencephalon				
Red nucleus		++++		++++
Substantia nigra, compact	+		++	+
Substantia nigra, reticular	++		++	
Retrorubral field			++	+
Subpeduncular tegmental nucleus			+	+
Deep mesencephalic nucleus	+++	+++	++++	+++
Dorsal raphe nucleus	++	++		
Periaqueductal grey, dorsomedial	+	+++		
Periaqueductal grey, lateral	++	++	++++	++++
Periaqueductal grey, ventrolateral	+++	+	++++	++
Cuneiform nucleus	+		++	+
Pons				
Reticulotegmental nucleus of the pons	++	+	+	
Basilar pontine nuclei	+++++	+++++		
Pontine reticular nucleus, oral		+	++	+
Principal sensory trigeminal nucleus	++	+		
Subcoeruleus nucleus, ventral	+++		+++	+
Mesencephalic trigeminal nucleus	+		++	+
Motor trigeminal nucleus	+			
Pontine reticular nucleus, caudal	++	++	++	++
Locus coeruleus	+	+	+	+
Kölliker-Fuse nucleus	++	++	+++	++
Parabrachial nuclei	+++	++	++++	+++
A5 noradrenergic cell group	+++	+	+++	++
Cerebellum				
Interposed cerebellar nucleus	++++		++++	+
Lateral (dentate) cerebellar nucleus	++		++	
Medial (fastigial) cerebellar nucleus	++	++	+	++++
Medulla oblongata				
Rostroventrolateral reticular nucleus	+	+	+	+
Raphe magnus nucleus	++	++++		
Raphe pallidus nucleus	++	++		
Raphe obscurus nucleus	+	++		
Vestibular nuclei	+	+	+	+
Dorsal paragigantocellular nucleus	+	+	+	+
Lateral paragigantocellular nucleus	++	++	++++	++++
Gigantocellular reticular nucleus, alpha	++	+	++++	++++

TABLE 1. (continued)

Location of labelled neurons	Small injection of CTb		Large injection of CTb	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Gigantocellular reticular nucleus, ventral	++	+	++	++
Gigantocellular reticular nucleus	++	++	+++	++
Intermediate reticular nucleus	++	+	+++	++
Dorsal reticular nucleus	+++	+++	++++	++++
Area postrema	+	++		
Nucleus of Roller			+	++
Hypoglossal nucleus	+	+	+	+
Dorsal motor nucleus of vagus	+	+	+	+
Nucleus of the solitary tract, ventrolateral	++++	++++	+++++	+++++
Nucleus of the solitary tract	++++	++++	+++++	++++
Nucleus cuneatus	++		+++	
Paratrigeminal nucleus	++++		++++	+
Spinal trigeminal nucleus	+++++	++	+++++	++
Ventral reticular nucleus	+	++	++	++
Caudal ventrolat. ret. form., lateral	+		+++	++
A1 noradrenergic cell group	++		++	++
Lateral reticular nucleus	++	+	++	+
Inferior olive		+++++		
Parvocellular reticular nucleus	+++++	+++	+++++	++++

Intensity of label: +++++, very dense; ++++, dense; +++, numerous; ++, few; + rare.

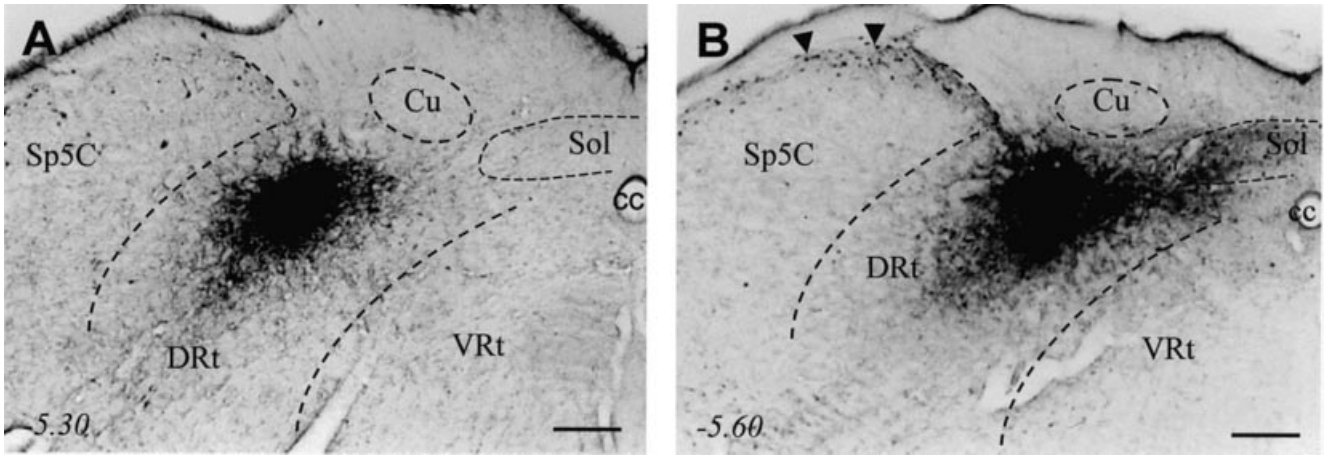

FIG. 1. Photomicrographs, each showing the coronal section presenting the largest tracer spread resulting from DRt injection with the retrograde tracer Ctb: (A) in a typical small rat (rat 691) and (B) in a typical large rat (rat 536). In both injections, CTb extends through the DRt but is restricted to the limits of the nucleus. In B, note the large number of retrogradely labelled neurons (arrowheads) located in the superficial layers of the Sp5C and in the Sol. Scale bar, 150 μm. For abbreviations, see list.

were occasionally reported in the eighties, was only recently subjected to detailed research (reviews by Fields & Basbaum, 1999; McNally, 1999; Millan, 1999; Urban & Gebhart, 1999; Lima & Almeida, 2002). Both inhibitory and facilitating descending actions were shown to be: (i) increased in the same nociceptive situation (e.g. chronic/neuropathic pain; Cervero *et al*., 1991; Schaible *et al*., 1991; Bian *et al*., 1998; Kauppila *et al*., 1998); (ii) triggered from the same brain areas (RVM, Fields *et al*., 1983; Zhuo & Gebhart, 1992, 1997; Wei *et al*., 1999; nucleus tractus solitarius, Ren *et al*., 1990; DRt, Bouhassira *et al*., 1992; Almeida *et al*., 1996, 1999a); and (iii) triggered from the same type of descending projection (e.g. noradrenergic descending pathways; Proudfit & Hammond, 1981; Yaksh, 1985; Fasmer *et al*., 1986; Tjølsen *et al*., 1991; Martin *et al*., 1999). Thus, it is a complex balance of facilitating and inhibiting descending influences upon the nociceptive transmission at the spinal dorsal horn level that determines pain modulation, the final action being dependent on the specific characterization of each painful condition (Lima, 1998; Fields & Basbaum, 1999; Lima & Almeida, 2002).

In order to ascertain the pattern of anatomical connections that may modulate the activity of the nociceptive-facilitating neurons present in the DRt, an extensive study of the brain areas that project to the nucleus was carried out. Some of the data have been presented previously in abstract form (Almeida *et al*., 1999b).

Materials and methods

The experiments followed the regulations of local authorities for handling laboratory animals, the European Community Council Directive 86/609/EEC and the ethical guidelines for the study of experimental pain in conscious animals (Zimmermann, 1983).

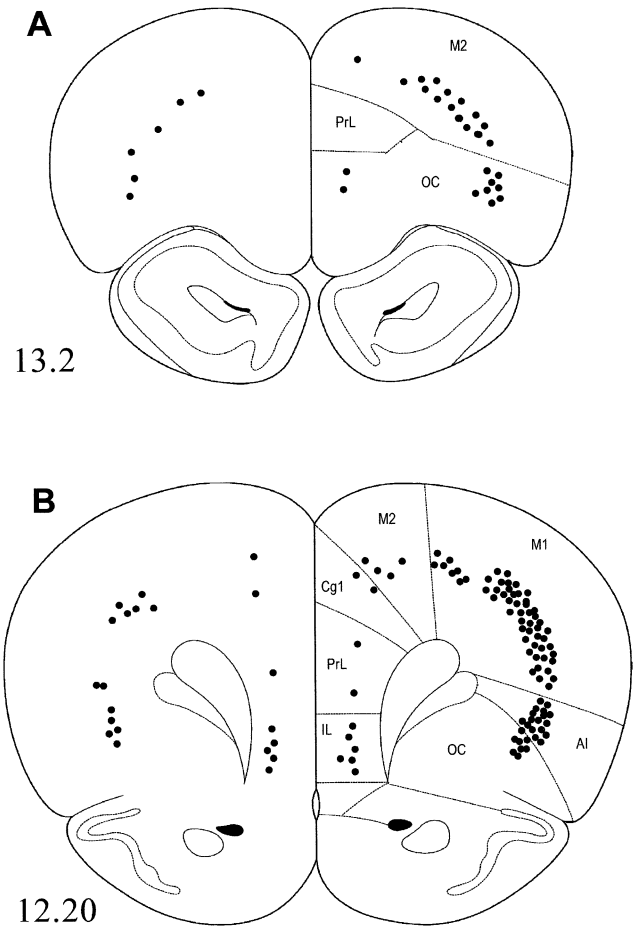

FIG. 2A and B. Locations of retrogradely labelled cells plotted on representative coronal sections through the brain of rat 536. The injection site is shown photographically in Fig. 1B. Each dot (●) and triangle (▲) represents 1 and 5 neurons, respectively. (A and B) Sections at 13.2 and 12.20 mm rostral to the interaural line. For abbreviations, see list.

Retrograde-tracing studies

In order to determine the brain nuclei projecting to the DRt, seven male Wistar rats (Gulbenkian Institute of Science, Lisbon, Portugal) weighing 270–320 g were anaesthetized with halothane (a gaseous mixture of 66% N_2O and 34% O2 containing halothane at 4% for induction and 1.5–2% for maintenance), and an iontophoretic injection of 1% cholera toxin subunit B (CTb; low salt, List Biological Products, Campbell, USA) was made in the left DRt following the stereotaxic parameters of Paxinos & Watson (1986), using a glass micropipette with 20–30 μm diameter tip and a positive DC current of 2.5 μA for 10 min (smaller injections; 5 rats) or 15 min (larger injections; 2 rats). After completion of the injections the micropipettes were left *in situ* for 10–15 min before being slowly retracted. One week later, animals were reanaesthetized with 35% chloral hydrate (1 mL/kg body weight) and perfused through the ascending aorta with 1000 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The entire brain was removed and immersed in the fixative for 4 h, then in 30% sucrose in PB at 4 °C for 2–3 days. Coronal sections of the entire brain were cut on a freezing microtome at 50 μm. One in every three brain sections was immunoreacted for CTb and counterstained using the formol–thionin technique (Donovick, 1974) and one in every three sections was only immunoreacted for CTb. CTb was revealed using the immunoperoxidase technique, as previously described (Almeida & Lima, 1997; Almeida *et al*., 2000). Briefly, sections were first incubated overnight at room temperature in a goat primary polyclonal antibody against CTb (List Biological Products, USA) at 1 : 40000 in 0.1 M phosphate buffer saline containing 0.3% Triton X-100 (PBST). After washing repeatedly in PBST, the sections were incubated for 1 h in PBST containing a biotinylated antigoat antibody raised in horse (1 : 200; Vector Laboratories, Burlingame, USA). Sections were then washed again and incubated for 1 h in PBST containing the avidin–biotin complex (1 : 200, Vector Laboratories, USA). After washing in 0.1 M tris-HCl, pH 8.2, peroxidase was revealed using 0.0125% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Immunochemicals, St Louis, USA) and 0.025% H_2O_2 in the same buffer. The sections were then dehydrated and mounted in Eukitt. Cytoarchitectonic diagrams of the injection sites were made with the aid of a camera lucida. Retrogradely labelled neurons in brain areas projecting to the DRt were located and superimposed on projection drawings of selected representative sections from the atlas of Paxinos & Watson (1998).

Anterograde-tracing control studies

Injection of the anterograde tracer biotinylated-dextran amine (BDA) was made in several DRt-projecting brain nuclei, in order to establish their termination areas in the nucleus. The paraventricular hypothalamic nucleus (Pa), the lateral hypothalamic area (LH), the ventrolateral periaqueductal grey matter (VLPAG), the red nucleus (RD) and the lateral cerebellar nucleus of male Wistar rats were injected iontophoretically in the left side of the brain, under halothane anaesthesia (see above), with a 10% PB buffer, pH 7.2, using a positive DC current of 2.5 μA for 20–30 min. Ten days later the rats were anaesthetized and perfused as described above. Serial 50 μm thick frozen coronal sections of the injection site and caudal medulla were immunoreacted using the avidin–biotin complex method and stained with DAB (see Almeida *et al*., 1995), the former sections being further counterstained with formol–thionin. Fibres and terminal arborizations labelled anterogradely in the DRt were plotted using camera lucida drawings.

Results

Injection sites

CTb injection sites showed a compact dark zone surrounded by a narrow rim without neuronal labelling, in which dark zones intermingled with lighter zones (Fig. 1). More peripherally, a few perikarya were labelled as a consequence of tracer uptake from the central area. The injection site was considered to encompass only the central dark area and surrounding rim. The injections were all placed inside the limits of the DRt and thus were all considered for analysis.

BDA injection sites also showed the compact dark central zone surrounded by a narrow rim of scarcely labelled neurons. In accordance with previous technical studies, only the central region and the rim were included in the injection site (Veenman *et al*., 1992; Wouterlood & Jorritsma-Byham, 1993). In the animals analysed, injections were placed inside the anatomical borders of the left Pa, LH, RD and VLPAG.

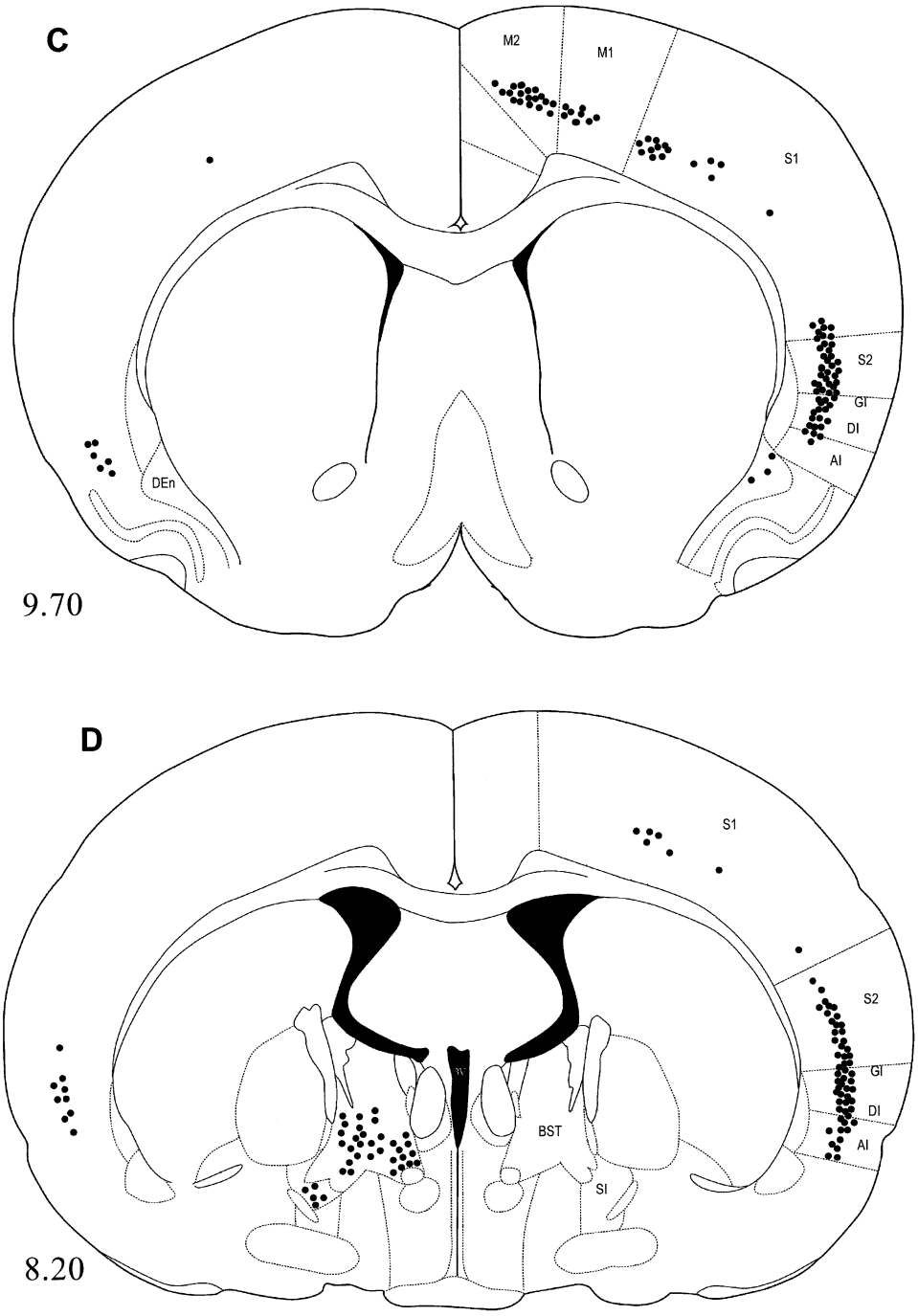

FIG. 2C and D. (Fig. 2 continued) Sections at 9.70 and 8.20 mm rostral to the interaural line. For details see legend to Fig. 2A and B, and abbreviations list.

Retrograde labelling

CTb-labelled neurons projecting to the DRt were distributed along the rostrocaudal extension of the brain, particularly in the cortex (contralateral) and brainstem, although high numbers of neurons were also found in some nuclei of the ventral telencephalon and diencephalon. A systematic analysis of the location and density of neurons projecting to the DRt is described in Table 1, whereas in Fig. 2 the precise locations of retrogradely labelled neurons are plotted on brain charts.

Telencephalon

In the prefrontal cortex, a bilateral projection to the DRt was shown to occur from the prelimbic (PrL), infralimbic (IL) (Figs 2A and B, and 3A), orbital (OC; Fig. 2A and B), and cingulate (Cg1) (Fig. 2B) cortices, although with a contralateral predominance. In the motor cortex, a higher number of neurons was present in the primary (M1) than in secondary (M2) motor cortices and, in both cases, they were predominantly located on the contralateral hemisphere (Fig. 2A–C). Regarding the somatosensory cortex, a large number of neurons were

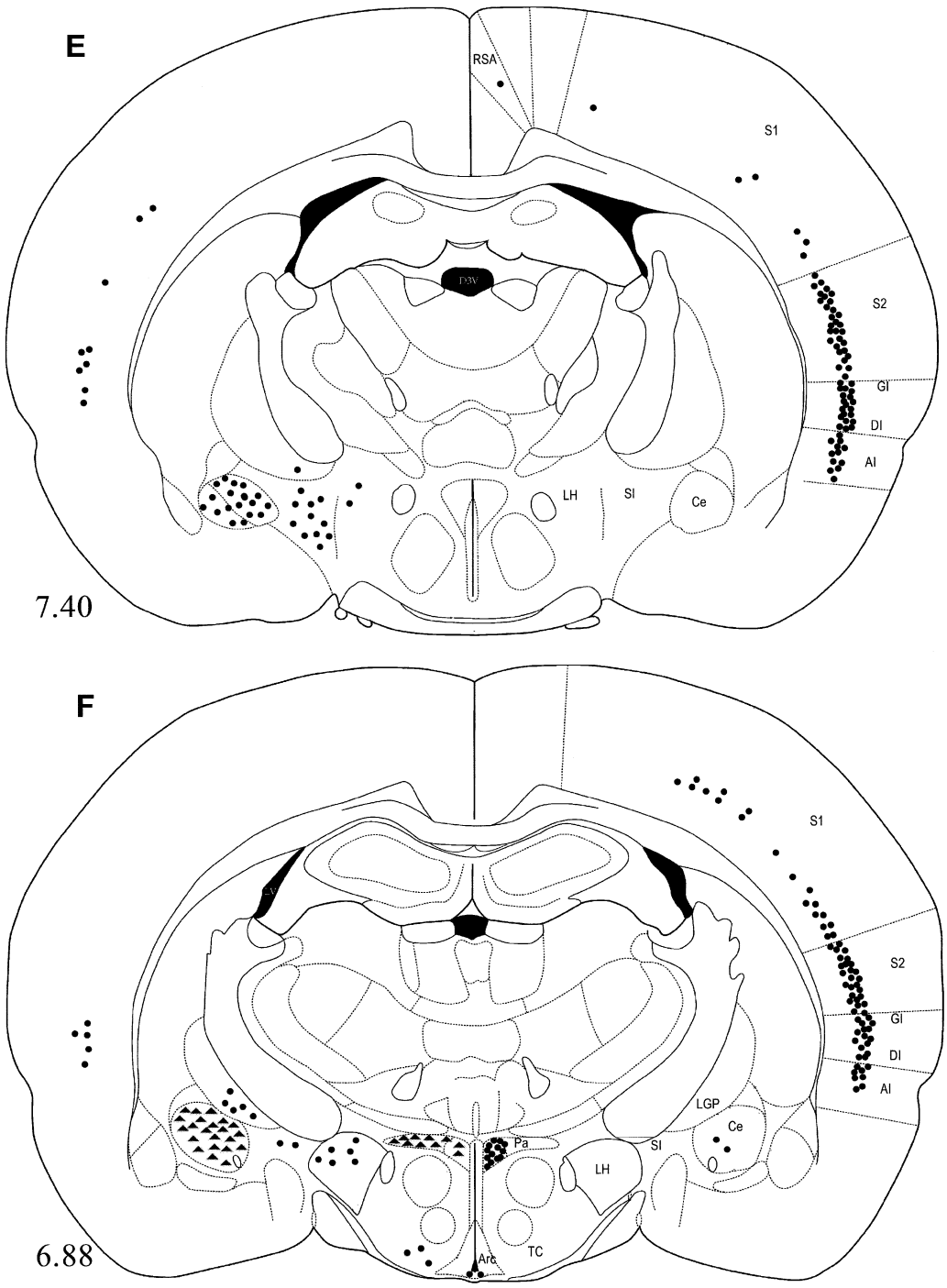

FIG. 2E and F. (Fig. 2 continued) Sections at 7.40 and 6.88 mm rostral to the interaural line. For details see legend to Fig. 2A and B, and abbreviations list.

present, mainly in the contralateral secondary somatosensory cortex (S2) and, in smaller numbers, in the contralateral primary somatosensory cortex (S1) (Fig. 2C–F), barrel field and facial regions. A small ipsilateral projection from the S_1 and S_2 cortical areas was also present (Fig. 2E). The forelimb, hindlimb and thorax regions showed very few or no cells. In the insular cortices, labelled neurons were present in all sections. Although the majority were located contralaterally (Fig. 2B–F), an ipsilateral projection was also consistently observed (Figs 2B–F and 3B).

In subcortical telencephalic areas, an impressive number of labelled neurons was located in the ipsilateral bed nucleus of the stria terminalis (BST; Fig. 2D). In the amygdala, a very strong projection was shown to occur, almost exclusively, along the entire rostrocaudal extent of the central amygdaloid nucleus (Ce),

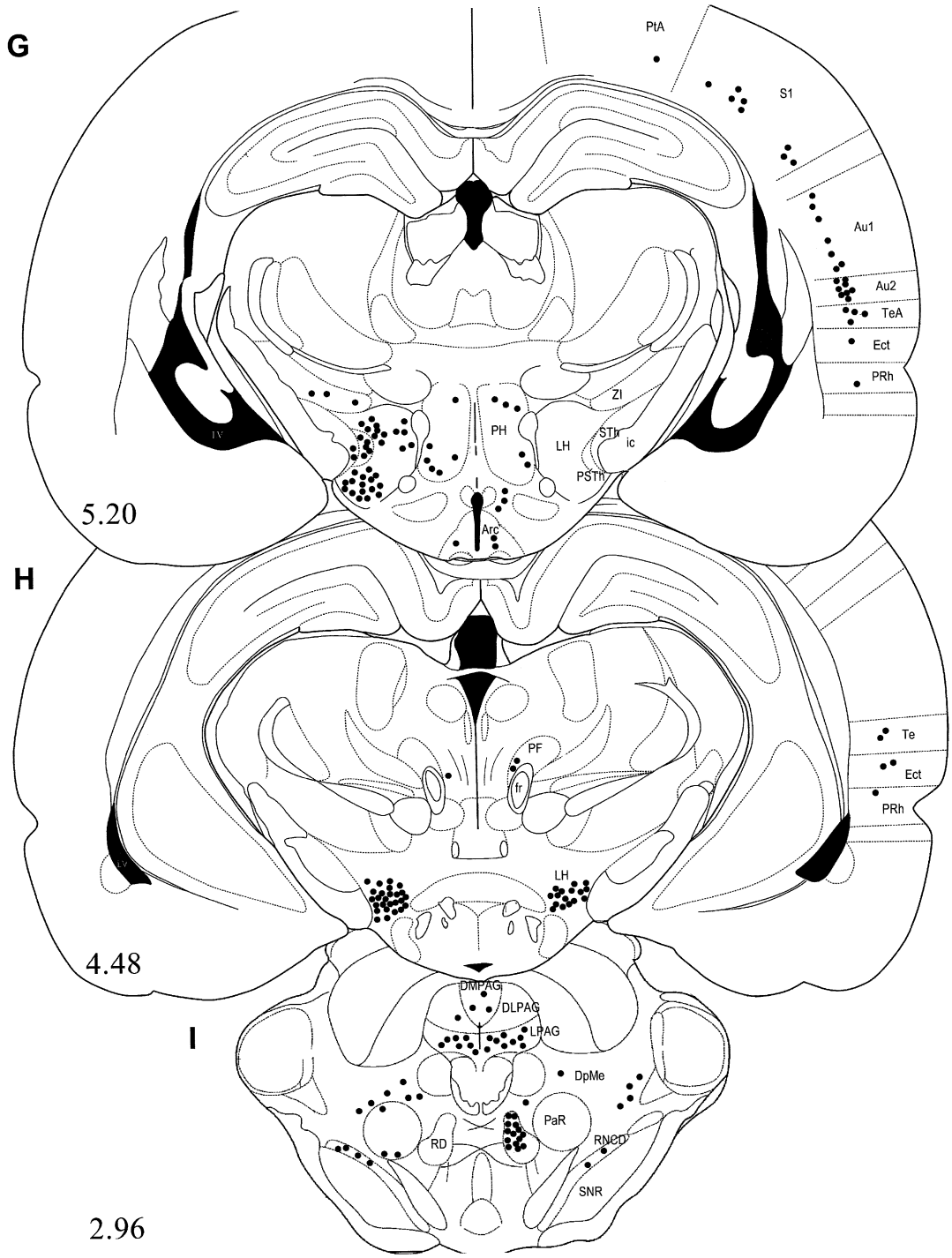

FIG. 2G–I. (Fig. 2 continued) Sections at 5.20, 4.48 and 2.96 mm rostral to the interaural line. For details see legend to Fig. 2A and B, and abbreviations list.

ipsilaterally (Figs 2E and F, and 3C). Numerous neurons projecting to the DRt were also noted in the ipsilateral substantia innominata (SI; Fig. 2D–F).

Diencephalon

In the thalamus, only the parafascicular nucleus (PF) presented some labelled neurons both medially and laterally to the *fasciculus retroflexus* (Fig. 2H). A small projection from the zona incerta (ZI) was present ipsilaterally (Fig. 2G). On the other hand, the hypothalamus showed a large number of labelled cells located bilaterally in different subnuclei of the Pa (Figs 2F and 3D). A great number of cells projecting to the DRt were also located in the medial and caudal portions of the LH, bilaterally (Fig. 2E–H). In the caudal hypothalamus, the posterior hypothalamic area (PH) showed a

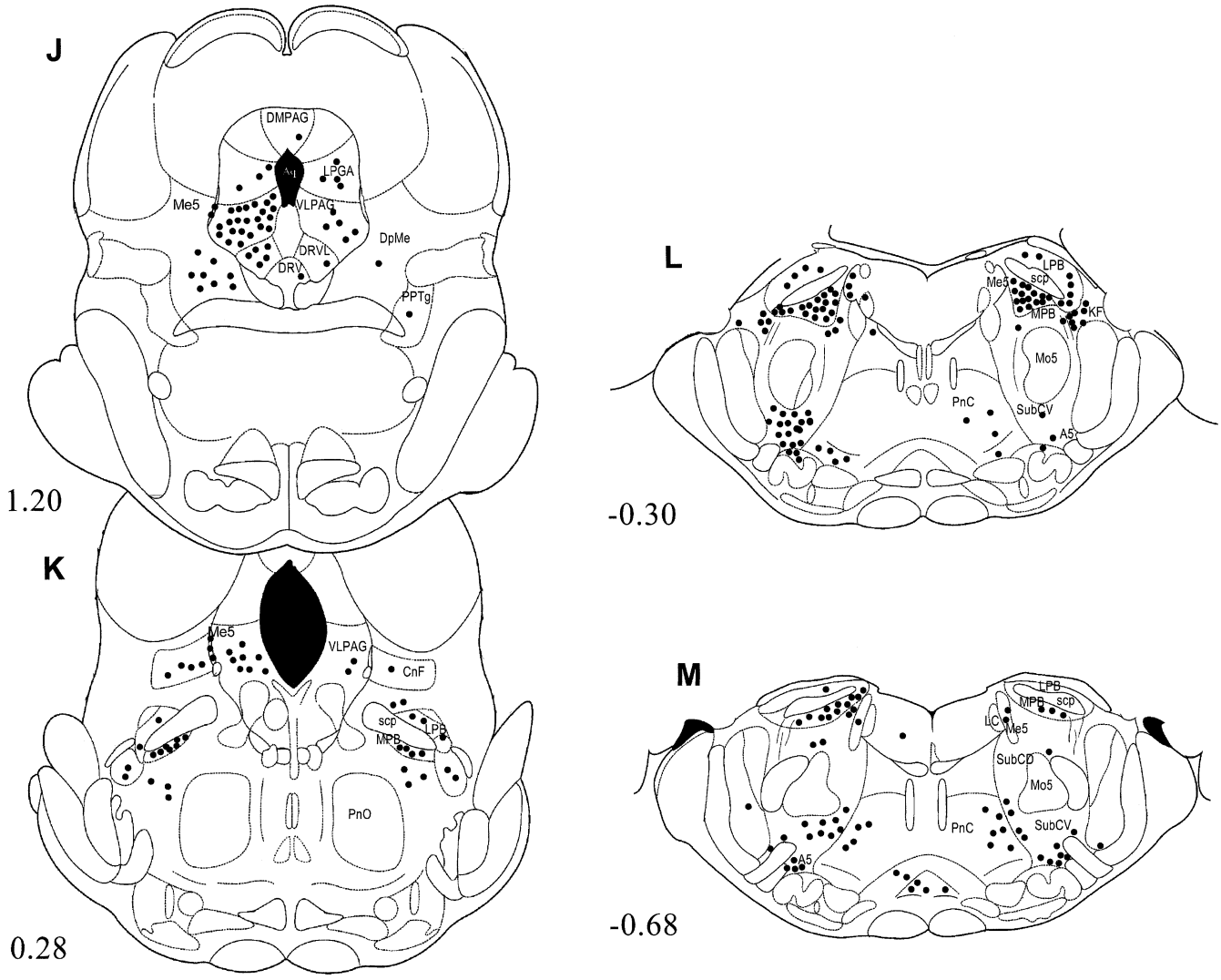

FIG. 2J–M. (Fig. 2 continued) Sections at 1.20 and 0.28 mm rostral, and 0.30 and 6.8 mm caudal to the interaural line. For details see legend to Fig. 2A and B, and abbreviations list.

moderate number of retrogradely labelled neurons, bilaterally (Fig. 2G). Fewer neurons were labelled in the arcuate nucleus (Arc) and ipsilateral tuberum cinerum (TC; Figs 2F and G).

Mesencephalon

Labelled cells were located in the caudalmost areas of the substantia nigra, bilaterally (Fig. 2I). In the RD, most of the large neurons labelled contralaterally were concentrated in its dorsal region (Fig. 2I). Several bilaterally located labelled neurons were observed through the rostrocaudal extension of the lateral and VLPAG (Figs 2I–K and 3E). Other areas of the PAG presented a small number of labelled neurons (Figs 2I and J). Labelled cells were scattered, mainly ipsilaterally, through the large area of the brainstem reticular formation forming the deep mesencephalic nucleus (DpMe; Figs 2I and J), and in the cuneiform nucleus (CnF; Fig. 2K). In the dorsal raphe, most neurons were present in the ventrolateral portion of the nucleus followed by the ventral part, whereas the dorsal part of the dorsal raphe was not labelled (Fig. 2J).

Pons

In one rat (number 691, small injection), the basilar pontine nuclei (BPN) were the areas of the pons presenting the most extensive distribution of bilaterally located cells, but no cells were labelled in the other rat described in Table 1 (numb 536, large injection). The CnF (Fig. 2K) and the caudal pontine reticular nucleus (PnC; Fig. 2L–N) showed a moderate number of labelled neurons. A highly packed set of cells projecting ipsilaterally to the DRt was present in the mesencephalic trigeminal nucleus (Me5; Fig. 2J–M). The A_5 noradrenergic group (A5), locus coeruleus (LC) and nucleus subcoeruleus (SubCV) were noradrenergic brainstem areas that presented a moderate number of labelled cells located mainly ipsilaterally (Fig. 2L–N). A large number of labelled neurons was distributed bilaterally through the subnuclei of the parabrachial nuclei (PBN), particularly in the medial PBN (MPB) and ventral part of the lateral PBN (LPB; Fig. 2K–M). Labelled neurons in the Kölliker–Fuse nucleus (KF) were present bilaterally in all the appropriate sections analysed (Figs 2K and L).

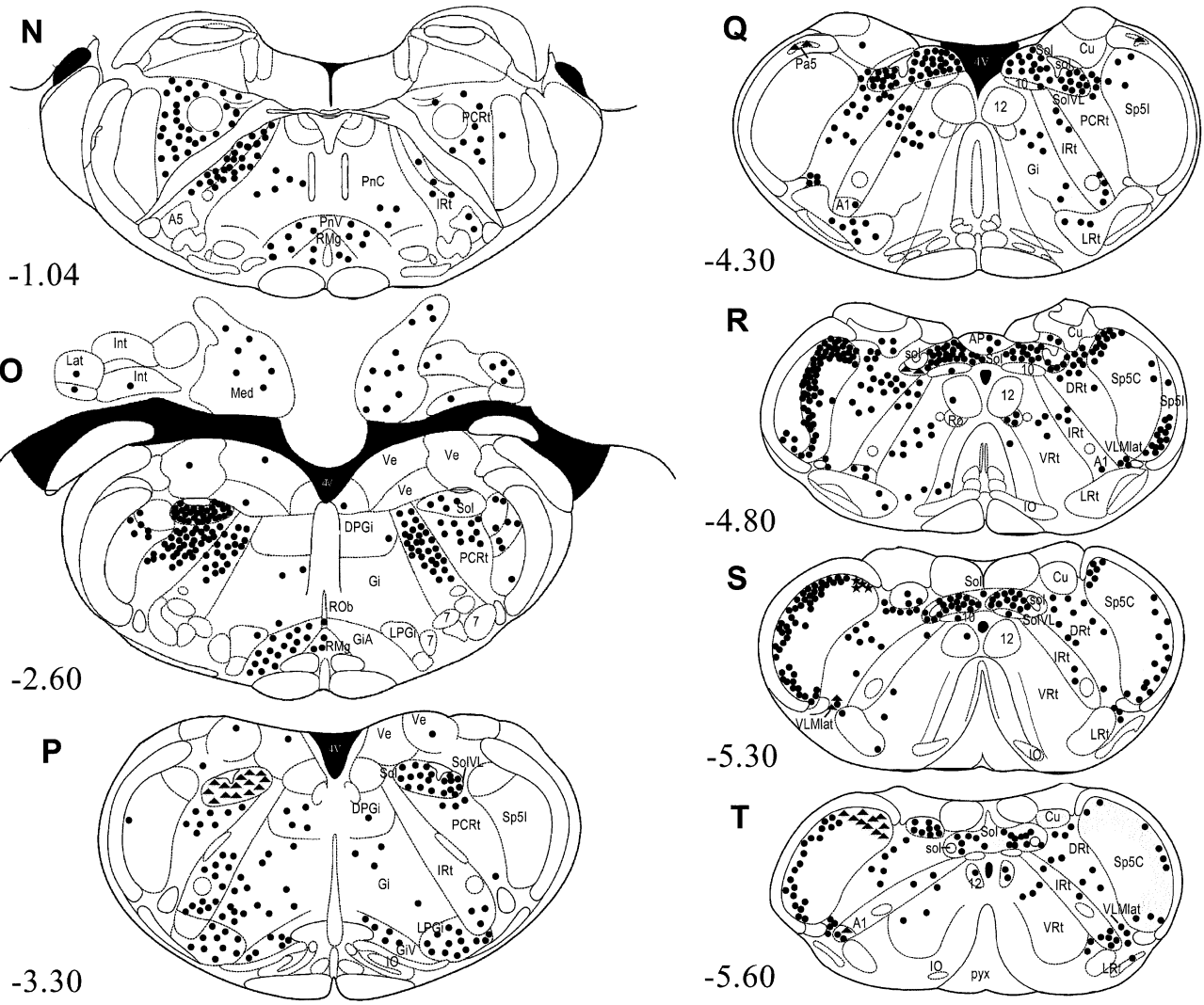

FIG. 2N–T. (Fig. 2 continued) Sections at 1.04 to 5.60 mm caudal to the interaural line. For details see legend to Fig. 2A and B, and abbreviations list. As before, each dot and triangle represents 1 and 5 neurons respectively. The stars in S each represent 10 neurons.

Cerebellum

All three major deep nuclei of the cerebellum presented a considerable number of bilaterally labelled neurons, particularly in the medial deep cerebellar nucleus (Fig. 2O).

Medulla oblongata

The gigantocellular (Gi, GiA, GiV), paragigantocellular (LPGi), lateral (LRt) and ventral (VRt) reticular nuclei presented a moderate number of labelled neurons, bilaterally (Fig. 2O–T). Although without consistent neuronal labelling in all sections analysed, all ponto-medullary raphe subdivisions contained neurons projecting to the DRt, with special emphasis on the nucleus raphe magnus (RMg; Fig. 2M–O). High numbers of neurons were located in both medial (SolM) and ventrolateral (SolVL) areas of the nucleus tractus solitarius (Sol), along its rostrocaudal extension (Figs 1B and 2O–T). Numerous neurons were also located bilaterally along the rostrocaudal ponto-medullary extension of the parvocellular reticular nucleus (PCRt; Fig. 2N–Q). In rat 691 (small injection), a high number of contralaterally located neurons was present in the medial portion of the inferior olive (IO), between interaural coordinates –3.30 and –4.68 mm (Paxinos & Watson, 1998), but no labelling in this area was observed in rat 536 (large injection). A small number of labelled neurons were located in the motor nucleus of the vagal nerve (10; Fig. 2Q–S) and hypoglossal nucleus (12; Fig. 2R–T). The lateral part of the ventrolateral medulla (VLMlat) showed a bilateral projection to the DRt (Fig. 2R–T). A large number of neurons were labelled along the ipsilateral rostrocaudal extension of the caudal portion of the spinal trigeminal nucleus (Sp5C), being mainly concentrated in the most superficial laminae (Figs 1A and B, and 2R–T). Numbers were much smaller in the interpolar part of the trigeminal nucleus (Sp5I), where the neurons were preferentially located in deeper laminae (Fig. 2O–Q). The DRt, the area injected with CTb and the focus of this study, presented neurons of local circuit, labelled ipsilaterally, and short-projecting neurons, labelled bilaterally (Figs 2R–T and 3F).

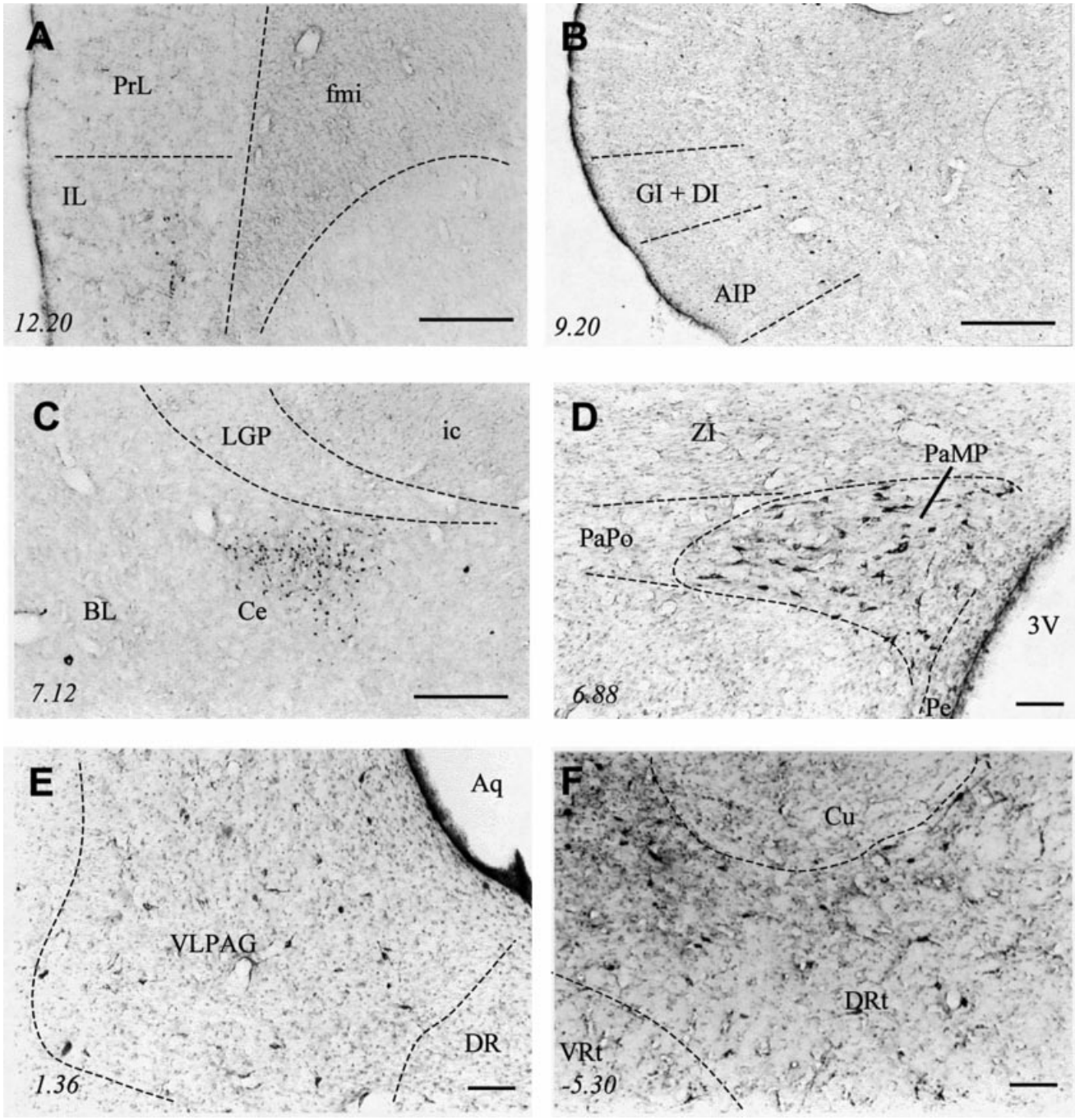

FIG. 3. Labelled neurons in the ipsilateral infralimbic (A) and insular (B) cortices, central amygdaloid (C) and paraventricular hypothalamic (D) nuclei, ventrolateral PAG (E) and contralateral DRt (F) following the large CTb injection shown in Fig. 1B. Scale bar, 150 μm. For abbreviations see list.

Anterograde labelling

Fibres and terminal boutons anterogradely labelled with BDA were observed mainly in the dorsal part of the DRt (DRtd) after cerebellar injections, and through the dorsoventral extension of the DRt [DRtd and ventral DRt (DRtv)] after hypothalamic (LH and Pa), VLPAG and RD (Fig. 4) injections.

Labelled fibres and terminals in the DRt occurred mainly ipsilaterally after cerebellar, hypothalamic and VLPAG injections, and exclusively contralaterally after RD injections.

Discussion

Technical considerations

Histologically, the DRt can be easily delimited from the Cu dorsally, the Sp5C laterally, the Sol dorsomedially and the VRt ventromedially. For the interpretation of the present findings, four possible sources of uncertainty inherent to the use of a tract-tracing technique were considered. First, the placement of CTb in the DRt; the small iontophoretic CTb injections resulted in restricted and perfectly defined injection sites, all located within the boundaries of the DRt

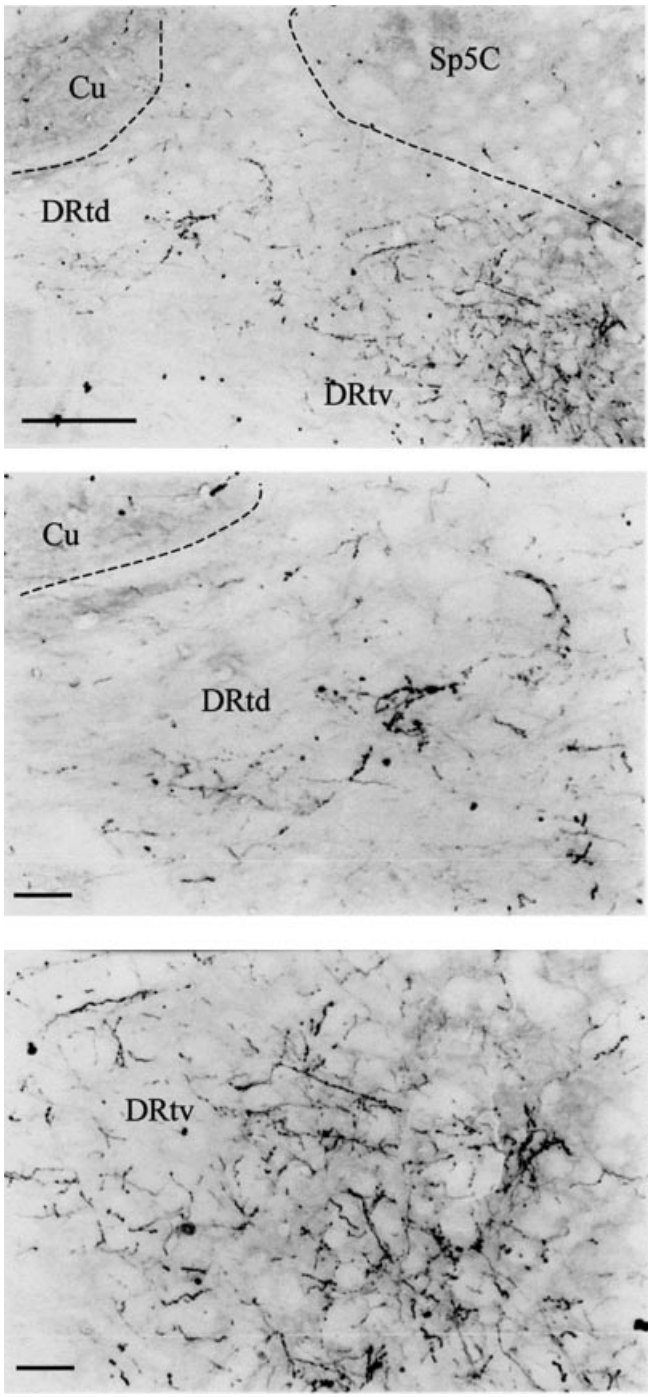

FIG. 4. Photomicrographs showing anterogradely labelled fibres and terminal boutons in the contralateral DRt following injections of BDA in the left red nucleus: (A) small magnification of the dorsoventral extension of the DRt, in which the location of the two subareas of the nucleus, the dorsal (DRtd) and the ventral (DRtv) Drt are indicated; (B and C) higher magnifications of the DRtd and DRtv areas, respectively, shown in A, which reveal details of the abundant anterograde labelling present in both areas of the nucleus. Scale bar, 150 μm. For abbreviations see list.

along the rostrocaudal extension of the nucleus. Larger injections, which were used in order to label larger numbers of cells, were also selected according to their inclusion inside the limits of the nucleus. Secondly, leakage of CTb: the micropipette track was examined and no leakage of CTb was detected, probably as a consequence of the small tip diameters of the micropipettes used and the time allowed for the micropipette to remain in the injection place after tracer administration. Thirdly, transneuronal transport: the possibility that trans-synaptic transport accounted for the labelling of cells receiving projections from the DRt can be ruled out, as CTb was shown not to be carried transneuronally after anterograde transport (Almeida *et al*., 1993). Fourthly, passing fibres: the possibility that fibres travelling in the injected DRt areas may contribute to some of the retrograde labelling observed does not need to be considered. In fact, previous observations have shown that no spinal labelling occurs after CTb injections into the spinothalamic tract (Lima *et al*., 1991), indicating that CTb is not picked up by passing fibres. Moreover, it has been demonstrated that, with CTb, anterograde or retrograde tracing by passing fibres does not occur when iontophoretic injections are made (Luppi *et al*., 1990; Angelucci *et al*., 1996). Taking into account all the above facts, it was concluded that DRt injections resulted exclusively in CTb retrograde labelling in the brain after tracer uptake by afferent terminals terminating in the DRt. Therefore, it was not considered necessary to make control CTb injections in medullary nuclei bordering the DRt.

A surprising result was the retrograde labelling present in the BPN and IO in rat 691 (Table 1). Spread of the tracer to nuclei close to the DRt cannot account for this observation as this rat received a small and well located injection. It is possible that this finding is due to the different rostrocaudal centre of the CTb injection (Fig. 1, interaural coordinates: –5.30 for rat 691 vs. –5.60 for rat 536) and, consequently, reflects different anatomical loci inside the DRt.

Brain afferents to the DRt: anatomical considerations

This is the first study addressing the systematic evaluation of the brain areas projecting to the DRt, through tracer injections applied directly to the nucleus. The only other work with retrograde tracer administration to the DRt was a detailed study on the cortical projections to the nucleus (Desbois *et al*., 1999).

Telencephalon

To the best of our knowledge, the projections from orbital cortex, globus pallidus and substantia innominata to the DRt were reported here for the first time.

Although our data are in general in accordance with the work of Desbois *et al*. (1999) concerning the dense afferent projection to the DRt from widespread contralateral cortical areas like the M1, M2, S1, S2 and insular cortices, we observed further labelling in other areas of the cortex. In fact, a small but consistent projection from the ipsilateral hemisphere of those cortical areas and an important projection from the contralateral prelimbic, infralimbic and orbital cortices are also described in the present study. The variation reported in these two studies probably reflect different sensitivities of the retrograde tracers used: wheat germ agglutinin–apo horseradish peroxidase–gold (WGA–ApoHRP–Au; Desbois *et al*. 1999) and CTb (the present study). If this is the case, we can conclude that CTb is a more sensitive tracer than WGA–ApoHRP–Au, especially if we take into account that in both studies the injections were restricted to the DRt and our small injections seem to be smaller than those in the other study. Although no direct comparisons have been made between the two tracers, this hypothesis is supported by retrograde-tracing studies which clearly showed that, in the spinothalamic and spino-DRt pathways, CTb labelled significantly more spinal cells than the tracers wheat germ agglutinin–horseradish peroxidase

(WGA–HRP) or free HRP after injections of similar size in the thalamus or DRt (Lima & Coimbra, 1988; Lima, 1990).

Diencephalon

To the best of our knowledge, the projections from the PF, posterior hypothalamic area and ZI, to the DRt were reported here for the first time. Interestingly, the PF is also one of the major efferent targets of the DRt in the brain (Villanueva *et al*., 1997), thereby suggesting the existence of a feedback loop between the DRt and PF.

The important projections originated in the Pa and LH have been referred to in previous anterograde tracing studies (Luiten *et al*., 1985; Allen & Cechetto, 1992); however, these only reported the strong ipsilateral projection. Such variability might be ascribed again to the injection site or tracer sensitivity. Indeed, it is possible that the injections of the anterograde tracer *Phaseolus vulgaris*-leucoagglutinin (PHA-L) in the Pa did not reach the neurons originating the contralateral projection (Luiten *et al*., 1985). In the case of the WGA–HRP injections in the LH, it is possible that the tracer lacks sufficient sensitivity to label the contralateral side of the projection (Allen & Cechetto, 1992).

Mesencephalon

To the best of our knowledge, the projections from the substantia nigra, RD, dorsal raphe nucleus and mesencephalic trigeminal nucleus to the DRt were reported here for the first time.

The ipsilateral projection from the DpMe to the DRt was also reported in a previous study (Jones & Yang, 1985; see also Veazey & Severin, 1980) but the contralateral projection, as seen here, was not detected, probably due to the lower sensitivity of the method employed (autoradiography). The bilateral projection from the caudal VLPAG terminating in the DRt was already mentioned (Chen & Aston-Jones, 1996; Odeh & Antal, 2001).

Pons

The projections from the basilar pontine nuclei, SubCV (see Westlund & Coulter, 1980) and reticulotegmental nucleus of the pons to the DRt were shown here for the first time.

The strong bilateral projection from both the medial and lateral PBN and KF (Saper & Loewy, 1980; Herbert *et al*., 1990; Krukoff *et al*., 1993) and the bilateral projections from the A_5 (Tavares *et al*., 1997) and LC (Jones & Yang, 1985; Fritschy & Grzanna, 1990) to the DRt have already been reported.

Cerebellum

In a previous study, the injection of ^{3}H-leucine/proline into the lateral and interposed cerebellar nuclei resulted in fibre and terminal labelling along all the ipsilateral dorsoventral extension of the DRt (Woodson & Angaut, 1984). Our findings extend these results by demonstrating a bilateral projection to the DRt from all the three groups of deep cerebellar nuclei (medial, interposed and lateral; see also Teune *et al*., 2000).

Medulla oblongata

To the best of our knowledge, the projections from the nucleus raphe magnus, inferior olive, paratrigeminal nucleus and ventral reticular nucleus to the DRt are reported here for the first time.

Our study confirms a very strong ipsilateral projection from the caudal part of the spinal trigeminal nucleus to the DRt (Esser *et al*., 1998), but also shows an important contralateral projection to the nucleus. Contrary to our results, the projection from the A_1 noradrenergic cell group was previously reported to be bilateral, probably due to the larger injection in the A_1 area in that study, which reached the nearby LRt (McKellar & Loewy, 1982). The latter has a bilateral projection to the DRt, as shown by the present study.

Functional considerations

The DRt seems to be an important recipient for the nociceptive information transmitted supraspinally as most DRt neurons (Villanueva *et al*., 1988) and superficial dorsal horn neurons projecting to the DRt (Almeida & Lima, 1997) are activated by noxious stimulation converging from all the body to the DRt (Villanueva *et al*., 1988). In addition, the nociceptive input is transmitted to the PF and ventromedial (VM) and reunions thalamic nuclei of the medial thalamus, to which the DRt projects massively (Villanueva *et al*., 1997; Monconduit *et al*., 1999). These nuclei project to the amygdala and hippocampus (Ottersen & Ben-Ari, 1979; Su & Bentivoglio, 1990), areas involved in emotional/affective and cognitive control (Lopes-da-Silva *et al*., 1990). They also project to large areas of the motor cortex (Donoghue & Parham, 1983) and/or dorsal (caudate–putamen complex) and ventral (nucleus accumbens) striatum (Berendse & Groenewegen, 1990). Altogether, these data indicate that the DRt belongs to the medial pain system (Melzack & Casey, 1968) and may be involved in processing the motor actions triggered by the motivational–affective afferent component of pain sensation.

The large spectrum of projections converging upon the DRt indicates that the activity of the nucleus is affected by, and involved in, several brain functions. However, in a classification effort, the brain areas projecting to the DRt were considered to belong to one or more of the following two broad functional systems.

Sensorimotor and pain control systems

A large spectrum of brain areas belonging to the supraspinal pain control system project to the DRt and may modulate its activity. Thus, areas shown to be antinociceptive, like the motor cortex, basal ganglia, amygdala, thalamus (PF, paraventricular thalamic nucleus, sensory thalamus), hypothalamus (LH, Pa, Arc, PH), the PAG–RVM circuit, noradrenergic brainstem areas (LC/subcoeruleus, PBN, KF, A_5, Sol, A_1) and the brainstem reticular formation (DpMe, CnF, VRt, VLMlat) were shown here to project to the DRt (reviewed by Lima & Almeida, 2002). On the other hand, areas traditionally known to be antinociceptive but presenting also pronociceptive actions, such as the RVM (Wei *et al*., 1999; Kovelowski *et al*., 2000) and Sol (Ren *et al*., 1990; Ness *et al*., 2000), also project to the DRt. Moreover, the ipsilateral and contralateral DRt itself, which has a primarily pronociceptive action (Almeida *et al*., 1996, 1999a), have short-projecting neurons inside its borders. Altogether, these data indicate that the DRt is a likely candidate to mediate the balance between inhibiting and facilitating nociceptive actions that may, respectively, turn off or turn on the descending facilitating action of the nucleus.

The DRt seems also to be implicated in the pain motor reaction itself, as it not only receives a strong projection from the motor cortex and several extrapyramidal motor areas (globus pallidus, substantia nigra, RD, basilar pontine nuclei, reticulotegmental nucleus of the pons, deep cerebellar nuclei, IO, PCRt and LRt), but also projects to the spinal ventral horn (Villanueva *et al*., 1995). Thus, it is possible that the DRt influences the motor reactions to noxious stimulation directly by modulating the activity of spinal motoneurons.

Autonomic and limbic systems

The activity of DRt neurons can also be influenced by the visceral motor system, which involves afferents from structures such as the dorsal motor nucleus of the vagus and the PrL and IL cortices. Other important forebrain autonomic centres (reviewed by Loewy, 1990) involved in the organism homeostasis and projecting to the DRt include the BST, Ce, Pa and LH. Most of the above autonomic centres belong also to the limbic system (where emotions are modulated; reviewed by Lopes-da-Silva *et al*., 1990), namely the prefrontal cortex (PrL, IL and OC), BST, Ce and SI. These data indicate that the DRt not only relays ascending nociceptive information related to the motivational–affective component of pain (see above), but its activity can also be modulated by the reaction triggered by that dimension of pain.

Brain afferents to the DRt: anterograde-tracing studies

We administered BDA to some of the brain areas that presented numerous retrogradely labelled cells after CTb injections in the DRt; all these injections confirmed, by the presence of abundant fibre- and bouton-labelling, that those areas project in fact to the DRt. Interestingly, the most dorsal portion of the DRt (DRtd; Almeida *et al*., 1995) is the only, or at least the main, area of the nucleus that receives projections from the various brain areas injected. The DRtd is the area of the nucleus that is reciprocally connected with the spinal cord lamina I through asymmetrical synapses, presumably excitatory (Almeida *et al*., 1993, 2000) and, thus, may underlie the descending facilitation of spinal nociception conveyed by the nucleus (Almeida *et al*., 1996, 1999a, 2000; Lima & Almeida, 2002). Moreover, the DRtd is the area that relays the transmission of ascending nociceptive transmission from the whole body to the VM (Monconduit *et al*., 1999; Desbois & Villanueva, 2001) which, in turn, projects diffusely to all areas of the ipsilateral cortex (Herkenham, 1979). Thus, one might conclude that the DRtd is an area of convergence of modulatory actions from the brain upon the ascending nociceptive input carried by the medial pain system.

Conclusion

In summary, the present findings, together with the current available literature, indicate that the DRtd might be at the centre of: (i) a spino-reticulo-thalamo-cortical nociceptive pathway that spreads the nociceptive information converging from all the body to large areas of the cortex; (ii) a supraspinal network that integrates information from areas of the autonomic, limbic, pyramidal, extrapiramidal and antinociceptive systems before triggering its output action; (iii) a descending nociceptive facilitating pathway that increases nociceptive transmission at the spinal dorsal horn level (Lima & Almeida, 2002).

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Abbreviations

3V, 3rd ventricle; 4V, 4th ventricle; 7, facial nucleus; 10, dorsal motor nucleus of the vagus; 12, hypoglossal nucleus; A1, A1 noradrenergic cell group; A5, A5 noradrenergic cell group; AI, agranular insular cortex; AIP, agranular insular cortex, posterior part; Aq, aqueduct of Sylvius; Arc, arcuate hypothalamic nucleus; Au1, primary auditory cortex; Au2, secondary auditory cortex; BDA, biotinylated-dextran amine; BL, basolateral amygdaloid nucleus; BPN, basilar pontine nuclei; BST, bed nucleus of the stria terminalis; cc, central canal; Ce, central amygdaloid nucleus; Cg1, cingulated cortex; CnF, cuneiform nucleus; CTb, cholera toxin subunit B; cu, cuneate fasciculus; Cu, cuneate nucleus; DAB, diaminobenzidine; DI, dysgranular insular cortex; DLPAG, dorsolateral periaqueductal grey; DMPAG, dorsomedial periaqueductal grey; DPGi, dorsal paragigantocellular nucleus; DpMe, deep mesencephalic nucleus; DRt, dorsal reticular nucleus; DRtd, dorsal reticular nucleus, dorsal part; DRV, dorsal raphe nucleus, ventral part; DRVL, dorsal raphe nucleus, ventrolateral part; Ect, ectorhinal cortex; fmi, forceps minor of the corpus callosum; GI, granular insular cortex; Gi, gigantocellular reticular nucleus; GiA, gigantocellular reticular nucleus, alpha part; GiV, gigantocellular reticular nucleus, ventral part; HRP, horseradish peroxidase; ic, internal capsule; IL, infralimbic cortex; Int, interposed cerebellar nucleus; IntA, interposed cerebellar nucleus, anterior part; IntPPC, interposed cerebellar nucleus, posterior parvicellular part; IO, inferior olive; IRt, intermediate reticular nucleus; KF, Kölliker–Fuse nucleus; Lat, lateral (dentate) cerebellar nucleus; LC, locus coeruleus; LGP, lateral globus pallidus; LH, lateral hypothalamic area; LPAG, lateral periaqueductal grey; LPB, lateral parabrachial nucleus; LPGi, lateral paragigantocellular nucleus; LRt, lateral reticular nucleus; M1, primary motor cortex; M2, secondary motor cortex; Me5, mesencephalic trigeminal nucleus; Medical, medial (fastigial) cerebellar nucleus; Mo5, motor trigeminal nucleus; MPB, medial parabrachial nucleus; OC, orbital cortex; Pa5, paratrigeminal nucleus; Pa, paraventricular hypothalamic nucleus; PAG, periaqueductal grey matter; PaMP, paraventricular hypothalamic nucleus, medial parvicellular part; PaPo, paraventricular hypothalamic nucleus, posterior part; PaR, pararubral nucleus; PB, phosphate buffer; PBN, parabrachial nuclei; PBS, phosphate buffer saline; PBST, phosphate buffer saline with triton; PCRt, parvocellular reticular nucleus; PF, parafascicular thalamic nucleus; PH, posterior hypothalamic area; PHA-L, Phaseolus vulgaris-leucoagglutinin; PnC, pontine reticular nucleus, caudal part; PnO, pontine reticular nucleus, oral part; PPTg, pedunculopontine tegmental nucleus; PRh, perirhinal cortex; PrL, prelimbic cortex; Pta, parietal association cortex pyx, pyramidal decussation; RD, red nucleus; RMg, raphe magnus nucleus; RNCD, substantia nigra, compact part; Ro, nucleus of Roller; ROb, raphe obscurus nucleus; RPa, raphe pallidus nucleus; RVM, rostroventromedial medulla; S1, primary somatosensory cortex; S2, secondary somatosensory cortex; scp, superior cerebellar peduncle (brachium conjunctivum); SI, substantia innominata; SNR, substantia nigra, reticular part; sol, solitary tract; Sol, nucleus of the solitary tract; SolM, nucleus of the solitary tract, medial part; SolVL, nucleus tractus solitarius, ventrolateral part; Sp5C, spinal trigeminal nucleus, caudal part; Sp5I, spinal trigeminal nucleus, interpolar part; STh, subthalamic nucleus; SubCV, subcoeruleus nucleus, ventral part; TC, tuber cinereum area; TeA, temporal association cortex; Ve, vestibular nuclei; VLMlat, ventrolateral medulla, lateral part; VLPAG, ventrolateral periaqueductal grey matter; VM, ventromedial thalamic nucleus; VRt, ventral reticular nucleus; WDR, wide-dynamic-range; WGA–ApoHRP–Au, wheatgerm agglutinin–apohorseradish peroxidase conjugate; WGA–HRP, wheat germ agglutinin–horseradish peroxidase conjugate; ZI, zona incerta.

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