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The projections of the midbrain periaqueductal grey to the pons and medulla oblongata in rats

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Abstract

It is now established that stimulation of the ventrolateral midbrain periaqueductal grey (PAG) evokes inhibition of nociceptive spinal neurons, which results in analgesia and a powerful attenuation of pain behaviour. It is postulated that the PAG exerts this inhibitory effect on spinal nociceptive functions through the activation of descending serotonergic and noradrenergic pathways that arise from the rostral ventromedial medulla (RVM) and pontine noradrenergic nuclei. To investigate the neuroanatomical substrate of this functional link between the PAG and RVM, as well as the pontine noradrenergic nuclei in the rat, we labelled axons that project from the ventrolateral PAG to various regions of the pons and medulla oblongata using the anterograde tracing substance, *Phaseolus vulgaris* leucoagglutinin. We demonstrated that some of PAG efferents really do terminate in the RVM and pontine noradrenergic nuclei, but a substantial proportion of them project to the intermediate subdivision of the pontobulbar reticular formation. Combining the axonal tracing with serotonin- and tyrosine-hydroxylase-immunohistochemistry, we also found that, in contrast to previous results, PAG efferents make relatively few appositions with serotonin- and tyrosine-hydroxylase-immunoreactive neurons in the RVM and pontine noradrenergic nuclei; most of them terminate in nonimmunoreactive territories. The results suggest that the ventrolateral PAG may activate a complex pontobulbar neuronal assembly including neurons in the intermediate subdivision of the pontobulbar reticular formation, serotonin- and tyrosine-hydroxylase-immunoreactive and nonimmunoreactive neurons in the RVM and pontine noradrenergic nuclei. This pontobulbar neural circuitry, then, may mediate the PAG-evoked activities towards the spinal dorsal horn resulting in the inhibition of spinal nociceptive functions.

Introduction

It has been known for more than a quarter of a century that the midbrain periaqueductal grey (PAG) plays a crucial role in endogenous pain attenuation mechanisms of the central nervous system (Fields *et al*., 1991; Sandküler, 1996; Mason, 1999). Independent discoveries demonstrated that electrical or chemical stimulation of the ventrolateral subdivision of the PAG suppresses a number of nociceptive reflexes and results in a profound analgesia (Gray & Dostrovsky, 1983; Lin *et al*., 1994; Gao *et al*., 1997; Waters & Lumb, 1997). These studies also demonstrated that the PAG presents a high degree of anatomical and functional organization. The most important functions that are associated with the PAG – defensive behaviour, cardiovascular functions or antinociception – are integrated by longitudinal columns of neurons that extend for varying distances along the rostrocaudal axis of the brainstem (Bandler & Shipley, 1994). From these longitudinally arranged cell assemblies, the ventrolateral cell column is associated with pain attenuation mechanisms.

It is postulated that the PAG exerts this powerful inhibition on nociceptive spinal neurons through a disynaptic, direct pathway. According to this theory, PAG efferents form monosynaptic contacts with spinally projecting, serotonergic and noradrenergic neurons in the RVM and pontine noradrenergic nuclei (Lakos & Basbaum, 1988; Reichling & Basbaum, 1990; Cameron *et al*., 1995; Bajic & Proudfit, 1999), and the monosynaptically activated descending serotonergic raphe–spinal and noradrenergic coerulo-spinal pathways terminate and release serotonin and noradrenaline in the spinal dorsal horn (Basbaum & Fields, 1984; Jones & Gebhart, 1986; El-Yassir & Fleetwood-Walker, 1990; Alhaider *et al*., 1991; Clark & Proudfit, 1991; Mason, 1999). The released serotonin and noradrenalin then produce a profound inhibition of nociceptive neurons in the spinal dorsal horn, resulting in a powerful attenuation of pain behaviour.

A growing body of experimental evidence, however, suggests that this attractive scheme outlining the neural basis of the PAG-evoked analgesia as a straightforward, disynaptic pathway may require re-evaluation. It has been reported that after electric stimulation of the nociceptive specific areas of the PAG, no serotonin-like cells were monosynaptically-activated in the rostral ventromedial medulla by single-pulse or train stimulation at antinociceptive intensities (Gao *et al*., 1997). These experiments suggest that monosynaptic excitation of serotonergic cells in the RVM is unlikely to be necessary for the antinociceptive effects of PAG stimulation.

However, if monosynaptic excitation from the PAG is not required for the activation of spinally projecting antinociceptive serotonergic and noradrenergic pathways, the following questions have to be raised. By which mechanism can the PAG activate the spinally projecting, antinociceptive serotonergic and noradrenergic pathways? What are the targets of the axons arising from the antinociceptive

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specific ventrolateral PAG? To answer these questions we studied the projections of the PAG to the pons and medulla oblongata in the rat. Preliminary observations from this experiment have been reported in abstract form (Antal & Odeh, 1998).

Materials and methods

Animals, injection of tracer and preparation of tissue sections

Experiments were carried out on seven Wistar–Kyoto rats (250–300 g, Gödöllõ, Hungary). All animal study protocols were approved by the Animal Care and Protection Committee at the University of Debrecen, Hungary, and were carried out in accordance with the European Communities Council Directives. The skull was opened with a dental drill under deep anaesthesia (35 mg/kg sodium pentobarbital, i.p.), while the animal was held in a stereotaxic frame. Glass micropipettes with a tip diameter of 20–30 μm were filled with a 2.5% solution of *Phaseolus vulgaris*-leucoagglutinin (PHA-L, Vector Laboratories, Burlingame, CA, USA), the highly sensitive anterograde tracing substance (Gerfen & Sawchenko, 1984). The tracer was injected unilaterally into the ventrolateral aspect of the PAG by iontophoresis, using positive direct current of 5 μA with a pulse duration of 7 s followed by 3 s intervals for a period of 15–20 min. The mediolateral, rostrocaudal and dorsoventral coordinates for the injection varied between 0.6 and 0.8 mm from the midline, 1.2 and 3.2 mm from the interaural line and 4.6 and 5.1 mm from the upper surface of the brain according to the atlas of Paxinos & Watson (1986). Each animal received two injections in a way that the two sites of injection were 0.5–1.0 mm distance from each other in the rostrocaudal direction.

In three animals, 3 weeks before the tracer application, we eliminated the central tegmental tract of descending hypothalamic fibres, again under deep sodium pentobarbital anaesthesia (35 mg/kg), by making a transverse lesion throughout the entire cross-sectional area of the PAG at the border of the diencephalon and mesencephalon.

After a 3-week survival period, the animals were reanesthetized and killed with an overdose of sodium pentobarbital (70 mg/kg) and perfused transcardially with Tyrode's solution, followed by fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The brainstem was removed, postfixed in the same fixative for 1–2 h, immersed, until it sank, in 10% and 20% sucrose dissolved in 0.1 M PB, frozen in liquid nitrogen, thawed in 0.1 M PB at room temperature and sectioned at 60 çm on a vibratome.

Immunocytochemistry

For immunocytochemical detection of PHA-L, free-floating sections of the brainstem were first incubated with biotinylated goat anti-PHA-L antibody (Vector Laboratories, Burlingame, CA, USA; diluted 1 : 1000) for 2 days at 4 °C. Then the sections were transferred into a solution of avidin–biotinylated-peroxidase complex (ABC; Vector Laboratories, Burlingame, CA, USA; diluted 1 : 100) for 4 h at room temperature. The immunoreaction was completed with a nickel-intensified diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) chromogen reaction (Hancock, 1984).

To reveal whether serotonergic and noradrenergic neurons in the brainstem establish close appositions with axon terminals arising from the PAG, a double-immunostaining procedure was performed in which the axonal tracing was combined with immunocytochemical detection of serotonin and tyrosine hydroxylase (TH), a good marker for catecholaminergic (including noradrenergic) neurons. First, the sections were incubated in a mixture of biotinylated goat anti-PHA-L antibody (Vector Laboratories, Burlingame, CA, USA; diluted 1 : 1000) and rabbit anti-serotonin (Chemicon Inc., Temecula, CA, USA; diluted 1 : 2000) or rabbit anti-TH antibodies (Eugene, Ridgefield Park, NJ, USA; diluted 1 : 1000). The immunological and immunocytochemical characteristics of anti-serotonin and anti-TH antibodies have been extensively tested and published earlier (Morrison-Graham *et al*., 1990; Vogel & Weston, 1990; English *et al*., 1992; Wang *et al*., 1992). Subsequently, the sections were transferred into a mixture of ABC (diluted 1 : 100) and goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; diluted 1 : 200) and left overnight at 4 °C. The PHA-L-labelled axons and axon terminals were visualized by a nickel-enhanced DAB chromogen reaction (Hancock, 1984). The sections were then treated with a rabbit-peroxidase anti-peroxidase complex (DAKO, Copenhagen, Denmark; diluted 1 : 100), and the immunostaining for serotonin or TH was completed with a chromogen reaction using DAB alone. Before the antibody treatments, sections were kept in 20% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 50 min. All of the antibodies were diluted in 0.01 M phosphate buffered saline (PBS, pH 7.4) to which 0.1% Triton X-100 and 1% normal goat serum (Vector Laboratories, Burlingame, CA, USA) were added. Between incubations in the antibody solutions, sections were rinsed three times for 30 min in the same buffer. Sections were mounted on gelatin-coated slides and covered with Permount neutral medium (Fluka, Buchs, Switzerland).

Three-dimensional reconstruction of the distribution of anterogradely labelled axon terminals and immunostained neurons

The distribution of PHA-L labelled axon terminals, and serotonin-immunoreactive (S-IR) and TH-immunoreactive (TH-IR) neurons in the brainstem were investigated in serial sections. Sections were cut from the site of PHA-L injection to the pyramidal decussation. Keeping their consecutive order, every fourth section was double-stained for PHA-L and serotonin, whereas, the consecutive ones were reacted for PHA-L and TH. By using the on-line digitizing mode of a Neurolucida 3-D reconstruction system, installed onto a Leitz Laborlux microscope with a motorized stage, the contours of these sections and the coordinates marking the location of the anterogradely labelled axon terminals, and also S-IR and TH-IR neurons, were fed into a computer (IBM PC 486). The individual sections were merged in consecutive order, and the stack of the sections with labelled axon terminals and immunostained neurons were rotated around axes of the three-dimensional coordinate system. The number of labelled axon terminals and S-IR and TH-IR neurons were counted. Close appositions between labelled axon terminals and immunostained neurons were also evaluated.

Results

Injection site of PHA-L

The tracer was delivered into the ventrolateral area of the midbrain PAG (Figs 1 and 2). In addition to the PAG, however, the tracer clearly spread into the adjacent midbrain tegmentum. As detected by the immunostaining, many cells incorporated PHA-L within the site of injection, but labelled cell bodies were only occasionally found outside this area. The appearance of the labelled cells, as well as the injection site, was similar to those reported in previous studies (Gerfen & Sawchenko, 1985; Wouterlood & Groenewegen, 1985).

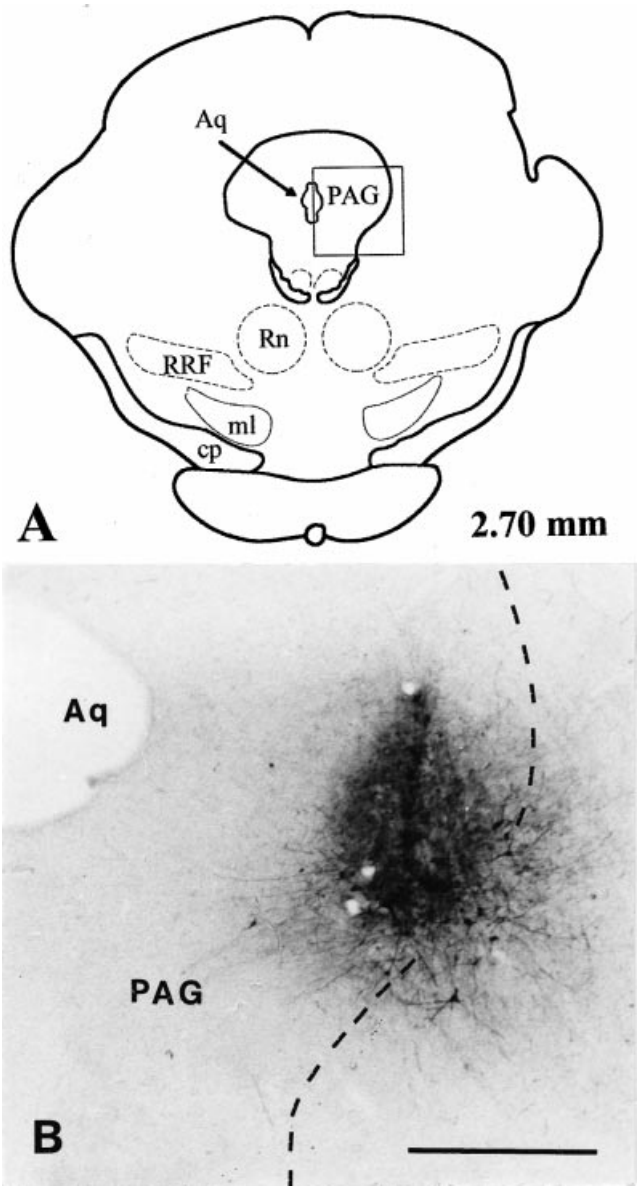


FIG. 1. (A) Camera lucida drawing of a transverse section of the mesencephalon at the level of the injection site of PHA-L. Rostrocaudal coordinate relative to the interaural line is indicated according to Paxinos & Watson (1986) (B) The photomicrograph shows an injection site (the area outlined in A) of PHA-L in animal PAG1. Aq, cerebral aqueduct; cp, cerebral peduncle; ml, medial lemniscus; PAG, periaqueductal grey matter; Rn, red nucleus; RRF, retrorubral field. Scale bar, 500 μm.

Distribution of PHA-L labelled axon terminals in the pons and medulla oblongata

Following unilateral PHA-L injections into the PAG, immunostained axons and varicose axon terminals (Fig. 3) were widely scattered in the pons and medulla oblongata (Fig. 4). Numerous axons descended and terminated ipsilateral to the site of injection, whereas, others crossed the midline at the level of the mesencephalon and projected to the contralateral side. The number of axon varicosities found on the ipsilateral side always outnumbered those that were recovered contralaterally. Although the numbers of the labelled axon terminals varied from animal to animal, presumably reflecting the differences in the sites and sizes of PHA-L delivery, the distribution of the terminals were very similar in the seven animals that were investigated in this study. For quantitative studies and 3-dimensional reconstruction we randomly selected three animals. Figure 2 illustrates sites of PHA-L injections in these animals, whereas Figs 4, 7 and 8 show experimental data obtained from one of them.

In the rostral pons (Fig. 4C), most of the labelled axon terminals were observed in the noradrenergic cell groups. Within the confines of these nuclei, most of the terminals were distributed in the ventral and dorsal subdivisions of the locus subcoeruleus and in the locus coeruleus, whereas, the density of labelling was lower in the alpha subdivision of the locus subcoeruleus and A5 cell group. In addition to this, a moderate density of varicose fibres was seen in the parabrachial nucleus, laterodorsal tegmental nucleus, pontine reticular nucleus, motor and principal sensory nuclei of the trigeminal nerve, and superior olive.

In the caudal pons and rostral medulla oblongata (Fig. 4D and E), a substantial number of terminals were recovered in the rostral ventromedial medulla (RVM). Within the confines of the RVM, most of the terminals were concentrated in the gigantocellular reticular nucleus pars alpha and lateral paragigantocellular nucleus, whereas, the raphe magnus and dorsal paragigantocellular nuclei were supplied only by a moderate number of terminals. In addition to the RVM, the intermediate reticular nucleus was also densely packed with labelled terminals, and a moderate number of axon varicosities was also seen in the parvocellular reticular nucleus and the motor nucleus of the facial nerve.

In the caudal medulla (Fig. 4F), labelled terminals were seen in almost equal densities in the following nuclei: nucleus of the solitary tract, nucleus of the Probst bundle, nucleus ambiguus, nucleus retroambiguus, ventrolateral reticular nucleus, dorsal and ventral medullary reticular fields.

Relationship between PHA-L labelled axon terminals and S-IR neurons

In addition to heavy labelling of PAG efferents and their terminals, we have obtained a substantial immunostaining also for serotonin (Fig. 5). Most of the S-IR dendrites could be traced from the immunostained perikarya for a distance of at least 200–300 μm. In many cases, immunostained dendrites left the section that contained their somata and entered the consecutive section. Close appositions made by PHA-L labelled axon terminals were also investigated on these distal dendritic segments.

Evaluation of sections double-stained for PHA-L and serotonin showed that S-IR neurons in the brainstem received close appositions from terminals of PAG efferents only in moderate numbers (Fig. 5). Most of the terminals were sitting on distal dendrites and only a few of them were apposed to proximal dendrites or somata of S-IR neurons (Fig. 5). Most of the serotonin-containing neurons that established close appositions with labelled terminals were seen within the confines of the RVM, but some of them were found also in the rostral pons and caudal medulla oblongata (Fig. 7). In order to estimate the strength of the PAG projection to the RVM and S-IR neurons in particular, varicosities along the PHA-L labelled axons, regarded as potential synaptic sites of the terminals, were counted in every fourth section of the pons and medulla oblongata in three animals. From the 8663, 23138 and 25190 terminals counted in the individual animals, only 9.0%, 21.1% and 20.8%, respectively, were found within the confines of the RVM, and even here most of these terminals were distributed in nonserotonin immunoreactive territories (Fig. 5). Only 0.8%, 1.0% and 0.7% of the total number of terminals (9.1%, 4.8% and 3.3%, respectively, of those that terminated in the RVM) were apposed to S-IR neurons of the RVM.

Relationship between PHA-L labelled axon terminals and TH-IR neurons

Immunostaining for TH was even stronger than that we have obtained for serotonin. In addition to somata, a substantial compartment of the dendritic tree of stained neurons also showed a strong immunoreactivity. Most of the TH-IR dendrites could be traced from their perikarya for several hundreds of micrometers. Similarly to S-IR dendrites, many of the TH-immunostained dendrites also left the section that contained their somata and entered the consecutive section. Close appositions made by PHA-L labelled axon terminals were also investigated on these distal dendritic segments.

Close appositions between PHA-L labelled PAG efferents and neurons immunoreactive for TH were only occasionally found in the pons and medulla oblongata (Fig. 6). Stained PAG terminals were apposed mostly to distal dendrites, whereas, axosomatic appositions were only sporadically seen (Fig. 6). Most of the TH-IR neurons that established close appositions with labelled

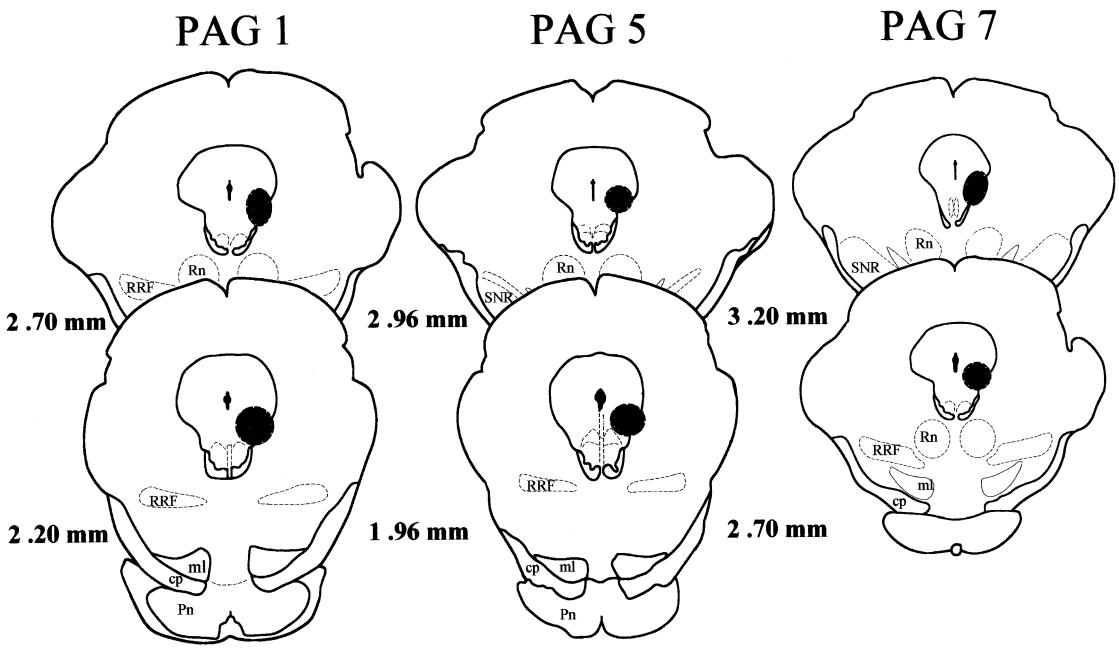


FIG. 2. Camera lucida drawings of frontal sections of the mesencephalon showing the locations of PHA-L deposits in three animals. Rostrocaudal coordinates relative to the interaural line are indicated according to Paxinos & Watson (1986). cp, cerebral peduncle; ml, medial lemniscus; Pn, pontine nuclei; Rn, red nucleus; RRF, retrorubral field and SNR, substantia nigra.

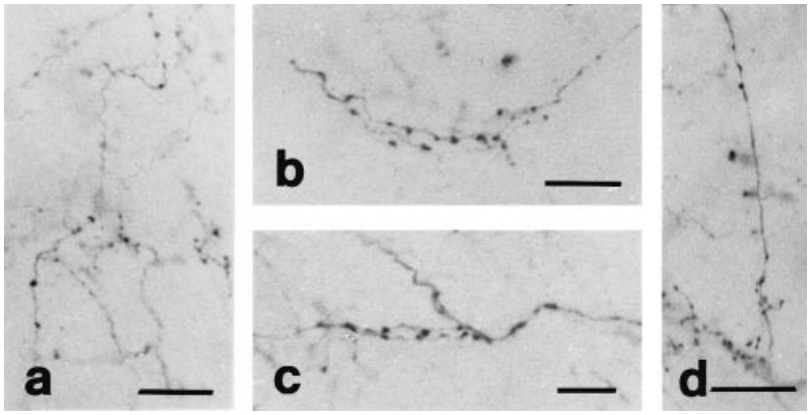
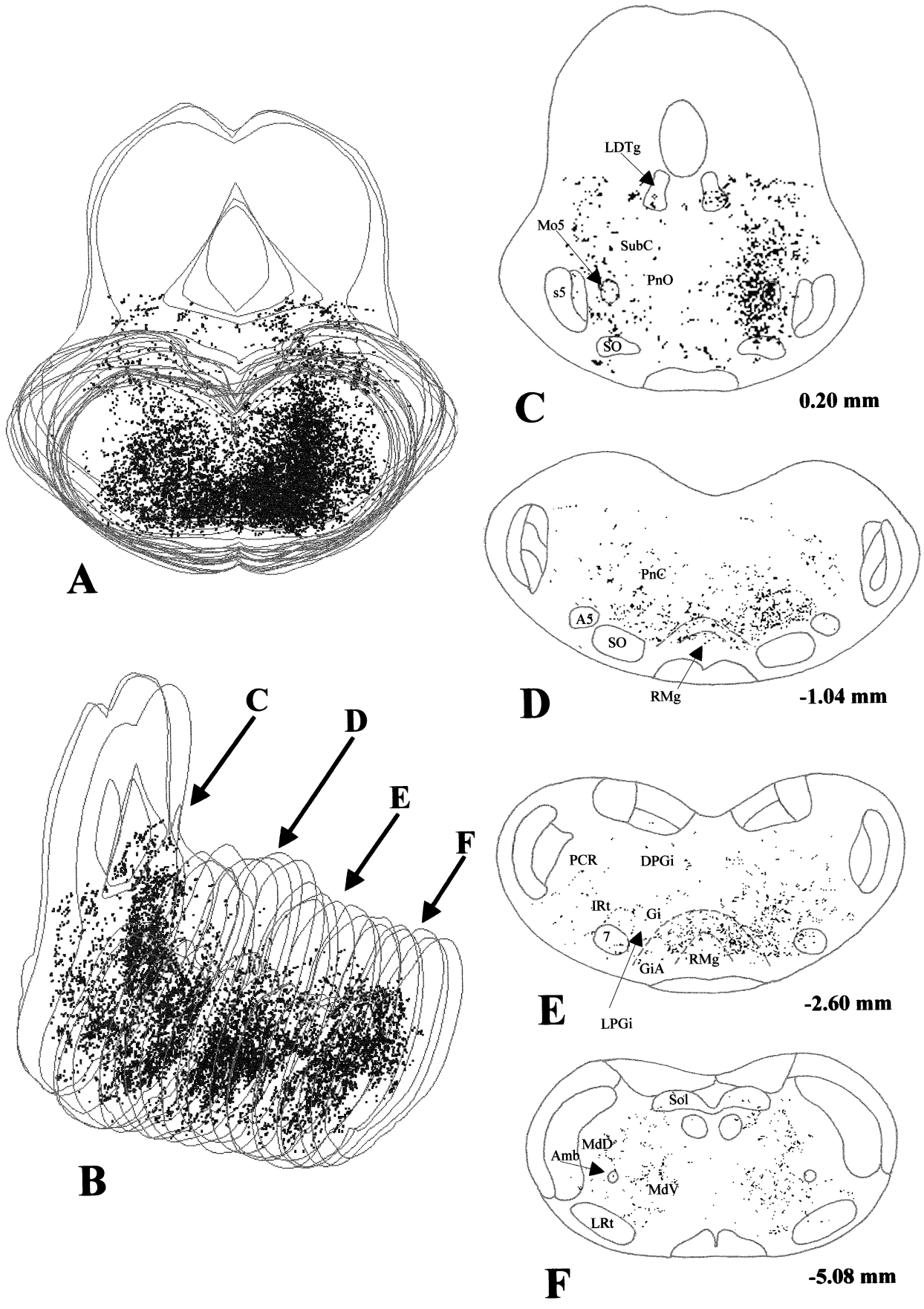


FIG. 3. Photomicrographs showing PHA-L labelled axons and axon terminals that arise from the ventrolateral PAG and terminate in various parts [(a) intermediate reticular nucleus; (b and c) lateral paragigantocellular nucleus; (d) dorsal paragigantocellular nucleus] of the pons and medulla oblongata. Scale bars, 50 μm.

FIG. 4. Schematic representation of the distribution of the PHA-L labelled axon terminals in 3-dimensional representations (A and B) and selected sections of the pons (C and D) and medulla oblongata (E and F). Each dot represents a labelled terminal. Data were obtained from animal PAG5. Rostrocaudal coordinates relative to the interaural line are indicated according to Paxinos & Watson (1986). Amb, ambiguus nucleus; A5, A5 noradrenergic cell group; DPGi, dorsal paragigantocellular nucleus; IRt, intermediate reticular nucleus; Gi, gigantocellular reticular nucleus; GiA, gigantocellular reticular nucleus pars alpha; LDTg, laterodorsal tegmental nucleus; LPGi, lateral paragigantocellular nucleus; LRt, lateral reticular nucleus; MdV, ventral medullary reticular nucleus; MdD, dorsal medullary reticular nucleus; Mo5, motor trigeminal nucleus; 7, motor facial nucleus; PCR, parvocellular reticular nucleus; PnC and PnO, pontine reticular nucleus (caudal and oral); RMg, raphe magnus nucleus; S5, sensory trigeminal nucleus; SO, superior olive; Sol, nucleus of the solitary tract; SubC, nucleus subcoeruleus.


A
B
C
D
E
F
LDTg
Mo5
SubC
PnO
s5
SO
0.20 mm
PnC
A5
SO
RMg
-1.04 mm
PCR
DPGi
IRt
Gi
7
GiA
RMg
LPGi
-2.60 mm
Sol
MdD
Amb
MdV
LRt
-5.08 mm

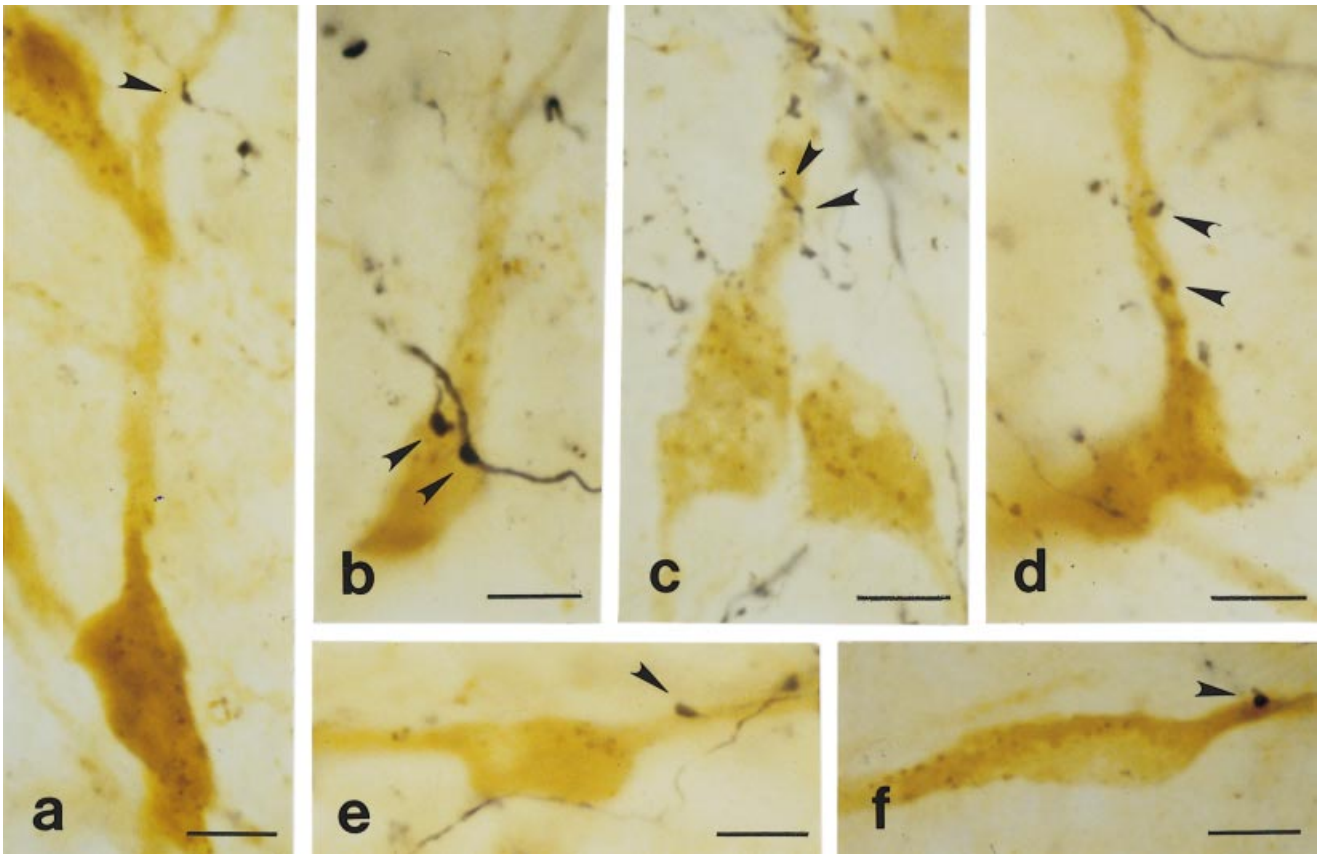


FIG. 5. Photomicrographs of sections double-immunostained for the simultaneous visualization of PHA-L labelled axons and axon terminals that arise from the ventrolateral PAG (dark blue or black) and S-IR neurons (brown) that are located in the raphe magnus (c and d), gigantocellular reticular nucleus pars alpha (a and b) and lateral paragigantocellular nucleus (e and f). Arrowheads point to labelled axon terminals that are in close apposition to dendrites or somata of S-IR neurons. Scale bars, 20 μm.

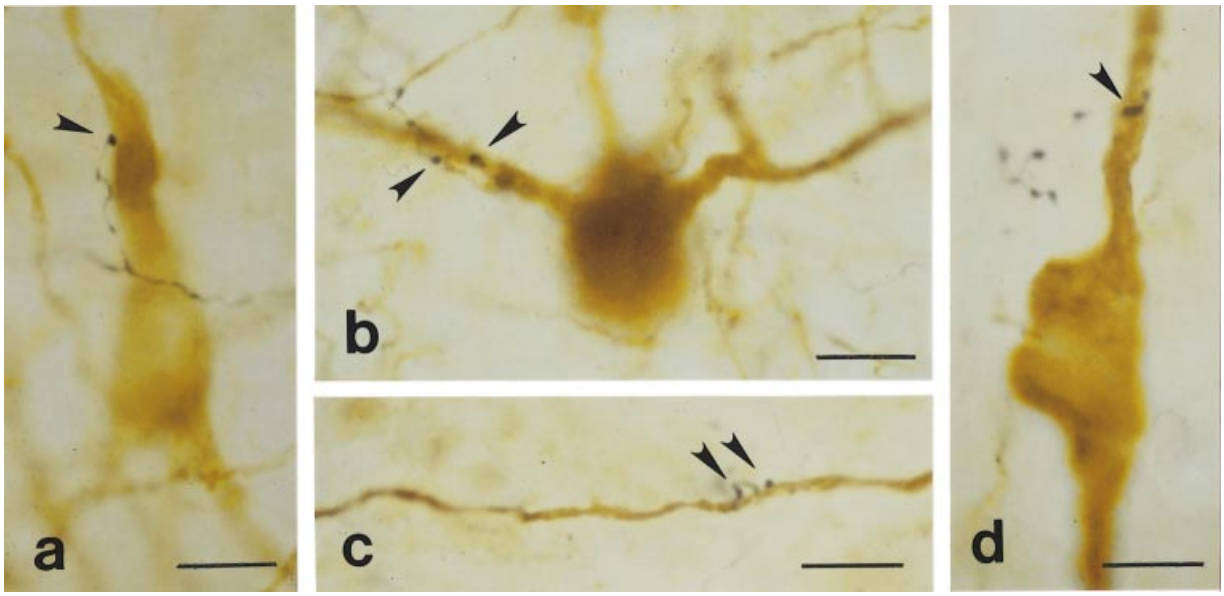


FIG. 6. Photomicrographs of sections double-immunostained for the simultaneous visualization of PHA-L labelled axons and axon terminals that arise from the ventrolateral PAG (dark blue or black) and TH-IR neurons (brown) that are located in the locus subcoeruleus (b–d) and A5 nucleus (a). Arrowheads point to labelled axon terminals that are in close apposition to dendrites or somata of TH-IR neurons. Scale bars, 20 μm.

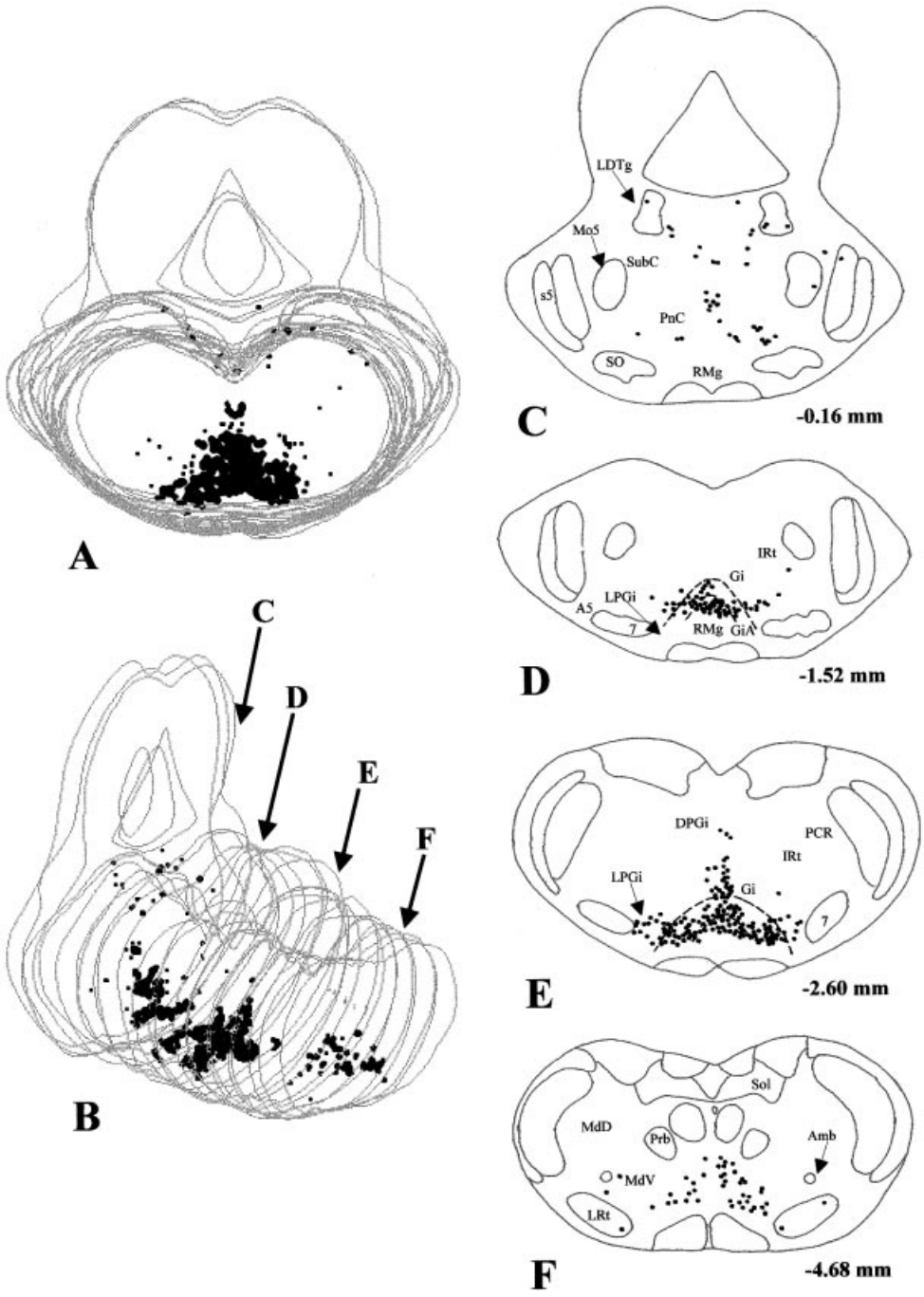


FIG. 7. Schematic representation of the distribution of PHA-L labelled axon terminals arising from the ventrolateral PAG that established close appositions with S-IR dendrites or perikarya in 3-dimensional representations (A and B) and selected sections of the pons (C and D) and medulla oblongata (Eand F). Each dot represents a PHA-L labelled axon terminal that established close apposition with a S-IR dendrite or perikaryon. Data were obtained from animal PAG5. Rostrocaudal coordinates relative to the interaural line are indicated according to Paxinos & Watson (1986). Amb, ambiguus nucleus; A5, A5 noradrenergic cell group; DPGi, dorsal paragigantocellular nucleus; IRt, intermediate reticular nucleus; Gi, gigantocellular reticular nucleus; GiA, gigantocellular reticular nucleus pars alpha; LDTg, laterodorsal tegmental nucleus; LPGi, lateral paragigantocellular nucleus; LRt, lateral reticular nucleus; MdV, ventral medullary reticular nucleus; MdD, dorsal medullary reticular nucleus; Mo5, motor trigeminal nucleus; 7, motor facial nucleus; PCR, parvocellular reticular nucleus; PnC, pontine reticular nucleus (caudal); Prb, nucleus of the Probst's bundle; RMg, raphe magnus nucleus; S5, sensory trigeminal nucleus; SO, superior olive; Sol, nucleus of the solitary tract; SubC, nucleus subcoeruleus.

terminals were seen in the ventral subdivision of the locus subcoeruleus and the A5 cell group (Fig. 8). In order to estimate the strength of PAG projection to the pontine noradrenergic cell groups, and TH-IR neurons in particular, varicosities, in three animals, along the stained axons in the PHA-L/TH double-stained sections were counted in the same way as in the PHA-L/serotonin double-stained sections. Of the 10163, 14412 and 28141 terminals counted in the individual animals, only 14.0%, 13.3% and 10.9%,

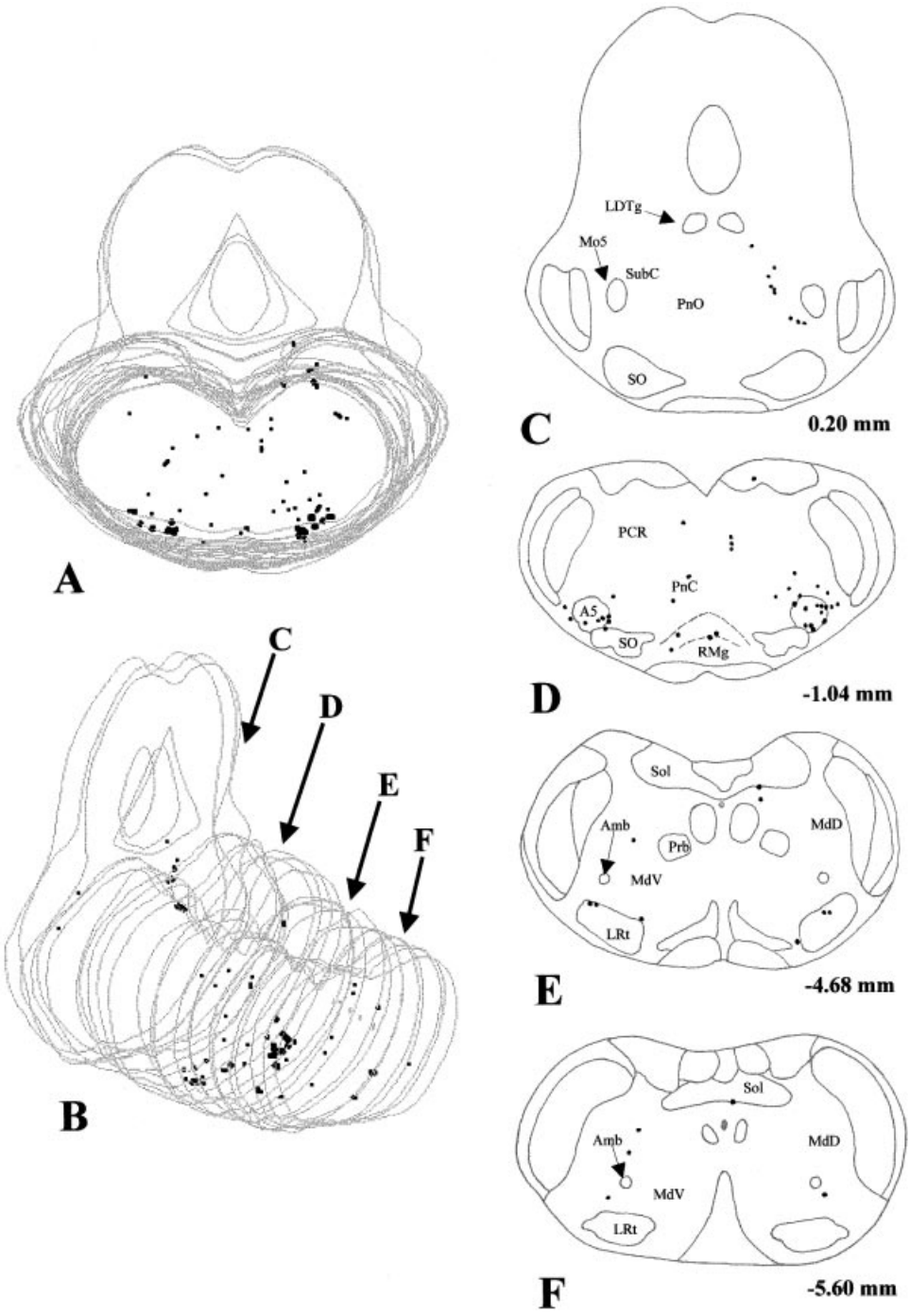


FIG. 8. Schematic representation of the distribution of PHA-L labelled axon terminals arising from the ventrolateral PAG that established close appositions with TH-IR dendrites or perikarya in 3-dimensional representations (A and B) and selected sections of the pons (C and D) and medulla oblongata (E and F). Each dot represents a PHA-L labelled axon terminal that established close apposition with a TH-IR dendrite or perikaryon. Data were obtained from animal PAG5. Rostrocaudal coordinates relative to the interaural line are indicated according to Paxinos & Watson (1986). Amb, ambiguus nucleus; A5, A5 noradrenergic cell group; DPGi, dorsal paragigantocellular nucleus; LDTg, laterodorsal tegmental nucleus; LRt, lateral reticular nucleus; MdV, ventral medullary reticular nucleus; MdD, dorsal medullary reticular nucleus; Mo5, motor trigeminal nucleus; PCR, parvocellular reticular nucleus; PnC and PnO, pontine reticular nucleus (caudal and oral); Prb, nucleus of the Probst's bundle; RMg, raphe magnus nucleus; SO, superior olive; Sol, nucleus of the solitary tract; SubC, nucleus subcoeruleus.

respectively, were found within the confines of the pontine noradrenergic cell groups (locus coeruleus, locus subcoeruleus and A5 cell group), where most of the terminals were distributed in non-TH-IR territories (Fig. 6). Only 0.5%, 0.6% and 0.4% of the total number of terminals (3.6%, 4.3% and 4.0%, respectively, of those that terminated in the pontine noradrenergic cell groups) established close appositions with TH-IR neurons of the pontine noradrenergic cell groups in the individual animals.

Discussion

Labelling of PAG efferents with Phaseolus vulgaris *leucoagglutinin*

To label axon terminals of PAG efferent neurons in the pons and medulla oblongata, PHA-L, the highly sensitive anterograde tracer was injected iontophoretically into the antinociceptive specific ventrolateral area of the PAG. A long line of experimental evidence indicates that after iontophoretic application, PHA-L is internalized almost exclusively by perikarya and dendrites of neurons located within the region of the injection site (Gerfen & Sawchenko, 1984, 1985; Wouterlood & Groenewegen, 1985; Wouterlood *et al*., 1987). Consequently, most of the labelled axon terminals that we encountered in this study may represent terminals of neurons that are located within the confines of PHA-L injection sites. However, it has also been reported that some local axon terminals and fibres, that originate outside the injection site and run through the area infiltrated by the tracer, can also take up and transport PHA-L (Cliffer & Giesler, 1988; Chen & Aston-Jones, 1998). Therefore, in principle, in addition to axons of neurons that are located within the area infiltrated by PHA-L, axons of neurons in the posterior hypothalamus, that are reported to descend in the lateral/ventrolateral regions of the diencephalomesopontine PAG (Cecheto & Saper, 1988; Vertes & Crane, 1996), could also take up and transport the lectin causing unspecific labelling in the brainstem. However, it is highly probable that this nonspecific labelling was very weak in our experiments. First, the labelling of fibres of passage has always been found to be poor in the central nervous system of mammals (Gerfen & Sawchenko, 1984, 1985; Wouterlood & Groenewegen, 1985; Wouterlood *et al*., 1987). Second, in three animals in which we eliminated the central tegmental tract of descending hypothalamic fibres (by making a transverse lesion throughout the entire cross-sectional area of the PAG at the border of the diencephalon and mesencephalon 3 weeks before the tracer application) the distribution and density of the labelled axon terminals were identical to those that were obtained in animals with intact descending hypothalamic projections. Therefore, it seems that the unspecific labelling of fibres of passage does not interfere with the major findings of the present study.

In addition to the PAG, the tracer clearly spread into the adjacent midbrain tegmentum. Therefore, it is necessary to raise the question whether the labelling of axon terminals of neurons in the midbrain tegmentum adjacent to the PAG might cause confusion in the interpretation of the results. A long line of experimental evidence indicates that a partial labelling of the medial midbrain tegmental field should not result in any misinterpretation of the results obtained in this study. It has been demonstrated that the ventrolateral PAG and the adjacent area of the midbrain tegmentum form a functionally homogeneous territory (Gray & Dostrovsky, 1983; Holstege, 1989; Bernard *et al*., 1995; Craig, 1995; Keay *et al*., 1997). The electric stimulation of both fields evokes profound inhibition of nociceptive-specific and wide dynamic range neurons in the spinal dorsal horn (Carstens *et al*., 1980, 1981; Gray & Dostrovsky, 1983), and the two areas may also work together in the initiation of vocalization (Holstege, 1989). The spinal antinociceptive effects of the tegmentum, e.g. inhibition of heat-evoked discharges of spinal neurons, are even stronger than that evoked by the activation of the ventrolateral PAG (Carstens *et al*., 1980). In addition, the ventrolateral PAG and the adjacent midbrain tegmentum receive identical ascending sensory inputs from the spinal cord (Bernard *et al*., 1995; Craig, 1995; Keay *et al*., 1997). It appears that the medial midbrain tegmental field can be regarded as an area that is closely related in function to the ventrolateral PAG, thus, a partial labelling of this territory is unlikely to lead us to dubious conclusions concerning the distribution of the genuine population of PAG efferents in the pons and medulla oblongata.

The termination pattern of ventrolateral PAG efferents in the pons and medulla oblongata

The termination pattern of efferent fibres arising from the PAG has been investigated extensively in the pons and medulla oblongata using degeneration (Hamilton, 1973) and various anterograde tract tracing methods (Mantyh, 1983; Holstege, 1989; Meller & Dennis, 1991; Cameron *et al*., 1995; Bajic & Proudfit, 1999). Most of the results obtained in the present experiment are in general agreement with the findings of these previous observations. However, here we also presented data that advanced our understanding of possible functional links between the PAG and RVM, pontine noradrenergic nuclei as well as other territories of the brainstem. We have shown that in contrast to previous results, PAG efferents make relatively few appositions with S-IR and TH-IR neurons in the RVM and pontine noradrenergic nuclei, most of them terminate in non-S-IR and non-TH-IR territories. We have also demonstrated that 20–30% of axon terminals arising from the ventrolateral PAG project to the intermediate subdivision of the pontobulbar reticular formation, suggesting that efferent fibres to this area of the reticular formation may represent a functionally important part of the projection system of the ventrolateral PAG.

Similar to our results, it has been demonstrated that the ventrolateral PAG innervates the parabrachial nuclei and suggested that this innervation functions as a behavioural state-dependent filter system that modulates ascending nociceptive information as it is relayed through the parabrachial nuclei to forebrain sites (Krout *et al*., 1998). It has also been shown that a number of neurons in the ventrolateral PAG send efferent fibres to the trigeminal sensory complex, presumably suppressing the activity of nociceptive neurons in the trigeminal system (Li *et al*., 1993). Confirming results of previous studies, here we also demonstrated that the ventrolateral PAG projects to the branchiomotor nuclei (motor nuclei of the trigeminal, facial, vagus and accessory nerves) and nucleus retroambiguus via which the PAG may produce excitation of motoneurons involved in vocalization (Holstege, 1989; Ennis *et al*., 1997; Holstege *et al*., 1997). Previous anatomical and physiological studies have also shown, similar to our present observations, that the ventolateral PAG innervates the rostral and caudal ventrolateral medulla through which it may regulate cardiovascular and respiratory functions (Van Bockstaele *et al*., 1991; Chen & Aston-Jones, 1995, 1996; Henderson *et al*., 1998).

There is general agreement in the literature that the ventrolateral PAG projects to the RVM and pontine noradrenergic cell groups, through which it exerts profound effects on somatomotor (Jankowska *et al*., 1968; Lai & Siegel, 1990), cardiovascular (Lovick, 1993; Bandler & Shipley, 1994) and nociceptive (Basbaum & Fields, 1984; Fields *et al*., 1991; Bandler & Shipley, 1994; Sandküler, 1996; Waters & Lumb, 1997; Mason, 1999) information processing mechanisms of the spinal cord. It has also been demonstrated, however, that the termination patterns of PAG efferents within the confines of the RVM, and especially the noradrenergic nuclei, show a wide variety among the different strains of rats (Clark & Proudfit, 1991; Cameron *et al*., 1995; Bajic & Proudfit, 1999). In the light of this, our present findings are quite unique, as to the best of our knowledge, this is the first account on this matter in Wistar–Kyoto rats. On the one hand, we found that the ventrolateral PAG projects strongly to the ventral and dorsal subdivisions of the locus

subcoeruleus and the locus coeruleus, whereas, the alpha subdivision of the locus subcoeruleus and A5 cell group is supplied weakly by terminals of the ventrolateral PAG in Wistar–Kyoto rats. On the other hand, within the RVM, most of the terminals were recovered in the gigantocellular reticular nucleus pars alpha and lateral paragigantocellular nucleus, whereas, the raphe magnus and dorsal paragigantocellular nuclei were supplied only by a moderate number of terminals.

According to the most widely accepted theory, most of the PAG efferents that project to the RVM and pontine noradrenergic cell groups form monosynaptic contacts with spinally projecting serotonergic and noradrenergic neurons (Lakos & Basbaum, 1988; Reichling & Basbaum, 1990; Bajic & Proudfit, 1999) and excite them through NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor mechanisms (Aimone & Gebhart, 1986; Wiklund *et al*., 1988). In support of this idea, serotonin receptor antagonists attenuate the antinociceptive effects of PAG stimulation on dorsal horn cells (Yaksh, 1979; Carstens *et al*., 1981; Yezierski *et al*., 1982; Paul & Phillips, 1986; Peng *et al*., 1996). Neurotoxic depletion of serotonin also results in the attenuation of PAG-evoked inhibition of dorsal horn neurons (Carstens *et al*., 1981). Combining experimental degeneration of PAG efferents with immunocytochemical detection of serotonin, however, Lakos & Basbaum (1988) found that in addition to serotonergic neurons, nonserotonergic cells in the RVM also receive monosynaptic inputs from the PAG. In addition, a recent study, in which the effects of PAG stimulation was tested on RVM neurons, provided little evidence for the existence of monosynaptic connections between PAG and serotoninergic-like cells in the RVM (Gao *et al*., 1997). No serotonin-immunorecative cells were activated by single pulse or train stimulation of the PAG at antinociceptive intensities. The results of the present experiment appear to be in a good agreement with the findings of Gao *et al*. (1997). We have observed most of the PAG efferents in non-S-IR territories of the RVM, and only $< 10\%$ of the labelled terminals recovered within the confines of the RVM were seen to establish close appositions with S-IR neurons. This suggests that monosynaptic activation of serotonergic cells in the RVM is unlikely to be necessary for the nociceptive modulatory effects of PAG stimulation, at least in Wistar–Kyoto rats.

Here we have also demonstrated that the projection of the ventrolateral PAG to pontine noradrenegic cell groups and TH-IR cells is even weaker than that to the RVM and S-IR neurons. As we found $< 5\%$ of the PAG efferents that terminate in the pontine noradrenergic cell groups may establish close appositions with TH-IR, presumably noradrenergic, neurons. Confirming our results, several other reports did not find any significant projections from the ventrolateral PAG to locus coeruleus in the rat, using anterograde tracing with PHA-L (Ennis *et al*., 1991; Van Bockstaele *et al*., 1991) or retrograde tracing with wheat germ agglutinin conjugated to horseradish peroxidase (Ennis *et al*., 1991). This indicates that, as is the case with serotonergic cells in the RVM, monosynaptic activation of noradrenergic cells in the pontine noradrenergic cell groups is also unlikely to play a substantial role in the mediation of signals from the ventrolateral PAG to the spinal cord. However, we have to concede that the relationship between PAG afferents and noradrenergic cells in the pons does not appear so clear-cut as was suggested above. That is, it has recently been reported that the ventrolateral PAG projects strongly to the pontine noradrenergic cell groups and a substantial number of terminals arising from the PAG establishes close appositions with TH-IR neurons within these nuclei in the Sasco substrain of Sprague–Dawley rats (Bajic & Proudfit, 1999). It is difficult to explain the differences between our results and those of Bajic & Proudfit (1999), but it is likely that the reported differences in the projections of the ventrolateral PAG to pontine noradrenergic cell groups may arise from at least two sources. First, injections made by Bajic & Proudfit (1999) were restricted to the ventrolateral PAG, whereas, in addition to the PAG the tracer clearly spread into the adjacent midbrain tegmentum in our experiments. The differences in the precise locus of PHA-L may well account for the reported differences in the termination fields of labelled axon terminals. Second, it may reflect real differences between neural circuits in the Sasco substrain of Sprague–Dawley and in other strains of rats. This notion is reinforced by other reports that document fundamental differences in the spinal projections (Clark & Proudfit, 1991, 1992; Clark *et al*., 1991; Proudfit & Clark, 1991) and physiological functions (West *et al*., 1993; Graham *et al*., 1997) of noradrenergic neurons in Sasco and other substrains of Sprague–Dawley rats.

Although the illustration of previous reports show unequivocally, that in addition to the nuclei discussed above, the PAG also projects to various territories of the pontobulbar reticular formation, these researchers have given little attention to these areas until now. In contrast to this, here we would like to emphasis that according to our findings, the ventrolateral PAG sends a substantial projection to the pontomedullary intermediate reticular nucleus, and also innervates the pontine reticular nucleus, the parvocellular reticular nucleus of the pons and the dorsal medullary reticular field. As we found 20–30% of the PHA-L labelled axon terminals in these reticular fields, it appears to be highly probable that efferent fibres to these areas of the pontobulbar reticular formation may represent a functionally very important part of the projection system of the ventrolateral PAG. The parvocellular reticular nucleus of the pons and the pontine reticular nucleus have been shown to send a strong projection to the RVM (Hermann *et al*., 1997). It has also been demonstrated that by making lesions in the dorsal medullary reticular field, through local application of electric current or quinolinic acid, formalin-evoked pain behaviours can be attenuated substantially (Almeida *et al*., 1999). These observations suggest that the areas of the pontobulbar reticular formation that receive direct inputs from the ventrolateral PAG might turn out to be organic parts of the descending somatomotor, cardiovascular and antinociceptive pathways. Neural circuits of these areas might be activated by volleys arising from the ventrolateral PAG and in case of suprathreshold activation they may influence the spinal somatomotor, cardiovascular and nociceptive apparatus either directly by sending descending fibres to the spinal cord or indirectly by activating neural circuits in the RVM.

The pontobulbar projections of the ventrolateral PAG as a part of the endogenous descending antinociceptive pathway of the CNS

Our results suggest that neural activities of the PAG may influence the nociceptive information processing mechanisms of the spinal cord through an intricate interneuronal circuit. Most probably, signals from the PAG influence substantially the excitation level of the medial subdivision of the pontomedullary reticular formation including the intermediate reticular nucleus, pontine reticular nucleus, parvocellular reticular nucleus of the pons and the dorsal medullary reticular field. Here, the PAG signals may activate neural circuits that forward neural activities towards the spinal cord (Almeida *et al*., 1999) or more likely towards the RVM and pontine noradrenergic cell groups (Hermann *et al*., 1997). A second group of PAG efferents terminate within the RVM and pontine noradrenergic cell groups on nonserotonergic and non-noradrenergic neurons. Some of these neurons may represent cells of origin of raphe-spinal and coerulospinal pathways (Skagerberg & Björklund, 1985; Bowker & Abbott,

1990; Jones *et al*., 1991; Jones & Light, 1992; Antal *et al*., 1996), others might be local interneurons. The activated interneural circuits presumably further process the incoming volleys, and then transmit the signals to spinally projecting neurons. A third group of efferent fibres may establish monosynaptic contacts with serotonergic and noradrenergic neurons among which there might be some spinally projecting cells (Basbaum & Fields, 1984; Lakos & Basbaum, 1988; Reichling & Basbaum, 1990; Fields *et al*., 1991; Bajic & Proudfit, 1999; Mason, 1999). The spinally projecting neurons, both serotonergic and noradrenergic and also nonserotonergic and non-noradrenergic (Skagerberg & Björklund, 1985; Bowker & Abbott, 1990; Jones *et al*., 1991; Jones & Light, 1992; Antal *et al*., 1996), then presumably integrate the monosynaptic inputs and signals coming from the activated intra- and extranuclear interneural circuits, and in case of suprathreshold activation, they may conduct volleys to the spinal dorsal horn. In the superficial dorsal horn, the terminals of the descending fibres may release serotonin, noradrenalin, GABA, glycine and various neuropeptides including enkephalin (Basbaum & Fields, 1984; Fleetwood-Walker *et al*., 1985; Jones & Gebhart, 1986; El-Yassir & Fleetwood-Walker, 1990; Alhaider *et al*., 1991; Clark & Proudfit, 1991; Fields *et al*., 1991; Antal *et al*., 1994, 1996; Mason, 1999), and the released neurotransmitters may evoke inhibition in spinal neural circuits underlying nociceptive information processing, that results in analgesia and attenuation of pain behaviour (Gray & Dostrovsky, 1983; Lin *et al*., 1994; Gao *et al*., 1997; Waters & Lumb, 1997).

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Abbreviations

DAB, diaminobenzidine; PAG, midbrain periaqueductal grey matter; PB, phosphate buffer; PHA-L, *Phaseolus vulgaris*-leucoagglutinin; RVM, rostral ventromedial medulla; S-IR, serotonin-immunoreactive; TH, tyrosine hydroxylase; TH-IR, tyrosine hydroxylase-immunoreactive.

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