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Serotonin Immunoreactive Boutons Form Close Appositions With Respiratory Neurons of the Dorsal Respiratory Group in the Cat

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ABSTRACT

The aim of this study was to examine the location of serotonin immunoreactive boutons on both the soma and dendrites of neurons in the dorsal respiratory group by using a combination of intracellular recording and labelling and immunohistochemistry. Inspiratory neurons in the ventrolateral nucleus of the solitary tract (vl-NTS) were intracellularly labelled with horseradish peroxidase (HRP) in anaesthetised adult cats. The morphology of 23 neurons, all antidromically activated from the contralateral C_3 spinal segment, was examined. Six neurons displayed pronounced dendritic arborizations outside the vl-NTS, with prominent dorsal and/or medial projections. The dendrites of the remaining neurons were almost entirely confined to the vl-NTS. Intramedullary axon collaterals were detected in four of nineteen examined axons. Serotoninergic fibres were immunohistochemically demonstrated in the NTS, and the apposition of immunoreactive boutons to the HRP-filled neurons examined at the light microscopic level. Boutons were identified in close apposition with the somata, proximal and distal dendrites of these neurons. However, the density of contacts was found to be substantially less than in a previous study of phrenic motoneurons (Lipski et al: *Soc. Neurosci. Abst.* 14:379, '88; Pilowsky et al: *J. Neurosci.* in press, '90). The relative paucity of contacts of serotonin immunoreactive boutons with premotor inspiratory neurons of the dorsal respiratory group indicates that the serotoninergic system affects respiratory pathways mainly at the level of respiratory motoneurons or at brainstem sites outside the vl-NTS.

Key words: intracellular labelling, nucleus of the solitary tract, respiratory control, immunohistochemistry

Although a number of anatomical, physiological, and pharmacological studies indicate that central serotoninergic neurons are involved in respiratory control (Olson et al., '79; Eldridge and Millhorn, '81; Lalley, '86b; Holtman et al., '87; Lipski et al., '88), it is not known if the effect is principally mediated through an action on spinal or cranial respiratory motoneurons, on premotor respiratory neurons, or through effects on other neurons that in turn project to premotor neurons. Anatomical and immunohistochemical data point towards a direct influence on motoneurons (e.g., Holtman et al., '84a,b; Jiang and Shen, '85; Holtman et al., '87; Holtman, '88; Lipski et al., '88; Holtman and Maley, '88; Pilowsky et al., '90), whereas other results suggest that serotonin-containing neurons influence these motoneurons indirectly, by affecting premotor respiratory neurons in the brainstem (Holtman et al., '86; Lalley, '86a; Millhorn, '86). The latter possibility is supported by the presence of serotonin or serotonin immunoreactive varicosities, localised at the gross anatomical level, in the nucleus of the solitary tract (NTS) and the nucleus ambiguus (Fuxe, '65; Palkovits et al., '74; Maley and Elde, '82a,b; Steinbusch, '84; Schaffar et al., '88; Thor et al., '88). These regions are known to include spinally projecting neurons of the dorsal and ventral respiratory groups respectively (e.g., Feldman, '86). However, since these

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medullary regions are functionally heterogeneous (e.g., Kalia and Mesulam, '80; Bieger and Hopkins, '87), it is not known whether the serotoninergic projections affect the premotor respiratory neurons or other neurons in these nuclei.

In the feline medulla, the cell bodies of neurons of the dorsal respiratory group (DRG) are confined to the ventrolateral nucleus of the solitary tract (vl-NTS) (Feldman, '86; Long and Duffin, '86). Berger et al. ('84, '85) reported that the dendritic arbors of these neurons remain almost exclusively within this subnucleus, a region in which Maley and Elde ('82a,b) described only a moderate density of serotonin immunoreactive fibres. The purpose of our study was to examine the relationship between serotonin immunoreactive boutons and intracellularly labelled neurons of the DRG at the light microscopic level. In the initial stage we re-examined dendritic projections of these neurons, focusing on possible projections outside the confines of the vl-NTS. Subsequently, we investigated possible contacts between serotonin immunoreactive boutons and labelled neurons.

Preliminary results were reported in abstract form (Voss et al., '88; Voss and Lipski, '89).

METHODS

Animal preparation

Experiments were performed on 20 adult cats (1.6–3.5 kg), anaesthetised with sodium pentobarbital (Nembutal, 35–40 mg/kg, i.p.). The level of anaesthesia (slow and regular "central" breathing, narrow pupils) was maintained with supplementary doses of Nembutal of 3–6 mg/kg, i.v. Cannulae were inserted in a femoral artery and vein for arterial blood pressure measurement and drug administration, respectively. A tracheostomy was performed to facilitate artificial ventilation and to monitor tracheal pressure. The animal was paralysed with pancuronium bromide (Pavulon, 0.4 mg/kg/hr) and artificially ventilated with oxygen-enriched air at 60/min (tidal volume about 15 ml, inflation pressure up to 5 cm H_2O). Following bilateral pneumothoraces, a positive end-expiratory pressure of 1–2 cm of water was imposed. End-tidal CO_2 was monitored and maintained between 4 and 5% by adjusting the tidal volume. In some experiments the recording stability was improved by using high-frequency (800–1,000/min) ventilation (Duffin and Lipski, '86). Rectal temperature was maintained within the limits 36–38°C by a servo-controlled heating blanket. The C_5 phrenic root was exposed low in the neck, cut distally, and prepared for standard bipolar recording. The dorsal surface of the medulla was exposed by an occipital craniotomy, and the cerebellar vermis removed by suction to improve the exposure of the brainstem. Bilateral parietal craniotomies were made to further improve neural recording stability (Merrill et al., '83). A dorsal cervical laminectomy was performed to expose the C_3 spinal segment.

Recording and stimulation

The signal recorded from the C_5 phrenic root was amplified, filtered (50 Hz–3 kHz), full-wave rectified, and integrated (τ – 100 ms, third order Paynter filter). Intracellular recordings were made using glass microelectrodes (resistance, 20–40 MΩ, tip diameter less than 1 μm) filled with a freshly prepared 10% solution of horseradish peroxidase (HRP, 1230 U/mg, Serva) in 0.1 M Tris-HCl buffer (pH 7.6) containing 0.3 M KCl. Neurons were classified as bulbospinal if they responded antidromically following electrical stimuli applied to the contralateral spinal cord at the C_3 segment using a pair of metal electrodes. The following criteria were used to classify action potentials as antidromic: a) an all-or-none response at near threshold intensity of stimulation; b) action potentials evoked without preceding excitatory postsynaptic potentials; and c) short (generally less than 2.0 ms) and constant latency to the onset of evoked responses following stimuli applied in the same phase of respiratory cycle (Lipski, '81). Horseradish peroxidase was ejected iontophoretically by using 3–10 nA positive current pulses (80% duty cycle, 2 Hz). The minimum charge transfer accepted for this study was 40 nA·min, (range, 40–100 nA·min). When charge transfer was greater than 60 nA·min, the quality of staining was optimal, with clear brown colour showing evenly down to very fine dendritic branches. The criteria used to determine which neurons were filled with HRP are described in Results.

The location of the column of vl-NTS inspiratory neurons within the medulla was established by making a series of recording tracks in the coronal plane from 1.8 to 2.6 mm lateral to the midline, 0.8 mm rostral to obex, using low resistance (1–2 MΩ) micropipettes filled with 3 M NaCl. Once strong extracellular multi-unit inspiratory discharge had been located, and individual units could be antidromically activated from the C_3 segment of the contralateral spinal cord, the low resistance microelectrode was replaced with a high resistance HRP-filled micropipette. Tracking was continued rostral or caudal to the coordinates of maximal inspiratory activity (0–2.5 mm rostral to obex, 1.4–2.5 mm below the dorsal surface of the medulla).

Histology and immunohistochemistry

At least 1 hour after the last neuron was injected, the cat was perfused transcardially with 0.9% NaCl and then with 4% formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Vibratome sections (50 or 75 μm) were cut either in the transverse (12 animals), or oblique parasagittal plane (8 animals). In the latter case, the sectioning was made at approximately 20° to the midsagittal plane, approximately parallel to the solitary tract. In most experiments (n – 18), the morphology of the HRP-filled neurons was revealed with the Hanker-Yates reaction (Hanker et al., '77). In two experiments, in which serotoninergic immunoreactivity was not examined, we used the nickel-intensified diaminobenzidine reaction (0.02% diaminobenzidine, 0.6% nickel amonium sulphate, 0.003% hydrogen peroxide). The reactions were terminated after 15 minutes. Most sections were counterstained with 0.5% neutral red after detailed reconstructions had been carried out, to establish the location of the major nuclei.

After the Hanker-Yates reaction, the sections chosen for immunohistochemistry were washed three times in a buffer containing NaCl, 120 mM; KCl, 5 mM; NaH_2PO_4, 1.5 mM; Na_2HPO_4, 8.5 mM; Tris base, 10 mM; 0.3% v/v Triton X-100; and 1 mM sodium ethylmercurithiosalicylate (pH 7.4) for 10 minutes. The sections were then incubated in a rabbit antibody to serotonin (Dr W. Watkins, Auckland) diluted 1:2,000 in the same buffer containing 20% heat-inactivated porcine serum (Sigma) for 48 hours at room temperature. Following a further three 10 minute washes, the sections were incubated overnight in a biotinylated sheep antirabbit antibody (Sigma B9140) in 1% porcine serum. After a further three washes the sections were incubated for 1 hour in

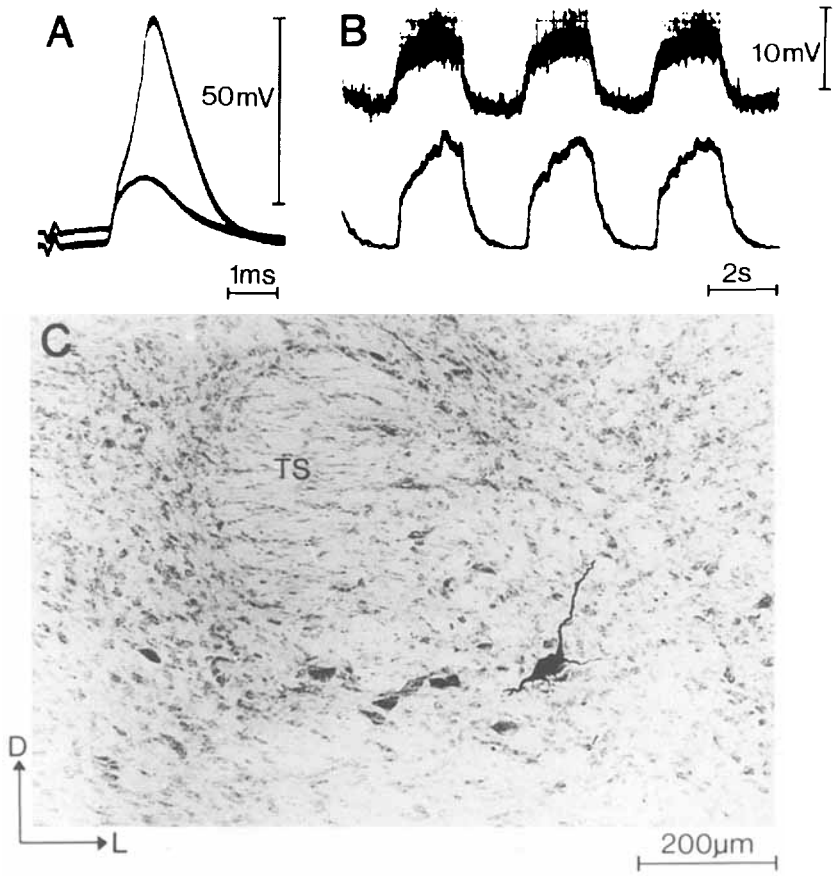

Fig. 1. Example of intracellular recordings from and morphology of a DRG neuron. **A:** Antidromically evoked action potentials during inspiration and expiration. Two superimposed sweeps recorded during inspiration (both the initial segment and soma-dendritic spikes present) and three superimposed sweeps during expiration (only the initial segment spike present). **B:** Changes of the membrane potential (upper trace) and the integrated discharge of the C_5 phrenic root (lower trace). The absolute value of the membrane potential (during expiration) was approximately −55 mV. **C:** Photomicrograph of a transverse section through the medulla oblongata 1.0 mm rostral to the obex. An HRP labelled DRG neuron in close proximity to neutral red counterstained large cell bodies of other vl-NTS neurons. TS = tractus solitarius; D = dorsal; L = lateral.

an avidin-peroxidase conjugate (1:1,000, Sigma) and washed. The location of serotonin immunoreactive nerve terminals was revealed by incubating the sections in 50 mM Tris-HCl buffer (pH 7.6) containing 0.05% diaminobenzidine, 10 mM imidazole-4-acetate and 0.03% hydrogen peroxide (Straus, '82). After further washes, the sections were mounted onto gelatin coated slides, dehydrated, cleared in xylene, and mounted with DePex.

Morphometric procedures and measurements

HRP-filled neurons were reconstructed by using a standard camera lucida technique. Low power reconstructions (at 10× objective for axonal trajectories and 25× objectives for dendritic trees) were made to assess dendritic arborization and axon trajectories. Fifty and 100× objectives were used to search for axon collaterals. No compensation was made for tissue shrinkage. Possible appositions of serotonin immunoreactive boutons with HRP labelled neurons were examined with a 100× oil objective, and strict criteria were defined for their identification. A serotoninergic terminal was said to be in "close apposition" with an HRP filled neuron if the elements were separated by a distance of less than 1 μm, as assessed by differential focusing, or if there was no discernible space between boutons and dendrites apposed side by side in the same focal plane. Only varicosities forming part of a string were considered when analysing close appositions. Isolated boutons (i.e., boutons not in continuity with other terminals through an axon fibre) were disregarded so as to avoid their possible confusion with tissue artefacts. These criteria may have caused a slight underestimation of the number of immunoreactive boutons in close apposition with labelled neurons. Calculations of contact density were carried out by counting the number of contacts on three regions of labelled neurons and dividing each figure by the surface area over which the contacts were distributed (see "Results"). The units for bouton density were boutons/(100 μm)^2.

RESULTS

Identification of DRG neurons

Neurons which fulfilled the following criteria were defined as DRG neurons and injected with HRP: a) appropriate location, as described in Methods; b) stable recording

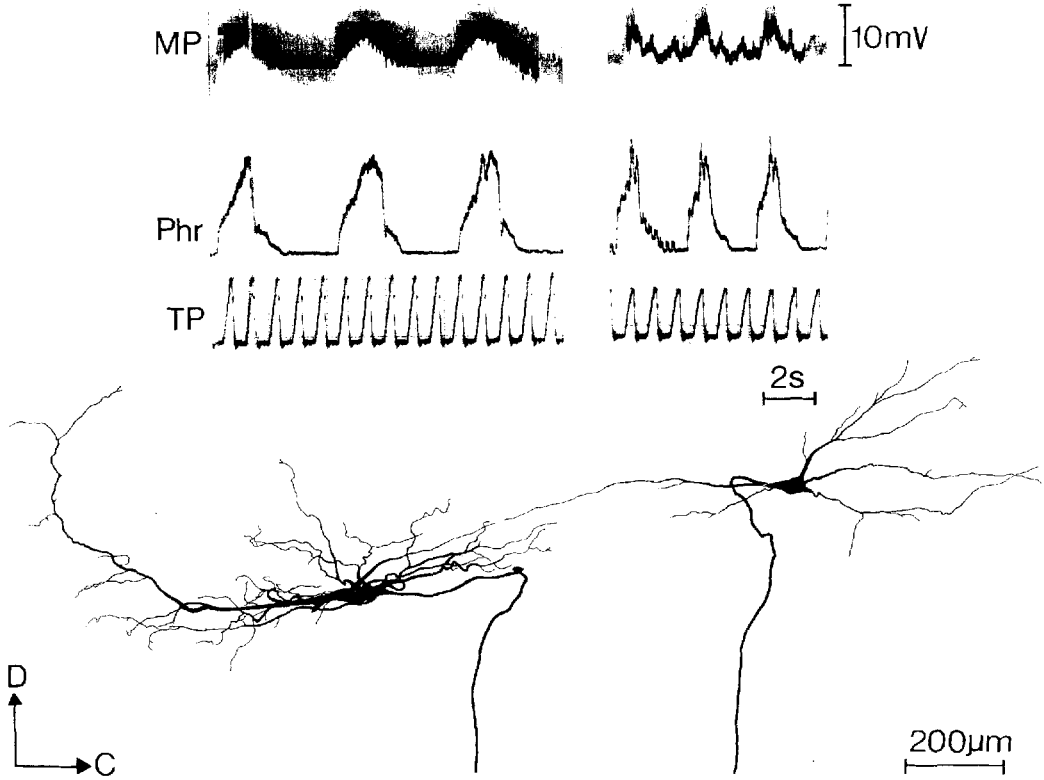

Fig. 2. Morphology and electrophysiological properties of two DRG neurons labelled in the same hemimedulla. **Above,** chart recordings; **below,** low-power reconstructions in the oblique parasagittal plane. The neuron on the right (Rβ) shows depolarizing shifts of the membrane potential with phrenic nerve discharge and increases in tracheal pressure. The depolarization induced by lung inflations were unlikely to be the result of movement artefact, since no ventilation-related instabilities were observed in this animal during intracellular recording from neurons in the dorsal tegmentum outside the NTS. The neuron on the left (Rα) shows depolarising shifts of the membrane potential only with phrenic nerve discharge. MP = membrane potential; Phr = integrated discharge of the C_5 phrenic root; TP = tracheal pressure; D = dorsal; C = caudal.

with an initial membrane potential of at least −40 mV; c) antidromic response following stimuli applied to the C_3 spinal segment (antidromic latency: mean, 2.1 ms; range, 0.8–4.0 ms); and d) depolarizing shifts of the membrane potential (range, 5–15 mV) during phrenic nerve discharge. An example of an intracellular record from a neuron fulfilling these criteria, together with a micrograph showing its location in relation to the solitary tract, is shown in Figure 1. All injected and recovered neuronal cell bodies were located in the vl-NTS.

Although systematic tests with lung inflations of variable volume and duration were not performed, our observations suggest that both Rα and Rβ neurons (e.g., Lipski et al., '83) were included in this study. Tentative classification was made on the basis of the presence or absence of membrane depolarization during lung inflations produced by artificial ventilation (Fig. 2). Ten of 23 injected cells, likely to be the Rα type, showed no clear depolarizations during inflation, while six displayed such depolarization (range, 2–5 mV) and were classified as Rβ. The remaining seven neurons could not be classified due to use of high-frequency ventilation. Since no clear differences were observed between dendritic trees of these groups (cf. also Berger et al., '85), all labelled inspiratory bulbospinal neurons were pooled for subsequent morphological analysis.

Of 23 labelled inspiratory bulbospinal DRG neurons, 12 were reconstructed in the transverse plane and 11 in the oblique parasagittal plane. Figure 2 shows a reconstruction in the oblique parasagittal plane of an Rα and an Rβ neuron labelled in one animal. The dendritic arbors of these two neurons, as well as those of 15 other neurons, were confined to the vl-NTS as described by Berger et al. ('84, '85). The dendrites travelling parallel to the long axis of the solitary tract projected up to 1.15 mm from the cell body. Some neurons (6/23; 1Rα, 1Rβ, 4 not classified) also displayed significant dendritic projections outside this subnucleus, prominently in the dorsal and medial directions. These dendrites projected medially as far as the dorsal motor nucleus of the vagus and nucleus intercalatus, or dorsally to a position dorsolateral to the solitary tract. Examples of both types of dendritic projection are shown in Figure 3. The average number of primary dendrites for the whole population was 5.3 (range, 4–8). Dendritic spines and appendages like those described by Berger et al. ('84) were observed.

As recurrent axon collaterals of intracellularly labelled neurons could be confused with immunoreactive fibres (see Discussion), it was necessary to examine neurons for the presence of such collaterals. Nineteen of the 23 neurons (10 reconstructed in the transverse and 9 in the oblique parasagittal plane) exhibited axons sufficiently well stained to

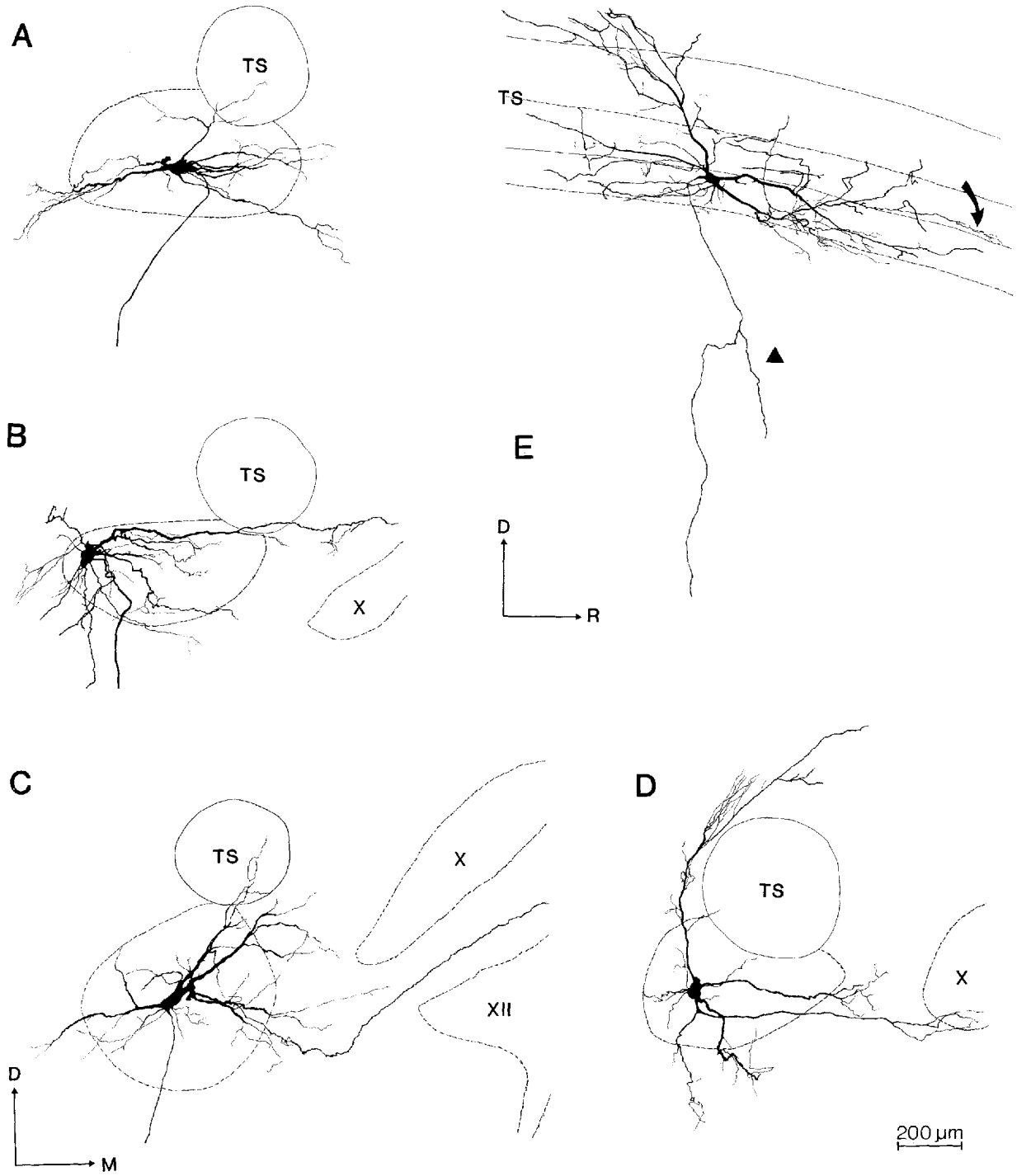

Fig. 3. Low power reconstructions of five DRG neurons (A–D, transverse; E, oblique parasagittal plane) labelled in different animals. The extent of the vl-NTS is indicated by dotted line. **A:** Dendritic tree largely confined to the vl-NTS. **B–E:** Prominent dendritic arborizations outside the vl-NTS. Medial projections towards the dorsal motor nucleus of the vagus (B, D, and arrow in E) and C) to the nucleus intercalatus. D and E show also projections lateral and dorsal to the solitary tract. An axon with a collateral is shown in E (arrowhead). TS = tractus solitarius; X = dorsal motor nucleus of the vagus; XII = hypoglossal nucleus; D = dorsal; M = medial; R = rostral.

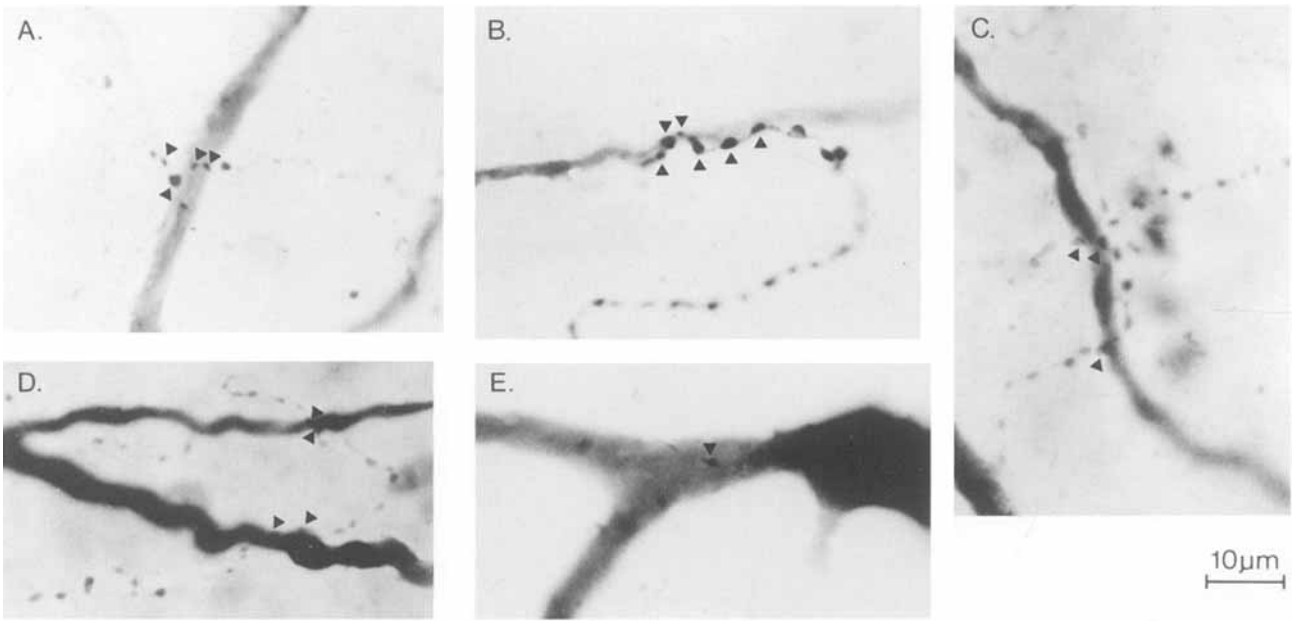

Fig. 4. Photomicrographs showing serotonin immunoreactive boutons and parts of HRP filled DRG neurons. Examples of close appositions are indicated by arrowheads. The leftmost arrowhead in A points at a bouton which is in close apposition with an out-of-focus dendritic branch. This close apposition can be seen in reconstruction shown in Figure 6A. **A–D:** Immunoreactive boutons in close apposition with distal dendrites. **E:** Close apposition on a proximal dendrite adjacent to the soma.

allow detailed examination for collaterals. On average, the axons could be traced 2.4 mm from the cell body (range, 1.0–4.4 mm). Their trajectories showed minor variations, but generally axons travelled ventrally and medially. We were unable to trace them into the contralateral medulla, due to the gradually declining intensity of staining.

Intramedullary axon collaterals were observed in 21% (4/19) of the axons studied (see Fig. 3E). The collaterals arose between 0.5 and 2 mm from the cell body. Three travelled caudally and dorsally, and one ventrally and slightly rostrally. Due to the fine diameter of these collaterals (0.8–1.3 μm) and the reduction in labelling intensity at increasing distances from their point of origin, we were unable to trace them to their sites of terminal arborization.

Serotonin immunoreactivity

Serotonin immunoreactive fibres were found throughout the NTS. In the vl-NTS a region of low density of these fibres was found close to the solitary tract, with higher densities laterally and ventrally within the subnucleus. This result is in general agreement with the findings of Maley and Elde ('82a). Nine well labelled DRG neurons were recovered and reconstructed in sections subjected to immunohistochemical processing for serotonin. The surface of these neurons was examined for close appositions with immunoreactive boutons. Only relatively small numbers of such contacts were identified on all parts of the labelled neurons. Examples of serotonin immunoreactive boutons in close proximity to labelled neurons are shown on photomicrographs in Figure 4.

The density of close appositions with labelled neurons was calculated for the somata and proximal and distal dendrites. For evaluation of the apposition density on the soma,

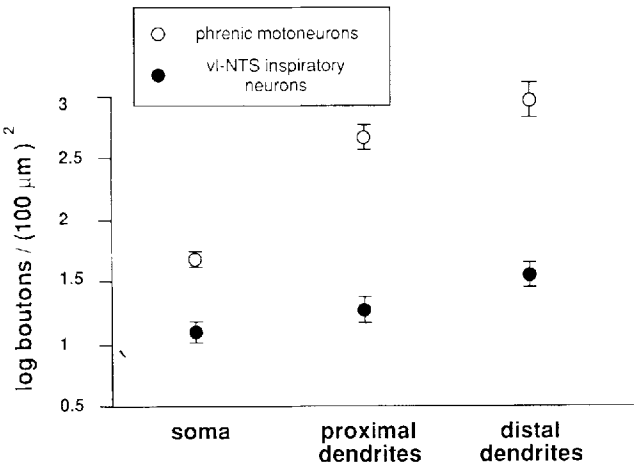

Fig. 5. Density of close appositions between serotonin immunoreactive boutons and HRP labelled DRG neurons (filled circles) in three regions of cells. Nine proximal and 9 distal dendritic sites were examined, one of each for each of the neurons. Open circles: density of close appositions between serotonin immunoreactive boutons and HRP labelled phrenic motoneurons (data from Pilowsky et al., '90). Data are means and standard errors of decimal logarithm of contact density.

the central part was treated as flat and close appositions counted within a measured region. For the dendrites, 200 μm long segments were arbitrarily selected (the middle of each segment being 120 and 350 μm from the soma for proximal and distal dendrites respectively) and contacts counted. Curvature of dendrites was taken into consideration when

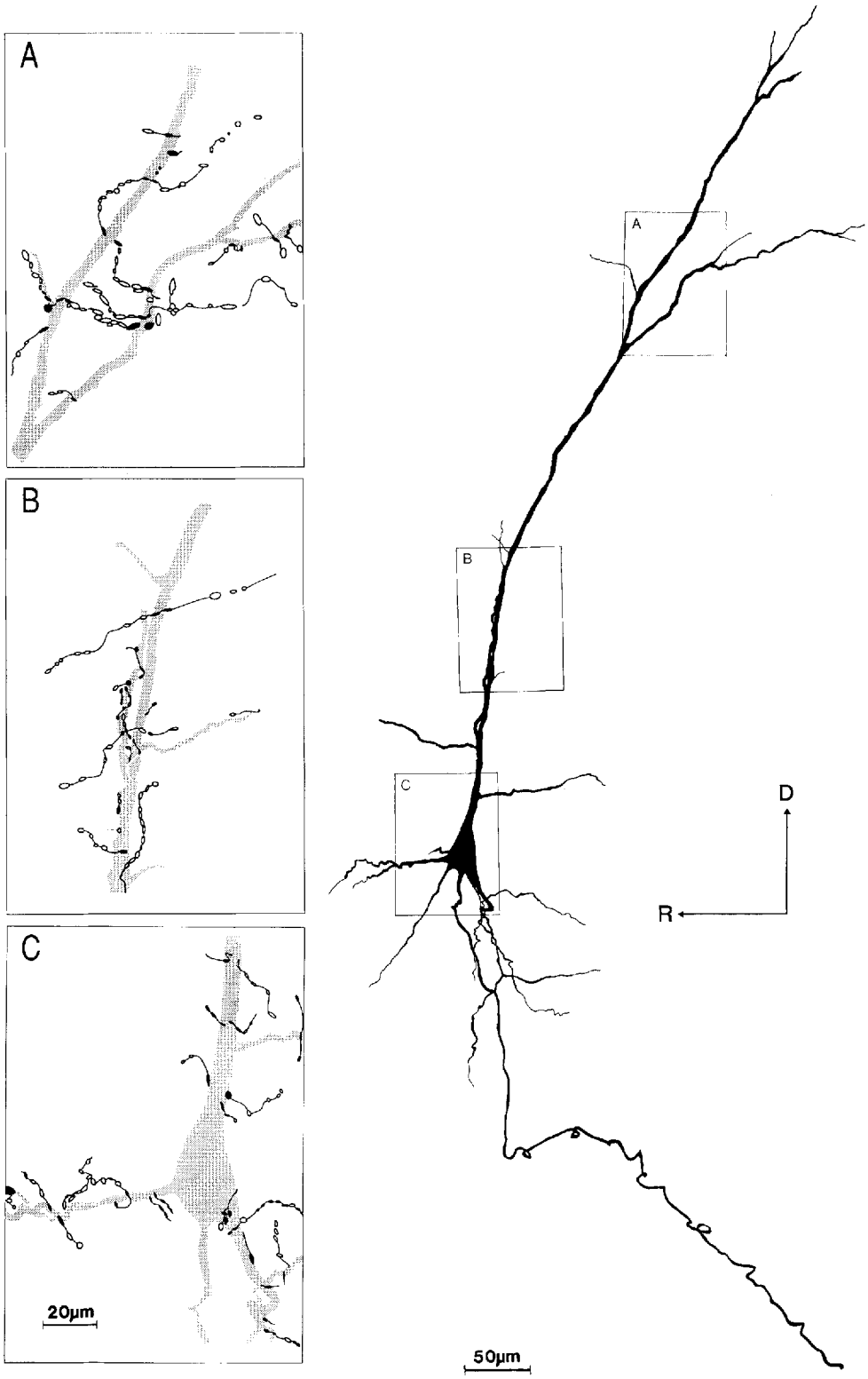

Fig. 6. Distribution of serotonin immunoreactive boutons in contact with a single DRG neuron. **Right:** A low-power reconstruction in the oblique parasagittal plane. **Left:** High-power reconstructions of **A**) a distal portion of the dorsally projecting dendrite, **B**) a proximal part of the same dendrite, and **C**) the soma. Filled shapes represent boutons in "close apposition." A photograph of part of inset A is shown in Figure 4A. D = dorsal; R = rostral.

measuring distances from the soma. The dendritic diameter was measured and averaged for each examined segment, and the contact density expressed in terms of the surface area of a 200 μm long cylinder of this diameter. Figure 5 indicates the distribution of contacts in three regions of nine analysed DRG neurons. The data from nine neurons was analysed statistically by analysis of variance after log transformation because of heterogeneity of the original variances. The difference in contact density at the 3 sites was significant ($F_{2,26} = 7.2$, $P < 0.005$). For comparison, data is included from a recent study in our laboratory (Pilowsky et al., '90) on density of close appositions of serotonin immunoreactive boutons on phrenic motoneurons. We did not attempt to analyze the distribution of contacts over the entire neuronal surface because of the large size of the dendritic trees of these neurons. Immunoreactive boutons in close apposition with a labelled DRG neuron are illustrated by Figure 6. The windows show contacts at three regions of a prominent, dorsally projecting dendrite.

The diameter of serotonin boutons in close apposition with DRG neurons was measured. A histogram of their size distribution is given in Figure 7. The majority of boutons in contact with labelled neurons had a diameter between 0.5 and 1.0 μm. No serotonin immunoreactive cell bodies were found in the solitary nucleus.

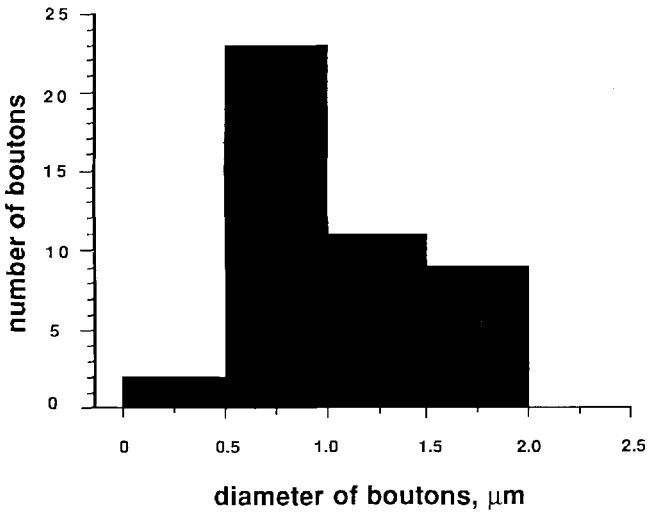

Fig. 7. Size distribution of serotonin immunoreactive boutons in close apposition with DRG neurons. The histogram represents the measurement of diameter of 44 boutons on 8 neurons.

DISCUSSION

A number of studies have described the presence of serotonin immunoreactive fibres in various regions of the medulla oblongata, including the NTS (Maley and Elde, '82a,b; Steinbusch, '84; Pickel et al., '84; Schaffer et al., '88; Thor et al., '88). To our knowledge, this is the first report to examine possible contacts of serotonin immunoreactive nerve terminals with single, functionally identified and intracellularly labelled neurons in the medulla. Previously we have employed a similar technique to examine serotonin immunoreactive boutons in close apposition to labelled phrenic motoneurons (Lipski et al., '88; Pilowsky et al., '90). The results of the present study provide a comparable analysis of serotonin immunoreactive boutons in close apposition with vl-NTS premotor respiratory neurons, which are known to excite phrenic and external intercostal motoneurons monosynaptically (e.g., Cohen et al., '74; Lipski et al., '83; Duffin and Lipski, '87). In order to relate the position of the immunoreactive boutons to various parts of labelled vl-NTS neurons, it was first necessary to examine details of their dendritic and axonal morphology. Subsequent light-microscopic analysis was used to examine possible contacts between labelled neuronal profiles.

Three other laboratories have reported on the morphology of respiratory neurons in the vl-NTS using intracellular labelling, one with Procion Yellow (von Euler et al., '73), and two with HRP (Berger et al., '84, '85; Otake et al., '89). Only the results of the studies using HRP can be compared with the material presented here, as staining with Procion Yellow does not result in adequate labelling of neuronal processes (e.g., Kreuter et al., '77).

In agreement with Berger et al. ('84, '85), we observed that the dendritic arborizations of most of the labelled neurons had a preferential orientation parallel to the long axis of the tractus solitarius (Fig. 3E), remaining largely within the confines of the vl-NTS (Fig. 3A). However, a significant proportion of neurons reconstructed in our study (6/23) displayed extensive dendritic projections outside this subnucleus. This observation, in agreement with recent results by Otake et al. ('89), is relevant in light of previous reports (Maley and Elde, '82a,b) that the density of serotonin immunoreactive boutons is higher in more medial and dorsal regions of the solitary complex than in the vl-NTS. In addition, the existence of dendritic projections outside the anatomical boundaries of the vl-NTS implies that DRG neurons may be involved in the processing of information transmitted by afferent fibres terminating outside this subnucleus.

The other major difference between our results and those of Berger et al. ('84) is in the presence of intramedullary axon collaterals. These were found in 4 out of 19 well-stained axons, while Berger et al. ('84) described axonal bifurcations but found no evidence of collateralization along the course of seven labelled axons. This difference can be explained by the larger number of axons examined in the present study. Recently, Otake et al. ('89) reported the presence of axon collaterals in 4 of 28 axons of DRG neurons.

Axon collaterals of both Rα and Rβ DRG neurons, projecting predominantly to the region of the ipsilateral ventral respiratory group, were identified in an early antidromic mapping study by Merrill ('74). Although figures are not given, it was concluded that this projection appeared to be extensive (cf. also Merrill, '81; von Baumgarten and Kanzow, '58). The possibility of contralaterally projecting axon collaterals of DRG neurons arises from a cross-correlation study by Cohen ('76). The function of collaterals of DRG neurons has yet to be elucidated. They may be involved in transmission of the inspiratory drive to motoneurons via other groups of respiratory neurons. Alternatively they may form part of a feedback loop, and hence play a role in the formation of the pattern of respiratory activity.

As no attempt was made in this study to differentiate between HRP-filled and immunoreactive neuronal elements on the basis of the colour of the HRP reaction product (cf. Hancock, '86), the possibility occurs of confusion between the immunoreactive profiles and the filled neurons.

This problem was not encountered for two reasons. Firstly, no serotonin-immunoreactive somata were observed in the examined part of the NTS. Secondly, in the few cases (4/19) when axon collaterals were present, they could not be traced to sites of terminal arborizations.

In contrast to studies based on retrograde labelling (e.g., Holtman et al., '84a; Holtman, '88) the combination of intracellular labelling and immunohistochemistry enables an examination of the distribution of immunoreactive boutons over the entire surface of the dendritic tree. Our analysis of contacts with labelled neurons was limited to the light microscopic level, using high magnification, an approach similar to that used in many studies of non-respiratory neurons (e.g., Miletic et al., '84; Lawrence et al., '85; Hylden et al., '86; Ohta et al., '88). A limitation of this light-microscopic, double-label technique is uncertainty as to whether "close appositions" represent synapses. Although a definite conclusion must await studies involving electron microscopy, it is likely that these appositions do represent synapses for the following reasons: a) In an electron microscopic study of serotonin immunoreactive fibres in the solitary complex, Maley and Elde ('82b) did find genuine synaptic contacts. As discussed by these authors, the small number of such contacts detected in their study may have been due to the small number (n = 5) of serially sectioned boutons. This possibility is supported by the results of another study performed in the cerebral cortex (Papadopoulos et al., '87) which showed that when systematic analysis of serial ultrathin sections is performed, at least 90% of labelled serotoninergic boutons formed specialised junctional appositions characteristic of synapses. b) The majority of varicosities shown in our study to be in close apposition with DRG neurons had a diameter of 0.5–1.0 μm. In the ultrastructural study by Maley and Elde ('82b), terminals of a similar size were shown to be more often in synaptic contact than larger boutons measuring 1.0–2.0 μm in diameter. c) In a recent study conducted in this laboratory (Pilowsky et al., '90), a similar approach was used to characterise the serotoninergic input to phrenic motoneurons. Similar criteria were used to identify immunoreactive boutons in apposition to labelled motoneurons. The diameter of boutons in contact with labelled neurons was similar to that seen in this study. The presence of synaptic specialisations was confirmed at the ultrastructural level (cf. also Holtman and Maley, '88).

Overall, the density of close appositions of serotonin immunoreactive boutons with DRG neurons was substantially smaller than that described recently in our laboratory for intracellularly labelled phrenic motoneurons (Lipski et al., '88; Pilowsky et al., '90). However, we observed a similar tendency for density of contacts to increase with increasing distance from the cell body. In the study by Maley and Elde ('82b) no synaptic contacts were found on cell bodies.

It is not known which group of serotonin neurons project to DRG neurons. No serotonin immunoreactive neural perikarya have been identified within the feline solitary complex in this or other studies (however, see Calza et al., '85 in the rat), implying that the cell bodies of these serotonin-containing neurons must be located outside this region. It still has to be established whether at least some of this projection originates from serotonin-containing neurons in the petrosal or nodose ganglia (Thor et al., '88). In one double-labelling study performed in the rat, serotonin immunoreactive neurons that project to the NTS were found in the raphe magnus, obscurus, pallidus, and in the rostral medulla lateral to the pyramidal tract (Thor and Helke, '87). In another study (Schaffar et al., '88) most of the double-labelled perikarya were observed in the nucleus raphe magnus, the adjacent part of the paragigantocellular nucleus, and the nucleus raphe dorsalis. These data must, however, be used cautiously when interpreting results obtained in the cat, particularly with regard to the possibility that projecting fibres affect DRG neurons. Electrophysiological studies in the rat indicate that few respiratory neurons can be identified in the region of the vl-NTS (Ezure et al., '88, but cf. Saether et al., '87). Similarly, anatomical studies have found that there is little (Onai et al., '87) or no (Yamada et al., '88) projection from the vl-NTS to the phrenic nucleus in the rat.

Functional implications

A comparison of the present results with data obtained in our laboratory on the distribution of serotoninergic contacts on intracellularly labelled phrenic motoneurons (Lipski et al., '88; Pilowsky et al., '90) demonstrates that the density of close appositions is significantly less in the DRG (Fig. 5). One interpretation of this observation is that the respiratory effect of the central serotoninergic system is mediated mainly at the level of motoneurons. On the other hand, our results do not exclude the possibility that there is an interaction between serotonin containing fibres and brainstem respiratory neurons other than those within the vl-NTS. Other subdivisions of the NTS showing higher densities of serotonin immunoreactive fibres (Maley and Elde, '82a,b) have a respiratory function, for example, P-cells found ventromedial and dorsolateral to the solitary tract (Davies et al., '87). In addition, the region of the ventral respiratory group has been shown to contain serotonin and serotonin immunoreactive fibres (Palkovits et al., '74; Steinbusch, '84; Holtman, '88), originating, in the rat, from caudal raphe nuclei and the paraolivary nucleus (Connelly et al., '88; Holtman et al., '89). Finally, it is not known whether respiratory neurons involved in the generation of respiratory rhythm located outside the dorsal and ventral groups of respiratory neurons (Feldman, '86) are directly affected by serotoninergic projections.

Although a number of studies have shown that manipulation of the level of activation of central serotonin receptors alters respiration, it is not clear if serotonin has a predominant excitatory or inhibitory effect. In some studies (e.g., Armijo et al., '74; Lundberg et al., '78; Olson et al., '79; McCrimon and Lalley, '82) an inhibitory effect was observed, while others (Millhorn et al., '80; for review see Eldridge and Millhorn, '81) suggest that serotonin has a tonic facilitatory effect. The inconclusiveness of results obtained at the whole animal level is reflected in experiments in which serotonin has been applied microiontophoretically onto single respiratory neurons. Electrophoretic administration on phrenic motoneurons produced little or no effect (Lalley, '86b), while administration to medullary respiratory neurons resulted in depression in some neurons and excitation in others (Champagnat et al., '79; Fallert et al., '79; Sessle and Henry, '85). These discrepancies can, at least partly, be explained if serotonin acts as a "gain setting" neuromodulator (e.g., McCall and Aghajanian, '79) rather than as a classical neurotransmitter. In this way serotonin could modulate the action of both excitatory and inhibitory neurotransmitters. The net effect would depend on which type of synaptic input predominated in each experimental situa-

tion. Alternatively, excitatory and inhibitory actions of serotonin might be mediated through different subsets of serotonin receptors (e.g., Gillis et al., '89). Certainly, further functional studies are required in order to elucidate the action of serotonin on respiration.

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