Electrophysiological and Morphological Characterization of Propriospinal Interneurons in the Thoracic Spinal Cord

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Saywell SA, Ford TW, Meehan CF, Todd AJ, Kirkwood PA. Electrophysiological and morphological characterization of propriospinal interneurons in the thoracic spinal cord. J Neurophysiol 105: 806 – 826, 2011. First published November 24, 2010; doi:10.1152/jn.00738.2010. Propriospinal interneurons in the thoracic spinal cord have vital roles not only in controlling respiratory and trunk muscles, but also in providing possible substrates for recovery from spinal cord injury. Intracellular recordings were made from such interneurons in anesthetized cats under neuromuscular blockade and with the respiratory drive stimulated by inhaled CO2. The majority of the interneurons were shown by antidromic activation to have axons descending for at least two to four segments, mostly contralateral to the soma. In all, 81% of the neurons showed postsynaptic potentials (PSPs) to stimulation of intercostal or dorsal ramus nerves of the same segment for low-threshold (≤5T) afferents. A monosynaptic component was present for the majority of the peripherally evoked excitatory PSPs. A central respiratory drive potential was present in most of the recordings, usually of small amplitude. Neurons depolarized in either inspiration or expiration, sometimes variably. The morphology of 17 of the interneurons and/or of their axons was studied following intracellular injection of Neurobiotin; 14 axons were descending, 6 with an additional ascending branch, and 3 were ascending (perhaps actually representing ascending tract cells); 15 axons were crossed, 2 ipsilateral, none bilateral. Collaterals were identified for 13 axons, showing exclusively unilateral projections. The collaterals were widely spaced and their terminations showed a variety of restricted locations in the ventral horn or intermediate area. Despite heterogeneity in detail, both physiological and morphological, which suggests heterogeneity of function, the projections mostly fitted a consistent general pattern: crossed axons, with locally weak, but widely distributed terminations.

INTRODUCTION

The physiology and the anatomy of motor systems in the thoracic spinal cord are generally much less well understood than in either the lumbar or the cervical segments. However, there is a growing interest in the thoracic segments, particularly with regard to spinal cord injury. Partly this stems from the occurrence of disordered reflexes involving control of the bladder and autonomic nervous system (reviewed by Schramm 2006; Weaver et al. 2006), but partly also because these segments are a favored site for experimental injury and repair. Moreover, they may also be a favored site for clinical trials of repair strategies because possible deleterious effects of treatments may have fewer serious consequences for the patient than those at cervical levels (Féron et al. 2005). The properties of thoracic interneurons are an important factor for each of these aspects. For reflexes, this is self-evident; for spinal cord repair one potential role of the interneurons is their participation in detour circuits, created via plasticity (Arvanian et al. 2006; Bareyre et al. 2004; Courtine et al. 2008). Another potential role is as either receivers or providers of axonal regeneration (Conta and Steltzner 2004). These interneurons could be particularly important because of the limited distance over which axonal regeneration of long descending systems has been achieved to date, i.e., up to about 25 mm in the rat (Fawcett 2002). Thus the thoracic interneurons, many of which are propriospinal, with descending axons of several segments in length (Kirkwood et al. 1988; Schmid et al. 1993) may be well suited to providing replacements for the loss of long descending (or ascending) axons.

For the thoracic cord, the physiological properties of motoneurons, particularly as they relate to respiratory movements, have been relatively well described (for review, see De Troyer et al. 2005; Saywell et al. 2007), their locations documented (see Meehan et al. 2004 for references), and morphological studies have been published (see Lipski and Martin-Body 1987; Pullen and Sears 1983), but there have been only a few similar studies of interneurons in the thoracic ventral horn. To date, studies using extracellular recording have focused on the roles of these interneurons in respiration (see Kirkwood et al. 1988, 1993 for references). In addition, a series of studies using intracellular recordings has concentrated on peripheral afferent and reticulospinal inputs (Gokin 1970; see Gokin 1978 for other references). The intention here was to extend these previous studies by combining measurements of synaptic inputs to these neurons from intracellular recording with morphological data obtained by labeling individual neurons. This study thus addresses particular issues arising from the earlier studies, but it also provides specific control data for a subsequent study using similar methods for an equivalent population of interneurons following experimental injury (Meehan et al. 2003).

In previous publications from this laboratory (Kirkwood et al. 1988, 1993; Schmid et al. 1993) the thoracic interneurons were defined as "respiratory interneurons." This was natural because one of the aims was to search for those that were expected to be part of respiratory pathways (De Troyer et al. 2005), although with extracellular recordings in anesthetized animals, the only active (firing) interneurons are likely to be those with respiratory discharges. Indeed, in those previous studies a strong respiratory drive was used to promote this. For consistency, we have used similar conditions here, but we have...
eliminated the bias of recording only from neurons that are firing, by the use of intracellular recordings, so that we can investigate how general the respiratory activation of thoracic interneurons actually is. Further, we have made no restriction to analyzing specifically a respiratory population. To do this would have been inappropriate with regard to providing a control population for interneurons in an injured cord because such a property would depend on connectivity that is likely to change as a consequence of injury.

Intracellular recordings also provide a more sensitive measure of peripheral afferent input to the interneurons than is possible with extracellular methods. By analogy with connections in the lumbosacral cord, such inputs would be expected to be common, but little evidence of this, particularly with regard to low-threshold afferents, was apparent in the extracellular recordings of Kirkwood et al. (1988). Gokin (1970) addressed this issue, but only using a broad general characterization. The experiments reported here make a more detailed analysis and allow a correlative approach with regard to the other neuronal properties examined.

Finally, most projections of the interneurons identified to date have been contralateral (Kirkwood et al. 1988; Schmid et al. 1993), but two specific issues remain, which the morphological data can address. First, previous investigations of the terminal fields, by extracellular spike-triggered averaging (Kirkwood et al. 1993; Schmid et al. 1993) suggested individually weak projections, although this observation was limited by relatively restricted sampling, especially along the rostrocaudal axis. The limitation is overcome here by the anatomical mapping of the axon collaterals of individual axons. Second, although the presence of contralateral collaterals was shown in the previous studies, it remained unknown whether the same cells also had ipsilateral projections, as might be expected for neurons involved in a bilaterally organized movement such as respiration, or whether the projections of the interneurons with crossed axons were exclusively contralateral.

Methods

Preparation

Experiments were conducted according to UK legislation [Animals (Scientific Procedures) Act 1986]. The data come from 20 cats of either sex, weighing 2.2 to 3.7 kg, all but two of them having also contributed to the data of Saywell et al. (2007). Animals were anesthetized with sodium pentobarbitone (initial dose 37.5 mg·kg⁻¹, administered intraperitoneally, then intravenously [iv] as required) and a bilateral vagotomy was performed. Neuromuscular blockade, was achieved by the use of gallamine triethiodide (subsequent to surgery, iv, repeated doses 24 mg as required) and the animals were artificially ventilated via a tracheal cannula with oxygen-enriched air, to bring the end-tidal CO₂ fraction initially to about 4%. A low stroke volume and a high pump rate (53 min⁻¹) were used so that events related to the central respiratory drive could be distinguished from those due to movement-related afferent input. A bilateral pneumothorax was performed and the end-expiratory pressure maintained at 2–3 cm H₂O. CO₂ was then added to the gas mixture to raise the end-tidal level sufficient to give a brisk respiratory discharge in the midthoracic intercostal nerves (typically 6–7%). During neuromuscular blockade, anesthesia was assessed by continuous observations of the patterns of the respiratory discharges and blood pressure together with responses, if any, in either of these to a noxious pinch of the forepaw. Only minimal, transient responses were allowed before supplements (5 mg·kg⁻¹) of pentobarbitone were administered. The animal was supported by vertebral clamps, a clamp on the iliac crest, and a plate screwed to the skull. Rectal temperature was maintained between 37 and 38°C by a thermostatically controlled heating blanket. Mean blood pressures were >80 mmHg throughout, maintained in a few animals by occasional infusions of 5% dextran in saline.

Three nerves were prepared for stimulation via platinum wire electrodes on the left side of one segment or, most often, two segments to be used for intracellular recording: 1) a bundle of dorsal ramus nerves (Kirkwood et al. 1988); 2) the external intercostal nerve; small pieces of pia were removed from the dorsal columns of the segment(s) to be used for intracellular recording ranged from T5 to T9. The left external intercostal nerve of a rostral segment (most often T6) was prepared for recording efferrant discharges, used to define inspiration. In three of the animals the left dorsal roots were cut at some point during the experiment for one or both of the segments used for intracellular recordings. This was done to allow specific identification of Renshaw cells (Kirkwood et al. 1981), a number of which were penetrated and intracellually labeled (Saywell et al. 1998). Their physiological properties and axonal projections will be presented elsewhere, but the dorsal root section also meant that three of the neurons presented here could not be tested for peripheral afferent inputs.

A thoracic laminectomy was performed, the dura was opened, and small pieces of pia were removed from the dorsal columns of the segment(s) to be used for intracellular recording. Stimulation electrodes were inserted into the spinal cord (a pair of tungsten electrodes on each side, tips intended to be in the ventromedial and ventrolateral funiculi) two to four segments below the chosen segment, as described by Kirkwood et al. (1988), and a shaped pressure plate lightly applied to the cord dorsum of the chosen segment, to aid mechanical stability. The laminectomy and nerves were submerged in a single paraffin oil pool constructed from skin flaps. At the end of the experiment the animals were either killed with an overdose of anesthetic or perfused for histology (see following text).

Recording

Intracellular recordings were made from interneurons in the chosen segment by tracking in the left ventral horn with glass microelectrodes filled with 2–4% Neurobiotin and 0.5 M K⁺ acetate in 0.01 M Tris buffer (pH 7.4). Electrodes were introduced through the dorsal columns at an angle of 15° to the sagittal plane (Kirkwood et al. 1988) and with tracks 0.05 mm apart (microdrive: TransverteX, Sweden; step size 4 μm). Electrodes were pulled on a Model 753 electrode puller (Campden Instruments, Loughborough, UK) and bevelled to an impedance of 20–45 MΩ (micropipette beveler BV-10; Sutter Instrument, Novato, CA). To ensure that the recordings were made within the gray matter, tracks were located with respect to the positions of motoneurons as determined either by antidromic field potentials resulting from stimulation of the three nerves at fivefold nerve threshold (5T) or by intracellular recordings from the motoneurons (cf. Kirkwood et al. 1988). Nerve stimulation was monitored from the cord dorsum by platinum wire electrodes mounted within the pressure plate. Interneurons were sought at depths between 1.8 mm from the surface and the ventral tip of the ventral horn (usually 3.0–3.2 mm deep, as indicated by the motoneuron positions).

Interneurons were identified by a variety of criteria. First, a necessary condition was the absence of an antidromic spike from the segmental nerves, which were stimulated during tracking. Second, a sufficient condition was antidromic activation from the spinal cord electrodes in the more caudal segments. Test stimulation via these electrodes was made soon after penetration, using voltage pulses (0.1 ms, ±30 V) applied between either the left or the right pair of electrodes, both polarities being tested. Care was taken to observe responses at threshold so that distinction could be made between antidromic and orthodromic activation. Third, the position of the neuron was a useful guide (Kirkwood et al. 1988). Finally, many...
interneurons were identified by their typical high-frequency injury discharge, particularly at the moment of penetration. When checked against more formal criteria, including intracellular labeling, this final criterion was never wrong.

Physiological data were recorded first. Usually this included a short period of nerve stimulation and/or stimulation of the spinal cord, followed by a period with no stimulation, to allow assessment of the central respiratory drive potential (CRDP; Sears 1964c) and of the stability of the penetration. If the membrane potential was around −40 mV or better and the recording was somatic (or relatively close to the soma), iontophoretic injection of Neurobiotin was performed. Depolarizing current pulses (650 ms, 1 s$^{-1}$) of variable amplitude (typically 5 nA) were used. The antidromic spike or a synaptic potential from stimulation of a muscle nerve or the spinal cord was set to occur a few milliseconds before the start of each current pulse, so that the physiological state of injected neurons could be continuously monitored. Injection was terminated if the membrane potential declined to −20 mV or sooner if the physiological response deteriorated too much. The mean current integral for the cells reported here was $51.6 \pm 28.4$ nA/s (n = 18). Membrane potentials were confirmed on exiting from the cell.

Some penetrations were axononal, as was obvious from the spike shapes or the absence of synaptic activity. However, axonal penetrations were accepted, especially for the morphological part of the study, if they were sufficiently close to the soma, the criterion being the clear presence of synaptic noise or synaptic responses to nerve or spinal cord stimulation. Unintended labeling of unidentified neurons or of motoneurons was minimized by withdrawing from such penetrations as soon as possible. Following termination of current injection, the electrode was withdrawn from the spinal cord and further penetrations were restricted to positions ≥2 mm more rostral or caudal. The rostrocaudal positions of all injected cells were noted with respect to dorsal root entry positions or other surface marks.

All recordings were stored on magnetic tape. Data were acquired for computer analysis or display either on- or off-line (CED 1401 A-D interface and Spike2 software; Cambridge Electronic Design, Cambridge, UK). Both a low-gain DC version (amplification $\times 50$) and a high-gain, high-pass filtered version (amplification $\times 1,000$; time constant, 50 ms) of the membrane potential were included.

**Histological procedures**

Not more than 4 h after the first injection of a cell (to allow for transport of label), the animal was heparinized and perfused through the left ventricle with a saline rinse, followed by 2 L of 4% paraformaldehyde in phosphate buffer (pH 7.4). Relevant segments of spinal cord were removed and, according to the hardness of the tissue, stored in either phosphate-buffered saline (PBS) or the same fixative overnight at 4°C. If frozen sections were required, the solutions also contained 20% sucrose.

If frozen, the sections were reacted with diaminobenzidine, nickel ammonium sulfate, and hydrogen peroxide in Tris buffer (pH 7.6), the sections were reacted with diaminobenzidine, nickel ammonium sulfate, and hydrogen peroxide in Tris buffer to reveal the labeled neuron and its processes. The progress of the reaction was followed by viewing the sections under a dissecting microscope. Once a well-stained neuron was identified or the background staining became prominent, the sections were washed in cold 4% Triton X-100 buffer. The sections were mounted on gelatinized slides and air dried for ≥24 h. They were counterstained with neutral red (1% solution), serially dehydrated through acetone, and cleared in xylene. The slides were then coated with DePeX mounting medium (BDH Laboratory Supplies) and coverslipped.

In some animals alternate sections were kept as two separate series and one series reacted first. Selected sections were then mounted in glycerol and examined under the microscope to identify those containing terminal fields. A few sections adjacent to these were selected from the second series and set aside for immunohistochemical processing. The remaining sections were processed in an identical fashion to the first series. Sections were examined with a Zeiss Axioskop microscope. Identified terminals and selected terminal fields were reconstructed in the transverse plane via a drawing tube.

The selected sections were incubated at room temperature for 90 min in avidin conjugated to fluorescein isothiocyanate (Vector Laboratories, Peterborough, UK) and then for 3 days at 4°C in sheep antisera against glutamic acid decarboxylase (GAD, diluted 1:600; a gift from Dr. W. H. Oertel) and monoclonal mouse antibody against gephyrin (mAb 7a, diluted 1:100; Boehringer Mannheim, Mannheim, Germany), and then overnight in species-specific secondary antibodies (donkey anti-goat immunoglobulin G [IgG] conjugated to Cy5 and donkey anti-mouse IgG conjugated to lissamine rhodamine, both diluted 1:100; both from Jackson ImmunoResearch, West Grove, PA). The sections were then scanned through a ×60 oil-immersion lens with a Bio-Rad MRC 1024 confocal microscope (Bio-Rad, Hemel Hempstead, UK), equipped with a krypton–argon laser. Scanning with the 488-, 568-, and 633-nm laser lines was performed sequentially to avoid fluorescent bleedthrough.

**Statistics**

Latencies were measured to the nearest 0.05 ms and rostrocaudal positions (for defining segmental locations or for calculating conduction velocities) to the nearest 0.5 mm. Mean values are quoted as ±SD. In statistical tests, $P < 0.05$ was taken as significant.

**RESULTS**

**Physiological properties**

**GENERAL**. The properties of 45 interneurons were analyzed, all with membrane potentials of −40 mV or more negative at some time, whether in the first minute (commonly the time for the “best” membrane potential) or (also common) after the passage of positive current for Neurobiotin injection. Values for best membrane potential ranged from −40 to −62 mV, mean $48.6 \pm 5.9$ mV. An acceptability threshold of −40 mV was chosen because in many recordings the amplitudes of synaptic potential (spontaneous or evoked) were seen to decrease substantially with depolarization of only a few millivolts above this value, but appeared to be much less sensitive at more polarized levels. Values for amplitudes are quoted for the period with the best membrane potential wherever possible, particularly for the CRDP and the synaptic noise.

Peripheral nerve-evoked synaptic potentials were tested most often at the start of a penetration. Alternatively, for neurons firing at high frequencies, testing was performed after the cells had stopped firing, either because they hyperpolarized or had depolarized (spikes inactivated). Occasionally, hyperpolarization of the membrane by passage of negative current was used to prevent firing of the cells and to reveal synaptic potentials, but mostly this procedure was avoided, to prevent unwanted Neurobiotin labeling.

**IDENTIFICATION**. Objective criteria were required to separate recordings of interneurons from those of motoneurons. The
The most important criterion was antidromic activation from the caudally located stimulating electrodes, located on either side of the spinal cord. The usual indicators of antidromic activation were used (i.e., a sharp threshold and a constant latency), particularly if constant with variation of stimulus strength. However, on their own, these two indicators were not very reliable, since a high proportion of the cells showed short-latency EPSPs following the cord stimuli. These EPSPs could be very large (Fig. 1B) or could readily elicit an action potential. Thus apparent all-or-nothing firing with minimal temporal dispersion could occur with synaptic activation, including double responses to double stimuli 1–2 ms apart. Antidromic activation was therefore considered reliable only if the presumed antidromic spike occurred ≥0.25 ms earlier than any EPSP evoked from the spinal cord in that neuron (Fig. 1A, cf. Fig. 1C).

Using these criteria, 25 neurons were antidromically identified from stimuli delivered 15–43 mm caudally, with latencies 0.4–1.05 ms. Allowance of 0.15 ms for utilization time gave conduction velocities of 30–112 m·s⁻¹ (mean, 58.8 ± 19.3) for these 25 neurons. EPSP latencies in the same neurons ranged from 0.9 to 1.3 ms (n = 18). A further 7 neurons were classified as borderline for antidromic identification, having shown spikes with a fixed latency and a sharp threshold, but with those spikes being at latencies very close to the latencies of EPSPs in the same neurons (0.85–1.4 ms) (Fig. 1, C and D). Ten neurons could not be activated antidromically (7 of these showed EPSPs with latencies 1.0–1.3 ms) and 3 other neurons were classified asClarke’s column neurons and showed no central respiratory drive potential (CRDP).

**FIG. 1.** Illustration of antidromic identification criteria and examples of synaptic noise. A–D: responses in 3 different interneurons to stimulation from the caudally located spinal cord electrodes. A: superimposed sweeps at threshold for antidromic activation. The distinction is clear between the antidromic spikes (*) and the later, presumably synaptically evoked spikes. B: an example of a large EPSP evoked from the spinal cord and that triggers spikes in about half of the trials. Notice that both here and in A, the temporal dispersion of the synaptically evoked spikes is very similar to that of the antidromic spikes in A, C and D: responses from a single interneuron, depolarized in expiration (C) and hyperpolarized in inspiration (D). The 4 earliest evoked spikes in C (*) appear to take off about 0.15 ms before the start of the EPSP, most clearly delineated in D. However, this was not considered sufficiently reliable for identification as antidromic (see text). Arrows in A–D indicate time of stimulus. E–J: synaptic noise. E: example dominated by large spontaneous EPSPs. This cell was anatomically identified as a Clarke’s column neuron and showed no central respiratory drive potential (CRDP). F: more typical synaptic noise, with a mixture of EPSPs and IPSPs, although this was the largest amplitude noise observed. The cell showed a variable expiratory CRDP, maximum amplitude 5 mV. Illustration taken from inspiration. G–J: recordings from 2 expiratory interneurons, illustrated in expiration (G, I) and in inspiration (H, J). Top traces: efferent discharge in the external intercostal nerve. G and H: example of synaptic noise dominated by IPSPs. CRDP amplitude measured as 1 mV. Note the increase in IPSP frequency during inspiration. I and J: example of a recording from an axonal penetration, showing synaptic noise and spikes with moderately sized afterhyperpolarizations (cell F, Fig. 5, same cell as C and D, CRDP amplitude measured as 2 mV). Time calibration in D applies to A–D, that in F applies to E and F, and that in J applies to G–J. Interneuron spikes in C, D, I, and J are truncated.
were not tested. Thus a high proportion of the neurons showed descending axons several segments in length, with relatively fast conducting axons.

Sixteen of the 25 neurons were classified as contralaterally projecting, on the basis of showing a substantially lower threshold to stimuli on the right than on the left; 7 were similarly classified as ipsilaterally projecting; and 2 as projecting to either side (similar thresholds on the two sides). For the borderline antidromic group, one was classified as contralaterally projecting, 4 as ipsilaterally projecting, and 2 as projecting to either side. Note that the relative thresholds between the two sides cannot be absolutely accurate in determining the laterality of the axon, especially for axons running in the dorsomedial part of the ventral funiculus, since such axons could have been at similar distances from the electrodes on the two sides, whose tips were placed more ventrally.

The two other criteria, depth and firing frequency, were used to identify the 20 interneurons that were borderline or not antidromically identified. The depth criterion was that the penetration should have been located 0.2 mm more dorsal than any of the antidromically identified motoneurons recorded in the same segment of the animal. This identified 12 neurons (depths 1.8–2.8 mm; 9 of these <2.3 mm). The firing frequency criterion was the occurrence of firing at >100 impulses·s⁻¹, which identified 15 neurons (firing rates up to 700 impulses·s⁻¹; 8 neurons firing faster than 200 impulses·s⁻¹). Seven of the 20 interneurons fulfilled both criteria.

Confirmation that the identifications were correct came from the intracellular labeling (see Table 1 and later sections). The axons of all neurons physiologically identified as interneurons and subsequently labeled stayed within the spinal cord. A descending axon, with or without an additional ascending branch, was anatomically identified for all 11 antidromically identified neurons that were subsequently labeled. Further, all 3 labeled axons from neurons in the borderline antidromically identified group were observed as descending. All 5 of the labeled axons from the not antidromically identified group proved to be ascending.

Two of this last group (the only two with ipsilateral axons) were anatomically identified as dorsal spinocerebellar tract (DSCT) neurons, being located in Clarke’s column, with characteristic rostrocaudally orientated spiny dendrites tightly constrained within the column, and an axon without collaterals ascending just lateral to the intermediolateral cell column (Houchin et al. 1983; Randić et al. 1981). Depths for the recordings were 2.0 and 2.05 mm. Physiologically, these neurons were characterized by an absence of a CRDP, as in Tanaka and Hirai (1994); the presence of a low-threshold EPSP with short segmental latency (0.45 or 0.8 ms) from one of the peripheral nerves; and the presence of dramatic, EPSP-dominating synaptic noise, as in Kuno et al. (1973) (Fig. 1E). Since our aim was to study propriospinal interneurons, these two neurons were not considered further, except that their physiological characteristics may be useful as possible exclusion criteria for other similar recordings.

The group of interneurons to be considered for their physiological properties thus consists of 43 neurons, 31 of which were positively identified by their axonal trajectory, either physiologically or anatomically (22 contralateral, 8 ipsilateral, one side not determined).

**AXONAL PENETRATIONS.** Proper interpretation of synaptic potentials depends on the assumption that the penetrations are somatic, but we suspected that at least some penetrations were axonal. Indeed, anatomical evidence confirmed that four penetrations definitely were axonal. Three of these penetration sites were identified from the axonal morphology, characterized by lengths of fragmented axons coupled with signs of extracellular label and, in two instances, retraction bulbs (see Fig. 5), the sites also being confirmed by depth measurements and identification of electrode tracks. For these three, the distances from the soma to the recording site along the axon were 0.40, 0.48, and 0.65 mm. The fourth site was not located precisely, but the recording was made on the left side and the soma was found contralaterally. The axon traveled 0.63 mm rostrally before crossing the midline, giving a total distance of ≥0.8 mm from the soma to the recording site.

Although the recordings at these sites were at a distance from the soma, some synaptic potentials were seen, including EPSPs or IPSPs from stimulation of either or both peripheral nerve or spinal cord (amplitude 0.5–3.7 mV). Two of these recordings showed CRDPs.

**TABLE 1. Summary of interneuron identifications and comparisons with anatomical measurements**

<table>
<thead>
<tr>
<th>Antidromically identified</th>
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<tr>
<td>Contralateral electrodes (Anatomy)</td>
<td>Ipsilateral electrodes (Anatomy)</td>
</tr>
<tr>
<td>16 (Contra, 8; Ipsi, 0)</td>
<td>7 (Contra, 0; Ipsi, 2)</td>
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**Borderline antidromically identified**

<table>
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<th>Ipsilateral electrodes (Anatomy)</th>
<th>Electodes on either side (Anatomy)</th>
<th>Total</th>
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<tr>
<td>1</td>
<td>4 (Contra, 1; Ipsi, 0)</td>
<td>2 (Contra, 1; Ipsi, 1)</td>
<td>7</td>
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**Not antidromically identified**

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<th>(Anatomy)</th>
<th>(Anatomy)</th>
<th>(Anatomy)</th>
<th>Total</th>
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<tr>
<td>10 (Contra, 3; Ipsi, 2)</td>
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<td>10</td>
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**Not tested from caudal electrodes (includes one neuron not physiologically identified)**

<table>
<thead>
<tr>
<th>(Anatomy)</th>
<th>Total</th>
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<td>4 (Contra, 1; Ipsi, 0)</td>
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The numbers of interneurons in each category of antidromic identification (from the electrodes two or three segments caudal) are listed, together with the numbers of the same group with subsequent identification of their axonal destination after intracellular labeling (parentheses). Some of the descending axons also possessed an ascending branch. Interneurons in the last three categories were identified either by position or by firing frequency. Note that there is only one discrepancy between the physiological identification and the anatomy and that the interneuron concerned was, in any case, in the borderline category.
(amplitude: 2 and 1.5 mV). Three of the neurons showed synaptic noise, although of relatively small amplitude, <1 mV peak to peak for one and 1–2 mV peak to peak for the other two (Fig. 1G). Similarly, the spikes in three of these four axonal penetrations showed small afterhyperpolarizations (AHPs), of 1–2.5 mV in amplitude (Fig. 1, I and J). The spikes in the other penetration showed none. These AHPs presumably represented attenuated versions of somatic AHPs, since classic descriptions of axonal spikes (e.g., for central axons; Rudin and Eisenman 1954) do not include an AHP. The majority of penetrations here that would have been thought to be somatic, with fast rising synaptic potentials and large-amplitude synaptic noise, showed spikes with AHPs of 5–15 mV.

We cannot know whether the other 39 penetrations were somatic or how near to somatic they were, but the above-cited values can nevertheless inform the interpretation of the observations in the following text.

SYNAPTIC NOISE. Spontaneous synaptic noise was a prominent feature of many of the recordings. In three neurons the synaptic noise was dominated by EPSPs, similar to the two confirmed DSCT neurons mentioned earlier. However, two of the three had identified descending axons, so only one (depth 2.2 mm, not tested from the caudal electrodes) might actually have been a DSCT neuron. In another five neurons the noise was dominated by IPSPs (Fig. 1, G and H), the remaining majority showing a mixture of EPSPs and IPSPs (e.g., Fig. 1F). For nine neurons the noise was clearly modulated during respiration (Figs. 1, G and H and 2, B, D, and G). Three of the five neurons whose noise was dominated by IPSPs were in this modulated group; all three showed expiratory CRDPs, with the noise increasing in inspiration (Fig. 1, G and H).

The peak-to-peak amplitude of the synaptic noise was also assessed visually for each recording. The maximum amplitude

![Fig. 2. Respiratory activity in interneurons. A–G: intracellular recordings from interneurons (bottom trace). Middle trace (3rd trace in A, top trace in C): external intercostal nerve discharge. In A, E, and F, the top trace is the firing rate of the interneuron displayed as instantaneous frequency. In A, the 2nd trace is a rate meter record for the interneuron (0.3-s bins). In B, D, and G the top trace is the high-pass filtered version of the intracellular record. A: a relatively large amplitude expiratory CRDP. Note the irregular discharge. B: an inspiratory CRDP. Note the modest increase in synaptic noise during inspiration, visible in the top trace. C: a low-amplitude expiratory CRDP. D–F: 3 different epochs from the recording of a single interneuron. The CRDP was weakly expiratory at the start of the penetration (D), although the synaptic noise increased in inspiration. The neuron subsequently hyperpolarized (E) and fired tonically, but with inspiratory phasing (note the irregularity of the discharge). During the passage of 1 nA hyperpolarizing current (F), the inspiratory CRDP was clearly revealed. G: a variable CRDP. For the first 5 cycles the neuron depolarized during inspiration; in the next 2 cycles, it hyperpolarized. Note that the synaptic noise (including its inspiratory modulation) increased as the inspiratory CRDP increased, but decreased again for the last 2 cycles. H and I: 2 successive epochs, 24 min apart, from an axonal recording from an interneuron. The intracellular record is not shown; instead, the top trace shows the interneuron firing frequency. Bottom trace: external intercostal nerve. Middle trace: multiunit firing frequency for spikes in this nerve. Firing frequency displayed in 0.1-s bins. The baseline tonic discharge of the interneuron was relatively constant (40–60 impulses·s⁻¹), but the inspiratory modulation varied significantly, without much change in the intensity of the nerve discharge. Time calibration in I also applies to H. The higher-frequency wave in B (4 × respiratory rate) is a movement artifact from the ventilator. Interneuron spikes in D–F are truncated.
was 10 mV (Fig. 1F); otherwise, 10 neurons showed amplitudes of 3–5 mV, 11 of 2–3 mV, 18 of 1–2 mV, and the remaining 3 of <1 mV. Two of those assessed as 1–2 mV are shown in Fig. 1, G–J, the second of these (Fig. 1, I and J) being one of the identified axonal recordings.

**CRDPs.** Thirty-six of the neurons showed CRDPs. Frequently, these were variable, waxing and waning in amplitude, often independently of changes in the membrane potential. The variability was most often seen with small CRDPs and could even involve a reversal of the CRDP. Examples of variable amplitude are shown in Fig. 2, D–F and in G. Amplitudes and time courses quoted in the following text apply to the largest-amplitude CRDP (averaged over a few respiratory cycles) for each neuron. Most of the CRDPs were measured when the neurons were not firing or were firing at slow rates (e.g., Fig. 2, A and F), but a few could be measured only while the neuron was firing at a relatively high rate. The amplitudes of these must be considered approximate.

The largest CRDP amplitude was 8 mV. Nine neurons showed CRDPs of amplitude ≥4 mV, 16 of amplitude 2–3.9 mV, and the rest <2 mV. Respiratory phasing of an interneuron was defined by the phase when it was most depolarized (or most active), following the three-phase description of the respiratory cycle by Richter (1982), with the external intercostal nerve discharge taking the place of the more usual phrenic discharge (Kirkwood et al. 1988). The most common type of CRDP was expiratory (19 neurons; mean CRDP amplitude: 2.4 ± 1.8 mV). These were mostly dominated by a sharp hyperpolarization during inspiration, almost certainly the result of postsynaptic inhibition (Kirkwood et al. 1992). Only one of these (amplitude: 7 mV; Fig. 2A) showed an incremental ramp of depolarization during expiration, most clearly evident in the figure as the ramp of firing frequency. The others were either flat during expiration or showed a relatively small (often variable) depolarization in postinspiration, declining through the rest of expiration. An example of the most common type of low-amplitude expiratory CRDP is shown in Fig. 2C. Despite the presence of declining expiratory depolarization, the clearest defining feature remained the inspiratory hyperpolarization. This therefore distinguishes these CRDPs from the pronounced decrementing depolarization, often with very little separate hyperpolarization in inspiration, which was evident in a high proportion of the recordings from hindlimb motoneurons (Ford and Kirkwood 2006) and we have therefore avoided the terminology used there (E_{dec}).

Eight neurons depolarized in inspiration (e.g., Fig. 2B) and a further four showed some depolarization in inspiration, but included an additional depolarization in postinspiration. The postinspiratory component was often variable, sometimes dominating. The mean amplitude for these 12 neurons was 3.1 ± 1.8 mV.

The remaining five neurons comprised a heterogeneous group with very variable CRDPs (mean amplitude: 3.1 ± 2.0 mV), including one neuron that was mostly expiratory at the start of the recording (CRDP of ∼1 mV, Fig. 2D), but later became clearly inspiratory, with a CRDP of 5 mV amplitude (Fig. 2, E and F). For this example, the change from an expiratory to an inspiratory one might be explained by concurrent excitation and inhibition during inspiration (cf. de Almeida and Kirkwood 2010), the inhibition becoming more dominant with depolarization of the cell (membrane potential being around ∼35 mV in Fig. 2D, but around ∼48 mV in Fig. 2E). However, that explanation cannot apply to the neuron illustrated in Fig. 2G, where the cell shows progressively more inspiratory depolarization during the first five cycles, but then inspiratory hyperpolarization in the next two cycles. In this case the apparent excitation is more prominent when the cell is most depolarized (the reverse of the previous example) and the synaptic noise shows a gradually increasing tonic plus inspiratory time course, which then fades away for the latter part of the figure.

**Firing patterns.** In the analysis of Kirkwood et al. (1993) an important feature was the division of the extracellularly recorded firing patterns of respiratory interneurons into two groups: phasic versus tonic. However, although the interneuron discharges here often demonstrated rather strong respiratory modulation, generally corresponding to the time courses seen in the CRDPs, it was generally not possible to make a meaningful distinction between tonic and phasic patterns from the present data. This was because of the presence of presumed injury discharges in a high proportion of the cells. Often this occurred as very high frequency firing at the start of the penetration, which subsequently settled down to a steady level. Conversely, some neurons with a CRDP were initially silent when well polarized, but progressed through a phasic to a tonic firing pattern as they gradually depolarized. In other cases, depolarized neurons, where the spikes had inactivated, repolarized and showed a similar sequence in reverse. Many of the high values of firing frequencies quoted earlier came from presumed injury discharges. Motoneurons (Saywell et al. 2007) never showed such high-frequency discharges.

For axonal penetrations, the firing frequencies might be expected to be less dependent on depolarization at the recording site than for those near the soma. For two of the identified axonal recordings where the neurons showed strong persistent discharges, this was indeed the case: the firing frequencies seemed to be scarcely affected by depolarization resulting from electrode movement and were thus quite unlike typical injury discharges. In one (recorded 0.4 mm from the soma), an expiratory pattern was present, with a hyperpolarization of about 2 mV during inspiration, which silenced the cell. This cell was therefore classified as showing a 2 mV CRDP. In the second (≥0.8 mm from the soma), no CRDP was detected, but the firing pattern was clearly inspiratory and/or postinspiratory. In this example it was also apparent, as illustrated in Fig. 2, H and I, that the degree of modulation itself varied over a very wide range (note especially the variation in the postinspiratory component), similar to the variation seen in many of the CRDPs. This axonal recording thus confirms that such variation is a physiological feature rather than a result of disturbance by the electrode. In another neuron, which was not a confirmed axonal recording, but where no CRDP was detected, respiratory modulation was also detected in the firing pattern. In this case, the neuron started out as firing preferentially in expiration, but by the end of the recording the pattern had shifted to be clearly inspiratory (plus postinspiratory). In this instance, the inspiratory drive monitored in the T6 external intercostal nerve discharge had gradually increased over this period.

An additional property of the interneuron discharges recorded here was that their firing was notably irregular, on an
interval-by-interval basis, as reported by Kirkwood et al. (1988) for extracellularly recorded thoracic interneurons. This is clear in the recordings illustrated (Fig. 2, A and E).

PERIPHERAL AFFERENT INPUTS. Synaptic potentials evoked by ipsilateral peripheral nerve stimulation were tested in 37 neurons, all at 5T. Where possible, graded intensities were used below 5T to give information on the afferents responsible for responses. Segmental latencies were measured from the cord dorsum volleys, at the baseline crossing point, going from positive to negative, and were taken as monosynaptic if <1 ms (Fig. 3, A–D, H, and I).

Synaptic potentials were detected in 30 cells, as detailed in Table 2A. The external intercostal nerve, which is exclusively a muscle nerve (Sears 1964a), gave the fewest PSPs, which were detected in only 4 cells; these comprised 3 cells that showed monosynaptic EPSPs to all three nerves (Fig. 3, A–C) and one cell that showed IPSPs to all three nerves (at segmental latencies 1.2–1.6 ms). One of the 3 cells with EPSPs to all three nerves (illustrated in Fig. 3, A–C) was the one previously mentioned as a possible DSCT neuron.

The other two nerves, internal intercostal and dorsal ramus, both mixed nerves, gave EPSPs in respectively 18/36 (50%) and 16/37 (43%) of neurons tested. However, these two inputs differed in the proportion of EPSPs that were monosynaptic, 6 of 18 for the internal intercostal and 13 of 16 for the dorsal ramus (a significant difference, $\chi^2, P < 0.05$), as exemplified in Fig. 3, D and E. All of those with a monosynaptic input from the internal intercostal nerve also showed one to the dorsal ramus and, in each case, the amplitude of the EPSP (at 5T) was larger for the dorsal ramus than that for the internal intercostal nerve. The thresholds for the monosynaptic EPSPs were noted to be below 1.5T for 9 neurons, with 8 EPSPs being evoked from the dorsal ramus, 3 from the internal intercostal nerve and one from the external intercostal nerve, including in the one possible DSCT neuron. The thresholds for two of these EPSPs from the dorsal ramus were measured and found to be close to 1.0T (Fig. 3, H, I, and K). The 13 neurons with a monosynaptic EPSP were not concentrated in any one group according to axonal destination, the axons concerned including 5 antidromically identified as contralateral descending, 2 anatomically identified as contralateral ascending, and 3 antidromically identified as ipsilateral descending. The same was true for the 9 neurons where the EPSP threshold was confirmed as below 1.5T (3, 1, and 3 neurons, respectively, for the same categories).

**FIG. 3.** Synaptic potentials evoked from peripheral nerves. A–J: averaged responses (2–40 sweeps at the stimulus strengths indicated). Top traces: intracellular recordings. Bottom traces: cord dorsum volleys (displayed with negative down). A–C: responses from an interneuron showing a monosynaptic EPSP to all 3 nerves: dorsal ramus, external intercostal, and internal intercostal for A, B, and C, respectively. D and E: responses from an interneuron with a monosynaptic EPSP from the dorsal ramus (E) and a longer-latency EPSP from the internal intercostal nerve (E). F: short-latency inhibition in an interneuron, probably disynaptic, from the internal intercostal nerve. G: longer-latency inhibition in a different interneuron, from the internal intercostal nerve. H and I: monosynaptic EPSP in an interneuron from the dorsal ramus, demonstrating a threshold at 1.0T; H, response at 5T, including a later component with a higher threshold; I, response near threshold. The amplitudes of the EPSP and the cord dorsum volley for this interneuron are plotted against stimulus strength in K. J: responses from an interneuron to stimulation of the dorsal ramus with the dorsal roots cut. Four superimposed sweeps; stimulus delivered at the arrow.
The total number of neurons represented in part 3, and 4 (by adding values from different columns, e.g., for the total number of neurons with EPSPs from the dorsal ramus, add values from the first row in columns 1, IPSPs 0 0 2 0 EPSPs 0 4 4 1 3 IPSPs 0 3 5 1 EPSPs 4 6 9 3 7 Renshaw cells might contribute to the IPSPs in this cell. nature of some of the late components suggests that only a few dorsal roots cut (Kirkwood et al. 1981). The all-or-nothing discharges often seen in thoracic Renshaw cells with the features are consistent with the relatively short and variable burst response to the dorsal ramus were facilitated by stimulation of the external intercostal nerve (not illustrated). All these features are consistent with the relatively short and variable burst latency of approximately 1 ms, presumably disynaptic (Fig. 3F). In three of the cells, the IPSPs (latencies 2.0–4.2 ms) were seen only to the internal intercostal nerve; in five of the cells the IPSPs (latencies: 1.45–5.2 ms) were present for both the dorsal ramus and the internal intercostal nerve; and for the remaining cell, IPSPs were seen to all three nerves. This last cell and one of those with IPSPs from both the dorsal ramus and the internal intercostal nerve were among the five neurons noted as showing prominent IPSP synaptic noise.

In addition, another of those with prominent IPSP synaptic noise showed an IPSP to dorsal ramus stimulation, at 1.7 ms (Fig. 3J). This example was excluded from Table 2 and the above-cited list because it was recorded with dorsal roots cut in the segment concerned. This neuron therefore received recurrent inhibition, presumably via Renshaw cells. It was classified as borderline for antidromic activation from the caudal spinal cord, but was certainly not a motoneuron, discharging up to 240 impulses·s⁻¹ as the cell slowly depolarized soon after penetration. Note the two early IPSP components in the response, about 1 ms apart, plus the rather variable, periodic occurrence of later components. IPSPs were not seen to stimulation of the other two nerves, but the later components in the response to the dorsal ramus were facilitated by stimulation of the external intercostal nerve (not illustrated). All these features are consistent with the relatively short and variable burst of discharges often seen in thoracic Renshaw cells with the dorsal roots cut (Kirkwood et al. 1981). The all-or-nothing nature of some of the late components suggests that only a few Renshaw cells might contribute to the IPSPs in this cell.

Table 2B lists the PSPs seen in the neurons analyzed morphologically (see following text). This sample seems to be representative of the main group, although inevitably with this small population not all categories are included.

**Morphological properties**

Eighteen neurons intracellularly labeled with Neurobiotin were recovered. Seventeen of these belong to the population, earlier described physiologically; the eighteenth, although well labeled, was not deliberately injected and its identity was ambiguous among the cells recorded. One of the neurons was located more dorsally than the others, laterally in lamina VII. This neuron was noted earlier as showing a high-threshold monosynaptic EPSP from the internal intercostal nerve. Because of its unusual position, both with respect to the other neurons here and those recorded in lesioned spinal cords (Meehan et al. 2003), it is not considered further. Thus the population considered consists of 17 neurons. For 16 of these, the axon was sufficiently stained to identify the presence or absence of collaterals, but the staining of the somata and dendrites was not always complete. Ten cells were sufficiently stained for reconstruction of the dendritic tree and, in one other, some sections were not sufficiently stained for reconstruction, although the extent of the dendritic tree could be determined. For two cells, the somata were missing, although their positions were clear from some surviving labeled dendrites.

**SOMADENDRITIC FEATURES.** Considerable variation in somatic morphology was seen, as may be appreciated from the illustrations in Figs. 4, 5, and 11. The dendrites of all but one of the neurons (Fig. 4D) were more or less radially organized. The sizes of the somata covered a wide range. Ellipses fitted to the reconstructed somata (n = 15) had major diameters ranging from 25 to 70 μm (mean 40 ± 12 μm) and minor diameters from 13 to 38 μm (mean 26 ± 7.9 μm). The somata illustrated in Fig. 5, top (also shown in Fig. 4C) and Fig. 4E were the two largest (55 × 38 and 70 × 35 μm, respectively) and those in Figs. 5 (bottom) and 11 were two of the smallest (27 × 20 and 28 × 13 μm, respectively). Note that the cell illustrated in Fig.

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**TABLE 2. Summary of PSPs evoked from peripheral nerves**

<table>
<thead>
<tr>
<th>PSP Category</th>
<th>Only From Dorsal Ramus</th>
<th>Only From Internal Intercostal Nerve</th>
<th>From Dorsal Ramus and Internal Intercostal Nerve</th>
<th>From All Three Nerves</th>
<th>No PSPs From Peripheral Nerves</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. All neurons</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPSPs (latency &lt;1 ms)</td>
<td>4 (3)</td>
<td>6 (0)</td>
<td>9 (both: 3) (DR only: 4)</td>
<td>3 (all 3: 3)</td>
<td></td>
</tr>
<tr>
<td>IPSPs (latency &lt;2 ms)</td>
<td>0 (1)</td>
<td>3 (both: 1)</td>
<td>5 (all 3: 1)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>B. Neurons analyzed for morphology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPSPs (latency &lt;1 ms)</td>
<td>0 (0)</td>
<td>4 (both: 1) (DR only: 2)</td>
<td>4 (all 3: 1)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>IPSPs (latency &lt;2 ms)</td>
<td>0 (0)</td>
<td>2 (0)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The number of interneurons with PSPs from the various combinations of peripheral nerves stimulated at 5T are listed. Totals for any one nerve may be obtained by adding values from different columns, e.g., for the total number of neurons with EPSPs from the dorsal ramus, add values from the first row in columns 1, 3, and 4 (=16). The total number of neurons represented in part A is 37, one of the neurons appearing twice in the table, having shown a monosynaptic EPSP to the dorsal ramus and a long-latency IPSP to the internal nerve. The numbers for IPSPs are the numbers of interneurons with IPSPs with thresholds lower than any EPSPs. No interneurons were found for the combinations not included in the table (e.g., external intercostal nerve only). Numbers in parentheses refer to the responses interpreted as monosynaptic (for the EPSPs) or disynaptic (for the IPSPs).
4E was larger than all the Nissl-stained cells in six adjacent sections (Fig. 4F). At least some of these other cells must have been motoneurons. The identity of this cell as an interneuron was unequivocal, as it was for all of the anatomically described neurons here, by virtue of its axon being traced, in this instance into the dorsomedial part of the ipsilateral ventral funiculus where it divided into ascending and descending branches (arrow in Fig. 4E). Note that the somatic dimensions quoted earlier refer to reconstructions in the transverse plane. Lipski and Martin-Body (1987) noted that thoracic motoneurons were often elongated rostrocaudally. The same may well have been true for the interneurons here, but there is little direct evidence for this, except that some of the largest cells, whose somata could be found in three successive 50-μm sections, must have had somata extending for ≥70 μm rostrocaudally.

Numbers of primary dendrites varied from 4 to 11 (median 7, n = 15) and their terminal branches frequently extended into the white matter (Figs. 4E, 5, and 11). In none of the cells did we observe any tendency for dendrites to turn at the gray–white border to avoid entering the white matter, as described for thoracic motoneurons by Lipski and Martin-Body (1987). None of the dendrites crossed the midline, with the exception of one cell located in region X of Rexed (1954). Neurons with larger somata tended to have more dendrites, as shown in Fig. 6A (filled circles). Note that the one outlying point in this graph (4 dendrites) came from the one unusually shaped cell in Fig. 4D. Dendrites extended between 500 and 1,250 μm rostral to the soma (mean 940 ± 224 μm, n = 10) and between 450 and 1,600 μm caudal (mean 914 ± 306 μm, n = 11).

Dendrites of cells with larger somata generally had a larger rostrocaudal spread (Fig. 6A, open circles), although a similar relationship for the dendritic spread in the transverse plane was not obvious (compare the two examples in Fig. 5). A few dendrites were very thin and unbranched (Fig. 4B), similar to the microdendrites shown in thoracic motoneurons by Lipski and Martin-Body (1987) (cf. Russell-Mergenthal et al. 1986). Dendrites were generally smooth, with hardly any spines, or spine-like features, although a few such processes were observed in two cells (Fig. 4B). The terminal regions of some dendrites were beaded, but again such a characteristic was not common.

AXONAL FEATURES. Axons originated either from the soma or from a proximal dendrite. Most of these (n = 17) were directed toward the ventral commissure and then crossed the midline to

FIG. 4. Soma features for labeled interneurons. A and C: photomicrographs of single sections through the somata of 2 interneurons: A, one of the smallest (full reconstruction in Fig. 11); C, one of the largest (full reconstruction in Fig. 5, top). B: examples of 2 rare features, both from cell P (shown in A): top, a dendritic spine; bottom, a microdendrite. Each photomicrograph is a montage of photographs at several focal planes. Brightness and contrast adjusted in Photoshop (Adobe). D: reconstruction of the central part of an unusual cell, with a nonradial arrangement of dendrites. E: partial reconstruction (proximal parts of the dendrites only) of the largest soma observed (cell O). F: drawings of the outlines of all of the counterstained neuronal somata in the 6 adjacent sections used for the reconstruction, also including the outline of the soma of cell O. Note that this soma is larger than any of the counterstained somata.
descend (8), ascend (3), or bifurcate into ascending and descending branches (4). Two axons projected ipsilaterally, one descending and ascending within the dorsomedial ventral funiculus, the other looping dorsally then projecting directly ventrally to bifurcate and run rostral and caudal, just ventral to the tip of the ventral horn (Fig. 11). The axons that started dorsally in the ventral funiculus frequently drifted more ventrally as they coursed either rostrally or caudally. All but one of the crossing axons did so at the level of, or rostral to, the soma (Fig. 7, A–N), the exception crossing 250 μm caudally (Fig. 7L). This was true for both rostrally and caudally projecting axons, although two of the three rostrally projecting ones also showed a loop of axon caudal to the soma (Fig. 7, I and J). Axon diameters were measured before crossing or bifurcation, from a part of the axon that was uniformly stained and running within the plane of the section, which most often occurred in the dorsal part of the ventral horn gray matter. The diameters ranged from 1.8 to 4 μm (mean 2.7 ± 0.56 μm). One axon (that of the only neuron in Rexed’s region X) is included in the preceding description, but excluded from Fig. 7, and from the analysis in the following text because the presence of another labeled axon made the assessment of its collaterals ambiguous.

The axons were followed over a rostrocaudal distance of 2.3 to 16.7 mm (including the rostrocaudal length of the initial loops), as summarized in Fig. 7. The length of axon that we were able to follow depended either on the length of the block of tissue cut or on fading of the axon to an extent that detection of collaterals was unreliable. One axon appeared to divide into two ascending branches 3.5 mm rostral to the soma, the two branches being followed, running very close to each other in the dorsomedial part of the ventral funiculus for a further 5.6 mm (Fig. 7, cell O). Although the actual branch point was not visualized, there seemed to be no doubt that the two branches belonged to the same axon. The presumed branch point was in an undisturbed part of the cord, well rostral to any area of tracking with a microelectrode and no other axon was seen running rostrally in any of the 70 sections that contained the initial 3.5 mm of axon. Thus this morphology was unlikely to be a consequence of stray Neurobiotin labeling. No cells had axonal branches of any kind on both sides of the cord.

Collaterals (fine branches that left the stem axon and ran into the gray matter) were identified for 13 neurons, although for one of these (cell O), the only collaterals seen were at 6.55 mm rostral and 7.6 mm caudal to the soma and could be traced only a relatively short distance from their origins, as was the case for the more distal collaterals of other neurons. Most of the more proximally located collaterals (as well as some distal ones) showed axonal swellings, presumed to represent synaptic boutons (examples in Fig. 8A). These were approximately equally distributed between terminaux and en passant types, mostly ranging in size from 0.5 to 2.5 μm in diameter, with one or two as large as 3.5 μm. Occasionally, these were seen in close apposition to the counterstained neuronal somata.

The most strongly stained collaterals all showed numerous boutons. We have therefore indicated the occurrence of bou-
tons in Fig. 7, which summarizes the spacing and rostrocaudal extent of the collaterals for each axon. Those branches that terminated in boutons are indicated, as an assessment of the reliability of the measurements. All collaterals for the crossed axons originated contralaterally to the soma and projected only contralaterally. For two of the three bifurcating contralateral axons, the first collateral originated before the main axonal branch point, but still contralaterally. The collaterals for both of the neurons with ipsilateral axons projected only ipsilaterally. As is evident in Fig. 7, the spacing between collaterals was relatively wide (mean 3.01 ± 3.77 mm, median 1.75 mm, n = 10) and the rostrocaudal spread of individual collaterals was relatively narrow, so that there were only two instances where overlap between collaterals was observed.

Most collaterals coursed directly into the gray matter, but a few ran rostrocaudally in the white matter before turning and heading for the gray matter (indicated in Fig. 7). Reconstructed examples are shown in Fig. 8, B and C and a summary in Fig. 9. All the collaterals ramified in the ventral or intermediate horn, usually in relatively restricted areas. Note that the collateral branches in Fig. 8B all terminated in boutons and the very severely restricted locations for these two collaterals may thus be regarded as reliable. The main part of collateral 1 in Fig. 8C, extending across the ventral horn and about 0.6 mm rostrocaudally, was also well supplied with boutons, although this collateral also possessed one branch that ran rostrally for a further 1.94 mm as a single unbranched fiber with hardly any boutons. Note also that the other two collaterals for this axon also projected to this same area, particularly by ramifications in the lateral region, which is where the majority of boutons were located.

The results of Kirkwood et al. (1993) suggested that inhibitory thoracic respiratory interneurons, shown to be mostly phasic, generally projected more ventrally than the excitatory, generally tonic group. As explained earlier it is not possible to separate the interneurons here into tonic or phasic categories, but instead we have separated them by the amplitudes of their CRDPs, on the assumption that those with the largest CRDPs would be likely to correspond to the most phasic category and those with the smallest to correspond to the most tonic. However, in this small sample, no differentiation between the groups in the areas of projection was evident with this separation, as is clear by inspection from Fig. 9.

On the other hand, the projection areas are nevertheless highly likely to represent some specificity, in that, for all of the eight cells with more than one sufficiently well-labeled collateral, the different collaterals of a given neuron projected to the same areas of the ventral horn, as illustrated in Fig. 8, B and C (cf. Matsuyama et al. 2006). This was quite obvious for six of those with crossed axons (cells B, C, F, G, K, and N in Fig. 7), and could also be said to be true for two other axons. One of these two (cell P) uniquely showed an extensive ipsilateral projection, overlapping with its dendritic field. The other (cell L) had a crossed bifurcating axon and showed a strong projection (ascending) to the intermediolateral column. For each of these two neurons, the first collateral had rather widespread
projections and the second collateral, although projecting to the same area, was much less widespread and thus did not provide such a good test of the hypothesis. Note that for several axons, because they gradually adopted a deeper position in the ventral fasciculus with distance from the soma, the different collaterals, of necessity, took a different path to project to the same specific area of the ventral horn, as in Fig. 8C.

Structure–function relationships

One possible relationship that was not found—a relationship between the CRDPs and the projection areas—has already been mentioned. Another possibility—a relationship between the soma location and the CRDP—might also have been expected from the results of Kirkwood et al. (1993). To investigate this possibility the outlines of the ventral horns containing each of the somata were traced and scaled so that the outlines superimposed, as shown in Fig. 9, bottom right, where the different symbols represent different categories of CDRP. Although the numbers are small, the overall pattern is remarkably similar to the distributions represented in Fig. 8, A–C of Kirkwood et al. (1993), on the assumption that the larger CRDPs here were equivalent to phasic units there and the smaller CRDPs, tonic units. In particular, a horizontal line could be drawn to separate a more dorsal region, where only cells with low-amplitude inspiratory CRDPs were found, from a ventral region, where all CRDP types were seen (Fig. 9).

We have also looked for relationships between the occurrence of particular patterns of peripheral afferent inputs and the projection pattern of the neurons. Of the 16 neurons illustrated in Fig. 7, 4 were not tested for peripheral afferent inputs. The remaining 12 could be divided into four groups according to their afferent inputs. Despite the small numbers involved, some patterns were apparent in the projections displayed. The first group of neurons (category W in Fig. 9: cells N, F, M, and D) showed small EPSPs either to the internal intercostal nerve alone (three instances), or both to this and to the dorsal ramus, with latencies of 2.3–4.0 ms (i.e., typical of trisynaptic connections). All four of these showed descending or bifurcating axons with terminal fields located in the central ventral horn, at positions probably in the dorsal part of the motor nuclei (Kirkwood et al. 1988). All of their projections included the lateral part of the ventral horn; the two most similar to each other, cells F and D, both showed expiratory CRDPs and both showed EPSPs only to the internal intercostal nerve. The other

FIG. 8. Examples of collateral morphology. A: examples of boutons. Each photomicrograph is a montage of photographs at several focal planes. Brightness and contrast adjusted in Photoshop (Adobe). B: an axon showing 2 widely spaced collaterals, with very restricted areas of termination. All branches terminated in boutons. C: an axon with rather more widely spread terminations, but still well-spaced collaterals. Some branches terminated in boutons as illustrated in A. The most ventral branch in C, collateral 1, comprised a single process running 1.94 mm rostrally (see diagram in Fig. 7, cell F), with only one small side branch and hardly any boutons. It is shown broken up because of the difficulty of joining up the axonal segments from the large number of sections involved while still retaining the correct position in the ventral horn. Instead, these pieces were each traced by aligning the ventral horn outlines. Representative outlines of the ventral horn for B and C are shown in gray. CC, central canal. The scale bar in C also applies to B.
two were inspiratory, with bifurcating axons, one of which (cell M) gave the most dorsal of the projections.

The next group (category X: cells A and B) comprised the only two reconstructed neurons that showed IPSPs, in both instances to both the dorsal ramus and the internal intercostal nerve, at latencies 2.3–3.7 ms, again likely trisynaptic. Both of these cells showed inspiratory CRDPs; they were the two most laterally located neurons, with remarkably similar locations; and they were the two giving the smallest projection fields, both located dorsally in the medial ventral horn, both distant from the soma.

A third group (category Y, four neurons) all showed low-threshold, monosynaptic EPSPs, all with inspiratory CRDPs. Two of these (cells O and P) were the only two neurons with ipsilateral axons, both bifurcating. For cell O, the collaterals were too distant for good staining of their terminations. Cell P is described in the following text. The remaining two cells of this group had ascending contralateral axons, one (cell J)
without collaterals (≤7.05 mm), the other (uniquely) terminating only in the medial intermediate area (cell I). Neurons with ascending axons are obviously at risk of being ascending tract neurons. The likelihood of this being the case is increased (but by no means proven) by the presence of the monosynaptic EPSPs for cells I and J. The third neuron with an ascending axon (cell K) was not tested for peripheral afferent inputs and so does not appear in one of these groups.

Finally, a group of 2 neurons (category Z) could be defined by showing no responses to peripheral nerve stimuli. One of these (cell L) was unique in being the only one of the 16 that projected to the intermediolateral cell column (IML). The other (cell E), although strictly not belonging to this group since the internal intercostal nerve was not tested, was also unique in being the most ventrally located of the neurons and gave no collaterals.

It was thought possible that soma size might be related to afferent input, or to the CRDP, or to the axonal projections (see following text), and therefore the soma cross-sectional area has been noted on Fig. 9. However, inspection of the data in Fig. 9 indicates that no such relationship is obvious.

One other, more specific, functional aspect was investigated for three of the cells by immunohistochemical staining of their presumed synaptic boutons. Selected sections that included terminal fields were reacted with two antibodies, first to gamma-aminobutyric acid decarboxylase (GAD), using an antibody that recognizes both GAD 65 and GAD 67 (Kaufman et al. 1991), and second to gephyrin. From the first of the neurons, a sequence of successive optical slices containing a series of en passant boutons located on a single collateral branch is shown in Fig. 10. The Neurobiotin series illustrating the collateral branch is included as the image stack (bottom right). None of the 16 boutons examined for this collateral was positive for GAD (blue) nor were any of them clearly opposed to gephyrin puncta (red). Indeed, in the images of Fig. 10 the boutons tended to fit neatly between a series of gephyrin puncta, apparently arranged along a dendritic process running horizontally across the middle of the images. Occasional apparent contacts were seen between avidin-stained and gephyrin-stained elements (e.g., arrow in Fig. 10), but these occurred at the edges of the boutons (note that the gephyrin punctum indicated in Fig. 10 is larger in the earlier optical slices, but the bouton is larger in the later slices) and they were not interpreted as synaptic appositions. We thus conclude that this neuron, being negative both for GAD and, by inference, for glycine, was most likely an excitatory interneuron. This neuron (cell L) had a low-amplitude CRDP and thus could be said to correspond to the general pattern from Kirkwood (1993) of tonic, excitatory, dorsally projecting interneurons. It was one of the two mentioned earlier that showed no EPSPs to peripheral nerve stimulation. A second neuron (cell N) showed a similar pattern of staining (15 boutons), but its projection fitted more clearly the previous pattern for excitatory neurons. This projection was quite widespread, but included the most ventral contralateral ventral horn and its CRDP was of medium amplitude (2.5 mV). It showed EPSPs to nerve stimulation, but they were not monosynaptic.

In contrast, all 39 of the boutons examined for the third neuron (cell P; Fig. 11), which were again negative for GAD, were closely opposed to gephyrin puncta, as illustrated in Fig. 11. This neuron may therefore be concluded to be a glycinergic inhibitory neuron (Todd et al. 1995). It had one of the smallest somata. Since it had a large inspiratory CRDP (the largest observed, 8 mV), this neuron could also be considered consistent with the conclusion of Kirkwood et al. (1993) that phasically firing thoracic interneurons are mostly inhibitory. Note, though, that this conclusion was derived from contralaterally projecting neurons, whereas the neuron here projected ipsilaterally. This neuron is also of interest in that it was one of the four cited earlier showing monosynaptic EPSPs, in this case from all three nerves at 5T, with the threshold for the EPSPs from both the internal and the external intercostal nerves being shown to be <1.5T. By virtue of its glycinergic identification, it thus could be said to be equivalent to the inhibitory interneurons in the lumbar segments activated by group I muscle afferents.

DISCUSSION

Spinal cord interneurons in the ventral horn and intermediate regions are well known for being heterogeneous in terms of their input connections, their projections, and their functional roles, such that in only a few instances is there correspondence between these properties and the interneuron morphology or location (Jankowska 2008). Despite the recent advances in the...
identification of cell lineage from transcription factors, thus providing a variety of cellular markers (for review see Goulding 2009), this remains the case. For the thoracic cord, even less is known about the functions of the interneurons than for the lumbar or cervical segments. The present study, using intracellular recordings, nevertheless confirms previous observations from extracellular recordings of respiratory-phased firing neurons (Kirkwood et al. 1993) and extends the characterization of thoracic interneurons both physiologically and anatomically.

Confirmation of previous observations

The majority of the interneurons identified physiologically here were antidromically identified from two to four segments caudally and most of these from the opposite side. These are entirely consistent with previous measurements (Kirkwood et al. 1988; Schmid et al. 1993). For the intracellularly labeled sample here, the presence of a crossed descending axon was confirmed anatomically for 8/8 interneurons identified antidromically or 12/17 (71%) of all the interneurons physiologically identified. This latter should be compared with 29/43 (67%) that showed evidence for a contralateral axon by extracellular spike-triggered averaging in Schmid et al. (1993).

The anatomical measurements here showed that 6/15 of those with descending axons also had an ascending branch. This is a new observation, although one of the 3 axons tested physiologically by Kirkwood et al. (1988) also showed this feature. Similarly, the result that 3/18 of the anatomically identified axons ascended, without any descending branch (Table 1, excluding the 2 identified DSCT neurons), is also new, although the possibility was previously admitted that some of the units classified as interneurons could actually have been ascending tract neurons (Schmid et al. 1993). This remains a definite possibility for the 3 axons here. Two of them (cells I and K) gave collaterals in the contralateral ventral horn, but this does not mean that they were propriospinal interneu-

FIG. 11. Reconstruction and immunohistochemistry for a glycinergic interneuron (cell P). Reconstruction: soma and dendrites, black; axon and first collateral, green. b, bifurcation of the axon into rostral- and caudal-running branches; c, origin of the collateral. Left: series of 1-μm optical sections for part of this collateral, as in Fig. 10. Each of the boutons is in close apposition to one or more gephyrin puncta. Representative outline of the ventral horn is shown in gray. CC, central canal; vf, ventral fissure.
rons rather than ascending tract cells, since projections to a very similar area from initial axon collaterals of ventral spino-cerebellar tract neurons were reported by Bras et al. (1988) who should also be consulted for references to initial axon collaterals from other ascending tract cells. One must assume that a proportion of the interneurons identified only physiologically, but without antidromic identification, would similarly be at risk of being ascending tract neurons. The most likely of these would be those monosynaptically excited from nerve stimulation, of which there were 5, plus 3 not tested for this.

The picture that emerged previously was that the projections of individual contralateral collaterals of the thoracic interneurons were relatively weak and were likely to be well spaced (Kirkwood et al. 1993). This is also supported by the observations here. Whereas we could not be certain that all collaterals were completely stained to their terminations, there were notable examples (e.g., cells A and B; see Figs. 7–9) where the collaterals were well stained and had projections that were both very restricted rostrocaudally and widely spaced. With one exception (Fig. 9, cell L) the contralateral projections were also all fairly well restricted in the transverse plane. Although some collaterals (Fig. 7; cell F, rostral collateral; cell L, caudal collateral) appeared to be relatively wide ranging rostrocaudally (2.55 and 2.15 mm, respectively), most of that rostrocaudal spread was made up in each case by a single rostrocaudally running branch with few terminals along its length.

By electrophysiological means, Schmid et al. (1993) identified the proportion of interneurons that gave contralateral collaterals as 26/43 (60%), sampled between 0.3 mm rostral and 1.3 mm caudal of the unit recording site. Here, anatomical measurements showed that a similar proportion of interneurons (7/16, 44%, Fig. 7) possessed contralateral collaterals extending into this rostrocaudal region.

The conduction velocities of the interneuron axons here, 30–112 ms⁻¹, mean 59 ± 19, were similar to those of Kirkwood et al. (1988): 8–140 ms⁻¹, mean 53. The possible difference at the low end of the conduction velocity spectrum is understandable, since the relatively small cells or axons involved would have not been penetrated so readily here. The positions of the neurons, as far as was reliably known (i.e., of those labeled and recovered), were also similar to those in previous reports. It is striking that the position of the line on Fig. 9, separating a dorsal region containing cells with only low-amplitude inspiratory CRDPS from a ventral region with cells of various types, is in a very similar location to the line drawn on Fig. 8 in Kirkwood et al. (1993), which also separated a dorsal region, in this case dominated by the presence of tonic inspiratory units, from the remainder of the ventral horn.

Overall, therefore the populations studied here were both qualitatively and quantitatively similar to those reported previously from this laboratory, particularly with respect to their axonal and collateral projections. The differences that might be thought to exist may be explained by methodological factors. For instance, the proportion with identified descending axons is significantly higher for the present population than that for Kirkwood et al. (1988) (χ², P < 0.05). However, not all of the interneurons in Kirkwood et al. (1988) were tested for descending axons and this population may also have included some axonal recordings (thus possibly including long descending or ascending axons, not local interneurons). With regard to the contralateral terminal fields, the populations in the current study and in Schmid et al. (1993) appear remarkably similar. This may be partly because both are biased toward larger neurons, here by the use of intracellular recordings and in Schmid et al. (1993) by the selection of unit recordings, which were insensitive to electrode movement. Nevertheless, the agreement is particularly interesting because of the quite different method of neuron selection, here essentially randomly by the intracellular electrode, but previously by the unit’s spontaneous respiratory discharge. Further, the assessments of the projections in the two series were also quite different, previously physiologically, by extracellular spike-triggered averaging, here anatomically by Neurobiotin labeling. We therefore conclude that the pattern of the projections revealed—largely contralateral with relatively widely spaced, modestly projecting collaterals—is a general one for the interneurons of the thoracic ventral horn.

Most of the neurons showed a CRDP. Does this mean therefore (despite the fact that they were not selected for respiratory activity) that they should nevertheless be considered as respiratory interneurons, just like the populations previously described (Kirkwood et al. 1988; Schmid et al. 1993)? There is little meaning in making direct comparisons of the firing patterns of the current population with an extracellularly recorded group. We do not know what proportion of the present population would have been firing if recorded extracellularly. Further, we do not know how much modulation of the firing would result from the CRDPs as recorded, especially because some of the CRDPS, recorded axonally, must be regarded as attenuated versions of what would be seen at the soma. Nevertheless, it must be said that many of the CRDPS were both small and variable, which makes them rather like those seen in hindlimb motoneurons (Ford and Kirkwood 2006), and it would seem unnatural to describe these motoneurons as respiratory neurons. Thus we consider it to be most accurate to describe the current population as generalized thoracic propriospinal interneurons that, under the present experimental conditions, mostly showed a respiratory drive. Of course, some of them must have been part of the same population described previously as respiratory interneurons. The variability of the firing patterns (e.g., Fig. 2, H and I) is similar to what was previously described for the respiratory interneurons (Schmid et al. 1993).

Finally, the observation of the frequently large amplitude of the synaptic noise (e.g., Fig. 1F) may also be considered confirmatory of previous observations, in that it is entirely consistent with the noticeably irregular firing of thoracic interneurons, as noted previously (Kirkwood et al. 1988) and as seen here.

New observations

The most important new observation from the physiological measurements was that most of the interneurons (30/37, 81%) showed PSPs from low-threshold (<5T) peripheral afferents. Moreover, 13/22 (59%) of the interneurons that showed EPSPs included a monosynaptic component from at least one nerve and 3/9 of those that showed IPSPs included a probable disynaptic component (Table 2). These observations thus rectify the apparent anomaly that such inputs had been detected in hardly any of the thoracic interneurons that had been previously recorded extracellularly (Kirkwood et al. 1988). We are
describing this as an anomaly because interneurons activated by these afferents are usually easy to find in the lumbar-sacral segments: indeed such activation comprises the mainstay of their most common definitions, as reviewed critically by Jankowska and Edgley (2010). Intercostal muscle afferents are known to project to all regions of the gray matter in their segment of origin (Nakayama et al. 1998), so it is inconceivable that interneurons in these regions would not be targets. The explanation for the difference between the previous results and the present observations must be that the EPSPs in the thoracic interneurons are usually too small (at least in the barbiturate-anesthetized animal) to reach threshold. It is inappropriate from our data, with sometimes axonal recordings and sometimes relatively low membrane potentials, to put much reliance on the absolute values of EPSP amplitudes observed, but nevertheless these EPSPs were never large. For instance, compare the maximum monosynaptic component in Fig. 3, which was one of the largest EPSPs (~2.3 mV in amplitude), with that resulting from stimulating the spinal cord in the same cell (Fig. 1B, about 10 mV in amplitude).

The ventral horn interneurons recorded by Gokin (1970) included a much lower proportion of cells with inputs from low-threshold afferents than here (19/100, counted from his Fig. 3 for the same area as here, i.e., ventral to the central canal). However, his recordings included an (unstated) proportion of axonal recordings that, unlike ours, were not restricted to those showing PSPs and which thus may have been from neurons located in other segments. Later publications from Gokin’s group (see Gokin 1978 for references) seem to show a higher proportion of cells with low-threshold inputs. Perhaps these studies included fewer axonal recordings.

Why should the thoracic interneurons receive much smaller EPSPs than is often the case in the lumbar-sacral cord? First, we suggest that individual thoracic afferents may give single-fiber EPSPs that are just as large as those in the lumbar-sacral segments, as is the case for muscle spindle afferents to thoracic motoneurons (Kirkwood and Sears 1982), but that fewer afferents are available from a single intercostal segment than from many large hindlimb muscles. This relatively small degree of convergence, however, may be compensated by additional convergence of afferents from other nearby segments, as was deduced to be the case for the Renshaw cell input to thoracic motoneurons by Kirkwood et al. (1981). Second, perhaps the fairest comparisons against the lumbar cord might be with commissural interneurons in those segments, since the majority of our population falls into this category. In this case the difference may not be so great: Jankowska (2008) mentions the occurrence of only small EPSPs from any one source as a general property of such interneurons.

Which afferents provide the input? The only certainty is that a reasonable number of interneurons (nine, but not all being tested) received an input from afferents with thresholds ≤1.5T and thus may be assumed to include afferents equivalent to Group I for the hindlimb (cf. Sears 1964a,b). As to the extent of representation of Group Ia or Ib or of Group II, this remains unknown, since the relative thresholds of the different categories of afferents are not known for the thoracic nerves. One might be tempted to suggest that the inhibitory neuron with an ipsilateral axon illustrated in Fig. 11 (cell P) might be a Ia reciprocal inhibitory interneuron. However, it should be remembered that reciprocal Ia inhibition has not been identified for thoracic segments (Sears 1964b). Note also that, of the three nerves stimulated, only the external intercostal nerve is a pure muscle nerve; the other two nerves both have cutaneous components. Nevertheless, it may be of interest that there was a general dominance of the dorsal ramus nerves in giving monosynaptic EPSPs, including those with a low threshold that most likely arise from muscle afferents. Perhaps this result reflects the dominant postural functions of the axial muscles innervated by these nerves.

One more new physiological observation is that of recurrent inhibition of an interneuron (Fig. 3). In the lumbar cord, the only types of interneurons receiving such inhibition are the Ia interneuron and the Renshaw interneuron itself (Windhorst 1996). Given the apparent absence of reciprocal Ia inhibition for thoracic motoneurons, this might suggest a different set of connections in these segments, unless reciprocal Ia inhibition actually is present, but for untested combinations, e.g., between contralateral muscle pairs, as in the sacral cord (Jankowska et al. 1978), although not for upper lumbar back muscles (Jankowska and Odutola 1980). However, this particular neuron was considered only borderline for antidromic identification as having a descending axon, so may instead have been a ventral spinocerebellar tract neuron, another neuronal category that has been shown to receive recurrent inhibition (Lindström and Schomburg 1973).

The most interesting new morphological observation is that none of the crossed axons gave any ipsilateral collaterals. The majority of the interneurons should thus be considered as true commissural neurons. This is of interest from a functional point of view. Although many of the actions of the thoracic muscles, such as respiration, are bilaterally synchronous, the thoracic interneuron network seems to have evolved to control these actions by unilateral rather than bilateral projections. One possible hypothesis to explain this is related to the multifunctionality of actions in which the muscles are involved, some unilateral, such as lateral flexion, some bilateral, such as lordosis. Perhaps the appropriate reconfiguration of the circuits (e.g., between walking and galloping) could become much simpler to organize if individual neurons do not generally have bilateral projections (cf. Jankowska et al. 2009). Note this is not the case for some descending projections. Individual expiratory bulbospinal neurons, which convey a large fraction of the expiratory drive to motoneurons in the segments investigated here (Saywell et al. 2007), have bilateral projections (Kirkwood 1995; Kirkwood et al. 1999). Moreover, these descending fibers have very wide ranging projections, including, as a group at least, projections to sacral and lumbar, as well as thoracic segments (Boers et al. 2005; Sasaki et al. 1994). Individual bulbospinal neurons make direct connections to motoneurons in both thoracic and upper lumbar segments (Road and Kirkwood 1993). Despite their strong direct connections to motoneurons these neurons with long descending axons may thus provide, as suggested by De Troyer et al. (2005) and Boers et al. (2005), only a basic, “baseline” activation during expiration, the details of characteristic regional activity patterns in the thorax and abdomen being provided by more local mechanisms, perhaps represented here by the exclusively unilaterally projecting interneurons.

That is not to say that the interneurons here have exclusively local projections. Note that none of the interneuron axons was followed to its termination: they are not segmental, but are
propriospinal, as discussed earlier. Further, the abovementioned logic might suggest that not only are the projections of individual interneurons at a given rostrocaudal location relatively weak, they could be highly specialized, for instance to motoneurons (or other interneurons), specifically controlling a particular region of the thorax (e.g., distal or proximal within a given intercostal space). This would make it even less surprising that so few interneuron–motoneuron connections have so far been revealed by cross-correlation or spike-triggered averaging (Kirkwood et al. 1987, 1993).

A similar dichotomy can be seen in comparing the reticulospinal axonal projections described by Matsuyama et al. (1997, 1999), a number of which showed bilateral projections, against the almost universally unilaterally projecting (one neuron being bilateral) lamina VIII commissural neurons in the lumbosacral cord described by Matsuyama et al. (2006). There is, indeed, a great deal of similarity between our neurons and those described by Matsuyama et al. (2006), especially for their longer projecting ascending examples with wide-spaced collaterals, in particular that each collateral of an individual axon should project to a similar region of the ventral horn, characteristic for that axon. Perhaps this should not be surprising. Most of the ventral horn at thoracic levels is designated as lamina VIII (Rexed 1954); so, although we have chosen not to separate the location of the interneurons as being in lamina VII or VIII, a large proportion must have been in lamina VIII. Matsuyama et al. (2006) should be consulted for further discussion on the properties and likely roles of such commissural interneurons.

We should consider which of our interneurons were excitatory, which inhibitory. First, it should be noted that the results of Kirkwood et al. (1993) showed commissural interneurons of both types, in approximately equal numbers, so there is no reason to expect the present population to be any different. Only three of the present population were identified directly (Figs. 10 and 11): one inhibitory, ipsilaterally projecting; two excitatory, commissural. However, the data represented in Fig. 9 are certainly compatible with the range of projections of both types of neurons illustrated in Kirkwood et al. (1993; their Figs. 6 and 8). Bannatyne et al. (2009) described a population of lamina VIII commissural interneurons with Group I and Group II afferent inputs and exclusively unilateral projections. Our interneurons might be seen to be equivalent to these, despite not all being activated by Group I and Group II inputs, especially since our population were mostly found in lamina VIII. However, by analogy with Kirkwood et al. (1993) our population will have included commissural inhibitory neurons, not observed by Bannatyne et al. (2009). Since all the interneurons here projected unilaterally, we have clearly not seen any equivalent to the bilaterally projecting excitatory lamina VII interneurons described by Bannatyne et al. (2009). However, there were only two confirmed lamina VII interneurons in our population (neurons I and K in Fig. 9) and only one of these (neuron I) was confirmed as having an input from low-threshold afferents, so our sample is too small to say whether such bilaterally projecting lamina VII interneurons are present in the thoracic segments.

What are the targets of the interneurons described here? Really, we are in the same position as Kirkwood et al. (1993) in saying only that these almost certainly include both motoneurons and other interneurons, without being much more specific. Perhaps not surprisingly, given the known locations of sympathetic premotor interneurons (Schramm 2006), only one of the interneurons, cell L, showed a collateral with significant terminations in the region of the IML, although cell I and perhaps cells G and M projected to the more medial areas also containing preganglionic sympathetic neurons. There were no particular physiological features to link these interneurons. Bannatyne et al. (2009) and Jankowska et al. (2009) were able both to define their commissural interneurons more precisely than we could (by their inputs) and also to positively identify excitatory and inhibitory interneurons as definitely synapsing on both motoneurons and interneurons. Nevertheless, they had to conclude that “considerable variability was found in each of (their) interneuronal subpopulations” and that this variability of both input and output appeared to be “an intrinsic property” of the interneurons. Little surprise, then, that in our relatively small population of neurons, relationships between physiological properties and projections were hard to find.

Two other morphological properties are worthy of comment. The first is the radial nature of the dendrites, extending well into the white matter in many instances and therefore unlike the thoracic motoneurons described by Lipski and Martin-Body (1987). The second is the large sizes of some of the interneurons (e.g., Figs. 4E and 5, top). It is not that such interneurons have not been recognized before. For example, see Fig. 19 in Matsushita (1970) for an interneuron almost identical in size and shape to cell M here (Fig. 5, top). It is rather that such observations are often ignored by authors seeking to identify motoneurons simply by size, without either a retrograde label or an appropriate marker. Perhaps the examples of Figs. 4E and 5, top are both rather dorsal to be confused with motoneurons, but note that the locations of the labeled interneurons in general (Fig. 9) include the areas of the motor nuclei, confirming the physiological observations of Kirkwood et al. (1988). Note also that, although the motor nuclei shown as lamina IX in Rexed (1954) consisted of narrow, discrete columns, they actually comprise overlapping areas occupying all of the ventral 50% of what Rexed described as lamina VIII (Kirkwood et al. 1988; also see Lipski and Martin-Body 1987).

It will be apparent that one of the factors making the establishment of relationships between morphological and functional properties for these interneurons even harder than it is for the lumbar cord is that there are few reliable functional definitions for the interneurons in the thoracic cord. Will the new definitions of interneurons, which are now being derived via transcription factors and the genetic manipulations of neuronal specification, help matters? In fact, in the lumbar cord, correspondence between these definitions and the classical physiological/pharmacological definitions has been found in only one or two instances. Goulding (2009) expresses optimism that the new techniques will soon provide understanding of the operation of the mammalian locomotor central pattern generator (CPG). Perhaps that might be the case, except one problem still to be faced in the lumbar cord is that the locomotor CPG is not readily separated from the interneurons that more logically might be thought of as pattern-shaping neurons transmitting the output of the CPG to the motoneurons. This difficulty does not arise for the circuits controlling respiratory movements because the CPG in this case is firmly established as being located in the medulla. Thus the interneurons in the thoracic cord, such as those described here, are
readily defined in the pattern-shaping category rather than being part of the (respiratory) CPG. Thus it could well be profitable to make the correlations between the new definitions against the functional roles for these interneurons first, or at least in parallel with the studies of locomotor-related interneurons in the lumbar or cervical enlargements. Studies of the thoracic interneurons during fictive behaviors such as locomotion and scratching could also reveal valuable correlative data with respect to the neurons of these other segments.

Finally, the most important reason for making the morphological measurements is that these data provide control measurements for comparison with a similar population of interneurons in animals with spinal cord lesions, where dendritic and probably axonal plasticity had occurred. More quantitative details of the morphology of the neurons here will be included in this comparison, which will be presented in a subsequent publication.

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DISCLOSURES

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