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Networks of inhibitory and excitatory commissural interneurons mediating crossed reticulospinal actions

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Abstract

Axonal projections and neurotransmitters used by commissural interneurons mediating crossed actions of reticulospinal neurons were investigated in adult cats. Eighteen interneurons, located in or close to lamina VIM in midlumbar segments, that were monosynaptically excited by reticulospinal tract fibres and projected to contralateral motor nuclei were labelled by intracellular injection of tetramethylrhodamine-dextran and Neurobiotin. The nine most completely labelled interneurons were analysed with combined confocal and light microscopy. None of the stem axons gave off ipsilateral axon collaterals. Seven cells had axon collaterals that arborized in the contralateral grey matter in the ventral horn of the same segments. Transmitters were identified by using antibodies raised against vesicular glutamate transporters 1 and 2, glutamic acid decarboxylase and the glycine transporter 2. The axons of two cells were immunoreactive for the glycine transporter 2 and hence were glycinergic. Three cells were immunoreactive for the vesicular glutamate transporter 2 and hence were glutamatergic. None of the axons displayed immunoreactivity for glutamic acid decarboxylase. Electron microscopy of two cells revealed direct synaptic connections with motoneurons and other neurons. Axonal swellings of one neuron formed synapses with profiles in motor nuclei whereas those of the other formed synapses with other structures, including cell bodies in lamina VII. The results show that this population of commissural interneurons includes both excitatory and inhibitory cells that may excite or inhibit contralateral motoneurons directly. They may also influence the activity of motoneurons indirectly by acting through interneurons located outside motor nuclei in the contralateral grey matter but are unlikely to have direct actions on interneurons in the ipsilateral grey matter.

Keywords

cat; glutamate; glycine; interneurons; motoneurons; reticular formation

Introduction

Descending reticulospinal systems have an important role in the control of posture and movement (Peterson *et al.*, 1978,1979; Grillner & Dubuc, 1988; Drew & Rossignol, 1990a,b). However, relatively little is known about the organization of neurons that are involved in this control, especially those influencing the activity of contralateral motoneurons. Recently, it has been demonstrated that excitation and inhibition of contralateral motoneurons by reticulospinal

neurons is mediated by interneurons that are concentrated within and around lamina VIII in midlumbar segments of cats (Jankowska *et al.*, 2003): we shall refer to cells of this type as RF commissural interneurons. It was concluded that these actions are most often evoked disynaptically, i.e. by reticulospinal neurons monosynaptically activating RF commissural interneurons, which in turn monosynaptically excite or inhibit contralateral motoneurons. Additional trisynaptic actions of reticulospinal neurons on contralateral motoneurons could also be evoked in two ways. They might either involve disynaptic actions of commissural interneurons via other neurons or be due to disynaptic excitation of commissural interneurons by reticulospinal fibres. Furthermore, disynaptic actions of commissural interneurons could be evoked via axon collaterals given off either ipsilaterally or contralaterally. Figure 1 illustrates the most likely organization of pathways between reticulospinal tract neurons and contralateral motoneurons via commissural interneurons. These hypothetical connections were investigated by analysing the axonal projections of individual RF commissural interneurons.

The principal aims of this study were to investigate the morphological substrates of the direct (monosynaptic) and indirect (disynaptic) actions of RF commissural interneurons on contralateral motoneurons, and to define the transmitters used by these neurons. In particular we addressed four questions: (i) Do RF commissural interneurons project to contralateral motor nuclei and form direct synaptic contacts with motoneurons as the electrophysiological evidence suggests? (ii) Do RF commissural interneurons project to other regions of the grey matter apart from contralateral motor nuclei (for example do they make synaptic connections with potential premotor interneurons)? (iii) Do RF commissural interneurons also target cells located in the ipsilateral grey matter? (iv) What neurotransmitters are used by RF commissural interneurons (i.e. do they form a mixed population of excitatory and inhibitory cells and, if so, do excitatory and inhibitory cells project to similar areas)?

In order to answer these questions, we used intracellular markers to label electrophysiologically identified interneurons that were both monosynaptically excited from ipsilateral reticular formation and antidromically activated by stimuli delivered in the contralateral motor nuclei in the L7-S1 segments. We developed a method that enabled detailed examination of the same axonal terminals with combined confocal and light microscopy along with the identification of amino acid transmitters within these terminals. In one of the experiments, cells were also processed for electron microscopy. The presence of excitatory and inhibitory transmitters was investigated in axons of labelled cells by identifying specific transmitter-associated proteins. To our knowledge, this is the first attempt to perform this type of analysis on functionally identified cells obtained *in vivo*. A preliminary account of this work has been published as an abstract (Bannatyne *et al.*, 2003).

Materials and methods

The experiments were performed on six deeply anaesthetized adult cats (2.4–3.1 kg). Anaesthesia was induced with sodium pentobarbital (40 mg/kg i.p.), maintained during surgery with intermittent doses of pentobarbital (1–2 mg/kg i.v. up to a total dose of 45 mg/kg), and subsequently with α -chloralose (Rhône Poulenc Santé, France; about 3 mg/kg/h i.v.) as required to maintain anaesthesia at a level at which no withdrawal reflexes were present. During recording, neuromuscular transmission was blocked by pancuronium bromide (Pavulon, about 0.2 mg/kg/h i.v.) and the animals were artificially ventilated. The depth of anaesthesia was then assessed by continuously recording blood pressure and heart rate and by monitoring pupil diameter. If there was any increase in the blood pressure or heart rate, or if the pupils began to dilate, additional doses of chloralose were given. The mean blood pressure was kept at 100–130 mmHg and the end-tidal concentration of CO₂ at about 4% by adjusting parameters of artificial ventilation and of the rate of a continuous infusion of a bicarbonate buffer solution with 5% glucose (1–2 mL/h/kg). The core body temperature was kept at about 38 °C by servo-

controlled infrared lamps. All procedures were approved by the University of Göteborg local ethical review committee and conformed with EU and NIH animal care guidelines.

Preliminary dissection included cannulation of the trachea (for artificial respiration and for continuous monitoring of end-tidal CO₂), a carotid artery (for continuous monitoring of blood pressure) and left and right cephalic veins (for intravenous injection of anaesthetics and other fluids). A number of peripheral hindlimb nerves were dissected, transected and mounted on stimulating electrodes (including left (ipsilateral) quadriceps (Q), and sartorius (Sart) and contralateral Q and gastrocnemius and soleus (GS)). The cerebellum was exposed to allow insertion of a stimulating electrode into the medial longitudinal fascicle (MLF) or at the border between the MLF and the medullary reticular formation at the level of the vagus nerve (for details see Jankowska *et al.*, 2003). Laminectomies exposed the spinal cord at thoracic (T12–T13) and lumbar (L2–L7) levels. Two electrodes were inserted within the lumbar enlargement. A tungsten electrode was placed in the right GS motor nucleus to stimulate axons of neurons projecting to this and nearby nuclei (using stimuli up to 50 μ A) and remained there stationary. A glass micropipette filled with a mixture of equal parts of (5%) tetramethylrhodamine-dextran (Molecular Probes Inc, Eugene, OR, USA) and (5%) Neurobiotin (Vector Laboratories, Peterborough, UK) in saline (pH 6.5) was used to record and to label the selected interneurons. Three pairs of silver ball-tipped electrodes were used to record afferent and descending volleys from midlumbar segments and to stimulate long ascending tract fibres at T12–T13 to exclude ipsi- and contralateral ascending tract neurons from the sample of the labelled neurons (for details see Jankowska *et al.*, 2003).

Spinal interneurons to be labelled were searched for in the 3rd, 4th and 5th lumbar (L3, L4, L5) segments. A space of about 1 mm was left between the labelled cells. Three main sampling criteria were used. The first was antidromic activation from the contralateral GS motor nucleus (verified by collision with synaptically evoked spikes, indicating a projection of the axon to the motor nuclei in the lower lumbar/upper sacral segments). The second criterion was a failure to be activated by T12–T13 stimuli, indicating that the neuron was not an ascending tract cell. The third criterion was activation by MLF stimuli at segmental latencies compatible with monosynaptic coupling (up to 1.2 ms extracellularly, <1 ms intracellularly, see Jankowska *et al.*, 2003). Once the selected cells were penetrated and their identification confirmed, they were labelled by passing a constant positive current (5 nA for up to 10 min). In successfully labelled cells, the total product of nA $\times$ minutes ranged between 18 and 42.

At the termination of experiments animals were perfused through the aorta with 4% formaldehyde in phosphate buffer (for combined confocal and light microscopy studies; five cats) or with 4% formaldehyde, 0.2% glutaraldehyde and picric acid (15mL saturated solution/L) in phosphate buffer (for combined confocal, light and electron microscopy; one cat). The spinal cord was divided into blocks (4–6 mm) that were sectioned in the transverse plane (50 μ m) with a Vibratome. All sections were collected in strict serial order and mounted on glass slides with Vectashield (Vector Laboratories). Sections were scanned with epifluorescence and individual cells labelled with tetramethylrhodamine-dextran were identified. Short series of sections (8–30) containing identified cells were selected and incubated in avidin-rhodamine (1:1000; Jackson Immunoresearch, Luton, U.K) for 3 h with Triton X-100 (for LM only) or overnight with no Triton X-100 (for material to be processed for electron microscopy) in order to reveal the Neurobiotin. Neurobiotin provides much greater detail of axonal processes than rhodamine-dextran but cannot be visualized directly with epifluorescence. Sections were then re-mounted in serial order. All labelled processes belonging to a cell were scanned with a Bio-Rad MRC 1024 confocal microscope (Bio-Rad, Hemmel-Hempsted, UK) equipped with a krypton-argon laser using a $\times 20$ lens at z intervals of 1 μ m and a preliminary reconstruction was made of the cell and its axonal arbor.

When the reconstruction was complete, sections containing labelled axonal processes were selected for immunocytochemical processing. At least two sections from each cell were reacted according to the following protocols: one section was incubated in a combination of rabbit anti-glutamate decarboxylase to identify GABAergic terminals (GAD; 1:1000; Sigma-Aldrich, Poole, UK) and a guinea-pig antibody raised against the vesicular glutamate transporter 2 to identify glutamatergic terminals (VGLUT2; 1: 5000; Chemicon International, Harrow, UK); the second section was incubated with sheep anti-glycine transporter 2 to identify glycinergic terminals (GlyT2; 1: 1000; Chemicon International) and a guinea-pig antibody raised against the vesicular glutamate transporter 1 to identify glutamatergic terminals (VGLUT1; 1:5000; Chemicon International). Sections were incubated for 48 h. The presence of antibody-antigen complexes was identified by using secondary antibodies that were raised against immunoglobulins of the species from which the primary antibodies were obtained. Anti-guinea-pig antibodies coupled to cyanine 1.58 were used to detect VGLUT1 and VGLUT2; an anti-rabbit antibody coupled to fluorescein (FITC) was used to detect GAD and an anti-sheep antibody coupled to FITC was used to detect GlyT2. All secondary antibodies were used at a dilution of 1:100 and incubations lasted for 3 h (all secondary antibodies were obtained from Jackson ImmunoResearch). Both primary and secondary antibodies were diluted in phosphate-buffered saline (PBS) with Triton X-100. Once these incubations were complete, sections were remounted and scanned with three-colour confocal microscopy. Axonal swellings were examined in short series of optical sections that were gathered with a Fluor $\times 40$ oil-immersion lens (numerical aperture 1.3), with a zoom factor of $\times 2$ and at z intervals of 0.5 μm . Individual terminals were examined in merged series of sections to determine if they displayed immunoreactivity for the amino acid markers. Sections from the animal that was perfused with glutaraldehyde were pretreated with borohydride prior to performing immunoreactions. The staining properties and specificity of primary antibodies used in this study have been discussed elsewhere (Mackie *et al.*, 2003; Todd *et al.*, 2003). In control experiments, tissue was processed according to the protocols described above except that individual primary antibodies were omitted. Under these circumstances no fluorescence was observed for the omitted antibody.

When confocal microscopic analysis was complete, sections were reacted with ABC reagent (5 μL + 5 μL ; Vector Elite), which was followed by a reaction with 3, 3'-diaminobenzidine (DAB) in order to render Neurobiotin-labelled structures visible with transmitted light. For sections to be processed for light microscopy only, all sections in the series were reacted for 3 h in the presence of Triton X-100. These were dehydrated and mounted on slides with DPX. This preserved labelled cells on a permanent basis and enabled further detailed reconstruction to be performed with the aid of a drawing tube. Particular attention was paid to the reconstruction of axonal arbors. Selected sections were also prepared for electron microscopy from the animal that was perfused with glutaraldehyde-containing fixative. These sections were incubated in ABC reagent for 3 days and no Triton X-100 was used. Following DAB reaction, sections were treated with osmium tetroxide, dehydrated and embedded in resin between cellulose acetate foils. Once the resin had polymerized, labelled structures could be examined with standard transmitted light microscopy and drawn and photographed. Areas of interest in sections (i.e. those containing labelled axon terminals) were attached to cured resin blocks with a droplet of resin. When the resin had polymerized, the block was trimmed and series of ultrathin sections were collected on single-slot grids. Sections were contrasted with lead citrate and viewed with a Phillips CM100 Biotwin electron microscope (FBI, Eindhoven, The Netherlands). Locations of individual labelled boutons, which characteristically contained dense DAB reaction product, were identified in electron microscope sections with the aid of the drawings and photographs. Boutons were followed through series of sections to determine if they formed synaptic relationships with adjacent structures. When necessary, sections were tilted with a goniometer to resolve synaptic specializations.

Results

In total, 18 properly identified (see Materials and methods) commissural interneurons that were antidromically activated from the contralateral GS motor nuclei were labelled successfully. However, only nine of these neurons were selected for analysis because axons of the remaining cells were not sufficiently well labelled. The relatively small proportion of labelled neurons retrieved (18 out of about 30 into which Neurobiotin was injected) and even smaller proportion of neurons with sufficiently well-labelled axon terminals precluded the use of a larger sample for analysis.

All cells labelled in this study were situated within lamina VIII or in the medial region of lamina VII adjacent to the border between lamina VIII and VII (Fig. 2) and had dendrites that projected radially and extended into lamina VII, VIII and motor nuclei (see reconstructions in Figs 3C, 5C, 7B and 9B). In addition to prominent monosynaptic excitatory postsynaptic potentials (EPSPs) evoked from the reticular formation, polysynaptic effects were evoked in five of the cells by group II afferent fibres from the quadriceps and/or sartorius nerves (inhibitory postsynaptic potentials (IPSPs) in three cells; EPSPs in two cells). In two cells stimulation of group II afferents had no obvious effect (see Table 1).

Ipsilateral projections

We found no evidence of axon collaterals given off ipsilaterally in any of the nine neurons in which the axons were traced to the opposite side of the spinal cord. The sample includes two interneurons where the axon could only be traced to the contralateral white matter as well as seven interneurons where the stem axon gave rise to axon collaterals that arborized in the contralateral grey matter and could be followed to their terminals.

Contralateral projections

All of the interneurons analysed in this study had axonal projections to the contralateral gastrocnemius-soleus motor nuclei of the 7th lumbar segment as shown by antidromic activation from these nuclei (Figs 3B, 5B, 7A and 9A). Reconstruction of terminal arbors of three of the cells revealed that they also projected to motor nuclei in the 4th and 5th segments (Figs 3C and 9B). Because different motor nuclei are located in these segments, this implies that single interneurons may contact motoneurons of different muscles. The same cells also gave rise to additional terminals in areas of the spinal grey matter outside motor nuclei. Terminal arbors of these and the four other cells were found within various laminae including laminae VII and VIII (Figs 3C and D, 5C and D, 7B and C, and 9B and C) as summarized in Table 1. Arbors of single axon collaterals extended for approximately 300 µm in the rostrocaudal plane. The majority of axonal swellings formed by these axonal arbors were *en passant*, but occasional terminal swellings were also observed. Electron microscopy confirmed that these swellings were synaptic boutons and that they formed contacts with neuronal profiles within motor nuclei and the surrounding grey matter (Figs 8 and 10; see below).

Transmitters contained within axon terminals

Selected sections of all seven cells with terminals in the contralateral grey matter were reacted with the series of antibodies described in the Methods to reveal the amino acid content of their axons (Table 1). Three cells had axons that were positively labelled for VGLUT2 but were not immunoreactive for GAD, VGLUT1 or GlyT2. Figure 4 (cell 4/1 in Table 1) illustrates the typical pattern of VGLUT2 immunoreactivity in the axon terminals of one of these neurons. Figure 7D (cell 3/3, Table 1) shows further examples of VGLUT2-immunoreactive boutons from one of the cells that was also analysed with electron microscopy (see below). In addition to lamina IX, VGLUT2-immunoreactive terminals were found in laminae VII and VIII (Figs 3C and D, and 7B and C). The commissural neurons might thus excite contralateral

interneurons and in turn have actions on the contralateral cord through this less direct pathway, for example they could mediate the trisynaptic excitation seen in contralateral motoneurons, as outlined in the circuit diagram in Fig. 1A.

Two of the cells had axon terminals that were immunoreactive for GlyT2 but were not labelled with the other three markers. Figure 6 shows a typical example of GlyT2-immunoreactive axonal swellings from one of these neurons (cell 1/5, Table 1). Characteristically, immunoreactivity for GlyT2 surrounds the intracellularly labelled boutons as the transporter is located within the plasma membrane of glycinergic boutons rather than in the cytoplasm (Spike *et al.*, 1997).

These terminals were located in lamina VII and VIII where some of them formed appositions with cell bodies (Fig. 6C–E). The type of neuron that might be inhibited by the labelled cells could not be identified.

Electron microscopic analysis was performed on the terminals of two of the cells with terminal branching in lamina VII and in lamina IX, respectively. The first of these was a glutamatergic neuron (cell 3/3, Table 1) with terminals that were immunoreactive for VGLUT2 (Fig. 7D). The terminals of this cell formed asymmetric synaptic contacts (five boutons analysed in serial sections) onto dendrites and cell bodies (Fig. 8), thus supporting the conclusion that it has an excitatory action (see Uchizono, 1965). The second cell processed for electron microscopy (cell 3/5, Table 1) was immunonegative for GAD, VGLUT1 and VGLUT2. Immunostaining for GlyT2 did not work in tissue fixed with glutaraldehyde. Nevertheless, the axon terminals of this cell formed symmetric synaptic junctions (13 boutons analysed in serial sections) with motoneurons and large dendritic profiles in lamina IX (Figs 9 and 10) and, as symmetric junctions are considered to be inhibitory (see Uchizono, 1965), it may have a direct inhibitory action on motoneurons, as suggested in Fig. 1B.

Discussion

The identification of spinal interneurons is most often based on input connectivity and/or morphology (e.g. see Jankowska, 1992). However, with the exception of a small number of specific classes of interneuron, such as Renshaw cells, Ia inhibitory interneurons and a subpopulation of Ib interneurons (see Jankowska, 1992), very little is known about the post-synaptic actions of most electrophysiologically investigated interneurons (i.e. whether they are likely to have inhibitory or excitatory actions or whether their actions are pre- or post-synaptic). The method we have developed in this study enables a detailed morphological analysis of interneuronal axonal terminals at the light and electron microscopic levels along with the identification of the transmitter substances contained within them and creates new possibilities for defining properties of selected functionally characterized interneurons and their rôle in neuronal networks. By using this method we have been able to answer all of the major questions outlined in the Introduction.

The interneurons investigated in this study included only those with monosynaptic input from the ipsilateral reticular formation. As shown in Table 1, none of them was monosynaptically excited by group II muscle afferents and they therefore belong to a distinct population of commissural interneurons that is different from those described previously, which include commissural neurons with group I input (Harrison *et al.*, 1986) or with group II input (Jankowska & Noga, 1990). Properties of these types of interneuron will be described separately (B. A. Bannatyne *et al.*, in preparation, and S. A. Edgley *et al.*, in preparation).

Axonal projections

We confirmed that electrophysiologically identified RF commissural interneurons project to motor nuclei and have shown that they do indeed form synaptic contacts with motoneurons. We have shown also that these interneurons are likely to evoke both excitation and inhibition of contralateral motoneurons by direct actions, as proposed previously (Jankowska *et al.*, 2003) and outlined in Fig. 1. As we found no evidence for the existence of ipsilateral axon collaterals, despite good labelling of axons and contralateral axon collaterals, our results suggest that the predominant, if not the only, action of these neurons is on the contralateral side of the cord. These interneurons would thus resemble commissural neurons mediating crossed actions from the vestibular nuclei in the cervical segments (Sugiuchi *et al.*, 1995) but differ from other commissural neurons in the lumbosacral enlargement described previously, which have bilateral projections. The lumbosacral commissural neurons described in anatomical studies (Cajal, 1909; Scheibel & Scheibel, 1966) were obtained from spinal cords of young animals including the chick, mouse and kitten, thus raising the question of whether bilateral projection may be a characteristic of developing neurons. Nevertheless, bilateral projections from some dorsal horn interneurons (Bras *et al.*, 1989) and ventral spinocerebellar tract neurons (Bras *et al.*, 1988) were also found in adult cats and therefore this pattern of projection may be related to the functional type of neuron. The axonal projections we observed suggest that the main function of RF commissural neurons with input from the reticular formation might be to co-ordinate the activity of contra-laterally located neurons. For instance, rather than co-ordinate activity of neurons located on both sides of the spinal cord, they may be important in the selection of motoneurons in different segments and hence in the recruitment of groups of contralateral muscles that subserve a variety of motor synergies. Even though they directly influence the activity of neurons on only one side of the spinal cord, RF commissural neurons might contribute to alternating movements of left and right legs during locomotion, as suggested by Jankowska & Noga (1990) and Matsuyama & Mori (1998). This could occur by successive activation of interneurons on the two sides by various axon collaterals of the same reticulospinal neurons or by various subpopulations of these neurons. The selection of reticulospinal neurons could then be made by neurons in the mesencephalic locomotor region and/or in the fastigial nucleus, which activate them effectively (Mori *et al.*, 1998). Activation of different subpopulations of RF commissural interneurons may also depend on other neuronal systems, e.g. the vestibulospinal tract (for further discussion of these points see Krutki *et al.*, 2003).

Terminal branching within laminae VII and VIII in addition to motor nuclei indicates that RF commissural interneurons not only have the capacity to excite and inhibit motoneurons directly but also indirectly via interneurons that in turn influence activity of motoneurons. Excitatory commissural interneurons may in this way amplify the disynaptic actions of reticulospinal fibres on contralateral motoneurons by tri- or polysynaptic actions mediated through such interneurons. This arrangement might be similar to the neuronal networks recently described in pathways from group II afferents (Jankowska *et al.*, 2002). By exciting inhibitory interneurons they may also enhance inhibitory actions needed for proper co-ordination of contralateral muscles. It will be shown separately (E. Jankowska and K. Matsuyama, in preparation) that at least some of the inhibitory interneurons that are target cells of RF commissural interneurons are interneurons that mediate Ia inhibition. Because these interneurons are located around motor nuclei, the observation that commissural neurons project to this area is consistent with this possibility. Actions of inhibitory commissural interneurons are more difficult to predict because their target interneurons could not be identified. Nevertheless, it might be hypothesized that they adjust activity of both excitatory and inhibitory contralateral premotor interneurons. In this way they may influence activity of contralateral motoneurons not only directly, as indicated in Fig. 1, but also indirectly.

Our results show that it is unlikely that commissural interneurons with monosynaptic input from RF contribute to disynaptic excitation of other interneurons of the same population (either excitatory or inhibitory, see Fig. 1) because ipsilateral axon collaterals were not found. We therefore propose that disynaptic excitation of commissural interneurons is predominantly, or even exclusively, a result of actions of other interneurons located in the ipsilateral grey matter. We further propose that trisynaptic actions of RF neurons on contralateral motoneurons are a result partly of disynaptic excitation of commissural interneurons and partly of disynaptic actions of commissural interneurons that are mediated by contralateral premotor interneurons.

Types of commissural interneurons

Immunocytochemical investigation of labelled RF commissural interneurons suggests that these cells use glutamate or glycine as neurotransmitters because separate populations of cells had axon terminals that were immunoreactive for either VGLUT2 or GlyT2. Both of these markers are known to be associated with glutamatergic and glycinergic neurons, respectively, and have been used in a variety of studies (Spike *et al.*, 1997; Varoqui *et al.*, 2002; Todd *et al.*, 2003). However, as far as we are aware, this is the first study that has attempted to look for the presence of these markers in terminals of electrophysiologically identified neurons that were labelled by intracellular injection, *in vivo*. The VGLUT2 transporter is found predominantly in terminals of spinal interneurons whereas the VGLUT1 transporter is found mainly in terminals of primary afferents (Varoqui *et al.*, 2002; Todd *et al.*, 2003) and therefore our finding that VGLUT2 is present in terminals of our population of neurons is consistent with the fact that they function as interneurons. We have not found evidence so far to show that commissural neurons use GABA because none of the axons examined was immunoreactive for GAD. The antibody we used recognizes both isoforms of the enzyme and therefore both isoforms should have been detected. This evidence suggests that, in contrast to other types of spinal interneuron (e.g. Todd & Sullivan, 1990; Maxwell *et al.*, 1997), the glycinergic commissural interneurons examined in this study do not appear to use GABA as a co-transmitter. One of the cells (cell 3/5) prepared for combined confocal and electron microscopy was immunonegative for VGLUT1 and 2 and GAD but no immunoreaction for GlyT2 was obtained as the GlyT2 antibody was found not to tolerate glutaraldehyde fixation. Nevertheless, it is possible that this cell is also glycinergic as terminals of its axon formed symmetric synaptic junctions with dendrites and cell bodies; symmetric synaptic junctions are believed to be indicative of inhibitory synapses (Uchizono, 1965). Another cell (cell 2/8) that was fixed with paraformaldehyde had axon terminals that were immunonegative for all four markers. This result could have arisen for a technical reason, i.e. that the immunostaining was incomplete. However, this seems unlikely as good immunostaining was obtained for all four markers in sections containing this cell. This then raises the possibility that some commissural interneurons use other neurotransmitters not examined in the present study. Nevertheless, bearing these technical issues in mind, it is probably justified to conclude that the majority of RF commissural interneurons use either glutamate or glycine as a neurotransmitter.

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Abbreviations

DAB	3,3'-diaminobenzidine
EPSP	excitatory postsynaptic potential
FITC	fluorescein isothiocyanate
GAD	glutamate decarboxylase
GlyT2	glycine transporter 2
GS	gastrocnemius and soleus
IPSP	inhibitory postsynaptic potential
MLF	medial longitudinal fascicle
Q	quadriceps
RF	reticular formation
Sart	sartorius
VGLUT1	vesicular glutamate transporter 1
VGLUT2	vesicular glutamate transporter 2

Fig 1.

Possible connections in disynaptic and trisynaptic pathways between neurons in the reticular formation and contralateral motoneurons. Hypothetical circuits based on electrophysiological findings reported by Jankowska *et al.* 2003. (A) Connections in excitatory pathways. (B) Connections in inhibitory pathways. In both A and B, disynaptic connections are mediated via commissural interneurons (labelled C) that form synapses with contralateral motoneurons (co MN) whereas trisynaptic connections are either via ipsilateral interneurons (1) and the commissural interneurons represented by the lower neuron or via those represented by the upper commissural interneurons and other premotor interneurons including other commissural interneurons, contralateral excitatory interneurons (2) or contralateral inhibitory interneurons (3). Dotted vertical lines indicate the midline. White cells, excitatory; black cells, inhibitory.

Fig 2.
Locations of cell bodies of RF commissural interneurons. (A and B) Locations of somata of interneurons located in the L4–L5 segments. Those used for confocal and EM analysis are indicated with filled circles.

Fig 3.

Records and axonal projections of a glutamatergic interneuron. (A and B) Records from an interneuron (cell 4/1, Table 1) showing that it was monosynaptically activated from the reticular formation (RF; arrow indicates time of stimulus) and antidromically activated from the contralateral GS motor nucleus (co GS MN). Stimulation of group II afferents (from the quadriceps nerve stimulated at five times the threshold for fibres with the lowest threshold of activation: Q 5T) produced a polysynaptic IPSP. Averages of ten single records. In all of the cells illustrated, full spikes were generated only within the first few seconds after the penetration and the remaining blocked spikes usually consisted of an M spike or of M and IS spikes (of less than 10 mV). Upper traces show intracellular records and lower traces show records from the cord dorsum. Dotted lines in A indicate the first component of the descending volley from the reticular formation and the onset of the EPSP; note that latency of the EPSP with respect to the volley, i.e. the segmental latency, was <1ms as required for monosynaptic coupling. Dotted lines in B indicate the stimulus artefact and blocked antidromic spike potential evoked from the motor nucleus. Note that the latency was about 1 ms and too short for synaptically evoked responses. An additional record showing the RF volley at a higher amplification is shown below. Voltage calibrations (rectangular pulses at the beginning of records) and time calibrations (horizontal bars) are as indicated. (C) The location of the cell body and the course of its main axon (L, left; R, right side of cord). (D) Reconstruction of the axon collateral contained within the boxed area shown in C. Scale bar, 50 µm. The small boxed

area (orientated by rotating clockwise by approximately 30°) contains the terminals examined with the confocal microscope shown in Fig. 4.

Fig 4. VGLUT2 immunoreactive axon terminals. (A) A projected confocal series of the collateral terminations (red) demarcated by the box in Fig. 3D. (B) A single optical section taken from the same series. (C) A single optical section showing immunoreactivity for VGLUT2 in the same plane as B (green). (D) A merged view of B and C. All axonal swellings viewed in this plane are immunoreactive for VGLUT2 but regions of preterminal axon do not contain the transporter. Arrows indicate individual boutons in A that are immunoreactive for VGLUT2 in the single optical section (C). Scale bar, 10 μ m.

Fig 5. Records and axonal projections of a glycinergic interneuron. (A and B) Records from an interneuron (cell 1/5, Table 1) showing that a monosynaptic EPSP was evoked from the reticular formation (arrow indicates time of stimulus) and a blocked antidromic spike was evoked from the contralateral GS motor nucleus whereas stimulation of Q group II afferent fibres produced no response. Averages of ten single records. Format and abbreviations as in Fig. 3. Note that the latency of the monosynaptic EPSPs with respect to the first components of the descending volleys (< 1 ms) and the latency of the antidromic spike from the shock artefact (about 1 ms) were the same as for the cell illustrated in Fig. 3. Voltage calibrations (rectangular pulses at the beginning of records) and time calibrations (horizontal bars) are as indicated. (C) The location of the cell body and the course of its main axon (L, left; R, right side of cord). (D) Reconstruction of an axon collateral (boxed area in B). Scale bar, 50 μ m.

Terminals contained within the boxed area (1) were examined with the confocal microscope (Fig. 6).

Fig 6.

GlyT2 immunoreactive axon terminals. (A) A confocal image showing a projected series of a collateral termination (red) contained within the boxed area shown in Fig. 5D. Arrows indicate boutons that appear in B–G. (B) A single optical section taken from the same series. Again the axon arbor is shown in red. (C) A further single optical section from the same plane showing immunoreactivity for GlyT2 (green). (D) A merged view of B and C. All axonal swellings in the single optical section are immunoreactive for GlyT2 (arrows), which typically is found on the axonal membrane surrounding boutons. Note that many of the axonal swellings arising from this collateral are clustered around the soma of a cell in lamina VII. (E–G) Single optical sections at higher magnification of boutons labelled 1–4 in C. Upper panels in each show interneuron boutons (red) and lower panels immunoreactivity for GlyT2 (green). Scale bars, 10 μ m (A–D); 2.5 μ m (E–G).

Fig 7.

Records, axonal projections and immunocytochemical characteristics of a glutamatergic interneuron also examined with the electron microscope. (A) Records of monosynaptic EPSPs evoked in an interneuron (cell 3/3, Table 1) from the reticular formation (arrows indicate times of stimuli) and of a blocked antidromic spike evoked from the contralateral GS motor nucleus. Both were followed by IPSPs. Averages of ten single records. Format and abbreviations as in Fig. 3. Note that the latency of the monosynaptic EPSPs with respect to the first components of the descending volleys (<1 ms) and the latency of the antidromic spike from the shock artefact (about 1 ms) were the same as for the cell illustrated in Fig. 3. Voltage calibrations (rectangular pulses at the beginning of records) and time calibrations (horizontal bars) are as indicated. (B) The location of the cell and the course of its main axon (L, left; R, right side of cord). (C) A reconstruction of the axon collateral (scale bar, 100 μm). The main axon gives rise to the collateral, which forms numerous terminations in lamina VII. Terminals contained within the boxed area (1) were examined with the confocal microscope. (D) The series of images shows single optical sections of two axonal swellings (red) and immunoreactivity for the vesicular glutamate transporter 2 (VGLUT2; green). The merged two-colour image shows

that the terminals are VGLUT2-immunoreactive and hence that the cell is glutamatergic (scale bar, 10 μm). Terminals contained within the boxed area (2) were examined with the electron microscope (see Fig. 8).

Fig 8.

Electron microscopy of glutamatergic terminals. (A) A light micrograph showing a cell contained within the boxed area 2 in Fig. 7C. A labelled collateral terminal is closely associated with it (arrow; scale bar, 10 μ m). Two blood vessels are indicated (*) that are also present in the electron micrograph of the same area (B), which also shows the terminal (arrow; scale bar, 10 μ m). C is a magnified view of the terminal (scale bar, 0.5 μ m) and D shows details of the synaptic area (boxed area in C). The bouton forms an asymmetric synaptic arrangement with the cell (arrowheads).

Fig 9.

Records and axonal projections of an interneuron examined with the electron microscope. (A) Records of monosynaptic EPSPs evoked in an interneuron (cell 3/5, Table 1) from the reticular formation (upper trace: arrows indicate times of stimuli), polysynaptic IPSP evoked from group II afferents and a blocked antidromic spike evoked from the contralateral GS motor nucleus (lower trace). Averages of ten single records. Note that the latency of EPSPs with respect to the first components of the descending volleys and of the spike potential with respect to the shock artefact was the same as for the cell illustrated in Fig. 3. Format and abbreviations as in Fig. 3. Voltage calibrations (rectangular pulses at the beginning of records) and time calibrations (horizontal bars) are as indicated. (B) The location of the cell and the course of its main axon (L, left, R, right side of cord). (C) Reconstruction of the axon collateral (boxed area in B). Note that this collateral ramifies principally in laminae VIII and IX. Axons within the boxed area (1) are illustrated in the electron micrographs shown in Fig. 10. Scale bar, 50 µm.

Fig 10.

Electron micrographs of axon collaterals in lamina IX. (A) Low-power micrograph from the boxed area shown in Fig. 9C showing an axon terminal (arrow) adjacent to a motorneuron cell body (MN) in lamina IX. Scale bar, 10 μ m. (B) A higher power image of the terminal with synaptic details (boxed area) shown in the inset (arrowheads). Scale bar, 0.5 μ m. (C and D) Images taken further through serial sections of the same motorneuron (MN) around the point of origin of a large dendrite (DEN) showing a second labelled bouton associated with the cell (arrows; scale bars, 5 μ m). The bouton formed a symmetrical synaptic contact (boxed area) with the dendrite (E, scale bar, 0.5 μ m), details of which are shown in the inset (arrowheads).

These synapses were studied in serial sections and sections were tilted to confirm that specializations were symmetrical.

Table 1
Characteristics of RF commissural interneurons analysed in the study

Cat ID	Cell ID	Input		Immunoreactivity				
		RF	GpII	Axon arbor	VGLUT1	VGLUT2	GlyT2	GAD
1	1/5	EPSP, monosynaptic	Not found	LVII, VIII	–	–	+	–
1	1/6	EPSP, monosynaptic	IPSP, polysynaptic	LVII, VIII	–	–	+	–
2	2/8	EPSP, monosynaptic	EPSP, polysynaptic	LVII, VIII	–	–	–	–
3*	3/3	EPSP, monosynaptic	Not found	LVII	–	+	0	–
3*	3/5	EPSP, monosynaptic	IPSP, polysynaptic	L VIII, IX	–	–	0	–
4	4/1	EPSP, monosynaptic	IPSP, polysynaptic	LVII, VIII, IX	–	+	–	–
4	4/4	EPSP, monosynaptic	EPSP, polysynaptic	LVII, VIII, IX	–	+	–	–

Synaptic actions evoked from the reticular formation (RF) and group II afferent fibres in muscle nerves (GP II) along with patterns of termination in laminae of the contralateral grey matter and immunocytochemical properties of cells are shown. VGLUT1, vesicular glutamate transporter 1; VGLUT2, vesicular glutamate transporter 2; Gly T2, glycine transporter 2; GAD, glutamic acid decarboxylase.

* Cat fixed with glutaraldehyde; +, positive immunoreaction in axon terminals; –, negative immunoreaction in axon terminals; 0, no immunoreactivity throughout spinal cord.