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INHIBITORY INPUTS TO FOUR TYPES OF SPINOCEREBELLAR TRACT NEURONS IN THE CAT SPINAL CORD

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Abstract—Spinocerebellar tract neurons are inhibited by various sources of input via pathways activated by descending tracts as well as peripheral afferents. Inhibition may be used to modulate transmission of excitatory information forwarded to the cerebellum. However it may also provide information on the degree of inhibition of motoneurons and on the operation of inhibitory premotor neurons. Our aim was to extend previous comparisons of morphological substrates of excitation of spinocerebellar neurons to inhibitory input. Contacts formed by inhibitory axon terminals were characterised as either GABAergic, glycinergic or both GABAergic/glycinergic by using antibodies against vesicular GABA transporter, glutamic acid decarboxylase and gephyrin. Quantitative analysis revealed the presence of much higher proportions of inhibitory contacts when compared with excitatory contacts on spinal border (SB) neurons. However similar proportions of inhibitory and excitatory contacts were associated with ventral spinocerebellar tract (VSCT) and dorsal spinocerebellar tract neurons located in Clarke's column (ccDSCT) and the dorsal horn (dhDSCT). In all of the cells, the majority of inhibitory terminals were glycinergic. The density of contacts was higher on somata and proximal versus distal dendrites of SB and VSCT neurons but more evenly distributed in ccDSCT and dhDSCT neurons. Variations in the density and distribution of inhibitory contacts found in this study may reflect differences in information on inhibitory processes forwarded by subtypes of spinocerebellar tract neurons to the cerebellum.

Key words: motor control, inhibition, neuronal networks, neurotransmitters, intracellular recording, morphology.

INTRODUCTION

It is well established that different populations of spinocerebellar tract neurons forward information from different sources (see e.g. Oscarsson, 1965; Bosco and Poppele, 2001) to the cerebellum. This information is primarily provided by excitatory input which is modified by postsynaptic inhibition of various origins, as well as by presynaptic filtering. However, in some spinocerebellar neurons inhibition may play a more essential role because a reduction or even arrest of firing in these neurons may be a source of information for their cerebellar target cells itself (Lundberg, 1971; Hammar et al., 2011). This may apply particularly to the subpopulation of ventral spinocerebellar neurons in which peripheral input is mainly or almost exclusively inhibitory (Burke et al., 1971a; Jankowska et al., 2010; Shakya-Shrestha et al., 2012).

Postsynaptic and presynaptic inhibitory actions on various populations of spinocerebellar neurons may also vary as much as the excitatory ones, both in origin and degree (Lundberg and Weight, 1971; Burke et al., 1971a; Edgley and Jankowska, 1988; Jankowska and Puczynska, 2008; Jankowska et al., 2010; Krutki et al., 2011). The aim of the present study was therefore to extend our previous investigation of excitatory input to four types of spinocerebellar tract neurons based on immunohistochemical analysis of nerve terminals in contact with labelled neurons (Shakya-Shrestha et al., 2012) by making a similar analysis of inhibitory input to these neurons.

The spinocerebellar neurons examined in this study include two populations of dorsal spinocerebellar tract (DSCT) neurons and two populations of ventral spinocerebellar tract (VSCT) neurons which were identified and characterised electrophysiologically before they were injected with an intracellular marker. These populations consisted of: (1) DSCT neurons located in Clarke's column (ccDSCT neurons) with dominant excitatory input from group Ia afferents and inhibitory input from group Ib and II afferents; (2) DSCT neurons located in the dorsal horn (dhDSCT neurons) with both excitatory and inhibitory input from group II afferents and skin afferents but not group I afferents (Edgley and Jankowska, 1987); (3) VSCT neurons located at the border between the white and grey matter in the ventral horn, either with excitatory peripheral input mainly from group Ia afferents or devoid of such input (referred to as spinal border, SB, neurons) and inhibitory input from group Ia, Ib, II and high-threshold muscle, skin and joint afferents; (4) VSCT neurons located in the medial part of

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Abbreviations: 4-AP, 4-aminopyridine; ANOVA, analysis of variance test; ccDSCT, dorsal spinocerebellar tract neurons located in Clarke's column; dhDSCT, dorsal spinocerebellar tract neurons located in dorsal horn; DSCT, dorsal spinocerebellar tract; GAD, glutamic acid decarboxylase; IPSP, inhibitory postsynaptic potential; L, lumbar; MLF, medial longitudinal fascicle; PB, phosphate buffer; PBS, phosphate-buffered saline containing 0.3 M NaCl; SB, Spinal border; SD, standard deviation; VGAT, vesicular GABA transporter; VGLUT, vesicular glutamate transporter; VSCT, ventral spinocerebellar tract.

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lamina VII, with excitatory peripheral input mainly from group Ib afferents (referred to as Ib-VSCT, or for the sake of brevity as VSCT neurons) and similar inhibitory input to SB neurons. The first aim of the study was to compare the number and the distribution of inhibitory (glycinergic and/or GABAergic) and excitatory (glutamatergic) terminals in contact with these neuronal populations and the second aim was to determine the proportion of contacts formed by glycinergic and GABAergic/glycinergic terminals on them.

EXPERIMENTAL PROCEDURES

Ethical approval

The study was carried out on a sample of 20 neurons labelled in seven adult cats. Cats (2.5–3.8 kg) were bred and housed under veterinary supervision at the Laboratory of Experimental Biomedicine at Sahlgrenska Academy where the electrophysiological experiments were carried out. All experimental procedures on these animals were approved by the Ethics Committee for Animal Research at the University of Gothenburg (Göteborgs Djurförsöksetiska Nämnd). All procedures comply with National Institutes of Health and European Union guidelines for animal care.

Surgical procedures

General anaesthesia was induced with sodium pentobarbital (Apoteksbolaget, Stockholm, Sweden; 40–44 mg/kg, i.p.) and maintained with intermittent doses of α-chloralose (Rhône-Poulenc Santé, Antony, France; 5 mg/kg; i.v., administered every 1–2 h up to 30 mg/kg and every 2–3 h up to 55 mg/kg thereafter). Additional doses of α-chloralose were given when motor reactions were evoked during dissection and when increases in the continuously monitored blood pressure or heart rate were evoked by experimental procedures. Atropine (0.05–0.2 mg/kg, i.m.) was occasionally administered during the preliminary surgical procedures to reduce tracheal secretion. During recordings, neuromuscular transmission was blocked by using pancuronium bromide (Pavulon, Organon, Sweden; about 0.2 mg/kg/h, i.v.) and the animals were artificially ventilated. The effectiveness of synaptic transmission was increased by intravenous administration of the potassium (K^+) channel blocker 4-aminopyridine (4-AP; Sigma, St. Louis, USA) at doses of 0.1–0.2 mg/kg, i.v. These doses were expected to result in a plasma concentration of 4-AP of about 1 μM and could be compared to clinically used doses of 10 mg, corresponding to 0.14 mg/kg in a 70-kg patient with minimal side effects (for latest references see Alvina and Khodakhah, 2010). 4-AP was expected to increase the duration of action potentials in presynaptic fibres and thereby enhance the release of transmitter from terminals and thus to increase the probability of synaptic activation. Mean blood pressure was maintained at 100–140 mm Hg and the end-tidal concentration of CO_2 at about 4% by adjusting the parameters of artificial ventilation and the rate of a continuous intra-arterial infusion of a bicarbonate buffer solution with 5% glucose (1–2 ml/h/kg). Core body temperature was kept at about 38 °C by servo-controlled infrared lamps.

Laminectomies were performed to expose the spinal cord from the third to the sixth lumbar (L3–L6) segments. The dura mater remained intact except for small holes (about 1 mm^2) over the dorsal columns through which recording electrodes were inserted. The caudal part of the cerebellum was exposed to allow insertion of tungsten electrodes (30–200 kΩ) used to activate antidromically axons of spinocerebellar neurons and to stimulate reticulospinal axons running in the medial longitudinal fascicle (MLF).

Cerebellar stimulation sites were located ipsilateral to DSCT and contralateral to VSCT and SB neurons, just rostral to or within the Nucleus Interpositus (at Horsley–Clarke coordinates about P 7, L 3.0–3.5, H 0 to −1). At the end of experiments they were marked by electrolytic lesions and reconstructed histologically. Axons within the MLF were stimulated ipsilateral with respect to the location of the neurons recorded from. The stimuli were applied at Horsley–Clarke coordinates P 7–9, L 0.5–0.8, H about −5. The final electrode position was adjusted while recording descending volleys from the surface of the spinal cord at the Th11–Th12 level. The electrodes were left at locations from which distinct descending volleys were evoked at thresholds of 10–20 μA; they were near maximal at 50–100 μA for cerebellar stimulation sites and at 100–150 μA for MLF stimulation sites.

In order to identify peripheral inputs, several left hindlimb nerves were dissected free, transected and mounted on stimulating electrodes. They included quadriceps (Q) and sartorius (Sart) branches of the femoral nerve mounted in subcutaneous cuff electrodes and the posterior biceps and semitendinosus (PBSt), anterior biceps and semimembranosus (ABSM), sural, superficial peroneal (SP), gastrocnemius-soleus (GS), plantaris (PL) and/or flexor digitorum and hallucis longus (FDL) branches of the sciatic nerve mounted on pairs of silver electrodes in a paraffin oil pool.

Stimulation

Peripheral nerves were stimulated with constant-voltage stimuli at intensities expressed in multiples of threshold (T) for the activation of the most excitable fibres. Afferent volleys following these stimuli (recorded from the dorsal root entry zone at the L5 segmental level) were used to determine the central latencies of postsynaptic potentials (PSPs) recorded once neurons had been penetrated. Intracerebellar axonal branches of spinocerebellar neurons were stimulated using 0.2-ms constant-current pulses at intensities ⩽100 μA. Axons of these neurons within the ipsilateral or contralateral funiculus at the Th11–Th12 level were stimulated extradurally, with two silver ball electrodes in contact with its surface, using constant-current pulses of 100–500 μA. The cerebellar and thoracic stimuli served to select neurons projecting to the cerebellum. Antidromic activation was first ascertained extracellularly and subsequently confirmed intracellularly, even if only blocked IS or M antidromic spikes could be recorded in strongly depolarised neurons where the spike-generating mechanisms were inactivated during ionophoresis of intracellular markers.

Criteria for identification of different populations of spinocerebellar neurons and intracellular labelling

DSCT neurons were searched for in the L3–L4 segments: ccDSCT neurons were found at the medial border of the dorsal horn at depths of 2–2.5 mm from the surface of the dorsal columns at which large field potentials were evoked from group I muscle afferents at thresholds < 2T and latencies of ⩽1 ms. dhDSCT neurons were found more laterally and more superficially, at 1.2–1.8 mm from the surface of the dorsal columns and often caudal to Clarke's column. In this area, large synaptic field potentials were evoked by group II, but not group I muscle afferents (Edgley and Jankowska, 1987). In addition, dhDSCT neurons displayed a characteristic input from group II muscle afferents and cutaneous afferents (Edgley and Jankowska, 1988); from muscle nerves at 3–5T and latencies of 2–3 ms and from cutaneous afferents at minimal latencies of 1–2 ms.

VSCT neurons were searched for in the L4 and L5 segments. The SB subpopulation of these neurons were identified by their location within a 100–200-μm-wide strip at the border between the lateral funiculus and the ventral horn (usually at depths 1.8–2.2 mm from the surface of the lateral funiculus at an angle of 10–20° from the vertical) and, following penetration, by

monosynaptic input from reticulospinal neurons (MLF; Hammar et al., 2011) and inhibitory input from group I and high-threshold muscle afferents (Lundberg and Weight, 1971; Burke et al., 1971a). Excitatory input from primary afferents, when present, was evoked from group Ia afferents, but we only labelled SB neurons with exclusive inhibitory input from peripheral nerves (see the last section of the Introduction) or where very small excitatory postsynaptic potentials were evoked from group I afferents. The more medially distributed Ib-VSCT neurons were identified by their location at depths 2.4–3.9 mm from the surface of the dorsal columns (at an angle of about 0°), at which focal field potentials were evoked from both group I and II, or only group II muscle afferents (Hammar et al., 2002), and by their characteristic excitatory input from group Ib afferents and inhibitory input from group I and high-threshold muscle afferents (Eccles et al., 1961). Representative examples of PSPs from each of these four neuronal populations are shown in Fig. 1.

Once the neurons were identified and penetrated, they were labelled intracellularly by ionophoresis from the same micropipettes (''sharp'', with tips broken to about 1.5 μm, impedance of 7–20 MΩ) that were used for extracellular and intracellular recordings. As in previous studies (see e.g. Liu et al. 2010; Shakya-Shrestha et al., 2012), the micropipettes were filled with a mixture of equal proportions of 5% tetramethylrhodamine-dextran (Molecular Probes, Inc., Eugene, OR, USA) and 5% Neurobiotin (Vector Laboratories, Peterborough, UK) in saline or KCl (pH 6.5) ejected by passing 5–10 nA of positive constant current for up to 15 min, or as long as PSPs were recorded from the neuron.

At the conclusion of the experiments, a lethal dose of pentobarbital was administered to the animals and they were perfused, initially with physiological saline and subsequently with paraformaldehyde (4%) in 0.1 M phosphate buffer (PB). Blocks of lumbar spinal cord containing labelled cells were removed and placed initially in the same fixative (for 8 h at 4 °C) and then in PB or in 30% sucrose in PB.

Immunohistochemical procedures

L3–L6 segments were cut into 50-μm-thick transverse sections with a Vibratome (Oxford Instruments, Technical Products International Inc., Austin, Texas, USA) after which they were placed immediately in an aqueous solution of 50% ethanol for 30 min to enhance antibody penetration. Thereafter the sections were washed several times with 0.1 M phosphate-buffered saline containing 0.3 M NaCl (PBS) and mounted with anti-fade medium, Vectashield (Vector Laboratories, Peterborough, UK) on glass slides.

Sections were collected and mounted in serial order to enable reconstruction of the labelled cells. Initially, the sections were examined for labelled spinocerebellar neurons with a fluorescence microscope and those containing labelled neurons were washed several times in PBS. They were then incubated in Avidin-Rhodamine (1:1000, Jackson ImmunoResearch, Luton, UK) for 3 h and mounted in Vectashield on glass slides. Sections were re-scanned using fluorescence microscope and those containing well-labelled neurons were processed for immunocytochemistry. Neurons that were only weakly labelled, or displayed different degrees of damage were excluded from the analysis.

Comparison of the density and distribution of inhibitory and of excitatory terminals in contact with the four populations of spinocerebellar neurons

Data from 12 neurons were used in this part of the study. Inhibitory and excitatory terminals in contact with labelled neurons were analysed immunohistochemically. In order to differentiate between them we used antibodies against vesicular GABA transporter (VGAT) which is known to be highly expressed in both GABAergic and glycinergic nerve endings (Burger et al., 1991; McIntire et al., 1997; Chaudhry et al., 1998; Wojcik et al., 2006; Aubrey et al., 2007) and a combination of antibodies against vesicular glutamate transporters 1 (VGLUT1) and 2 (VGLUT2) which should label most excitatory terminals (Varoqui et al., 2002; Todd et al., 2003; Alvarez et al., 2004). Selected sections containing intracellularly labelled neurons were first washed several times in PBS and thereafter incubated in a combination of primary antibodies containing rabbit anti-VGAT and guinea-pig anti-VGLUT1 + 2 for 72 h at 4 °C. Following several washes in PBS, the sections were incubated in a combination of secondary antibodies coupled to Alexa 488 and Dylight 649 for 3 h at room temperature to identify VGAT and VGLUT1 + 2 terminals respectively. They were rinsed in PBS and mounted in Vectashield. All antibodies were diluted in PBS containing 0.3% Triton (PBST) and incubated for 72 h at 4 °C (see Table 1 for details of antibodies used).

Comparison of proportions of glycinergic and GABAergic/glycinergic terminals forming contacts with different populations of spinocerebellar neurons

Data from eight additional neurons were used for this comparison. GABA and glycine are the two major inhibitory neurotransmitters in the spinal cord. It is widely accepted that antibodies directed against glutamic acid decarboxylase (GAD) can be used as reliable markers for axon terminals using GABA as neurotransmitter (Kaufman et al., 1991; Soghomonian and Martin, 1998) whereas antibodies against gephyrin have been established as markers of postsynaptic glycine and GABA receptor clusters (Todd et al., 1995; Alvarez et al., 1997). In order to examine whether the contacts formed by inhibitory terminals are glycinergic or GABAergic or both glycinergic/GABAergic, we incubated the cat spinal cord sections containing well-labelled cells in the following combination of primary antibodies: rabbit anti-GAD and mouse anti-7A (which recognises gephyrin) for 72 h at 4 °C. Following several washes in PBS, the sections were incubated in a combination of secondary antibodies coupled to Dylight 649 and Alexa 488 for 3 h at room temperature to identify GAD terminals and gephyrin puncta respectively. The sections were then rinsed in PBS and subsequently mounted in Vectashield. The sections were scanned using confocal laser microscopy and contacts were analysed as described in the section below. It was not possible to make an accurate estimate of the number of pure glycinergic contacts by counting and quantifying gephyrin puncta not apposed by GAD terminals because gephyrin labels multiple active sites associated with a single presynaptic bouton. However, it was possible to estimate the numbers of pure glycinergic contacts by utilising quantitative data obtained for overall contact density of inhibitory terminals which were VGAT immunoreactive (see Aim 1) and deducting the overall contact density of terminals immunolabelled with GAD apposed to gephyrin puncta.

Confocal microscopy, reconstruction and analysis

Sections containing cell bodies and dendrites of intracellularly labelled cat neurons were initially scanned at low power (20× lens, zoom factor 1.2) and this image was used as a frame of reference for the location of labelled processes within each section and to make preliminary reconstructions of the cells. Following this, individual cells were scanned at a higher magnification by using a 40× oil immersion lens at a zoom factor of 2 at an increment of 0.5 μm. Series of confocal images were gathered and a montage was constructed for

each cell. Usually the processes of cells extended within six or seven sections and 60–80 series of images were collected from each section.

Cells were reconstructed three dimensionally from series of 50-μm transverse sections by using Neurolucida for Confocal software (MicroBrightField, Colchester, VT, USA) and contacts on the labelled cells were mapped. Reconstructions were always started from the stacks of single optical section containing the cell body. Once the reconstruction of the cell body was complete, dendritic processes were systematically added and contacts were plotted and recorded simultaneously with appropriate markers from the subsequent stacks until the dendritic tree was fully reconstructed. Only contacts in close apposition with the cell in the same focal plane and with no intervening black pixel space were counted.

Contact densities were calculated using data generated by Neurolucida and expressed as numbers of contacts per unit area (100 μm^2) of neuronal surface. The surface area of the cell body was estimated by measuring the perimeter of each labelled cell body from projected confocal images using Image J software (National Institutes of Health, Bethesda, Maryland, USA) and calculating the surface area of an equivalent sphere. The distribution of contacts was analysed using Sholl analysis with Neurolucida Explorer. The number of contacts within a series of 25-μm concentric spheres from the centre of the cell body of each cell was expressed as numbers per 100 μm of dendritic length. Numbers of contacts within each sphere were averaged for the four types of cell and shown as Sholl plots.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Multi-group comparisons were made by using an analysis of variance test (ANOVA) followed by a post hoc Tukey's analysis as appropriate and two-variable comparisons among the same population were made by using Student's t test. A $p < 0.05$ was considered to be statistically significant.

RESULTS

Comparison of the density and distribution of inhibitory and excitatory terminals in contact with the four populations of spinocerebellar neurons.

GABAergic/glycinergic and glutamatergic terminals on intracellularly labelled cat neurons

Samples of spinocerebellar neurons. The sample of analysed neurons included 12 neurons from seven adult cats. Only well-labelled neurons without signs of damage caused by penetration or ionophoresis which displayed inhibitory postsynaptic potentials (IPSPs) in accordance with the typical origin of IPSPs previously demonstrated (see above) were selected for analysis. The analysis was performed on three SB neurons (cells 1–3), three VSCT neurons (cells 4–6), three ccDSCT neurons (cells 7–9) and three dhDSCT neurons (cells 10–12).

Individual neurons were classified as belonging to one of the four subpopulations of spinocerebellar tract neurons on the basis of antidromic activation by stimulation within or close to the Nucleus Interpositus, and from either the contralateral or ipsilateral lateral funiculus, on the basis of synaptic input from primary afferents and these neurons and according to their anatomical location in the lumbar grey matter, as described above in Experimental procedures. Series of electrophysiological recordings from four neurons illustrating antidromic activation from the cerebellum and inhibitory postsynaptic potentials evoked from muscle nerves are shown in Fig. 1. These records illustrate strong inhibition from group I (early) and group II (late) afferents in SB and VSCT neurons, from mainly group I afferents in the ccDSCT and group II afferents in dhDSCT neurons.

All neurons were located within midlumbar segments. The locations of cell bodies of these neurons within the L3, L4 and L5 segments are shown in Fig. 2. The cell bodies of SB neurons (red circles[1]) were located within the most lateral part of the grey matter in lamina VII while the cell bodies of VSCT neurons (green circles) were within more medial parts of lamina VII. The cell bodies of the ccDSCT neurons (purple circles) were contained within Clarke's column whereas the dhDSCT neurons (blue circles) had their cell bodies in either lamina V or VI. Both the location and morphology of analysed neurons was fully consistent with the categorisation of these cells based on their physiological properties.

As described in the Introduction, immunoreactivity for the VGAT was used to reveal inhibitory axonal terminals containing GABA and/or Glycine and a combination of antibodies against both VGLUT1 and VGLUT2 was used to reveal excitatory contacts independently of their source of origin. The results revealed marked differences in proportions and distribution of VGAT and VGLUT1 + 2 axonal contacts on the SB, VSCT, ccDSCT and dhDSCT neurons. Some of these differences are apparent in the examples of single confocal optical sections shown in Fig. 3 while other differences, based on reconstructions of contact locations over the cell bodies and the whole dendritic trees of the four populations of these neurons are illustrated in Fig. 4. The emerging patterns of contacts on these neurons are (i) the greater numbers of VGAT contacts (green in Fig. 4) when compared with VGLUT1 + 2 contacts (red) on both soma and dendrites of SB neurons, (ii) similar proportions and more even distribution of VGAT and VGLUT1 + 2 contacts on soma and dendrites of VSCT, and dhDSCT neurons, (iii) the clustered distribution of contacts on ccDSCT neurons, suggesting a higher density of excitatory contacts close to the initial segment or on some dendrites and (iv) a generally smaller density of somatic contacts on ccDSCT neurons than on other neurons.

The results of a quantitative comparison of distribution of VGAT and VGLUT1 + 2 contacts at different distances from the soma are summarised in Fig. 5. They are based on Sholl analysis in which numbers of contacts in concentric areas around the soma are plotted against distances from the soma. The four charts show that the density of VGAT and VGLUT1 + 2 contacts on proximal dendrites of SB and VSCT neurons was higher than of ccDSCT and dhDSCT neurons but similar at longer

[1] For interpretation of colour in Fig. 2, the reader is referred to the web version of this article.

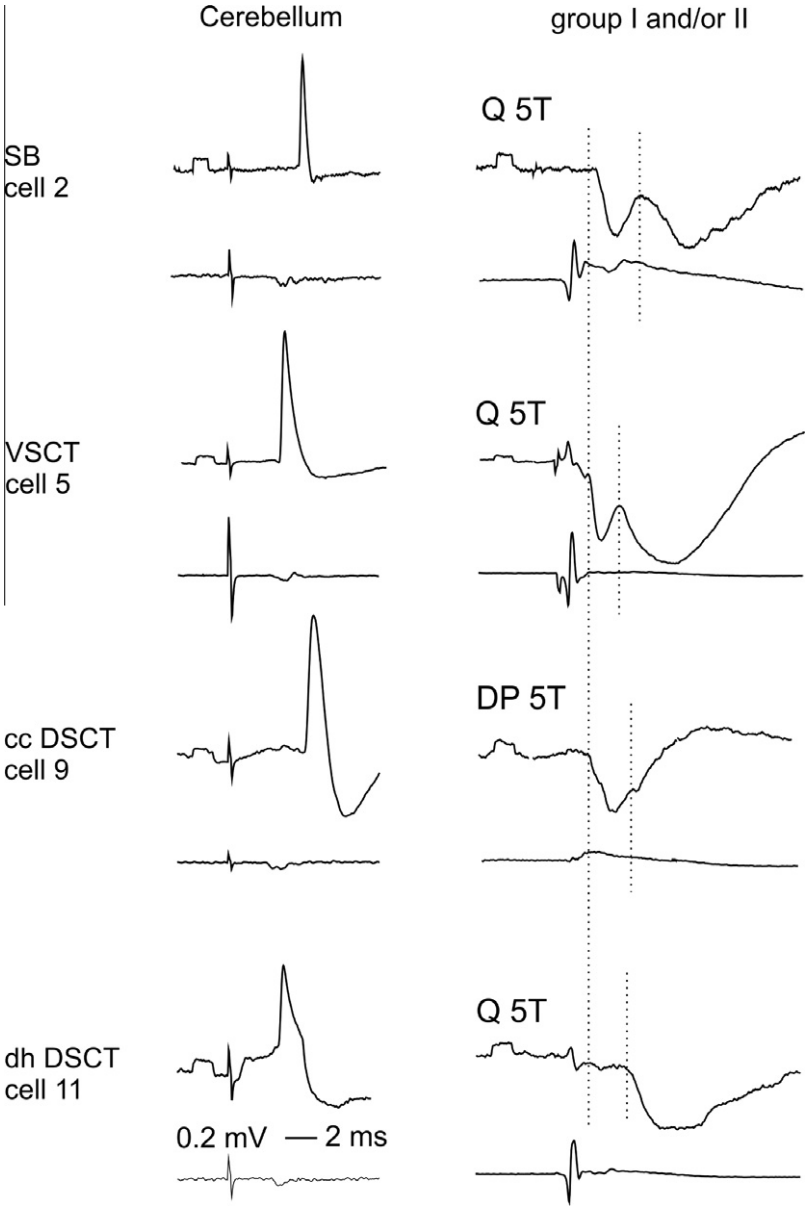

Fig. 1. Examples of records from the four populations of lumbar spinocerebellar neurons analysed morphologically. In each pair of traces, the upper traces are intracellular records (averages of 10 or 20 single recordings) from the cells identified as described in Experimental procedures, and the lower traces are from the surface of the spinal cord close to the dorsal root entry zone where both descending volleys evoked by cerebellar stimuli (left panels) and afferent volleys from the stimulated nerves (right panels) were monitored. Panels in the left column illustrate antidromic activation following the cerebellar stimulation when spikes could not invade the soma and dendrites during ionophoresis and a resulting strong depolarisation when only M or IS spikes of small amplitudes were recorded. Panels in the right column illustrate IPSPs evoked from muscle nerves by stimulation of both group I and II afferents in the quadriceps (Q) or deep peroneal (DP) nerves at intensity five times threshold (T) for group I afferents. For each neuron used in this study it was verified that IPSPs evoked in this neuron conformed with the characteristic origin of IPSPs previously found in the four populations of spinocerebellar neurons. The negativity is downward in intracellular records and upward in those from the cord dorsum. The dotted lines indicate the onset of the earliest group I and group II evoked potentials respectively. Rectangular calibration pulses at the beginning of traces are 0.2 mV.

distances from soma. The proportion of VGAT contacts within the first 75 μm was considerably higher than of VGLUT1 + 2 contacts for SB neurons, somewhat higher for VSCT and dhDSCT neurons and similar for ccDSCT neurons. A similar finding was also found for the initial axon segments of SB neurons where the contact density (2.19 ± 0.74/100 μm^2) was almost twice that associated with the other three types of spinocerebellar tract neuron (VSCT = 1.41 ± 1.11; ccDSCT = 1.15 ± 0.69; dhDSCT = 1.28 ± 0.52 per 100 m^2; however none of these values were significantly different, ANOVA, $p < 0.05$). For further quantitative comparisons see Fig 6, Table. 2 and next section.

Quantitative comparison of coverage of VGAT and VGLUT1 + 2 on intracellularly labelled spinocerebellar neurons

Table 2 summarises quantitative data for the four populations of intracellularly labelled spinocerebellar

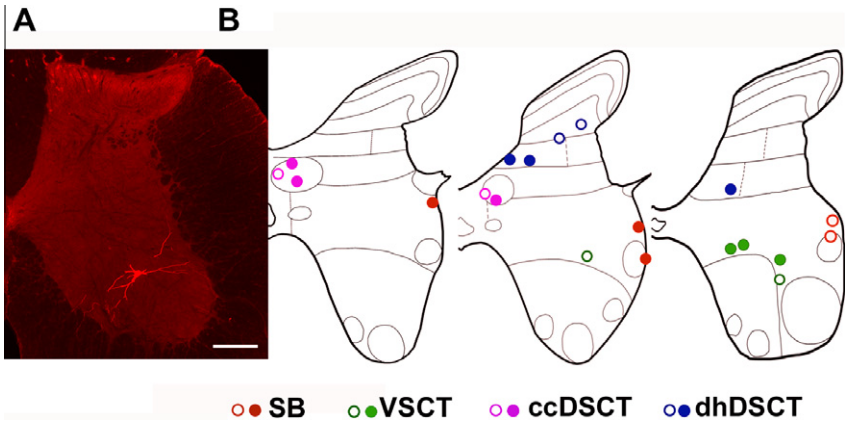

Fig. 2. Locations of 20 intracellularly labelled spinocerebellar neurons. (A) A low-magnification image of a transverse section of the L5 segment of the spinal cord showing the location of one of the VSCT neurons (cell 4). (B) Diagrams illustrating the locations of cell bodies of all 20 cells in the L3, L4 and L5 segments respectively on contours of the spinal cord from Rexed (1954). Closed circles indicate the location of the 12 cells analysed for both VGAT and VGLUT1 + 2 while open circles are for the eight cells analysed for GAD and gephyrin. Scale bar in panel A = 400 μm.

neurons, including the number and density of VGAT and VGLUT1 + 2 terminals in contact with them and the density of these contacts.

The overall average densities of VGAT and VGLUT1 + 2 contacts per 100 μm^2 for the somata and the dendritic trees of SB neurons were 5.84 ± 0.92 and 3.86 ± 0.73 respectively. The total contact density of VGAT terminals was therefore 1.5 times greater than that of VGLUT1 + 2 terminals. This difference was statistically significant (Student's *t*-test, $p < 0.05$). The difference was even greater (two times) for the soma. In contrast to SB neurons, VSCT neurons have similar overall contact densities of VGAT and VGLUT1 + 2 terminals, 3.95 ± 0.63 and 3.62 ± 0.54 per 100 μm^2 even though there was a somewhat higher (1.25 times, not statistically significant) density of VGAT contacts on cell bodies. Therefore this comparison suggests that the main difference between SB and VSCT neurons was in the overall density of VGAT contacts but as this difference was not statistically significant ($p > 0.05$; ANOVA) no firm conclusions can be drawn.

ccDSCT neurons, displayed the lowest overall average densities of VGAT and VGLUT1 + 2 contacts, 2.50 ± 0.85 and 3.15 ± 1.16 per 100 μm^2 respectively and the difference between them was not statistically significant ($p > 0.05$). In dhDSCT neurons, the overall average densities of these terminals were also relatively low and not significantly different, with a VGAT density of 2.76 ± 0.80 and a VGLUT1 + 2 density of 3.48 ± 0.77 per 100 μm^2. However, as shown in Fig. 6A, B, the overall contact density of VGAT terminals associated with SB neurons was significantly higher than that on either ccDSCT or dhDSCT neurons ($p < 0.01$; ANOVA). No such difference was found in the contact densities of VGLUT1 + 2 terminals among any of the population of neurons analysed in the current study. However, it should be noted that significant differences in the densities of separately labelled VGLUT1 and VGLUT2 terminals were found in our pervious study (see Fig. 5 in Shakya-Shrestha et al., 2012).

The results presented in Figs. 4–6 and in Table 2 show that synaptic actions of VGAT and VGLUT1 + 2 terminals may be evoked within all compartments of the dendritic trees of SB and VSCT neurons even though, as specified in our previous paper (Shakya-Shrestha et al., 2012) it is more likely that the actions of VGLUT1 terminals are focused upon proximal dendrites and the highest densities of VGAT terminals are on soma and proximal dendrites. Taken together our results indicate that the distribution of VGAT and VGLUT1 + 2 contacts within each class of spinocerebellar neuron is characteristic and differs from the distribution in other classes.

Comparison of proportions of glycinergic and GABAergic/glycinergic terminals forming contacts with different populations of spinocerebellar neurons

Following the quantification of contacts formed by VGAT terminals in different populations of spinocerebellar neurons as described above, eight additional intracellularly labelled spinocerebellar neurons (a–h) from three cats were examined to define which inhibitory neurotransmitters were contained within the VGAT-immunoreactive contacts. This was done by using markers for GABAergic terminals and glycine/ GABA receptors (see Table 1B). Examples of confocal images revealing the association of GABAergic terminals and gephyrin for each population of spinocerebellar tract neurons are shown in Fig. 7A–D; GABAergic terminals apposed to gephyrin are indicated by arrow heads while gephyrin puncta occurring separately are indicated by arrows. Analysis of distribution of GAD terminals apposed to gephyrin (green) and GAD terminals which were not associated with gephyrin (red) revealed that the great majority of GAD terminals apposed to all types of spinocerebellar neurons formed synaptic specialisations. Examples of these reconstructions are shown in Fig. 8.

In SB neurons, the overall mean densities of contacts formed by GAD terminals apposed to gephyrin were 0.70/100 μm^2 and of those GAD terminals which were not associated with gephyrin were 0.02/100 μm^2. Similarly, in VSCT neurons, the density of the contacts made by GAD-positive terminals associated with gephyrin puncta

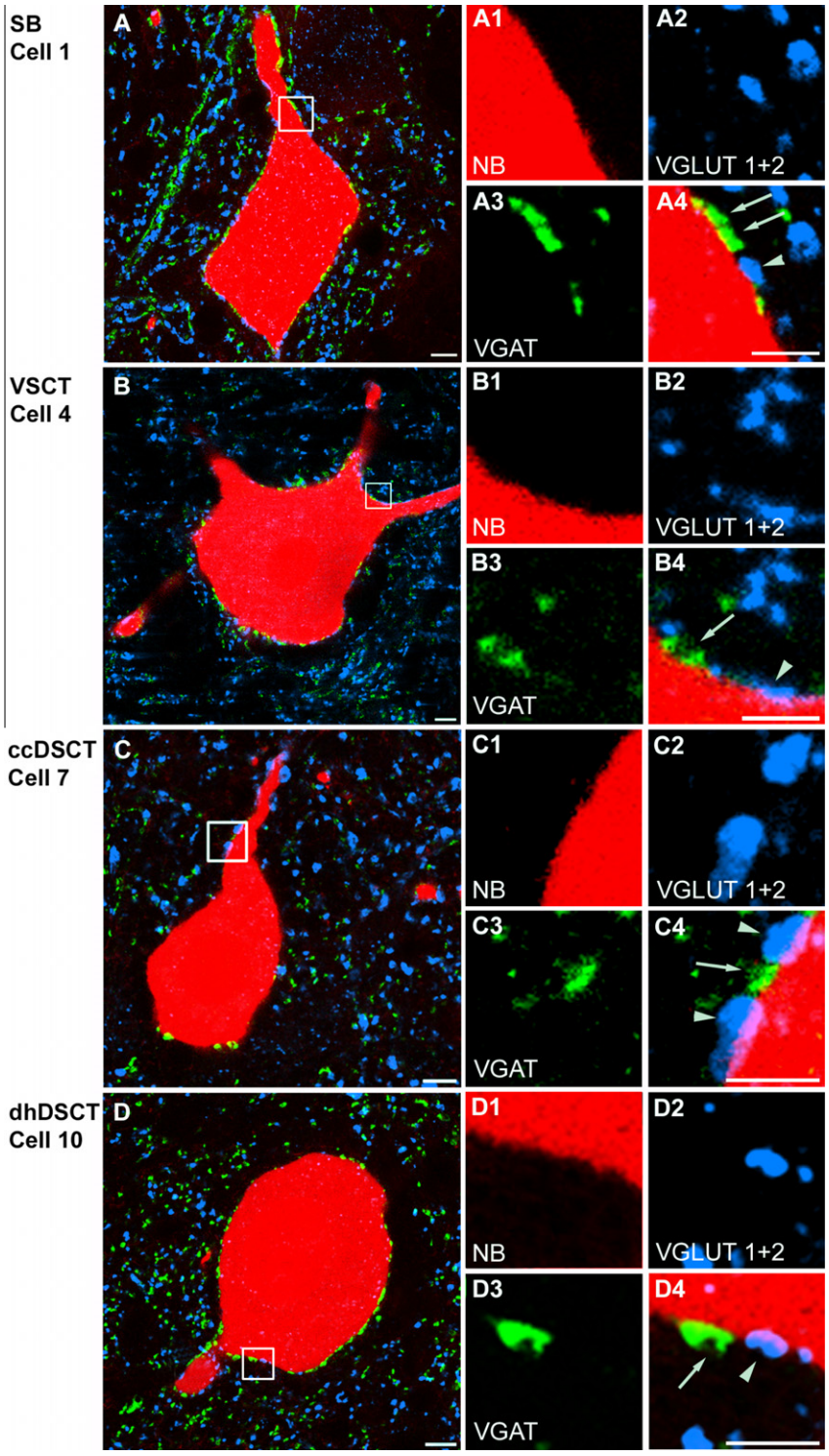

Fig. 3. Immunohistochemical characteristics of VGAT and VGLUT1 + 2 axon terminals in contact with intracellularly labelled spinocerebellar neurons. (A–D) Single optical sections through the cell bodies of representative SB (Cell 1), VSCT (Cell 4), ccDSCT (Cell 7) and dhDSCT (Cell 10) neurons showing the presence of VGAT- (green) and VGLUT1 + 2- (blue) immunoreactive axon terminals. The cell body and dendrites of intracellularly labelled cells are in red. (A1–A4; B1–B4; C1–C4 and D1–D4) Images of dendritic trunks from cells shown in panels A, B, C and D respectively (areas encompassed in the boxes) illustrating contacts made by VGAT- and VGLUT1 + 2-immunoreactive terminals with the cell indicated by arrows and arrowheads respectively. Scale bar in A–D = 10 μm. Scale bar in A4, B4, C4 and D4 = 5 μm.

was 1.08/100 μm^2 and that of GAD terminals only was 0.02/100μm^2. Hence, using the data in Table 2 it was possible to estimate the numbers of pure glycinergic contacts by deducting the overall contact density of terminals immunolabelled with GAD apposed to gephyrin puncta from the overall contact density of

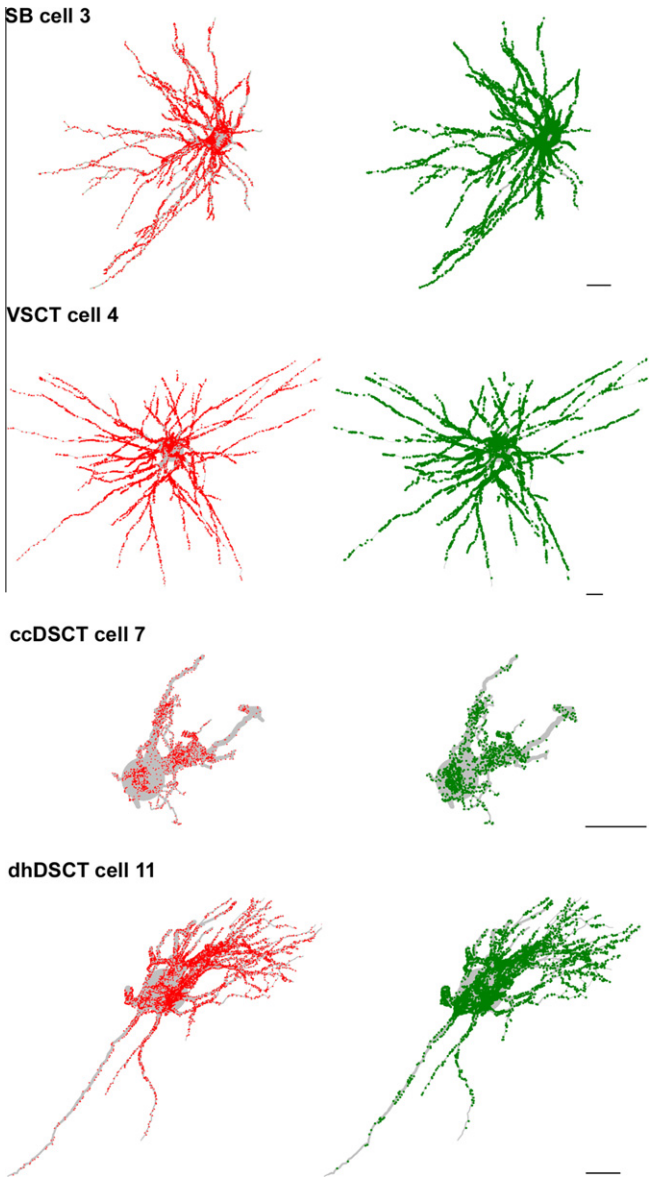

Fig. 4. Distribution of VGAT and VGLUT1 + 2 contacts on reconstructions of SB, VSCT, ccDSCT and dhDSCT neurons. Distribution of VGLUT1 + 2 (red) and VGAT (green) contacts formed with somata and dendrites is illustrated on two-dimensional projections of the same neuron of each class. The reconstructions were made with Neurolucida for Confocal. Cell bodies and dendrites are shown in light grey. Scale bars = 50 μm. All cells are oriented such that the midline is to the left and the lateral borders of the grey matter to the right, as in Fig. 2. Note the more even distribution of both VGLUT1 + 2 (red) and VGAT contacts on the soma and throughout the whole dendritic tree of SB and VSCT neurons and the patchy distribution of these contacts on both populations of DSCT neurons.

inhibitory terminals which were VGAT immunoreactive (see Experimental procedures). Thus the density of purely glycinergic axon terminals that apposed to SB and VSCT neurons was estimated as 5.12 and 2.85/100 μm^2 respectively.

In ccDSCT and dhDSCT neurons the overall contact densities of GAD-immunolabelled terminals associated with gephyrin puncta were 1.64 and 1.45/100 μm^2 respectively while the densities of the appositions formed by GAD-positive terminals not associated with gephyrin were 0.12 and 0.01/100 μm^2. On the basis of these data we estimated the density of contacts made by glycinergic terminals as 1.39 terminals/100 μm^2 in ccDSCT neurons and as 2.02 terminals/100 μm^2 in dhDSCT neurons.

More generally, together with the data on the overall density of VGAT contacts on the same populations of cells, our results show that the majority of the contacts made by inhibitory terminals are likely to be made by glycinergic interneurons.

DISCUSSION

The results of this study revealed considerable differences in the total number, density and distribution of

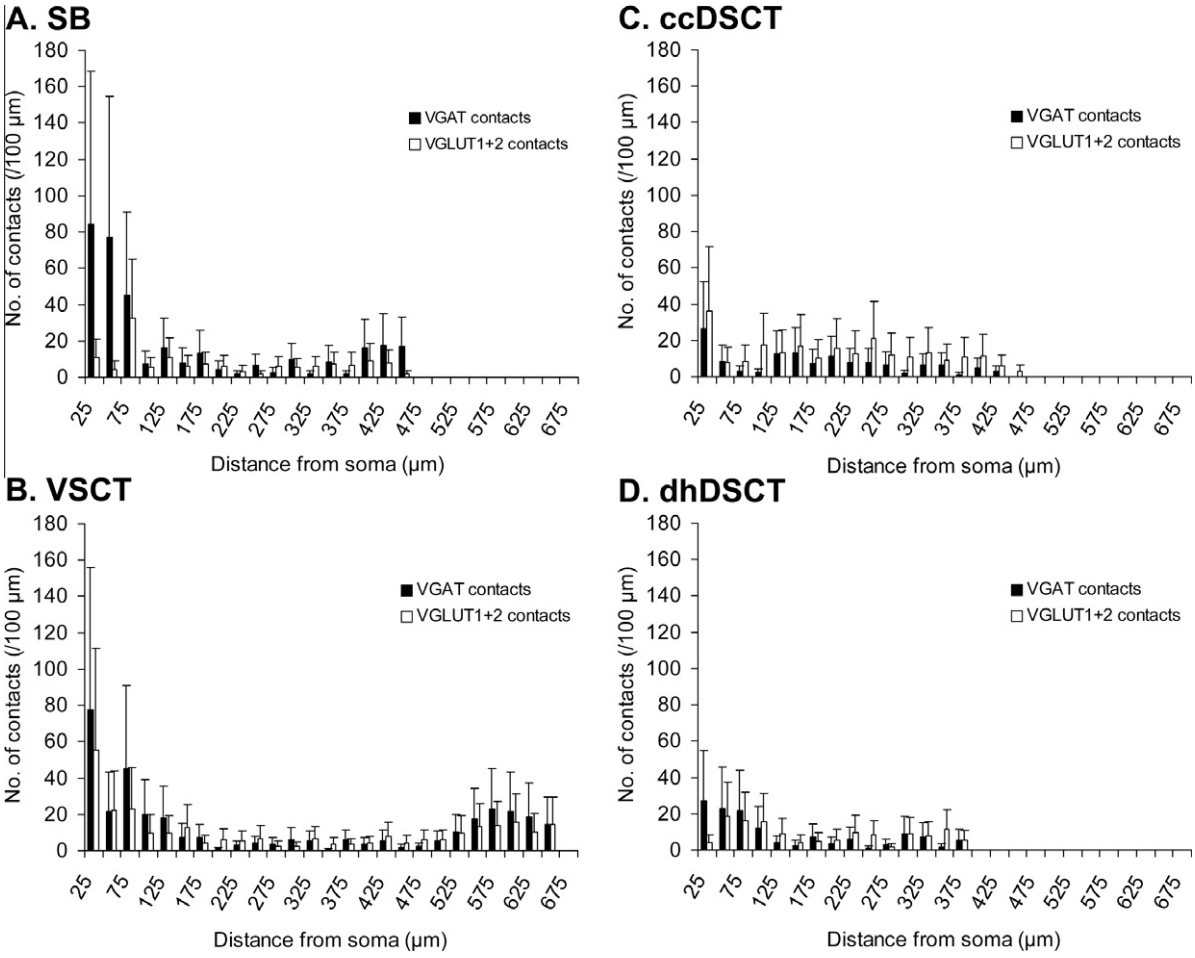

Fig. 5. Comparison of distribution of VGAT and VGLUT1 + 2 terminals along dendrites. Mean numbers of VGAT and VGLUT1 + 2 terminals forming contact with different populations of intracellularly labelled spinocerebellar tract neurons at different distances from soma derived from Sholl analysis. (A) SB neurons (cells 1–3), (B) VSCT neurons (cells 4–6), (C) ccDSCT neurons (cells 7–9) and (D) dhDSCT neurons (cells 10–12). The plots show the mean numbers of contacts per 100 µm of dendritic length contained within concentric spheres with radii which increase in 25-µm steps from the centre of the cell body. Bars represent the standard deviations.

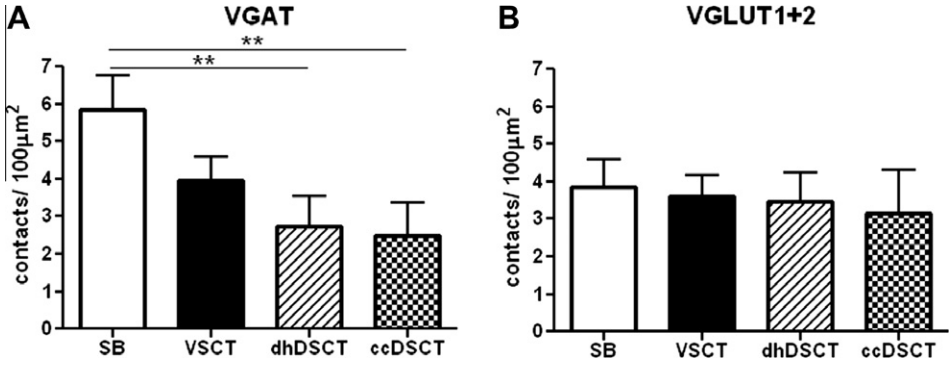

Fig. 6. Comparison between contact densities of VGAT and VGLUT1 + 2 axon terminals in different populations of spinocerebellar neurons. (A, B) Differences in overall contact density of VGAT and similar density of VGLUT terminals/100 µm^2 for SB, VSCT, dhDSCT and ccDSCT neurons. $^{**}p < 0.01$.

inhibitory contacts on SB, VSCT, ccDSCT and dhDSCT neurons. When taken together with the demonstration of differences in glutamatergic terminals relaying excitatory input to these neurons (Shakya Shrestha et al., 2012), our results provide a new basis for understanding the morphological substrates of the types of information

Table 1. Summary of the primary and secondary antibody combinations and concentrations used in the current study

	Primary antibody combination	Primary antibody concentration	Supplier	Secondary antibody combination	Secondary antibody concentration	Supplier
A	gpVGLUTI	1:5000	Chemicon, Harlow, UK	Dylight 649	1:500	Jackson ImmunoResearch, West Grove, PA, USA
	gp VGLUT2	1:5000	Chemicon, Harlow, UK	Dylight 649	1:500	Jackson ImmunoResearch, West Grove, PA, USA
	rbt VGAT	1:5000	Synaptic Systems, Goettingen, Germany	Alexa 488	1:500	Molecular Probes, Eugene, OR, USA
B	rbt GAD	1:1000	Sigma, UK	Dylight 649	1:500	Jackson ImmunoResearch, West Grove, PA, USA
	mo Gephyrin (7a)	1:1000	Synaptic Systems, Goettingen, Germany	Alexa 488	1:500	Molecular Probes, Eugene, OR, USA

All secondary antibodies were raised in donkey and conjugated to Dylight 649, Alexa 488, Alexa-fluor 488.
gp, Guinea pig; rbt, rabbit; mo, mouse; VGLUT, vesicular glutamate transporter; VGAT, vesicular GABA transporter; GAD, glutamic acid decarboxylase.

forwarded to the cerebellum by different populations of spinocerebellar neurons.

Methodological issues

Two methodological issues need to be taken into consideration before evaluating the results of this study. One of these issues is that we pooled the data on excitatory terminals with VGLUT1 and VGLUT2 together, in contrast to our previous study (Shakya-Shrestha et al., 2012) where distribution of these terminals was analysed separately. Terminals using glycine, GABA or co-expressing glycine and GABA, were all labelled with an antibody against VGAT which is highly expressed in both GABAergic and glycinergic nerve endings (Burger et al., 1991; McIntire et al., 1997; Chaudhry et al., 1998; Wojcik et al., 2006; Aubrey et al., 2007). This approach enabled us to maximise labelling of both excitatory and inhibitory terminals and thus allowed the proportions of inhibitory and excitatory terminals to be defined with confidence.

Another methodological issue is that of the small size of the samples of neurons used for analysis. However, individually labelled neurons identified electrophysiologically have a great advantage over neurons identified by other approaches because they represent distinct functional populations of neurons characterised according to well-established criteria and the morphological features of these neurons may therefore be reliably related to their functions. By using electrophysiological criteria to classify individual neurons while searching for cells to be labelled we were also able to select subpopulations of particular interest, e.g. neurons of a subpopulation of SB cells with a dominant inhibitory input from peripheral afferents and monosynaptic input from reticulospinal tract neurons (Burke et al., 1971a; Jankowska et al., 2010; Hammar et al., 2011). Furthermore, intracellular labelling provides a much more complete visualisation of the whole dendritic tree. These advantages to some extent compensate for the unavoidably restricted numbers of intracellularly labelled neurons in our samples.

Density and origin of inhibitory axonal contacts on different populations of spinocerebellar neurons

Among the four populations of spinocerebellar neurons, SB and VSCT neurons showed the highest densities of contacts formed by VGAT-positive terminals. As VGAT should label all inhibitory terminals containing GABA and/or glycine (Abzug et al., 1974; Todd and Sullivan, 1990; Chaudhry et al., 1998), a high density of VGAT contacts on SB and VSCT neurons and relatively small proportion associated with ccDSCT and dhDSCT neurons provides a morphological substrate for the differences in the inhibition of these neurons found in electrophysiological studies. Electrophysiological studies revealed particularly strong inhibitory input to SB and VSCT neurons which in some cells practically constitutes the exclusive input from the periphery (Eccles et al., 1961; Oscarsson, 1965; Lundberg and Weight, 1971; Burke et al., 1971a; Hammar et al., 2011; Shakya-Shrestha et al., 2012). Inhibition of these neurons is evoked to a great extent by collateral actions of premotor interneurons, (most of which would mediate disynaptic inhibition from group Ia, Ib and II afferents), Renshaw cells and high-threshold muscle, skin and joint afferents, in parallel with their actions on motoneurons (Hultborn et al., 1971; Lundberg, 1971; Lindstrom and Schomburg, 1973, 1974; Jankowska et al., 2010). The same interneurons would also mediate inhibition of spinocerebellar neurons from descending tract neurons (Baldissera and Roberts, 1976; Baldissera and ten Bruggencate, 1976; Hammar et al., 2011; Jankowska et al., 2011). As the total number of premotor interneurons in any of these pathways may amount to not more than a thousand (see Alvarez and Fyffe, 2007 for Renshaw cells) and many interneurons mediate inhibition from several sources, the finding of several thousands of inhibitory terminals on individual SB or VSCT neurons requires comment. This could indicate either multiple contacts of individual interneurons, or contacts of a considerable number of interneurons on individual spinocerebellar neurons, or both. In the cat, the source of inhibitory terminals could be limited to spinal interneurons or propriospinal neurons in view of

Table 2. The number and densities of VGAT and VGLUT1 + 2 axon terminals in apposition with the cell bodies and dendrites of different populations of spinocerebellar tract cells

Populations of spinocerebellar tract cells	Cell	Contacts (total number)		Soma					Dendrite					
				Contacts (*n*)		Surface area (μrn^2)	Density (*n*/100 μm2)		Contacts (*n*)		Total dendritic length (μrn)	Surface area (μrn^2)	Density (*n*/100 μm^2)	
		VGAT	VGLUT1 + 2	VGAT	VGLUT1 + 2		VGAT	VGLUT1 + 2	VGAT	VGLUT1 + 2			VGAT	VGLUT1 + 2
SB	Cell 1	4864	3371	164	59	8260.33	1.99	0.71	4700	3312	9286.9	63472.452	7.40	5.22
	Cell 2	6160	3780	542	237	12445.91	4.35	1.90	5618	3543	9013.4	93367.21	6.02	3.79
	Cell 3	6026	4061	449	245	12436.47	3.61	1.97	5577	3816	10588.1	109762.29	5.08	3.48
Mean							3.32	1.53					6.17	4.16
SD							1.21	0.71					1.17	0.93
VSCT	Cell 4	5723	5446	286	144	20467.14	1.45	0.40	5427	5302	16815.9	153591.14	3.53	3.45
	Cell 5	5297	4655	435	416	21347.05	2.04	1.95	4862	4239	9049.1	110283.64	4.41	3.84
	Cell 6	3252	3002	235	228	8948.45	2.63	2.55	3017	2774	7172	62676.9	4.81	4.43
Mean							2.04	1.63					4.25	3.91
SD							0.59	1.11					0.65	0.49
ccDSCT	Cell 7	1000	1267	54	22	6081.86	0.89	0.36	946	1245	2019	22654.74	4.18	5.50
	Cell 8	1196	1306	110	52	10548	1.04	0.49	1086	1254	3079.1	50451.69	2.15	2.49
	Cell 9	1543	2169	163	117	13919.93	1.17	0.84	1380	2052	5697.3	61025.78	2.26	3.36
Mean							1.03	0.56					2.86	3.78
SD							0.14	0.25					1.14	1.55
dhDSCT	Cell 10	2405	3104	443	196	12496.2	3.55	1.57	1962	2908	5141.6	66574.3	2.95	4.37
	Cell 11	3651	4245	446	278	17427.93	2.56	1.60	3205	3967	8229.2	90850.29	3.53	4.37
	Cell 12	2186	3052	244	330	18336.92	1.41	1.90	1942	2722	7459.3	99661.73	1.95	2.73
Mean							2.51	0.18					2.81	3.82
SD							1.07	0.18					0.08	0.95

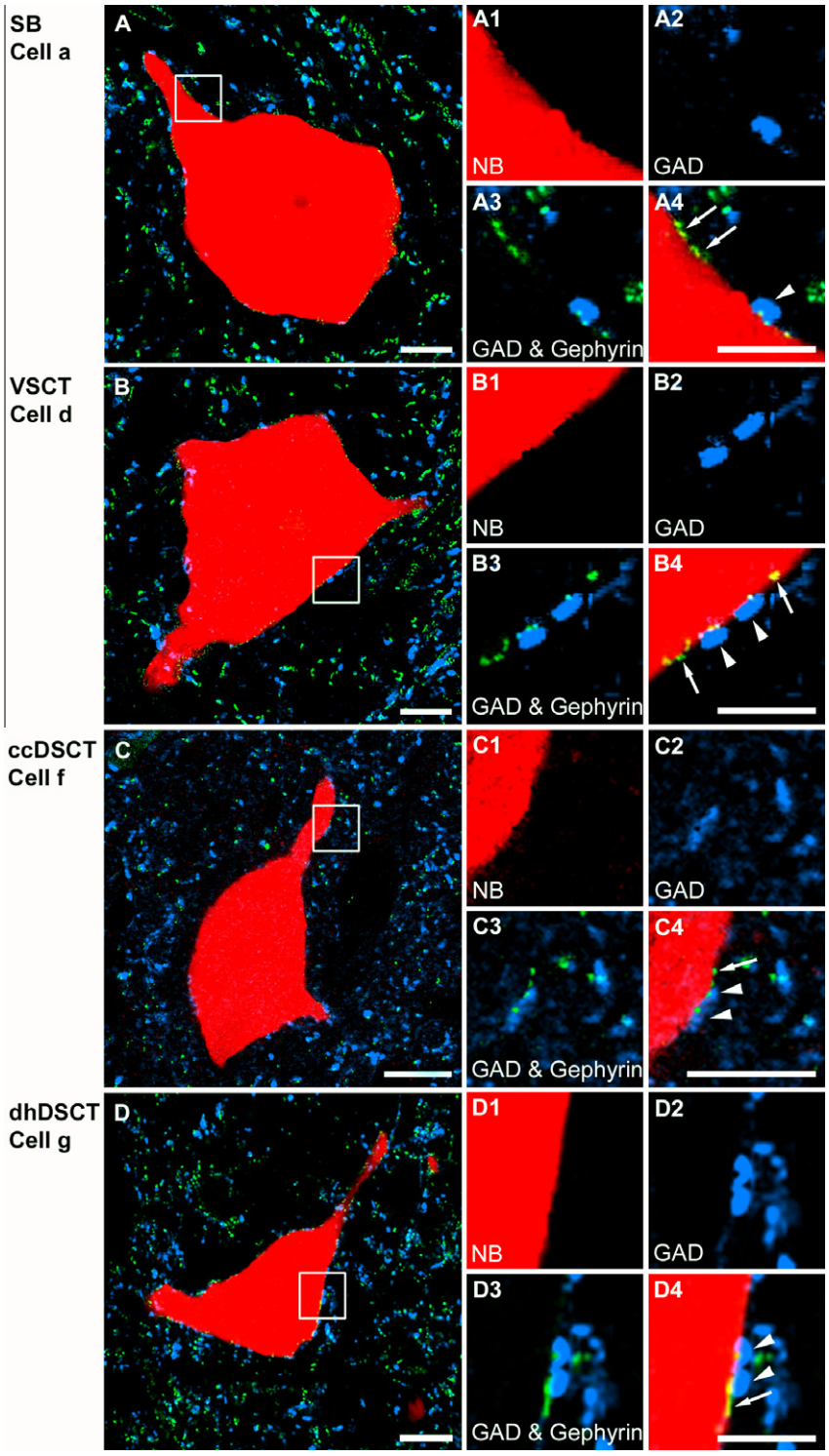

Fig. 7. Immunohistochemical characteristics of GAD axon terminals and gephyrin puncta in apposition with intracellularly labelled spinocerebellar neurons. (A–D) Single optical sections through the cell bodies of representative SB, VSCT, ccDSCT and dhDSCT neurons showing the presence of gephyrin puncta- (green) and GAD- (blue) immunoreactive axon terminals. The cell body and dendrites are in red. (A1–A4; B1–B4; C1–C4 and D1–D4) Projected images of cell bodies or dendritic trunks from cells shown in panels A, B, C and D respectively (areas encompassed in the boxes) illustrating contacts made by GAD-immunoreactive terminals associated with gephyrin puncta and GAD-negative gephyrin puncta associated with the cell indicated by arrowheads and arrows respectively. Note differences in the density of gephyrin associated with the surface of cell bodies and dendrites in all the four neuronal populations shown in panels A–D. Scale bar in A–D = 20 μm. Scale bar in A4, B4, C4 and D4 = 5 μm.

lack of evidence for projections of supraspinal inhibitory neurons to lumbar segments. However, in other species such as the rat some inhibitory terminals might also originate from a proportion of inhibitory reticulospinal

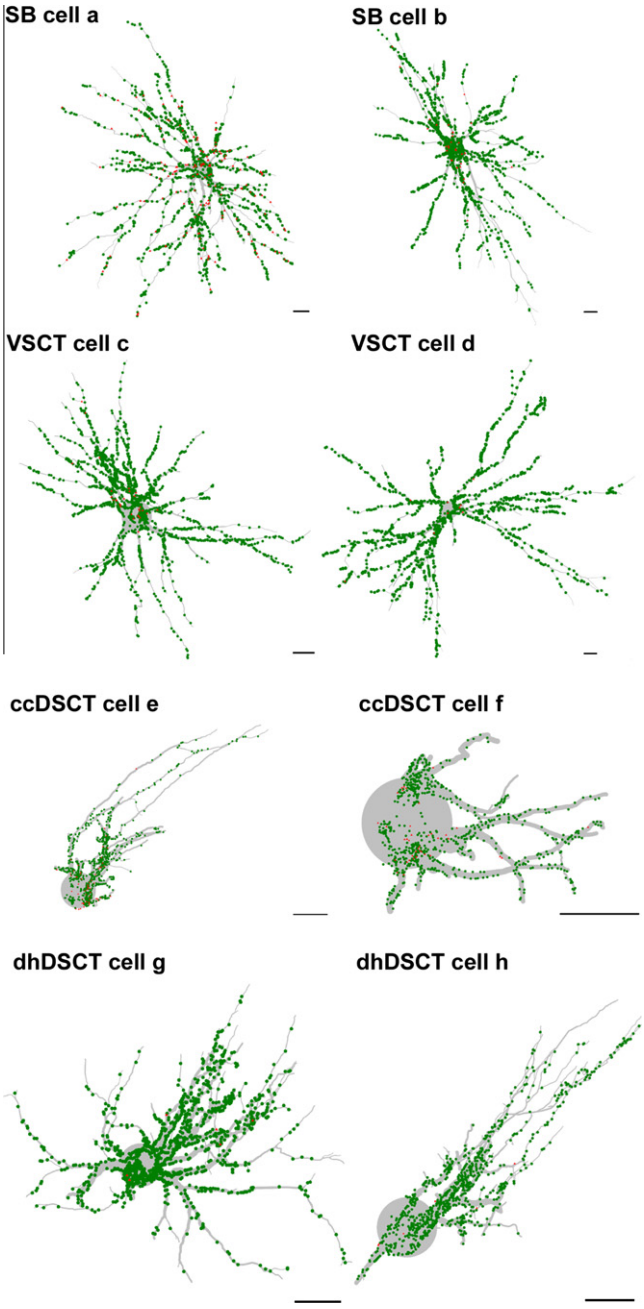

Fig. 8. Reconstructions of distribution of GAD terminals associated with gephyrin on four types of the spinocerebellar tract neurons. Reconstructions of spinocerebellar tract neurons showing the distributions of GAD terminals apposed to gephyrin puncta (green) and GAD terminals without association with gephyrin contacts (red) throughout the dendritic trees. The reconstructions were made with Neurolucida for Confocal. Cell body and dendrites are shown in light grey. Scale bars = 50 μm. All are oriented such that the midline is to the left and the lateral borders of the grey matter to the right, as in Fig. 2.

neurons projecting as far caudally as lumbar segments (Holstege and Bongers, 1991; Du Beau et al., 2012).

A variety of inhibitory interneurons and proprioneurons might contribute to the inhibition of individual spinocerebellar neurons. Premotor interneurons would include interneurons located in the ventral horn (e.g. Ia inhibitory interneurons and Renshaw cells) as well as in the intermediate zone (e.g. group I/II interneurons). This is indicated not only by electrophysiological evidence but also by morphological observations based on the demonstration of glycinergic inhibitory intermediate zone interneurons with inputs from group I and II afferents that have terminal projection areas in the intermediate zone and in laminae VII and VIII in addition to motor nuclei (Bannatyne et al., 2009). Another potential source of inhibitory terminals might be inhibitory dorsal horn interneurons activated by group II muscle afferents with terminal arborisations both contralaterally and ipsilaterally

(Bannatyne et al., 2006) and commissural interneurons with direct input from reticulospinal neurons which terminate outside motor nuclei in laminae VII and VIII (Bannatyne et al., 2003). Propriospinal neurons potentially could include inhibitory neurons located in the cervical 3rd and 4th segments (Alstermark et al., 1984) as well as neurons in forelimb segments with highly diverging coordinative actions (Fetz et al., 2002; Takei and Seki, 2010) or other intersegmentally operating inhibitory interneurons (Liu et al., 2010).

Inhibitory terminals on ccDSCT and dhDSCT neurons should likewise originate from spinal neurons but from a smaller range of interneuron populations including intermediate zone inhibitory premotor interneurons in pathways from group Ib and II afferents but not Ia inhibitory interneurons and Renshaw cells (Hongo et al., 1983; Jankowska and Puczynska, 2008). Another source of inhibitory input to these neurons might be dorsal horn interneurons relaying information from group II afferents which are shown to have terminal projections in laminae IV, V and VI of the dorsal horn (Bannatyne et al., 2006, 2009). However, in contrast to SB and VSCT neurons inhibitory input from supraspinal neurons, in particular reticulospinal neurons (see above) would not be expected either in ccDSCT or in dhDSCT neurons, because hardly any input from reticulospinal neurons has been found in them, at least in the cat (Hammar et al., 2011). The smaller numbers of inhibitory contacts on ccDSCT and dhDSCT neurons found in the present study might thus be related to generally weaker inhibitory input revealed in electrophysiological studies. Fig. 9 is a summary diagram based on findings from the present and our previous report (Shakya Shrestha et al., 2012) showing putative arrangements of inhibitory and excitatory inputs to the four classes of spinocerebellar tract neurons.

Proportions of glycinergic and GABA/glycinergic axon terminals on intracellularly labelled neurons in the cat

It is widely accepted that the two isoforms of the GABA-synthesising enzyme GAD 65 and 67 are found within GABAergic presynaptic terminals (Kaufman et al., 1991) and all GABAergic neurons in the spinal cord contain both isoforms of GAD (Soghomonian and Martin, 1998; Mackie et al., 2003). Similarly, there is strong evidence for the presence of gephyrin at glycinergic/GABAergic synapses (Mitchell et al., 1993; Todd et al., 1995). Therefore, GAD and gephyrin are known to be reliable markers of GABAergic axon terminals and glycinergic/GABAergic receptors respectively (Mackie et al., 2003). There is considerable evidence for the coexistence of GABA and glycine in axon terminals of the spinal cord (Ornung et al., 1994, 1996, 1998; Taal and Holstege, 1994; Mackie et al., 2003) but there is variation in the proportions of terminals containing both transmitters depending upon their location, origin, animal age and

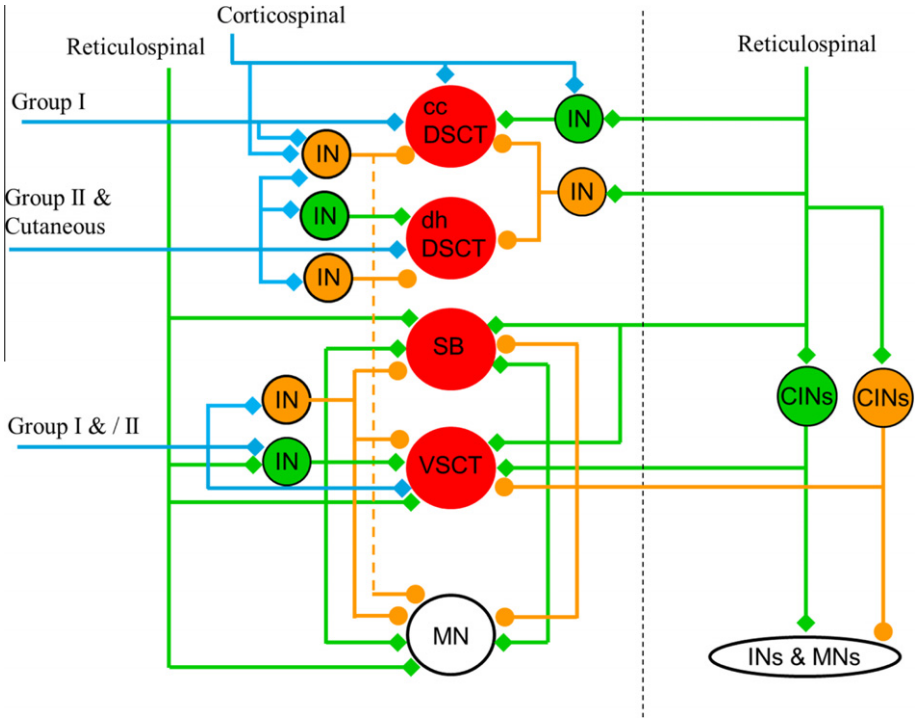

Fig. 9. Summary of patterns of inhibitory and excitatory contacts formed with the four different populations of lumbar spinocerebellar tract neurons indicated by results of the present and previous study. Red circles represent Clarke's column dorsal spinocerebellar tract neurons (ccDSCT); dorsal horn dorsal spinocerebellar tract neurons (dhDSCT), spinal border neurons (SB), Ib-ventral spinocerebellar tract neurons (VSCT). Green circles represent excitatory interneurons in reflex pathways to motoneurons (MN) activated by primary afferents and descending systems. Blue lines and diamonds represent neurons that express vesicular glutamate transporter 1 (VGLUT1) in their axon terminals. Green lines and diamonds represent neurons that express vesicular glutamate transporter 2 (VGLUT2) in their axon terminals. Orange lines and circles represent neurons that express vesicular GABA transporter (VGAT) in their axon terminals. CINs, commissural interneurons; INs, interneurons; MNs, motoneurons.

species. Todd et al. (1995) reported that the great majority of boutons that were presynaptic at gephyrin-immunoreactive synapses were glycine immunoreactive. However, it seems that gephyrin is not only present at glycinergic synapses as it is also found at the postsynaptic membrane of pure GABAergic boutons (Triller et al., 1987; Mitchell et al., 1993). Evidence for the presence of GAD-positive terminals in apposition to gephyrin puncta has been reported in the ventral horn of the adult rat (Triller et al., 1987). Such terminals were present on large cells which were presumed to be motoneurons. Ornung et al. (1996, 1998) found that almost all the GABAergic terminals contacting motoneurons also contained glycine in the adult cat spinal cord but that a sizable proportion of contacts were exclusively glycinergic and only a very small proportion contained GABA alone. The results of the present study suggest that inhibitory input to SB, VSCT, ccDSCT and dhDSCT neurons is primarily from glycinergic neurons as many gephyrin puncta which were not apposed by GAD terminals were observed. This was confirmed by calculating the contact density of GAD/gephyrin synapses and subtracting this from the contact densities calculated for VGAT. In keeping with the evidence discussed above, it is likely that most of the GABA/gephyrin synapses we observed represent synapses that use both GABA and glycine as neurotransmitters. Therefore at least two types of inhibitory neuron forming contacts with spinocerebellar tract cells may mediate different actions on these cells. The predominance of glycinergic synapses is predicted from previous anatomical studies of the adult cat which have shown that inhibitory interneurons in the dorsal horn activated by group II afferents as well as intermediate zone interneurons with input from group I/II afferents were almost entirely glycinergic (Bannatyne et al., 2006, 2009). Similarly, inhibitory commissural interneurons mediating crossed actions of reticulospinal neurons were glycinergic but not GABAergic (Bannatyne et al., 2003). Further evidence has also been provided for the presence of glycine-like immunoreactivity in axon terminals of Ia inhibitory interneurons and Renshaw cells (Fyffe, 1991a), both of which have collateral projections to motoneurons (Fyffe, 1991b; Schneider and Fyffe, 1992).

Functional consequences of differential distribution of inhibitory terminals

As judged by several features of IPSPs evoked in spinal motoneurons, IPSPs of different origin are evoked by inhibitory interneurons forming synapses at different subcellular domains. These different features include shapes of the IPSPs and their reversal potential, indicating that e.g. Renshaw cells terminate within the proximal dendrites of the motoneurons but contacts of Ia inhibitory interneurons are closer to or on somata (Curtis and Eccles, 1959; Burke et al., 1971b). A location close to the initial segment has been considered as particularly favourable for counteracting activation of the neurons. IPSPs evoked close to the initial segment may thus be most effective in preventing activation of the neurons, as for example, in the case of the arrest of firing of SB neurons during the rising phase and peak of IPSPs evoked in them by muscle afferents (Hammar et al., 2011). The concentration of inhibitory synapses within 75 μm from the soma of SB neurons illustrated in Fig. 5A and generally the highest density of inhibitory terminals on the soma and proximal dendrites of SB neurons could serve this purpose. However, high concentrations of inhibitory terminals were found within the region of the initial segment not only of SB but also of the other types of spinocerebellar neurons indicating that the activation of other spinocerebellar neurons might likewise be regulated in this way.

Distribution of inhibitory terminals along the whole surface of neurons could be used for the purposes of integration of excitatory and inhibitory inputs to spinocerebellar neurons, especially for tuning effects of excitatory terminals. The linearity of interactions between synaptic actions of the excitatory and inhibitory terminals would depend on distances between them (Rall et al., 1967). Close relations between inhibitory and excitatory terminals indicated in Figs. 3–5 along not only somata but also the whole length of the dendrites might be particularly relevant for such interactions. As illustrated in these figures excitatory and inhibitory terminals were often located side by side, making interactions between synaptic potentials evoked by them most efficient. The irregular, clustered distribution of inhibitory terminals on dendrites of ccDSCT neurons, which is best seen in Fig. 8, is dissimilar to the parallel distribution of inhibitory and excitatory terminals observed on the other neurons and may be indicative of a particular kind of processing of information by these neurons. Excitatory input to ccDSCT neurons comes largely from VGLUT1-containing primary afferents (Shakya Shrestha et al., 2012). These terminals form ‘giant’ boutons with ccDSCT cells that are under very limited presynaptic inhibitory control (Walmsley et al., 1985) and have very powerful ‘‘all or nothing’’ excitatory effects. Clusters of inhibitory terminals on specific regions of dendritic trees could produce powerful shunting inhibition that may selectively modulate specific excitatory inputs. The spatially close locations of glycinergic and glutamatergic terminals might also be of importance for effects of glycine on *N*-methyl-D-aspartate (NMDA) glutamatergic receptors (Berger et al., 1998). The predominance of either the excitatory or inhibitory terminals on some of the dendrites, especially of ccDSCT and dhDSCT neurons on the other hand might be an indication of specialisation of some dendrites of these neurons (Korogod et al., 2000).

Comparison of distribution of inhibitory terminals on spinocerebellar neurons and on other neurons

As far as we are aware this is the first report documenting the distribution of excitatory and inhibitory terminal contacts on extensively reconstructed dendritic trees of a specific group of neurons. Previous studies have concentrated on the cell bodies or short sections of dendrite (e.g. Ornung et al., 1996, 1998). Distribution of inhibitory terminals on neurons has often been reported

in fairly general terms previously. For example, Alvarez and Fyffe (2007) reported that Renshaw cells "uniquely displayed a high density of proximal inhibitory synapses". On spinal motoneurons, glycine-positive terminals were reported to be widespread, similar to spinocerebellar neurons analysed presently, but it is difficult to make a direct comparison with these findings as information is given in terms of the proportions of terminals on the somatic (18–38%) and proximal dendritic (17–45%) compartments (Destombes et al., 1992). Perhaps the best studied type of neuron with respect to inhibitory input is the CA1 pyramidal cell of the hippocampus (e.g. Klausberger and Somogyi, 2008). These cells are contacted by a diversity of inhibitory interneurons that target specific subcellular domains of pyramidal cells and have particular effects on their firing patterns. We can only speculate at present on the origin, subcellular targets and effects of the inhibitory terminals that contact spinocerebellar tract cells. In future studies it would be of interest to determine the origin and properties of inhibitory contacts on initial axon segments which control activation of neurons and compare this with dendritic contacts where inhibitory input is utilised primarily for tuning of excitatory input.

AUTHOR CONTRIBUTIONS

All authors were involved in designing the study. S.S.S., B.A.B. and D.J.M. analysed the immunochemistry of inhibitory terminals. E.J., I.H. and E.N. performed the electrophysiological experiments in which the spinocerebellar neurons were selected, identified and labelled. S.S.S. wrote the paper and prepared the illustrations, D.J.M., I.H. and E.J. contributed to editing.

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