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RESEARCH ARTICLE

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Crossed reciprocal inhibition evoked by electrical stimulation of the lamprey spinal cord

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Abstract Activation of a motoneuron pool is often accompanied by inhibition of the antagonistic pool through a system of reciprocal inhibition between the two parts of the neuronal network controlling the antagonistic pools. In the present study, we describe the activity of such a system in the isolated spinal cord of the lamprey, when a tonic motor output is evoked by extracellular stimulation (0.5–1 s train of pulses, 20 Hz) of either end of the spinal cord. With two electrodes symmetrically positioned in relation to the midline, stimulation with either of them separately elicited prolonged (1–5 s) ipsilateral ventral root activity. Activity could be abolished by stronger, simultaneously applied, stimulation of the contralateral side of the cord, suggesting that reciprocal inhibition between hemisegments operates when a tonic motor output is generated. Simultaneous stimulation of both sides of the spinal cord with a single electrode with a large tip (300–400 µm in diameter), positioned over the anatomical midline, elicited inconsistent right-side, left-side, or bilateral ventral root responses. A minor displacement (10–20 µm) to the left or right from the midline resulted in activation of ipsilateral motoneurons, whereas the contralateral motoneurons were silent. These findings indicate that a small asymmetry in the excitatory drive to the left and right spinal hemisegments can be further amplified by reciprocal inhibition between the hemisegments. Longitudinal splitting of the spinal cord along the midline resulted in reduced reciprocal inhibition between the hemisegments separated by the lesion. The reduction was proportional to the extent of the split. The inhibition was abolished when the split reached nine segments in length. From these experiments, the longitudinal distribution of the commissural axons responsible for inhibition of contralateral motor output could be estimated.

Key words Lamprey · Spinal cord · Reciprocal inhibition · Reticulospinal system · Turning response · Locomotion

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Introduction

As one group of muscles becomes activated, their antagonists often become inhibited, a condition referred to as reciprocal inhibition. Such reciprocal inhibition occurs between muscle groups when their motoneurons become activated sensory afferents (for example, stretch reflexes, Sherrington 1906; Eccles et al. 1957; Jankowska and Roberts 1972), descending commands (Grillner and Hongo 1972), or by central networks generating different patterns of motor behavior (Feldman and Orlovsky 1975; Deliagina and Orlovsky 1980; Dale 1985; Cowley and Schmidt 1995).

In the lamprey, a lower vertebrate, the neuronal networks in the left and right hemisegments of the spinal cord also exert a mutual inhibitory action upon each other. The inhibitory influences are addressed both to the antagonistic motoneuron pools and the interneurons constituting the central pattern generator for locomotion. Electrophysiological studies (Grillner and Wallén 1980; Buchanan 1982; Cohen and Harris-Warrick 1984; Wallén et al. 1993; McPherson et al. 1994; Buchanan and McPherson 1995) and computer simulations of the lamprey spinal locomotor network (Grillner et al. 1988; Buchanan 1992; Hellgren et al. 1992; Wallén et al. 1992; Wadden et al. 1997) have shown that crossed reciprocal inhibition plays an important role in the generation of locomotor rhythm. One of the neuron groups mediating the reciprocal inhibition has been identified. These are the CC interneurons (Buchanan 1982) which are relatively large cells, numbering 20–40 per nemisegment, with their axons

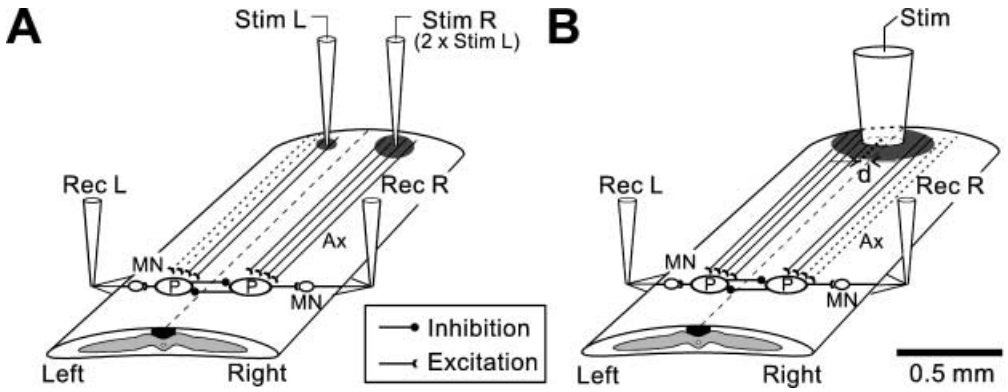


Fig. 1A,B Experimental arrangement. A piece of the spinal cord was positioned in the chamber, and motoneuron activity recorded bilaterally from ventral roots using suction electrodes (*Rec L, Rec R*). Long spinal and reticulospinal axons (*Ax*) were electrically stimulated, either with two stimulating electrodes (*Stim L, Stim R* in **A**) or with one large electrode (*Stim* in **B**). Stronger stimulation in one of the two electrodes (*Stim R* in **A**) or a small displacement (*d* in **B**) of the large electrode from the anatomical midline (*broken line*), activated a larger volume of tissue (indicated by the size of the *hatched circles*) and a larger number of axons on one side of the spinal cord. Axons not activated by the stimulation are indicated by *dotted lines*. Stimulated axons are thought to activate premotor networks (*P*) with excitatory connections to motoneurons (*MN*) and inhibitory connections to premotor networks on the opposite side of the spinal cord. Direct inputs to motoneurons from activated axons have been omitted in this figure. The positions of the gray matter (*hatched*) and the dorsal columns (*black*) are indicated on the cross-sections of the cord

crossing the midline and projecting caudally at distances up to 20 segments. Some of these neurons also have a short ascending branch. Another possible candidate is a large group (180–300/hemisegment) of smaller neurons with axons projecting rostrally or caudally for a few a segments after crossing the midline (Ohta et al. 1991).

Besides locomotion, the spinal networks in the lamprey generate a number of non-rhythmical motor patterns, including lateral bending at different sites along the body (Rovainen 1967; Ayers et al. 1983; McClellan and Grillner 1983; McClellan 1984; Deliagina et al. 1995; Ullén et al. 1995a,b). Such patterns may underlie lateral turns, associated or not with the undulatory locomotor pattern. However, information concerning the involvement of crossed reciprocal inhibitory systems in the generation of these motor patterns is lacking.

Our interest in this problem was stimulated by previous experiments where the population activity in the left and right reticulospinal (RS) pathways was recorded during locomotion in intact lampreys (Deliagina et al. 2000). It is known that the RS system is the main descending system in the lamprey (Tretjakoff 1909; Rovainen 1967, 1974; Ronan 1989; Swain et al. 1993; Davis and McClellan 1994). It is responsible for the control of steering and equilibrium during locomotion (Rovainen 1979; Deliagina et al. 1992a,b, 1995; McClellan and Hagevik 1997; Deliagina and Fagerstedt 2000). RS neurons activate ipsilateral motoneurons directly (Batueva and Belozerova 1970; Rovainen 1974), and bilateral motor output indirectly through the segmental locomotor networks (Buchanan 1982; Ohta and Grillner 1989; Deliagina et al. 2000).

When recording activity in RS pathways (Deliagina et al. 2000), we have found that rectilinear swimming was associated with symmetrical activation of the left and right RS pathways. During lateral turns, a slight asymmetry was observed with a predominance (ca 25%) of activity on the turning side. The asymmetry in motor output during turns, however, was strongly pronounced: the electromyogram of the trunk muscles on the concave side of the body was large and prolonged, while muscular activity on the convex side was absent. This finding suggests that the asymmetry in the supraspinal motor commands is further amplified in the spinal cord, possibly through a system of reciprocal inhibition between the spinal hemisegments.

To test this hypothesis, in the present study we elicited tonic activity in the spinal cord by bilateral electrical stimulation of long spinal pathways, including RS axons. An asymmetry in the activity of the left and right pathways could be produced by unequal stimulation of the two sides. This asymmetry was then compared to the asymmetry in the segmental motor output. It was found that the asymmetry of excitatory inputs to spinal segmental networks was considerably amplified when tonic motor output was generated. The longitudinal distribution of projections of the crossing interneurons mediating the reciprocal inhibition was estimated by performing midline lesions.

A preliminary account of this work has appeared in abstract form (Zelenin et al. 1997).

Materials and methods

Preparation and experimental arrangement

Experiments were carried out on adult lampreys, *Lampetra fluviatilis* (*n*=23) and *Ichthyomyzon unicuspis* (*n*=2). The animals were anesthetized with tricaine methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, Mo., USA; 200 mg/l water) and decapitated according to principles of laboratory animal care, as approved by the Swedish National Board for Laboratory Animals (CFN) and the European Communities Council directive 86/609/EEC. The spinal cord from the midbody area (usually segments 10–50) together with the notochord was dissected and pinned down in a silicone elastomer (Sylgard; Dow Corning, Midland, Mich., USA) lined chamber. The preparation was continuously perfused with cold (8–12°C) oxygenated Ringer's solution of the following composi-

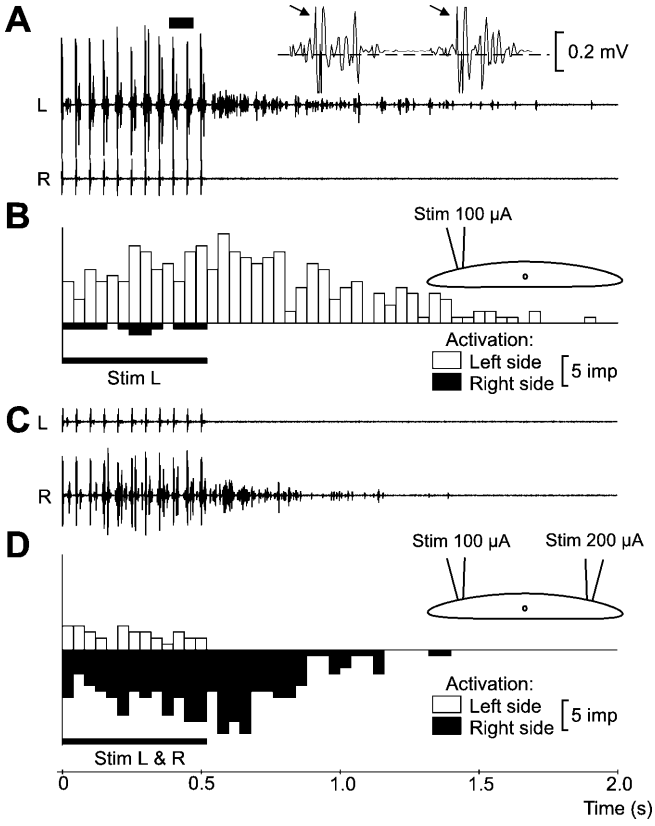


Fig. 2A–D Effects of spinal cord stimulation using two electrodes. **A,B** Stimulation (*Stim*, 20 Hz, 2 ms pulse duration, 100 µA) of the left side of the spinal cord (*inset* in **B**) elicited a long-lasting response in the left ventral root (*L*). A part of the recording in **A** (*bar*) is presented in the *inset* with higher time resolution with the threshold for spike discrimination (*broken line*) and stimulus artifacts (*arrows*) indicated. **C,D** When this stimulation was accompanied by a stronger (200 µA) stimulation of the right side (*inset*), the response in the left ventral root was inhibited, and a response in the right ventral root (*R*) appeared instead. Large peaks in **A** and **C** are stimulation artifacts. The raw data shown in **A** and **C** were processed and presented as histograms of the number of ventral root spikes (*imp*) during a bin interval (40 ms) in **B** and **D**, correspondingly. The histogram of the left-side activity is plotted above the time axis and that of the right-side below the time axis

tion (in mM: 138 NaCl, 2.1 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 0.5 L-glutamine, 2 HEPES, and 4 glucose), adjusted to pH 7.4 by 0.1 M NaOH (Zhang et al. 1996). Motoneuron activity was recorded bilaterally from the ventral roots in one or two of the spinal segments (usually segment 23–25 or/and 43–45) by means of suction electrodes (glass pipettes, ca 50 µm tip diameter; Fig. 1). Activity of individual long spinal axons was recorded intracellularly with sharp electrodes (glass pipettes filled with 3 M KCl). Extracellular stimulation of the spinal axons was performed either by one or two electrodes (see below). In some preparations, the medial area of the spinal cord, corresponding to the dorsal column, where numerous ascending afferent fibers are located (indicated in black in Fig. 1A,B), was transected between the stimulation and recording sites with a scalpel. In ten preparations, a longitudinal midline split of the spinal cord, extending up to nine segments, was performed (Fig. 5A), in order to cut crossing axons.

Stimulation with two electrodes

Glass pipettes (tip diameter ca 100 µm) were used as stimulating electrodes. They were positioned symmetrically on the dorsal surface of the spinal cord, close to the rostral or caudal end of the preparation (Fig. 1A). A series of pulses was applied to each of the electrodes (frequency, 20 Hz; pulse duration, 2 ms; duration of the series, 0.5–1 s; pulse current, 100–200 µA). An asymmetry in the activation of the left and right sides was caused by different stimulation current strength in the two electrodes. The difficulties assessing the real degree of asymmetry with this experimental design due to subtle electrode characteristics and local conditions (for example, the shape of the tip, the position of the electrode in relation to the spinal cord surface) could be overcome by using a large single electrode.

Stimulation with one electrode

A large stimulating electrode (glass pipette, 300–400 µm tip diameter) was positioned at the midline on the dorsal surface close to either end of the spinal cord (Fig. 1B). A series of pulses was applied to the electrode (frequency, 20 Hz; pulse duration, 2 ms; duration of the series, 0.5–1 s; pulse current, 200–400 µA; threshold 50 µA). An asymmetry in activation of the left and right sides of the spinal cord was produced when the electrode was slightly moved in either lateral direction (*d* in Fig. 1B) from the estimated anatomical midline.

Processing of data

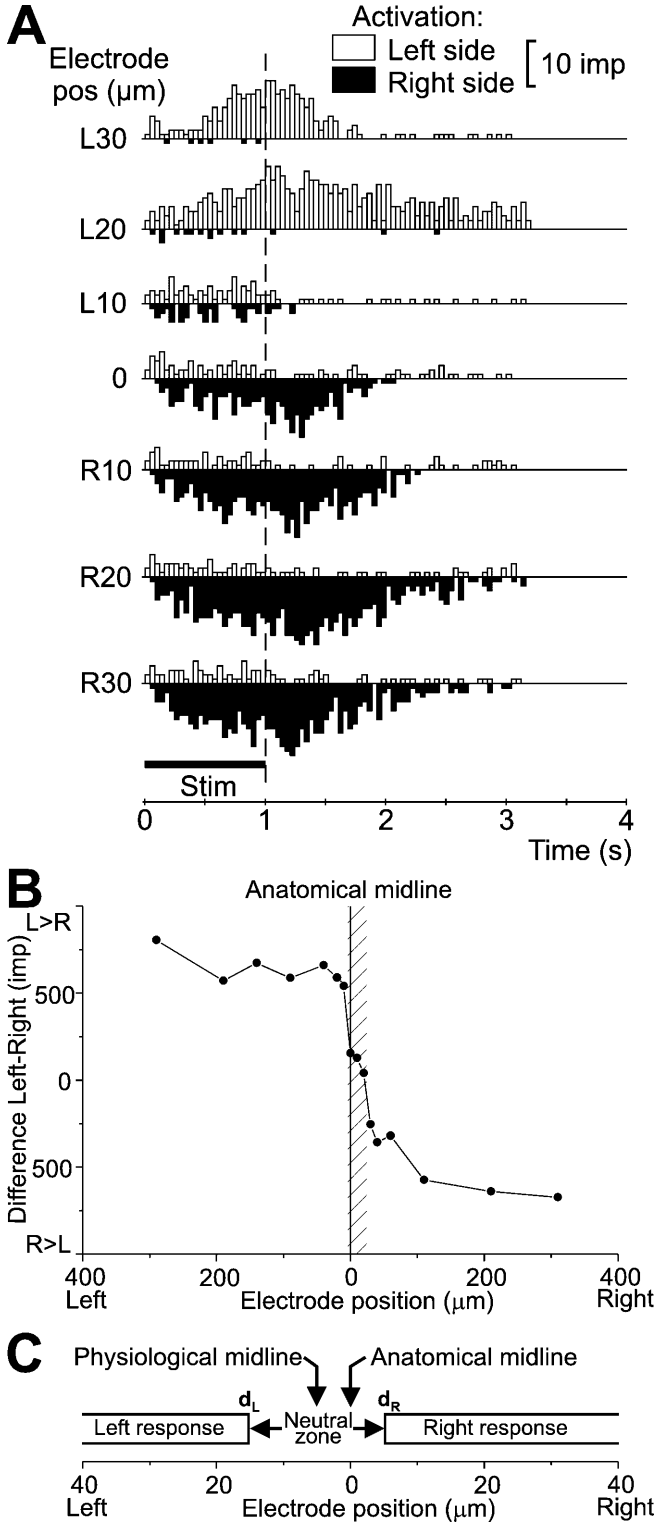
Ventral root activity was recorded and digitized online. All positive waveforms which exceeded the chosen threshold (usually 10–20 µV; see inset in Fig. 2A) were considered as spikes and represented in a form of histogram (bin width, 40 ms; Fig. 2B) using Datapac 2000 software (Run Technologies, Laguna Hills, Calif., USA). Stimulation artifacts (indicated by arrows in inset in Fig. 2A) were subtracted from bins. Statistical analysis was performed by one factor independent ANOVA tests followed by a post-hoc Dunnett's test. A value of $P<0.05$ was considered significant.

Results

Characterization of the system of reciprocal inhibition

In the experimental design with two stimulating electrodes (Fig. 1A), brief stimulation (1–5 pulses of 2 ms, 50 Hz) of the spinal cord by one of the electrodes could elicit a short-lasting ventral root response (50–100 ms, not illustrated). However, when a 0.5–1 s train of pulses was used, the effect changed dramatically (Fig. 2A,B). Initially, the left side was stimulated (inset in Fig. 2B). This stimulation caused a strong excitatory response of motoneurons on the left side that outlasted the stimulus duration by up to a few seconds. This prolonged ipsilateral response could be elicited by applying a stimulus at any lateral position of the electrode (up to 500 µm from the midline) except for the most medial area. The response could be recorded in the ipsilateral ventral roots at any distance from the stimulated site (up to 30 segments), both in the rostral and caudal directions. These findings suggest that the axons responsible for elicitation of the long-lasting response are long, and that they are distributed over the cross-section of the ipsilateral half of

the spinal cord. Intracellular recordings of individual long spinal axons during stimulation showed no sign of prolonged activity (*n*=6; conduction velocity 2.5–4 m/s; data not shown). The axons fired one action potential in response to each stimulus pulse, suggesting activation of spinal premotor networks (*P* in Fig. 1A,B) as the cause of the long-lasting response (see Discussion).



When an identical stimulation of the left side was combined with simultaneous, but stronger, stimulation of the right side (inset in Fig. 2D), motoneurons on the right side were activated, while the activity of motoneurons on the left side was decreased (Fig. 2C,D). This was presumably due to inhibitory influences of stronger activated networks in the right hemisegments on those in the left hemisegments.

To analyze this phenomenon in more detail, experiments were performed in which the spinal cord was stimulated by one large electrode, with a diameter of 300–400 μm (approximately 20% of the spinal cord width; Fig. 1B). Figure 3A shows histograms of responses in the left and right ventral roots evoked by stimulation at different electrode positions. Displacement of the electrode, in relation to the estimated anatomical midline, to the left by 20 μm or more resulted in complete dominance of the left-side motoneuron activity, whereas a displacement to the right by 10 μm or more resulted in a complete dominance of the right-side motoneuron activity.

The difference between the responses of the left and right motoneuron pools is plotted against the value of the electrode displacement in Fig. 3B. There is a narrow zone of stimulation, close to the anatomical midline, where the response switches from a left-side response to a right-side response (neutral zone; hatched in Fig. 3B). Repeated tests (with an interval of 5 min or more) showed that stimulation of the same site outside the neutral zone consistently elicited an ipsilateral response. Repeated stimulation within the neutral zone, however, evoked less consistent responses that could spontaneously alternate between the left and right side, or disappear altogether.

The neutral zone, and the immediate area next to it, correspond to the dorsal columns, which contain relatively thin (1–4 μm in diameter), densely packed, afferent fibers (Christenson et al. 1988). Transection of this region (black area in cross-section in Fig. 1A,B) between the stimulating and recording electrodes did not affect the prolonged response (two experiments), demonstrating that the effect is not dependent on dorsal column fibers.

The medial borders of the zones where consistent left-side and right-side responses could be evoked (d_L and d_R in Fig. 3C) were determined in one to six spinal segments each in 16 experiments. The following parameters

Fig. 3A–C Effects of spinal cord stimulation using one large electrode. **A** Histograms of responses (number of spikes per 40 ms) in the left (*empty bars*) and right (*black bars*) ventral roots recorded at different positions of the stimulating electrode in relation to the anatomical midline (*Electrode pos*). **B** The difference between the number of spikes in left and right responses calculated over a period of 3 s and plotted against the electrode position. The neutral zone is *hatched*. **A** and **B** are from different experiments. **C** Different characteristics of the responses presented in **A** and **B**: the zone of right-side response, the zone of left-side response, the neutral zone, the borders between the zones (d_L and d_R), the anatomical midline, and the physiological midline are indicated

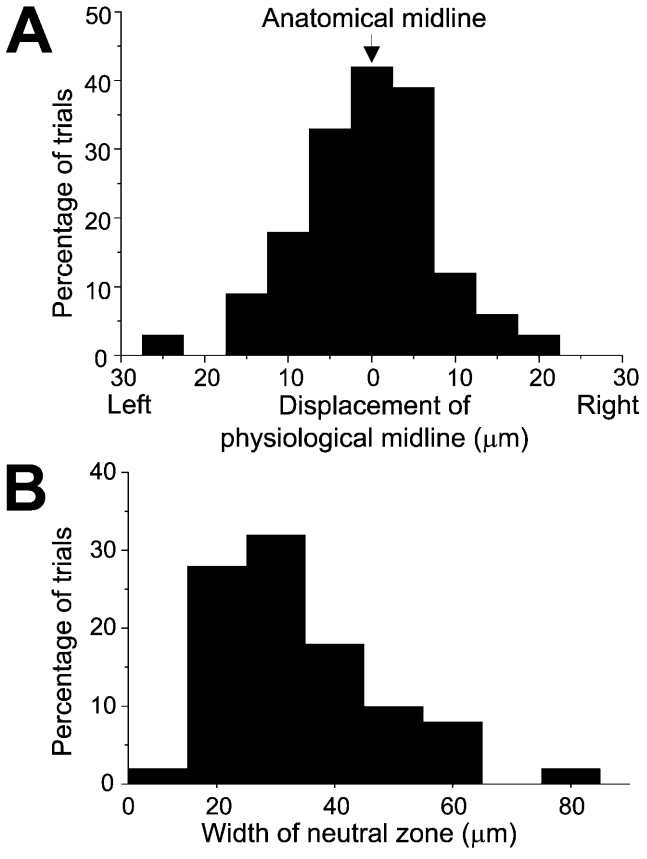


Fig. 4 **A** Distribution of the physiological midline in relation to the anatomical midline tested in 55 trials in 16 experiments (bin width 10 μm). The distribution is slightly skewed due to a minor bias in the initial positioning of the stimulus electrode in relation to the anatomical midline. **B** Distribution of the width of the neutral zone in the same experiments (bin width 20 μm)

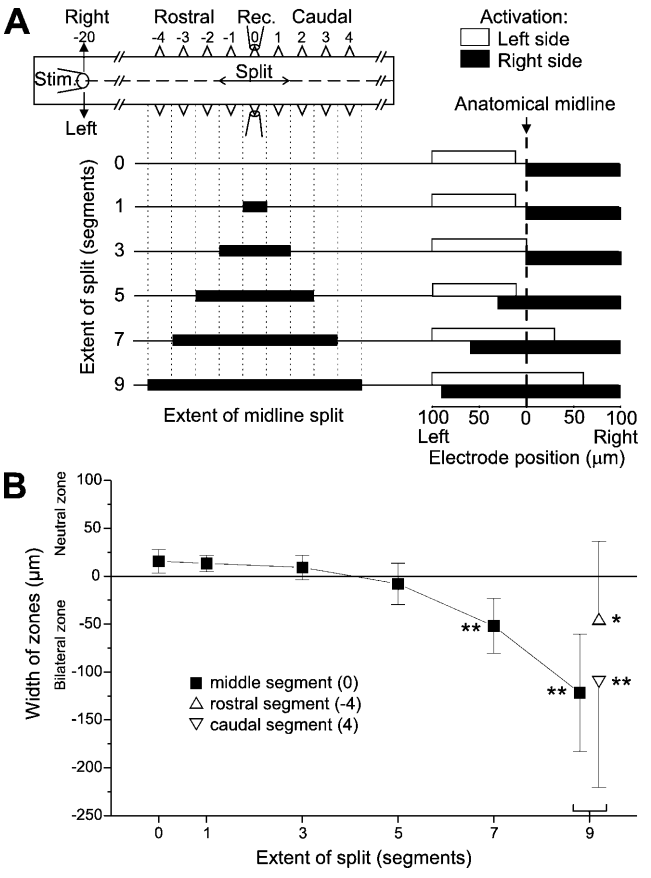


Fig. 5A,B Effects of a longitudinal split of the spinal cord. **A** The *left panel* shows the experimental arrangement. Positions of the stimulating (*Stim*) and recording (*Rec*) electrodes are indicated. Splitting of the cord was performed in steps; the extent of the split is indicated (number of segments). The *right panel* shows the zones of left and right responses observed with different lengths of splitting. **B** The curve (*black squares*) shows the width of the neutral and bilateral zones (mean ± SD), measured in the midpoint of the split (segment 0), as a function of the split length (summary of ten experiments, each length tested in five to eight experiments). For splits of seven and nine segments in length, the width of the zone significantly differed from that in the non-split spinal cord (extent of split=0). For the nine-segment split, the zone width was also measured in the rostral and caudal end-points of the split (segments –4 and +4, respectively) and shown by symbols Δ and ∇, respectively (mean ± SD). The absolute mean values are significantly different than that of the intact segments (**P*<0.05; ***P*<0.01)

were used to characterize the effects of stimulation: the width of the neutral zone (d_L–d_R), the physiological midline (the midpoint of the neutral zone), and the displacement of the physiological midline in relation to the anatomical midline. We have found that, in all experiments, the displacement of the physiological midline in relation to the anatomical midline was small, 1±8 μm (mean ± SD, *n*=55; Fig. 4A). The small mismatch between the physiological and anatomical midline presumably reflects a minor bias in the initial positioning of the stimulating electrode in relation to the anatomical midline and is not due to a lateralization of any of the animals. This finding indicates that the neuronal networks in the left and right hemisegments of the isolated spinal cord are similar to each other with regard to their excitability and mutual inhibitory action. The width of the neutral zone in all segments was also small (22±14 μm, mean ± SD, *n*=55; Fig. 4B), suggesting a strong contralateral inhibition caused by a minor asymmetric excitation of one side, i.e., a high gain in the pathways responsible for mutual inhibition of the left and right hemisegments.

Effect of longitudinal splitting of the spinal cord

To characterize the longitudinal extent of the contralateral projections mediating the reciprocal inhibition to a hemisegment, the spinal cord was split along its midline (*n*=10). The extent of the lesion was increased in steps from one to nine segments, symmetrically in relation to the recording electrodes (Fig. 5A, left panel). The right panel illustrates the results of one such experiment. With lengthening of the split from one to three segments, the neutral zone decreased and then disappeared. With further lengthening of the split (to five, and then to seven segments), a medial (bi-

lateral) zone where stimulation evoked coactivation of the two sides appeared instead, indicating a further reduction of the reciprocal inhibition. When the split was nine segments in length, the reciprocal inhibition was practically abolished, as indicated by the bilateral activation from all but the most lateral stimulation sites.

Results from ten such experiments are summarized in Fig. 5B, where the width of the neutral/bilateral zones is plotted against the length of the lesion (a curve indicated by black squares). Values of width above 0 correspond to the width of neutral zones, values below 0 correspond to the overlap between the zones where consistent left-side and right-side responses were evoked, i. e., the width of bilateral zones. There was a significant reduction in reciprocal inhibition between the hemisegments when the extent of the split reached seven segments. This indicates that a large proportion of the commissural axons causing reciprocal inhibition cross the midline at a distance of less than three to four segments rostrally or caudally to their target segment.

In five of the experiments described above, after having performed a split of nine segments, ventral root responses were recorded not only from the middle segment (segment 0; Fig. 5A), but also at the anterior and posterior endpoints of the split (segments –4 and +4). At both endpoints, stimulation caused bilateral activation within wide zones, –76±65 μm in the rostral segment and –104±98 μm in the caudal segment (mean ± SD; indicated by triangles in Fig. 5B). These two values did not differ significantly from each other (ANOVA, P=0.61), but both values strongly differed from the zone width before the lesion was performed, 16±12 μm (mean ± SD, ANOVA, rostral end P<0.05, caudal end P<0.01). These observations suggest that inhibitory input to a hemisegment is delivered by fibers crossing the midline both rostrally and caudally in relation to the hemisegment.

Discussion

Activation of the system of reciprocal inhibition

In the present study, unilateral electrical stimulation of the quiescent spinal cord in the lamprey evoked a long-lasting response (up to a few seconds) in the ipsilateral ventral roots throughout the whole extent of the spinal cord preparation (up to 40 segments). This finding suggests that the activation of the motor output is mediated by long spinal axons that affect all segments. The long-lasting activation of the motor output on one side of the spinal cord is accompanied by inhibition of the motor output on the contralateral side. This finding indicates that the system of reciprocal inhibition in the lamprey spinal cord may operate not only during rhythmical locomotor activity (Grillner and Wallén 1980; Kahn 1982; Wallén et al. 1993; Buchanan 1999), but also during the generation of tonic motor output.

The long-lasting response depends on the activation of spinal neurons since no prolonged activity could be observed in long spinal axons after stimulation (see Results). Both the gradually growing response during stimulation, and the maintenance of activity for seconds after the termination of stimulation, in motoneurons, which lack endogenous plateau properties (Teräväinen and Rovainen 1971), suggest that the direct activation of motoneurons by input from the long spinal axons is unlikely to cause the prolonged response. The most likely neuronal mechanism of this phenomenon is activation, via excitatory input from long spinal axons, of the segmental interneuron networks, including commissural interneurons, which in their turn will inhibit the interneuron network and motoneurons in the contralateral hemisegments.

Axons eliciting long-lasting response

Different groups of axons, both ascending and descending, traverse the spinal cord in the midbody area. A considerable part of the axons of sensory afferents are located in the dorsal column. However, in the present study it was found that stimulation with a thin electrode evoked a long-lasting response at any lateral position of this electrode (Fig. 2A,B), except for the most medial ones. Also, the response evoked by a large stimulating electrode positioned medially persisted after transection of the dorsal column. We therefore conclude that axons responsible for elicitation of the long-lasting response are distributed over the cross-section of the ipsilateral half of the spinal cord.

The most likely group of axons responsible for elicitation of the long-lasting response are the RS axons. About one thousand of these axons pass the midbody area of the spinal cord and are distributed over the whole cross-section of the cord (Bussières 1994). The RS axons predominantly exert an excitatory action on different groups of ipsilateral spinal neurons, including interneurons and motoneurons (Batueva and Belozerova 1970; Rovainen 1974; Buchanan 1982; Buchanan et al. 1987; Ohta and Grillner 1989; Wannier et al. 1995). The finding that the prolonged motoneuron response can be evoked from different lateral positions of the stimulating electrode suggests that this response can be elicited by different subdivisions of the RS system, although the participation of long propriospinal and ascending sensory pathways cannot be excluded.

Asymmetry in supraspinal motor commands may be amplified in the spinal cord

In a previous study (Deliagina et al. 2000), the question was addressed of how a command for a lateral turn is coded in the activity of the RS system. Recording of the population activity in RS axons (by implanted electrodes) showed that a relatively small difference (ca 25%) in the activity of the left and right subdivisions of the RS system appeared during lateral turns that preced-

ed rectilinear swimming. On the other hand, there was a pronounced asymmetry in the segmental motor output during these lateral turns. The trunk musculature on the concave body side was strongly activated whereas the musculature on the convex side was silent. The present study, in the quiescent spinal cord, gives a satisfactory explanation of this discrepancy: a small asymmetry in the excitatory inputs to the networks in the left and right spinal hemisegments may be considerably amplified due to the system of crossed reciprocal inhibition. One can estimate that the 10- to 20-µm lateral displacement of the stimulating electrode with a tip diameter of 300–400 µm, which resulted in a switch of the response between the two sides (Fig. 3), produced a 5–10% difference between the volume of nervous tissue stimulated on the left and right sides of the spinal cord. Since stimulation with the same parameters elicited a long-lasting response at any lateral positions of the electrode (see Results and Fig. 2A,B), one can assume that the axons eliciting response are distributed more or less evenly over the cross-section of the cord. If so, a difference of 5–10% in the number of axons activated on left and right side will be sufficient to cause a switch of the response.

Longitudinal extent of inhibitory contralateral projections

Experiments with longitudinal splitting of the spinal cord (Fig. 5) provided an estimate of the extent of the area where the axons, responsible for reciprocal inhibition, cross the midline. It was found that a hemisegment receives inhibitory inputs from both rostral and caudal neighboring parts of the spinal cord. The effect of reciprocal inhibition was considerably reduced after a longitudinal split of seven segments in length, symmetrical in relation to the recorded segment, and practically abolished when the lesion reached nine segments in length.

This finding indicates that commissural neurons with shorter axons play a predominant role in the reciprocal inhibition when tonic motor output is generated by the spinal cord. Similar results have been obtained for rhythmical motor output in experiments with a longitudinal split of the spinal cord (Buchanan and McPherson 1995; Buchanan 1999). This conclusion corresponds well to the fact that the majority of the commissural neurons in the lamprey are small cells projecting caudally or rostrally at distances less than five segments (Ohta et al. 1991). Both immunocytochemical and electrophysiological studies have shown that a part of these small cells are inhibitory neurons (Rovainen 1983; Fagerstedt and Wallén 1992; D. Parker unpublished observations).

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