Localization of the Spinal Network Associated With Generation of Hindlimb Locomotion in the Neonatal Rat and Organization of Its Transverse Coupling System

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Kremer, E. and A. Lev-Tov. Localization of the spinal network mainly by strychnine-sensitive glycine receptors with possible contribution of strychnine-resistant glycine receptors and/or GABA_A receptors. J. Neurophysiol. 77: 1155–1170, 1997. The segmental organization of the hindlimb locomotor pattern generators and the coordination of rhythmic motor activity were studied in isolated spinal cords of the neonatal rat. All lumbar segments and many thoracic and sacral segments of the cord exhibited an alternating left-right rhythm in the presence of serotonin (5-HT) and N-methyl-D-aspartate (NMDA). Other thoracic segments exhibited a synchronized left-right rhythm or an irregular bursting activity. Transection of the cord at the thoracolumbar or lumbosacral junction abolished the rhythmicity of nonlumbar segments and had no affect on the rhythmicity of lumbar segments. A fast alternating rhythm persisted in rostral lumbar segments after transection of the cord at mid-L1. A much slower alternating rhythm was found in the detached caudal lumbar segments after elevation of the NMDA concentration. These findings suggest that neurogenesis of hindlimb locomotion is not restricted to L1/L2, and that the lumbar pattern generators exhibit rostrocaudal specialization. An alternating left-right rhythm persisted in lumbar cords of midsagittally split preparations that were kept with either L1, L2, L3, or L4 as the only bilaterally intact segment. An alternating rhythm persisted also in preparations that were midsagittally split up to T13–T12, or down to L4. Extension of these lesions led to a bilaterally synchronous rhythm or to left-right independent rhythms in the lumbar cord. These results indicated that the transverse coupling system in the caudal-thoracic and lumbar segments is specialized and that left-right alternation in the lumbar cord can be carried out by the cross connectivity, which is relayed at least through the T12–L4 segments. Bath application of the glycine receptor antagonist strychnine, or the γ-aminobutyric acid-A (GABA_A) receptor blocker bicuculline, induced in the presence of NMDA and 5-HT a bilaterally synchronous rhythm in any intact or detached segment of the cord and in midsagittally split preparations with few bilaterally intact upper thoracic or lower sacral segments. A strychnine-resistant left-right alternating rhythm was found in the presence of 5-HT and NMDA in preparations that were treated with the non-NMDA receptor blocker 6-cyano-7-nitroquinoxaline (CNQX) before and during the application of strychnine. Subsequent washout of CNQX immediately induced a bilateral synchronous rhythm. These results suggest that the phase relation between the hemicords during the rhythm is determined by a dynamic interplay between the excitatory and inhibitory cross connectivity, and that this interplay can be modulated experimentally. Local application of strychnine to L2 kept bilaterally intact in midsagittally split preparations perturbed but did not completely block the alternating pattern of the rhythm induced by 5-HT and NMDA. Local application of bicuculline under the same conditions prolonged the cycle time and had no affect on left-right alternation. These results, together with those described above, suggest that left-right alternation is mediated mainly by strychnine-sensitive glycine receptors with possible contribution of strychnine-resistant glycine receptors and/or GABA_A receptors.

INTRODUCTION

Automatic locomotor activity in vertebrates is thought to be produced in spinal half centers consisting of antagonistic groups of motoneurons that are driven by interneuronal central pattern generators (CPGs) (Gossard and Hultborn 1991; Grillner 1981). Coordination within and between the half centers is assumed to be obtained by longitudinal and transverse coupling systems. The CPGs associated with swimming of the lamprey (Cohen 1987; Cohen and Wallen 1980; Grillner and Matsushima 1991; Hagevic and McClellan 1994) and Xenopus (Kahn and Roberts 1982) are localized along the rostrocaudal axis of the cord, and the transverse coupling between the left and right halves of the cord has been suggested to be carried out by glycineric reciprocal inhibitory connections (Alford and Williams 1989; Cohen and Harris-Warrick 1984; Dale 1985; Hagevic and McClellan 1994) and by weak cross-excitatory pathways (Hagevic and McClellan 1994). Studies of the spontaneous motor rhythm in the embryonic chick spinal cord are also consistent with the notion of rostrocaudal distribution of CPGs (Ho and O’Donovan 1993). In recent studies of the neonatal rat spinal cord, however, it has been suggested that the CPGs associated with hindlimb locomotion are localized to the L1/L2 segments of the cord, and that phasing of the rhythms in the left and right hemicords is obtained by reciprocal inhibitory and cross-excitatory pathways that are relayed from side to side only through L1, and L2 (Cazalets et al. 1995, 1996). These pathways have been suggested to project onto the caudal lumbar cord by biphasic connections that have been assumed to be monosynaptic (Cazalets et al. 1995, 1996). In the present study we reexamine the localization of the CPGs in the spinal cord of the neonatal rat, the segmental organization of the transverse coupling systems between the left and right hemicords, and the receptors associated with reciprocal inhibition.

On the basis of surgical manipulations of the cord and the use of bath applied or locally (pressure ejections) applied inhibitory amino acid (IAA) receptor blockers, our data reveal that the locomotor CPGs are not restricted to L1/L2; that the reciprocal inhibitory pathways are relayed from side to side through at least T12, T13, L1, L2, L3, and L4 and not...
only through L1/L2; and that the CPGs and the transverse coupling system exhibit regional specialization that is expressed in the excitability of the segmental CPGs and in the interplay between the excitatory and the inhibitory components of the transverse coupling system. The experiments in which IAA receptor antagonists were pressure ejected onto midsagittally split preparations with a single bilaterally intact segment suggest a major role for strychnine-sensitive receptors in mediating the reciprocal inhibition between the left and right hemicords. A possible contribution of strychnine-resistant glycine receptors and/or of γ-aminobutyric acid-A (GABA\(_{\text{A}}\)) receptors in the process is discussed.

Some of the preliminary findings have appeared in abstract form (Kremer and Lev-Tov 1995; Simon and Lev-Tov 1994).

### METHODS

#### Preparation

Experiments were performed on the en bloc spinal cord preparation (see Kudo and Yamada 1987; Smith and Feldman 1987) isolated from neonatal rats (postnatal days 0–4). Preparations of the spinal cord and the dorsal and ventral roots associated with it were isolated from ether-anesthetized rats in a dissection chamber superfused with cold (10°C) and oxygenated (95% O\(_2\)-5% CO\(_2\)) normal Krebs saline (composition, in mM: 128 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgSO\(_4\), 1 NaH\(_2\)PO\(_4\), 25 NaHCO\(_3\), and 30 glucose). The cervical cord and the three rostral thoracic segments were removed and the rest of the thoracolumbosacral cord (from rostral T5 down) was transferred to an experimental chamber that was continuously superfused at 10–15 ml/min with oxygenated normal Krebs saline, pH 7.3, at room temperature (24–26°C).

#### Stimulation and recordings

Homologous pairs of lumbar ventral roots were placed in suction electrodes and prepared for recordings. Extracellular recordings were performed with the use of a high-gain AC amplifier at 0.1 Hz to 5 kHz. Intracellular recordings were obtained from neurons that were impaled from the ventral aspect of the cord by 60- to 100-MΩ micropipettes filled with 3 M potassium acetate and identified as motoneurons by the presence of antidromic spikes. The rhythm was induced by serotonin (5-HT) and N-methyl-d-aspartate (NMDA) (Squalli-Houssaini et al. 1993) (see below). Figure 1A, left, shows simultaneous 10 Hz–5 kHz (top pair of traces) and 0.1 Hz–5 kHz (bottom pair of traces) AC recordings from the left and right (top and bottom trace in each pair, respectively) L4 ventral roots in the presence of 5-HT and NMDA. Figure 1A, right, shows simultaneous intracellular recordings from the left L4 segment (IC; top trace) and ‘slow potential’ recordings from the left and right L4 ventral roots (EC; bottom trace). The changes in the transmembrane potential of this and seven additional motoneurons recorded from the same segment (total of 8 of 9) were in phase with the slow potential recordings from the ipsilateral ventral root. Thus, depending on the homogeneity of the motoneuron population in a given segment (most lumbar segments are predominated by either flexor or extensor motoneurons) (see Kiehn and Kjaerulf 1996), the slow potential recordings can be used as an inference for both sub- and suprathereshold changes in the locomotor rhythm of the segmental motoneurons. This is demonstrated in Fig. 1B, in which 5 μM of the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid virtually blocked the suprathereshold activity (left) but did not stop the alternating rhythmic slow potentials (right). In addition, the slow potential recordings were found to be conducive for automatic time series analysis of long data segments (63–400 s in this study) at low A-D rates (~20 Hz; see below).

#### Pharmacological substances

A mixture of 5-HT (Sigma, 20–30 μM) and NMDA (Sigma, 1–6 μM) was used to induce locomotor-like activity in the spinal cord. 2-Amino-5-phosphonovaleric acid (Sigma) and 6-cyano-7-nitroquinoxaline (CNQX; Tocris) were used to block the NMDA and non-NMDA receptors, respectively. Strychnine and bicuculline methiodide (Sigma) were used to block the glycine and GABA\(_{\text{A}}\) receptors, respectively.

#### Local application of drugs

Strychnine (1 or 10 mM) or bicuculline (2 or 10 mM) dissolved in the experimental Krebs saline was ejected onto the ventral surface of the cord from glass micropipettes (10–50 μm tip diameter) at 2–25 psi for 2–25 s with the use of an electronically controlled pressure ejection device (Picospritzer II, General Valve). The ejection pressure, which depended on the diameter of the pipette, was adjusted before each ejection to assure the formation of a drop of solution around the tip of the micropipette. Ejections were performed in parallel with the rostrocaudally directed flow of the perfusate. Test ejections of Fast Green dissolved in normal Krebs saline onto the ventral cord revealed that a precise control of the territory affected by the ejection could be gained by application of suction through a micropipette (100 μm tip diameter) positioned laterally to the ejection site during and immediately after each ejection. The spread of the drug could be restricted under these conditions to about half the length of a spinal segment and half the width of the hemicord.

#### Surgical manipulations

Preparations were completely or partially split at the midsagittal plane with the use of a sharpened fine tungsten needle. Transverse sections of the cord were performed with the use of ultralfine microdissecting scissors with tip thickness of 8 μm and blade thickness of 75 μm (FST).

#### Data acquisition and analysis

Data (wideband recordings) were continuously recorded with the use of a high-speed (22–88 kHz) PCM recorder (Neurodata), filtered with the use of high- and low-pass filters, and stored for subsequent off-line computer analyses.

**TIME SERIES ANALYSES.** Slow potential data segments (63–400 s each) were replayed, sampled at 20–40 Hz with the use of an A-D converter (Digidata 1200A, Axon Instruments), and smoothed with the use of a five-point moving average. The correlation coefficients between a time series variable and its values \(t\) times periods earlier (lagged values of the variable) were estimated for lags 1 to \(k\) (\(k\) = the maximal lag specified), and the resultant autocorrelograms were plotted with means ± 2 SE (pointwise) to determine the lag beyond which all correlations are not significantly different from 0. The cycle time of the rhythm could be then extracted by multiplying the number of lag shifts that was required to describe a complete cycle by the duration of the lag. The use of autocorrelation analysis is demonstrated in Fig. 2. The analyses were performed on 100-s samples of slow potentials recorded from the left and right L4 ventral roots, before and after midsagittal section of the cord (Fig. 2A, left) with the use of \(k = 600\) lags, lag duration = 25 ms. These analyses revealed that a significant rhythmicity persisted after the midsagittal section (Fig. 2, B and C). The cycle time calculated from the autocorrelograms was 7.1 s (284 lag shifts) in the left and right L4 before the lesion.
The correlation between one time series (for example, the slow potential data recorded from the left L2 ventral root; Fig. 2A, top trace in each pair) at time $t$ and a second time series (for example, the slow potential data recorded from the right L3 ventral root; Fig. 2A, bottom trace in each pair) at time $(t + k)$ was estimated as a function of the lag $[lag = (-k)$ through $k]$. The resultant cross-correlogram was plotted with mean $\pm$ 2 SE (Fig. 2A, right) to determine the significance of the correlation between the two time series and their phase relation. A highly significant inverse correlation (phase shift $= 180^\circ$) was found between the activities in left and right L2 before the mid-sagittal section (thick line correlogram). The correlation between the two time series after the lesion (thin line correlogram) was not significantly different from 0. Thus the cross-correlation analyses were useful to assess whether the correlation and the phase relation between the time series variables are altered by surgical or pharmacological manipulations of the cord. Evaluation of the regularity of the rhythm required observation of the correlograms over a few cycles. This was obtained with the use of a number of lag shifts that was matched to exceed the cycle time of the rhythm by a factor of $\approx$2–3 (see Fig. 2A). Constant successive correlation peaks or moderately decaying successive peaks indicated highly regular and correlated rhythms (see Fig. 2A, thick line). An initially high correlation peak (at lag = 0) followed by a substantial decline of the successive peaks as a function of lag indicated highly correlated time series variables (left and right ventral root data, respectively), which are characterized by irregular rhythms (see Fig. 7, Bicuculline). Lack of a significant correlation peak (or trough) around the center of symmetry (lag shift = 0) of a cross-correlogram followed by an irregular and asymmetric correlogram pattern indicated that the two series were independent of each other (see Fig. 2A, thin line correlogram). This was correct even in cases where some of these correlation coefficients were significantly different from 0 (usually due to a regular rhythmicity of the 2 variables; see Fig. 10E, thick line correlogram). The occurrence of false significant correlations under these conditions could be minimized by increasing the sample sizes whenever possible.

### RESULTS

**Hindlimb pattern generators are not restricted to L2/L5**

Slow potential recordings from homologous pairs of ventral roots in different regions of the cord in the presence of 5-HT and NMDA are shown in Fig. 3. The recordings from the ventral roots of L2 (Fig. 3, A and C), L3 (Fig. 3B), and from L5 and T6 (Fig. 3D) are representative examples of the highly regular alternating rhythm observed in each of the lumbar segments of the cord under these conditions. Left-right alternating rhythmic activity with various degrees of regularity could also be recorded from sacral segments (Fig. 3A), from caudal thoracic segments (not shown), and from some midthoracic segments (Fig. 3C). Slow potential recordings from some other thoracic segments revealed bilaterally synchronous rhythm (Fig. 3B) or random bursting activity (not shown) in the presence of 5-HT and NMDA. The left-right alternating rhythm recorded from the nonlumbar segments described above did not persist when these segments were detached from the lumbar cord (9 experiments). Figure 3C, left, shows recordings from the left and right L2 and T6 ventral roots in the presence of 5-HT and NMDA. Both segments showed a regular left-right alternating rhythm. The rhythm recorded from the T6 ventral roots was completely abolished after the cord was transversely cut at the mid-T11 segment (Fig. 3C, right); the rhythm recorded from the L2 ventral roots under these conditions remained virtually unaltered. Similar results were obtained in five additional experiments (total = 6) in which the cord was transected between the thoracic and lumbar level, and in three other experiments in which rhythmically active sacral segments were separated from the lumbar cord (see Fig. 8C). The effect on the locomotor rhythm of transection of the lumbar cord into rostral and caudal parts is shown in Fig. 3D. Control records from L4 and L6 are shown in Fig. 3D.
left. A regular left-right alternating rhythm with a similar cycle time was observed in both segments. Transection of the cord at the mid-L3 level blocked the activity in the detached caudal lumbar cord and did not affect the alternating rhythm in the detached rostral lumbar part (not shown). Elevation of the concentration of NMDA in the bath under these conditions from 3 to 5 μM induced a left-right alternating rhythm also in the detached caudal lumbar cord. This rhythm (recorded from L5) was characterized by a longer cycle time compared with that observed for the L1 segment (Fig. 3D, right). Analysis of the five experiments performed in this series revealed that the cycle time measured from autocorrelograms of the data recorded from caudal lumbar segments after transection of the cord was longer by a factor of 1.7 ± 0.25 (SD) (n = 5) than that measured under the same conditions from the rostral lumbar segments.

To assess whether the rostrocaudal differences in rhythmicity described in our study (Fig. 3D) required cross connectivity between the two sides of the cord, we induced the locomotor-like rhythm by bath application of 5-HT and NMDA, split the cord in the midsagittal plane (8 experiments, see Fig. 2), and then transected the cord at the mid-L3 level (4 of these experiments). As is shown in Fig. 2, the rhythmicity induced by 5-HT and NMDA persisted in the left and right cord after the longitudinal midsagittal split. The longitudinal lesion could either increase or decrease the cycle time in the recorded segments by 1–80% [change of 23 ± 21.9%, mean ± SD; n = 16 (8 preparations, 2 sides each)]. Therefore the cycle time measured for the hemicords after midsagittal section (4.5 ± 1 s, mean ± SD; n = 16) did not differ significantly (paired 2-sample t-test for means) from that of the control series (4.6 ± 1.1 s, mean ± SD; n = 16). Transection of the midsagittally split preparations at the mid-L3 level (4 experiments) retained (after the adjustment of the NMDA concentration, usually from 2–3 to 5 μM; see above) a significant regular rhythmicity in the separated rostral and caudal lumbar segments (revealed by autocorrelation analysis). As in the case of the transverse cuts described in Fig. 3D, the cycle time of the rhythm recorded from the detached caudal lumbar hemisphere was much...
longer (by a factor of 2.07 ± 0.21; n = 4) than that recorded from the detached rostral lumbar hemisegments.

Cross connectivity required for left-right alternation is relayed at least through T12–L4

The effects of partial midsagittal section of the cord on left-right alternation of the locomotor-like rhythm were examined in 15 different spinal cord preparations. In 11 of these preparations the cord was longitudinally split in the midsagittal plane from its caudalmost region rostrally. The regular left-right alternating rhythm induced by 5-HT and NMDA was perturbed only as the lesion reached the rostral T11 segment (1 of 11 preparations), the caudal T12 segment (6 of 11 preparations), or the caudal T13 segment (4 of 11 preparations). In four additional experiments the cord was midsagittally sectioned in the rostrocaudal direction. In these preparations (4 of 4 cases) the alternating rhythm was perturbed only as the lesion reached the rostral L3 segment. Figure 4 shows the regular left-right alternating rhythm that was observed in a preparation that was split caudorostrally up to caudal T13 (Fig. 4A; recordings were obtained from the left and right L2 and L3 ventral roots) and in a different preparation that was split rostrocaudally down to rostral L4 (Fig. 4B; recordings were obtained from the left and right L3 and L4 ventral roots). A regular left-right alternating rhythm could also be recorded from lumbar ventral roots in midsagittally sectioned preparations in which only a single rostral-lumbar segment was kept bilaterally intact (Fig. 4C; L2 is the only bilaterally intact segment, recordings were obtained from the left and right L2 and L4 ventral roots). Similar results were also obtained in midsagittally split preparations in which either the L1, L3, or occasionally L4 was relayed at least through T12–L4 left as the only bilaterally intact segment (not shown).

The perturbations in the left-right alternating pattern of the locomotor-like rhythm that were induced by partial midsagittal sections of the cord were expressed as a bilateral synchronicity in 5 of 11 of the caudorostral and in one of four of the rostrocaudal midsagittal section experiments, and as left-right independent rhythms in 6 of 11 of caudorostral and in three of four of the rostrocaudal midsagittal section experiments. The appearance of bilateral synchronicity in midsagittally split preparations is demonstrated in Fig. 5. Recordings are from the left and right L2 ventral roots (Fig. 5; top and bottom pair of traces in each set are AC recordings at 0.1 Hz to 2 kHz and 10 Hz to 5 kHz, respectively) in a preparation that was first split caudorostrally up to caudal L3 segment (Fig. 5A) and then up to caudal T13 (Fig. 5B). The respective cross-correlograms (100-s data samples each) are shown superimposed in Fig. 5C. The regular left-right alternating pattern observed following the initial split (solid line correlogram) has been converted to a bilaterally synchronous rhythm as the section was extended up to caudal T13 (dotted line correlogram).

Figure 6 illustrates the appearance of left-right independent rhythms during gradual midsagittal section of the cord.

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**Fig. 3.** 5-HT-NMDA-induced rhythm in the isolated spinal cord preparation. Ventral root recordings (0.1 Hz to 5 kHz AC; top trace in each pair: left; bottom trace in each pair: right) from spinal cord preparations in the presence of 5-HT and NMDA. A: simultaneous recordings from L2 and S1 ventral roots show a left-right alternating rhythmicity. B: recordings from L3 ventral roots show an alternating rhythmicity, whereas the simultaneously recorded activity from T10 shows bilateral synchronicity. C: simultaneous recordings from L2 and T6 in the presence of 5-HT and NMDA before (left) and after transection of the cord at mid-T11 (right). The rhythmic activity in T6 was blocked after transection. D: ventral root recordings from L1 and L6 ventral roots in the presence of 20 μM 5-HT and 3 μM NMDA before (left) transection of the cord at mid-L3 level and in the presence of 20 μM 5-HT and 5 μM NMDA after the transection (right). For more details see text.
FIG. 4. Effects of partial midsagittal sections of the cord on the rhythm. Recordings (10 Hz to 5 kHz AC) of motoneuron firing from the left (top trace in each pair) and right (bottom trace in each pair) lumbar ventral roots in the presence of 5-HT and NMDA after caudorostral midsagittal split up to caudal T13 (A), rostrocaudal midsagittal split down to rostral L4 (B), and a complete midsagittal section with L2 as the only bilaterally intact segment (C). A regular left-right alternating rhythmicity was evident in each of the 3 preparations. The extent of the midsagittal split in each case is illustrated on the left. The segmented part in each illustration denotes the 6 lumbar segments of the cord.

A normal left-right alternating rhythm was observed after rostrocaudal split of the cord down to rostral L3 in the midsagittal plane (Fig. 6A; see also the solid line correlogram in Fig. 6D). Extension of the section down to mid-L4 (Fig. 6B) resulted in perturbed left-right alternation (Fig. 6B; dotted cross-correlogram in Fig. 6D). Further extension of the section to rostral L5 (Fig. 6C) induced left-right independent rhythms in the recorded ventral roots (Fig. 6D, dash/double-dotted cross-correlogram).

Bath applied bicuculline or strychnine induce synchronous rhythmic activity in the presence of 5-HT and NMDA

The mechanism of left-right alternation has been suggested to involve reciprocal inhibition between the left and right cords (Gossard and Hultborn 1991; Grillner 1981). The potential candidates for reciprocal inhibition are either glycineric or GABAAergic pathways. Although the involvement of glycine receptors in left-right alternation is widely accepted, the involvement of GABAA receptors is controversial. Cazalets et al. (1994) reported that bath application of the GABAA receptor blocker bicuculline (5 μM) in the presence of N-methyl-DL-aspartate (NMDA) did not affect the left-right alternating pattern of the rhythm. Cowley and Schmidt (1995), reported, however, that bath applied bicuculline induced a synchronous rhythm in the presence of NMA. This issue was therefore reexamined. Figure 7 shows the effects of bath applied bicuculline and of bath applied strychnine on the locomotor-like rhythm induced by NMDA and 5-HT. The regular left-right alternating rhythm observed during the control period has been converted to a bilaterally synchronous rhythm after addition of 4 μM bicuculline (left) or 2 μM strychnine (right) to the experimental chamber. The cross-correlograms (bottom) computed from the slow potential data recorded from the left and right homologous ventral roots under these conditions showed that the phase relation between the left and right cord was shifted from 180° in the control series to ~0° after addition of bicuculline (dotted line correlogram, bottom left) or strychnine.

FIG. 5. Effects of partial midsagittal section on the rhythm: appearance of a bilaterally synchronous rhythmicity. Recordings (top pair of traces, 0.1 Hz to 2 kHz; bottom pair of traces, 10 Hz to 5 kHz) from the left and right L2 ventral roots in the presence of 5-HT and NMDA after a caudorostral midsagittal split up to caudal L2 (A) and after the extension of the section to caudal T13 (B). C: respective cross-correlograms superimposed with means ± 3 SE. Sample size: 100 s each; lag = 50 ms.
nine (thick line correlogram, bottom right). These cross-correlograms also show that both antagonists affected the regularity of the rhythm mainly by introducing synchronous paroxysmal bursts (see Fig. 9A) of variable duration (composed of fast synchronous oscillations in the case of strychnine) (see Bracci et al. 1996; Cazalets et al. 1996; Cowley and Schmidt 1995). Similar results were obtained in 17 different preparations to which bicuculline (2–4 μM) was added and in 13 preparations to which strychnine (0.25–3 μM) was added.

*Bilateral synchronicity can be induced by bicuculline or strychnine in any intact or detached spinal segment, and in any segment of partially split cords*

To further characterize the neurochemically induced (5-HT and NMDA) locomotor-like rhythm in the presence of strychnine and bicuculline, a left-right alternating rhythm was induced by 5-HT and NMDA, the cord was midsagittally sectioned either rostrocaudally \( (n = 12) \) or caudorostrally \( (n = 3) \) to a level at which left-right alternation no longer existed in the lumbar cord, and strychnine or bicuculline was then added to the experimental bath. Figure 8 demonstrates that addition of 4 μM bicuculline or 2 μM strychnine under these conditions induced bilateral synchronicity in two different preparations that were midsagittally split in the caudorostral direction up to caudal T₆ \( (i.e., \text{the cord was left with 3 bilaterally intact segments}; \text{Fig. 8A, left and right}) \) and in another preparation that was midsagittally split in the rostrocaudal direction down to rostral L₅ \( (\text{Fig. 8B, left and right}; \text{for further details see the legend to Fig. 8}) \).

In another series of experiments we found that the bilateral synchronicity induced by strychnine or bicuculline in the presence of 5-HT and NMDA was not unique to the lumbar cord and that it could also be demonstrated in nonlumbar spinal cord segments that were detached from the lumbar cord by a transverse cut. Figure 8C shows data recorded \( (0.1–100 \text{ Hz AC recordings}) \) from the left and right L₂ and S₁ segments of the cord in the presence of 5-HT and NMDA (Control). Transection of the cord at the mid-L₆ segment abolished the rhythm recorded from the S₁ segment and did not affect the L₂ rhythm \( (\text{see similar effects in Fig. 2C for the rhythms in L}_{2} \text{ and } T_{6} \text{ following a mid-T}_{11} \text{ transection}) \). Addition of bicuculline under these conditions induced at the first stage \( (\text{Bicuculline 4 min}) \) an in-phase rhythmicity in the detached sacral cord, whereas the left-right alternating rhythm in the rostral lumbar cord was virtually unaffected. Six minutes later \( (\text{Bicuculline 10 min}) \) the alternating rhythm in L₂ has been converted to a synchronous rhythm, and the surgically separated sacral part retained the bilateral synchronicity acquired at the earlier stage. Figure 8D shows that a strychnine-induced rhythmicity can also be demonstrated in the presence of 5-HT and NMDA in rostral \( (L_{2}) \) and caudal \( (L_{4}) \) lumbar hemisegments that were separated from each other by transection of a midsagittally split preparation at the mid-L₄ level \( (\text{left}) \). This strychnine-induced rhythmicity was also retained in an isolated L₄ \( (\text{right}) \) or any other hemisegment. As in the case of transection of the cord in preparations that were not treated with strychnine and bicuculline (see above), the cycle time of the separated caudal lumbar segments was slower than that of the rostral lumbar segments.

**Strychnine-resistant left-right alternating rhythm is unmasked by CNQX**

The finding that the synchronous bursts induced by the IAA receptor antagonists could be generated at each level of the cord and transferred to split lumbar segments by descending and ascending pathways raises the possibility that such a massive synchronous activity might mask active reciprocal inhibitory processes that were not blocked by the IAA receptor antagonists. To check this possibility we had to minimize the effects of the ascending and descending cross excitation described above. Figure 9A shows that 10 μM of the NMDA receptor blocker CNQX was sufficient to block the nonrhythmic synchronous bursts induced by strychnine \( (2 \mu M) \) or bicuculline \( (\text{not shown}) \) in the absence of 5-HT and NMDA \( (\text{see also Bracci et al. 1996; Kremer and Lev-Tov 1995; Simon and Lev-Tov 1994}) \). Figure 9B shows that similar concentrations of CNQX \( (10 \mu M) \) de-
FIG. 7. Conversion of a left-right alternating to a synchronous rhythm by inhibitory amino acid (IAA) receptor antagonists. Recordings (10 Hz to 5 kHz AC) from the left (top trace in each pair) and right L3 ventral roots (bottom trace in each pair) in 2 different preparations in the presence of 5-HT and NMDA, are shown before (Control) and after the addition of 4 μM bicuculline (left) or 2 μM strychnine (right) to the bath. Cross-correlograms at bottom are those computed for the controls (thin line, left and right) and those computed for the data obtained after the addition of bicuculline (thick line, left) and strychnine (thick line, right). Sample size: 120 s (left), 80 s (right); lag = 50 ms. Correlograms were computed from 0.1 Hz–500 Hz AC recordings that were obtained simultaneously with the recordings shown above.

creased the background excitation and the locomotor-like drive induced by NMDA and 5-HT but preserved the rhythm and its left-right alternating pattern. With the use of these findings, we decided to test the effects of strychnine on the locomotor-like rhythm after pretreatment with CNQX. Figure 9C, left, shows recordings from a preparation in which the locomotor-like rhythm was induced by bath applied 5-HT and NMDA (Control). Addition of 10 μM CNQX decreased the locomotor drive but did not interfere with the regularity of the rhythm or with its left-right alternation (CNQX; see also the respective thin line cross-correlogram in Fig. 9C, right). Bath application of 3 μM strychnine under these conditions did not perturb the left-right alternating rhythm even 45 min after its administration (CNQX + Strychnine; see also the thick line cross-correlogram in Fig. 9C, right). Washout of the CNQX led to induction of a bilaterally synchronous rhythm (CNQX Wash). Similar results were obtained in each of the five experiments performed in this series. The use of the experimental protocol described above (addition of the IAA receptor blocker to CNQX-treated preparations) with bicuculline instead of strychnine was checked in five different preparations. The results were complicated by the strong effects of even low concentrations (2–4 μM) of bath applied bicuculline on rhythm generation: the rhythm became irregular (4 of 5 experiments) or blocked (1 of 5 experiments) after addition of bicuculline to the CNQX-treated preparations (not shown). Therefore concentrations of bicuculline that are required to block the GABA<sub>A</sub> receptors (10–20 μM) (see Bracci et al. 1996; Cowley and Schmidt 1995; Pinco and Lev-Tov 1994) could not be used in this series. Left-right alternation of the rhythm could be detected in the presence of 5-HT, NMDA, CNQX, and 2−4 μM bicuculline in three of the four preparations in which the rhythm was perturbed but not blocked (i.e., in 3 of 5 experiments performed in this series). In an additional preparation a left-right alternating rhythm could be detected only for the first 15 min after the addition of 2 μM bicuculline. The rhythm was then gradually abolished.

Locally applied strychnine perturbs the left-right alternation, whereas locally applied bicuculline does not

Another way to reduce the descending and ascending cross-excitatory effects induced by IAA receptor antagonists in the recorded segment was to apply the drugs to restricted regions of the cord by pressure ejection (see METHODS). To accomplish this we first added 5-HT and NMDA to the bath to induce the control locomotor-like rhythm and then ejected strychnine onto the ventral surface of the cord of a bilaterally
FIG. 8. IAA receptor antagonists induced rhythm. A: bilateral bursts recorded at 10 Hz to 5 kHz from left and right L₂ ventral roots in 2 different preparations that were midsagittally split in the caudorostral direction up to T₆ in the presence of 5-HT and NMDA, after addition of 4 μM bicuculline (left) and 2 μM strychnine (right). B: bilateral bursts recorded at 10 Hz to 5 kHz from left and right L₂ ventral roots in a preparation that was midsagittally split in the caudorostral direction down to rostral L₅ in the presence of 5-HT and NMDA, after addition of 4 μM bicuculline (left). After a 45-min wash of the bicuculline, bilaterally synchronous bursts were reinduced by bath applied strychnine (2 μM; right). C: recordings (0.1 ± 100 Hz AC) from L₂ and S₁ ventral roots in the presence of 5-HT and NMDA, shown before (Control) and after transection of the cord at the mid-L₆ level (Cut at mid-L₆). The effects of bath applied bicuculline (4 μM) are shown 4 and 10 min after its addition to the transected cord. The bicuculline-induced bilateral synchronicity appeared 1st in the S₁ ventral roots (Bicuculline 4 min) and only later in the L₂ roots (Bicuculline 10 min). D, left: recordings (10 Hz to 5 kHz AC) from the left and right L₂ and L₄ ventral roots in a spinal cord preparation that was 1st sectioned in the midsagittal plane and then transected at the mid-L₃ level in the presence of 5-HT and NMDA, shown after addition of 2 μM strychnine to the bath. D, right: regular rhythmic activity recorded from a surgically isolated left L₆ hemisegment in another preparation in the presence of 5-HT, NMDA, and 2 μM strychnine.

intact (illustrated in Fig. 10A, left; n = 4) or a midsagittally split preparation with a single bilaterally intact rostral-lumbar segment (illustrated in Fig. 10A, right; n = 9) with the use of micropipettes filled with 1 or 10 mM of drug. Ejections were repeated to maximize the effects and to ensure that the ejected drug was spread over the entire segment. The effect of strychnine was not instantaneous. It developed gradually within 10–15 min after the ejections and (unlike the case of bath applied of strychnine) could be washed out in most cases 30–40 min after the application. Figure 10B shows the control left-right alternating rhythm recorded from the left and right ventral roots of L₂ and L₄ (see also the thin line correlograms in Fig. 10D, L₂ and L₄). The effects of pressure ejection of strychnine (5 consecutive ejections, 10 s each, 5 min intervals) onto the ventral surface of the left and right L₄ segment of a bilaterally intact spinal cord preparation are shown in Fig. 10, B–D. The locally applied strychnine induced bilaterally synchronous bursts and intermittent left-right alternations mainly in the L₄ segment (Fig. 10C; thick line cross-correlogram in Fig. 10D, L₂ and L₄). The left-right alternating pattern of the rhythm recorded from the L₄ segment was affected to a lesser extent by the strychnine ejected onto L₄ (Fig. 10D, L₂, and thick line cross-correlogram), with the exception of transient interruptions of the rhythm corresponding to the occurrence of bilaterally synchronous bursts in L₄ (Fig. 10C, L₂).

To further weaken the cross-excitatory effects, preparations were split in the midsagittal plane and left with a single bilaterally intact rostral lumbar segment (Fig. 10A, right). Strychnine was then ejected onto the ventral surface of the bilaterally intact segment. Figure 10E shows that in one of these experiments (1 of 9), pressure ejection of strychnine onto the intact L₂ segment converted the left-right alternating locomotor rhythm induced by 5-HT and NMDA and recorded from the
left and right L₂ ventral roots (Fig. 10E, top 2 traces and thin line cross-correlogram) to left-right independent rhythms (Fig. 10E, bottom 2 traces and thick line cross-correlogram). In a different experiment (Fig. 10F), and in eight of nine experiments performed in this series, the rhythm recorded from the left and right L₂ ventral roots in the presence of 5-HT and NMDA (top 2 traces and thin line cross-correlogram) developed irregularities accompanied by erratic left-right alternation after pressure ejection of strychnine onto the left and right L₂ segment (bottom 2 traces and thick line cross-correlogram). Left-right alternation under these conditions (in 8 of 9 experiments), however, could not be completely blocked by strychnine, even in cases in which the ejections were prolonged and repeated many times.

Bicuculline was pressure ejected onto the ventral surface of eight different spinal cord preparations (midsagittally split preparations with a single bilaterally intact rostral-lumbar segment; see Fig. 10A, right) with the use of micropipettes filled with either 2 or 10 mM bicuculline. Prolonged (10–20 s) ejections of 10 mM bicuculline increased the tonic excitation and abolished the locomotor-like rhythm immediately (not shown). After a few minutes the rhythmic activity recovered. The recovery was characterized occasionally by few widely spaced bilaterally synchronous bursts (not shown). The effects of bicuculline were immediate and reversible. Figure 11A shows the control locomotor-like rhythm that was induced by bath applied 5-HT and NMDA and recorded from the left and right ventral roots of the intact L₂ segment. The rhythm under these conditions was regular and alternating (see the thin line cross-correlogram in Fig. 11E). The effects of pressure ejection of 2 mM bicuculline onto the ventral surface of the intact L₂ evolved almost instantaneously. Bicuculline first increased the background excitation, the locomotordrive, and the bursting activity (Fig. 11B). Later on, the cycle time and the burst duration were prolonged (Fig. 11, C and D). The left-right alternating activity under these conditions was unperturbed during and after the bicuculline application (Fig. 11E, thick line cross-correlogram). Recordings from the left and right ventral roots of L₂ and L₄ segments in the same preparation (Fig. 11F) revealed that the bicuculline-induced prolongation in the cycle time and burst duration was transmitted...
from the treated L₂ segment to the untreated L₄ segment, suggesting that the effects of bicuculline were mediated through an action on the networks of the intact segment.

**Discussion**

Our study deals with the segmental localization of the CPGs associated with hindlimb locomotion in the isolated thoracolumbosacral cord of the neonatal rat, with the organization of the transverse coupling system between the left and right hemicords, and with receptors involved in mediation of left-right alternating rhythm in this preparation. The results concerning these issues are discussed below.

**Localization of the CPGs involved with hindlimb locomotion**

As mentioned above, in vitro studies of the swimming rhythm in the lamprey (Cohen 1987; Cohen and Wallen 1980; Grillner and Matsushima 1991; Hagevic and McClellan 1994) and Xenopus (Kahn and Roberts 1982), and of the spontaneous motor rhythm in chick embryo (Ho and O’Donovan 1993) suggested that these rhythms were generated by CPGs that are distributed along the rostrocaudal axis of the cord. Recent studies of the locomotor-like rhythm in isolated spinal cord preparations of the neonatal rat have challenged this view and suggested that the locomotor CPGs are restricted to the L₁ and L₂ spinal cord segments, and that the caudal lumbar cord is driven by the CPGs located in the rostral cord (Cazalets et al. 1995, 1996). The present findings that a regular rhythm could be induced in the rostral and caudal parts of the lumbar cord in preparations that were transected at the mid-L₃ level (Fig. 3), together with the flexor-extensor alternating rhythmicity that has been reported to occur in a detached caudal lumbar (L₅/L₆) hemicord (Kudo and Yamada 1987), show that the
FIG. 11. Local application of bicuculline to the cord. Bicuculline was pressure ejected onto the ventral surface of L2 in a midsagittally split preparation with L2 as the only bilaterally intact segment (see Fig. 10A, right). A–D: control (A) and the development of the bicuculline effects (B–D) with the use of 0.1 Hz to 2 kHz recordings from the left and right L2 ventral roots in the presence of 5-HT and NMDA. E: cross-correlograms of the data shown in A and D (thin line: control; thick line: bicuculline). Sample size: 150 s; lag = 50 ms; reference lines are at r_k = 0 at mean ± 3 SE and at lag shift = 0. F: recordings (10 Hz to 2 kHz AC) from the left and right L2 and L4 ventral roots from the same experiment, before (top set) and after (bottom set) pressure ejection of bicuculline onto the ventral surface of L2. Note that the prolongation of the cycle time induced by ejection of bicuculline onto L2 was transmitted also to the untreated L4 segment.

CPGs are not restricted to L1 and L2 segments. Attempts to induce rhythmic activity in nonlumbar segments by 5-HT and high NMDA concentrations failed. The question therefore arises whether the CPGs in these segments are difficult to excite under these conditions or else are limited to the first three to four lumbar segments of the cord. Application of IAA receptor antagonists to spinal cord preparations in the absence of 5-HT and NMDA has been shown to induce nonrhythmic irregular synchronous bursts in the spinal cord (Bracci et al. 1996; Cazalets et al. 1996; Cowley and Schmidt 1995) (see Fig. 9). In the presence of 5-HT and NMDA, however, the IAA receptor antagonists strychnine or bicuculline induced a bilaterally synchronous rhythm in the cord (Cowley and Schmidt 1995) (see Fig. 7). It is therefore possible that the appearance of the regular rhythmic activity in detached spinal cord segments or hemisegments in the presence of 5-HT and NMDA after addition of strychnine or bicuculline (Fig. 8, C and D; note that even the differences in cycle time between the rostral and caudal lumbar cord persisted in the presence of strychnine) reflects an activation of “latent” CPGs in these segments by the increased excitability induced by strychnine and bicuculline. If this is correct, the CPGs in the neonatal rat spinal cord may be distributed along its rostrocaudal axis as has been shown for the lamprey, Xenopus, and the embryonic chick spinal cords. The notion of a distributed CPG is further supported by recent lesion experiments of the neonatal rat spinal cords. The notion of a distributed CPG is further supported by recent lesion experiments of the neonatal rat spinal cord in which rhythmic activity was demonstrated not only in L1–L2 but also in caudal thoracic and caudal lumbar segments (Kjaerulff and Kiehn 1996).

The present study also reveals rostrocaudal specialization of the pattern generation capability of the lumbar cord. The rhythm in the rostral lumbar cord (L1–L2) was maintained after its detachment from the caudal lumbar sacral cord (mid-L3 and down), whereas activation of the rhythm in the detached caudal part of the lumbar cord required elevation of the NMDA concentration in the perfusate. Moreover, activation of rhythmicity in detached nonlumbar segments in the presence of 5-HT and NMDA could be accomplished only after addition of strychnine or bicuculline to the perfusate (see above). In addition to the regional differences in excitability that are suggested by these findings, the cycle time in the isolated rostral lumbar cord (L1–L2) was shorter than that observed for the isolated caudal lumbar sacral cord. Thus, under normal conditions, the cycle time of the rhythm can be dominated by the more excitable rostral lumbar segments.
of the cord. Rostrocaudal specialization of the lumbar cord has also been reported for the embryonic chick spinal cord in which the rostral lumbar segments were more capable of generating the motor rhythm than the caudal ones (Ho and O’Donovan 1993). Specialization of the hindlimb enlargement of the cord is not unique to the in vitro isolated spinal cord preparations of the rat and chick. Lumbosacral specialization has been documented for cat locomotion (segments L6, L7, and S1 in this case) (Grillner and Zangger 1979) and for scratching in the cat (Arshavsky et al. 1984; Deliagina et al. 1983; Gelfand et al. 1988) and the turtle (Morton and Stein 1989). The rostrocaudal differences in excitability of the lumbar cord suggested by our study require an involvement of an efficient longitudinal coupling system to ensure a stable proximal-to-distal multijoint coordination during locomotion. This is achieved by the propriospinal pathways. The propriospinal pathways have been shown to develop early in ontogeny of the chick (Oppenheim et al. 1988) and rat (Altman and Bayer 1984), and were found to be functional in the embryonic chick (Bradley and Bekoff 1992) and the embryonic and neonatal rat (Bekoff and Lau 1980; Bekoff and Trainer 1979). Moreover, the synaptic projections of propriospinal fibers traveling in the ventrolateral white matter funiculus onto interneurons and motoneurons in the neonatal rat have been found to be characterized by high-efficacy excitatory synaptic transmission (Lev-Tov and O’Donovan 1995; Pinco and Lev-Tov 1994), thereby assuring a reliable longitudinal coupling in the system. The notion of an efficient rostrocaudal coupling in the lumbar cord is further supported by the studies of Cazalets et al. (1995, 1996) in which a short-latency biphasic projection system has been suggested to couple the rostral and caudal lumbar cord.

In summary, it is has been shown directly that the CPGs are not restricted to L1/L2 but are localized at least also in L3 and L4; it has been suggested that the CPGs are actually distributed along the axis of the cord; and it has been indicated that the CPGs in rostral lumbar region are more excitable than those in other segments of the cord and therefore may play a dominant role in neurogenesis of locomotion and in determination of the cycle time. An efficacious longitudinal coupling system is suggested to activate and synchronize the less excitable CPGs in the caudal lumbar cord.

Organization of the transverse coupling system

RHYTHMOGENESIS AND TRANSVERSE COUPLING. The left-right alternation of the motor rhythms described in the lamprey (Cohen and Harris-Warrick 1984; Grillner and Wallen 1980; Hagevic and McClellan 1994), Xenopus (Dale 1985; Soffe 1987; Soffe et al. 1984), and the neonatal rat spinal cord (Cazalets et al. 1996; Cowley and Schmidt 1995; Kudo et al. 1991) has been attributed to reciprocal inhibitory connections between the left and right hemisegments. Mutual inhibitory connections have also been suggested to mediate flexor-extensor alternation in the chick (O’Donovan et al. 1992; Sernagor et al. 1995), the mouse (Droge and Tao 1993), the rat (Cowley and Schmidt 1995), and the cat spinal cord (Perret 1983; Pratt and Jordan 1987). The existence of the motor rhythms in the lamprey (Alford and Williams 1989; Cohen and Harris-Warrick 1984; Grillner and Wallen 1980; Hagevic and McClellan 1984), Xenopus (Dale 1985; Soffe 1987), the chick spinal cord (Sernagor et al. 1995), and the neonatal rat spinal cord (Cowley and Schmidt 1995; Kudo et al. 1991; our results) in the presence of the IAA receptor antagonists strychnine and/or bicuculline suggests that inhibitory connections and IAA receptors may not be essential for generation of rhythmic activity in the spinal cord. The regular rhythmicity observed in the left and right hemisegments after a complete midsagittal section of the cord in the present study (Fig. 3) and in the study of Kudo and Yamada (1987) showed that the fibers that cross the cord from side to side and are associated with either the cross-excitatory or the reciprocal inhibitory pathways are not required for rhythmo-
genesis but are rather used for phasing the rhythms induced in the left and right hemisegments. Similar conclusions have been reached for the Xenopus spinal cord, in which midsagittal section stopped the left-right alternation and did not block the rhythm (Kahn and Roberts 1982), and for the respiratory system, in which separate mechanisms have been suggested to account for rhythmo genesis and pattern formation (Feldman and Smith 1989).

EXCITATORY AND INHIBITORY COMPONENTS OF THE TRANSVERSE COUPLING SYSTEM. Pharmacological studies of the left-right alternating rhythm in the lamprey (Alford and Williams 1989; Cohen and Harris-Warrick 1984; Hagevic and McClellan 1994) and the neonatal rat (Cazalets et al. 1996; Kudo et al. 1991) have shown that the out-of-phase rhythmicity has been converted to a bilaterally synchronous rhythm by bath applied strychnine, or strychnine and bicuculline (Cowley and Schmidt 1995; this study). If an alternating rhythm is indicative of a predominant reciprocal inhibition, and a bilaterally synchronous rhythm reflects a predominant cross excitation, then these findings suggest that the phase relation between the left and right hemisegments during the locomotor rhythm may be determined by an interplay between an in-parallel inhibitory and excitatory cross-coupling pathways. The present study shows that the interplay between the excitatory and inhibitory components of the transverse coupling system is dynamic and that it can be modulated experimentally. This was done in three ways. 1) Decreasing the cross excitation—the non-NMDA receptor blocker CNQX prevented the transformation of a left-right alternating rhythm to a bilaterally synchronous rhythm induced by the IAA receptor antagonists. 2) Increasing the cross excitation in remote spinal cord segments—local application of strychnine by pressure ejections perturbed the phase relation between the left and right hemisegments in untreated regions of the cord. This finding is also supported by data reported for the lamprey spinal cord, in which application of strychnine to the rostral cord (the cord was mounted in a dual chamber bath) induced an in-phase rhythmicity in the untreated caudal segments of the cord (Hagevic and McClellan 1994). 3) Weakening the reciprocal inhibition—reduction of the inhibitory component of the transverse coupling system in the caudal thoracic cord by midsagittal splitting of the lumbar cord allowed the cross-excitatory component to dominate and produce a bilaterally synchronous rhythm in the split lumbar cord in 40% of the experiments performed in that series (see Fig. 5). The two latter examples also show that the excitatory-inhibitory bal-
ance of the transverse coupling system and the resultant phase relation between the activities of the left and right hemicords in a given spinal cord segment is affected not only by the connectivity relayed from side to side in that segment, but also by projections from adjacent and from more remote segments. This view is further supported by the finding that a regular left-right alternation was found in split lumbar segments in the partial midsagittal section experiments (see Fig. 4), and that the bilateral synchronicity induced by IAA receptor antagonists could be transmitted by descending or ascending propriospinal pathways from the bilaterally intact rostral thoracic or lower sacral segments to the split lumbar segments (see Fig. 8).

The interplay between cross excitation and reciprocal inhibition and its role in determination of the phase relation between the left and right hemicords during locomotor activity is not limited to the experimental manipulations described above. During ontogeny, the locomotor-like activity that first appears on embryonic day 17 is characterized by synchronous left-right bursts (Greer et al. 1992). Before birth, the rhythm develops left-right alternation (Greer et al. 1992), which is probably timed to maturation of the reciprocal inhibitory pathways between the two halves of the cord.

**SPECIALIZATION OF THE TRANSVERSE COUPLING SYSTEM OF THE LUMBAR ENLARGEMENT OF THE CORD.** Assuming, as mentioned in the previous section, that an alternating left-right rhythm reflects a predominant reciprocal inhibition and that a bilaterally synchronous rhythm reflects a predominant cross excitation, it is suggested that the transverse coupling system between the two halves of the cord is regionally specialized. Under normal circumstances all lumbar segments of the cord (which are characterized by a regular left-right alternating rhythm) are therefore controlled by a strong reciprocal inhibition and a much weaker cross excitation, whereas the reciprocal inhibition in some nonlumbar segments is weaker in the way that it occasionally allows the weak cross-excitatory connectivity to dominate and induce bilateral synchronicity in these segments (see Fig. 3B). Moreover, the removal of the strong inhibitory projections from the transverse coupling system of the rostral lumbar cord to these nonlumbar segments after splitting the lumbar cord in the midsagittal plane induced descending or ascending cross excitation in the split lumbar segments of the cord in 40% of the experiments (see Fig. 5). In most (60%) of these experiments, however, the weak cross-excitatory connectivity was insufficient to synchronize the rhythms in the two hemicords after removal of the inhibitory projections that originated in the lumbar cord (see Fig. 6).

The specialization of the transverse coupling system of the lumbar cord (i.e., a predominant reciprocal inhibition in lumbar segments and a much weaker one in nonlumbar segments) is also supported by the finding that the transformation of tonic firing in detached sacral segments to bilateral synchronicity after bath application of bicuculline preceded the conversion of the left-right alternating rhythm in the detached rostral lumbar segments of the same preparation to a bilaterally synchronous rhythm (see Fig. 8C). Another line of evidence supporting the notion of the regional specialization suggested above originates from the partial midsagittal section experiments performed in this study. The reciprocal inhibitory connectivity relayed from side to side through a single bilaterally intact rostral (L₁, L₂, L₃, or in some cases L₄) lumbar segment was sufficient to maintain a regular left-right alternating rhythm in most lumbar segments by its rostrocaudal projections.

This latter finding brings us to the issue of the spinal cord segments that are used to relay the cross connectivity that is required to enable left-right alternation of the locomotor-like rhythm. On the basis of the observation that a left-right alternating rhythm persisted in the lumbar cord after a midsagittal section from the caudal end to L₂, Cazalets et al. (1995) suggested that the cross connectivity that is required for left-right alternation is relayed from side to side only through L₀/L₂. Our midsagittal section experiments showed that a normal left-right alternating rhythm persisted in the lumbar cord after caudorostral midsagittal splits up to caudal T₁₁ and after rostrocaudal splits down to L₄. Thus left-right alternation could be maintained in the lumbar cord by reciprocal inhibitory connections that are relayed from side to side through at least the T₁₂, T₁₃, L₁, L₂, L₃, and L₄ segments.

The question that arises in this regard has to do with the transverse coupling system in other lumbar or nonlumbar segments: are the in-parallel excitatory inhibitory cross connections relayed from side to side through each segmental level of the cord? This question could not be answered directly in this study because the detached caudalmost lumbar segments (L₄/L₀) or other nonlumbar segments were not rhythmically active in the presence of 5-HT and NMDA (see Figs. 2C and 8C). However, the in-phase left-right rhythmicity observed in these detached segments in the presence of 5-HT and NMDA after the addition of strychnine or bicuculline to the bath, suggests that at least the cross-excitatory component of the transverse coupling system is spread throughout the length of the cord, and that each segment is used to relay part of these excitatory pathways from side to side. Because cross excitation under these conditions could be induced either by blockade of reciprocal inhibitory connections between the hemicords, or by blockade of other nonreciprocal inhibitory input onto motoneurons or onto interneurons that are involved in cross excitation, or both, it could not be determined whether reciprocal inhibitory connectivity in nonlumbar regions in the intact preparation under normal conditions is relayed from side to side also through these regions or obtained by projections from the lumbar cord.

**IAA receptors and left-right alternation of the locomotor rhythm**

Left-right alternation of motor rhythms in vertebrates has been attributed to reciprocal inhibition between the two halves of the cord (Gossard and Hultborn 1991; Grillner 1981). The conversion of left-right alternating rhythm to a synchronous rhythm in the lamprey and Xenopus by strychnine and not by bicuculline led to the suggestion that reciprocal inhibition in these preparations is mediated by glycnergic but not by GABAergic pathways. GABA, receptors have been recently shown to be involved in flexor-extensor alternation of the spontaneous motor rhythm generated in the embryonic chick spinal cord (Sernagor et al. 1995), in
the inactivation of the locomotor CPGs in the neonatal rat spinal cord (Cazalets et al. 1994), and in coordination of locomotor activity (Alford et al. 1991; Tegner et al. 1993).

The involvement of GABA_\text{A} receptors in the reciprocal inhibition associated with left-right alternation in the rat is not clear. Cazalets et al. (1994) reported that left-right alternating rhythmicity was not affected by bath applied bicuculline, whereas Cowley and Schmidt (1995) have found that bath applied bicuculline induced bilateral synchronicity in the neonatal rat spinal cord and suggested a mutual contribution of glycine and GABA_\text{A} receptors to reciprocal inhibition. In the present study we showed that a bilaterally synchronous rhythm could be induced in the presence of NMDA and 5-HT by either strychnine or bicuculline (see Figs. 7 and 8). However, because cross-excitatory effects are so dominant and are easily induced even in cases where the receptors involved in reciprocal inhibition are not blocked (see the descending and ascending cross excitation in regions of the cord that were not exposed to IAA receptor blockers, Fig. 10) (see also Hagevic and McClellan 1994), or are only partially blocked (cross excitation can be induced by 0.25 \mu M strychnine or 2 \mu M bicuculline; concentrations are too low to block the IAA receptors), the appearance of bilateral synchronicity can mask the expression of active reciprocal inhibitory processes (see also the strychnine-resistant alternating rhythm, Fig. 9) and cannot be used as the only tool to determine whether a given class of receptors is involved in mediation of left-right alternation. The intracellular studies of motoneurons reported by Cazalets et al. (1996) revealed a strychnine-sensitive component of the locomotor drive potential. It is not clear, however, whether this component is associated with flexor-extensor or with left-right glycinergic reciprocal inhibition. The fact that bicuculline had no effect on the inhibitory component of the locomotor drive potential in that study does not rule out a possible contribution of GABA_\text{A} receptors to reciprocal inhibition. The side-to-side inhibitory pathways may simply project to interneurons associated with the pattern generators rather than to motoneurons.

The local application experiments performed on midsagittally split preparations with a single bilaterally intact segment allowed us to clarify the identity of the receptors involved in left-right alternation of the locomotor rhythm. These experiments showed that locally applied strychnine introduced perturbations and irregularities to the left-right phase relations, but could not block the alternation. Locally applied bicuculline under the same conditions did not interfere with the out-of-phase rhythmicity. It is therefore suggested that the side-to-side reciprocal inhibition is mediated primarily by glycine receptors. The bilateral synchronicity induced by bath applied bicuculline (see Figs. 7 and 8) may therefore reflect disinhibition of GABA_\text{A} receptors that are not necessarily associated with reciprocal inhibition.

The question arises as to the mechanisms allowing the strychnine-resistant left-right alternation in the presence of CNQX. Three main possibilities are considered in this regard. First, as discussed above, the possibility of mutual involvement of glycine and GABA_\text{A} receptors in the mechanism of left-right alternation of the rhythm does not seem highly likely but cannot be completely excluded. Second, the strychnine-resistant left-right alternation in the presence of CNQX and the inability of locally applied strychnine to completely abolish left-right alternation may have to do with an inadequate concentration of strychnine in the media. The concentration of strychnine that has been reported to block glycine-mediated outward currents, inhibitory postsynaptic currents, and inhibitory postsynaptic potentials in the neonatal rat spinal cord was ~1 \mu M in most cases (Bracci et al. 1996; Cazalets et al. 1996; Konnerth et al. 1990; Wu et al. 1995). The concentration of strychnine used for bath application in the series of our CNQX experiments varied between 2 and 3 \mu M. It therefore seems that the strychnine concentration used in this series was adequate. This gives rise to a third possibility: it has recently been shown that glycine-induced currents in sympathetic preganglionic neurons in the neonatal rat revealed, in addition to the strychnine-sensitive chloride channel mediated component, a strychnine-resistant component that is mediated by a mixed cationic conductance (reversal potential = −40 mV) (Wu et al. 1995). The inward current associated with these strychnine-resistant glycine receptors has been shown to reduce membrane excitability and to attenuate synaptic transmission (Wu et al. 1995). If glycine receptors in the ventral cord were to operate similarly, then the subpopulation of the strychnine-resistant glycine receptor/ion channel complex would be a good candidate to carry on the reciprocal inhibition in the presence of strychnine. The recent findings that a small depolarizing component of inhibitory postsynaptic potentials produced by propriospinal axons (which are the most likely candidates to mediate reciprocal inhibition between the two halves of the cord) (Ho and O’Donovan 1993) in lumbar motoneurons persists in the presence of 5 \mu M strychnine and 10 \mu M bicuculline (Pinco and Lev-Tov 1994) may provide a basis for such mechanism. Similarly, incomplete strychnine block has also been reported for recurrent inhibitory postsynaptic potentials in lumbar motoneurons of the neonatal rat (Schneider and Fyffe 1992).

In summary, we suggest that left-right alternation of the locomotor-like rhythm is carried out mainly by glycinergic pathways between the left and right hemisegments. These pathways are suggested to be mediated by strychnine-sensitive receptors, and possibly also by strychnine-resistant receptors. A more direct evaluation of the contribution of strychnine-resistant glycine receptors to the process and the possible contribution of GABA_\text{A} receptors (see above) should await further studies of the transverse coupling system.

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