Reversible Disorganization of the Locomotor Pattern after Neonatal Spinal Cord Transection in the Rat

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The central pattern generators (CPGs) for locomotion, located in the lumbar spinal cord, are functional at birth in the rat. Their maturation occurs during the last few days preceding birth, a period during which the first projections from the brainstem start to reach the lumbar enlargement of the spinal cord. The goal of the present study was to investigate the effect of suppressing inputs from supraspinal structures on the CPGs, shortly after their formation. The spinal cord was transected at the thoracic level at birth [postnatal day 0 (P0)]. We examined during the first postnatal week the capacity of the CPGs to produce rhythmic motor activity in two complementary experimental conditions. Left and right ankle extensor muscles were recorded *in vivo* during airstepping, and lumbar ventral roots were recorded *in vitro* during pharmacologically evoked fictive locomotion. Mechanical stimulation of the tail elicited long-lasting sequences of airstepping in the spinal neonates and only a few steps in sham-operated rats. *In vitro* experiments made simultaneously on spinal and sham animals confirmed the increased excitability of the CPGs after spinalization. A left–right alternating locomotor pattern was observed at P1–P3. Both types of experiments showed that the pattern was disorganized at P6–P7, and that the left–right alternation was lost. Alternation was restored after the activation of serotonergic 5-HT_2 receptors *in vivo*. These results suggest that descending pathways, in particular serotonergic projections, control the strength of reciprocal inhibition and therefore shape the locomotor pattern in the neonatal rat.

Key words: **central pattern generators; locomotion; development; descending pathways; serotonin; spinal cord transection**

Introduction

It is well established that the basic rhythmic activity underlying locomotion is generated by interneuronal networks within the spinal cord (Grillner and Wallén, 1985), defined as the central pattern generators (CPGs). These are functional at birth in the rat, as shown by experiments on *in vitro* spinal cord preparations isolated from neonates (Cazalets et al., 1992). Pharmacological activation of the CPGs evokes a fictive locomotor pattern consisting of alternation both between the motor bursts on the left and right sides of the spinal cord and between flexors and extensors on one side (Kiehn and Kjaerulff, 1996). The same kind of experiments made on embryonic day 16 (E16; i.e., 5 d before birth) reveal a motor pattern with all bursts in phase (Iizuka et al., 1998). The transition from left–right synchrony to alternation occurs at E18 (Iizuka et al., 1998) and may be attributable to the maturation of reciprocal inhibitory connections between the two sides, more precisely to the shift of glycine-evoked potentials from excitation to inhibition (Wu et al., 1992).

These major changes in locomotor network operation occur shortly after the arrival in the lumbar enlargement of the first axons descending from the brainstem, suggesting that these pathways may contribute to some extent to the maturation of spinal networks (Vinay et al., 2000). Projections arising from the raphe nuclei are among the earliest axons to reach the upper lumbar segments in the rat (Lakke, 1997). They are the source of almost all the serotonin (5-HT) in the lumbar spinal cord (for review, see Schmidt and Jordan, 2000). Serotonergic fibers start to innervate the gray matter by E17 (Bregman, 1987; Rajaofetra et al., 1989). 5-HT_2 receptors play a key role in the modulation of motor function and its recovery after spinal cord injury. First, they are found primarily in the ventral horn (Marlier et al., 1991; Thor et al., 1993). Secondly, their activation restores the extensor excitability in the spine of the cat (Barbeau and Rossignol, 1990; Miller et al., 1996) and enhances locomotor function in rats that received neural transplants after neonatal spinal transection (Kim et al., 1999).

In this study, we investigated the effect of suppressing inputs from supraspinal structures on the CPGs shortly after their formation. We examined during the first postnatal week the ability of rats to produce rhythmic motor activity with their hindlimbs after a complete spinal cord transection at the thoracic level on the day of birth. Animals were analyzed in two complementary experimental conditions: (1) Airstepping, which suppresses postural constraints, enabled us to examine *in vivo* the production of rhythmic motor output despite the marked postural immaturity at this age (Fady et al., 1998; Brocard et al., 1999). (2) *In vitro* experiments enabled us to evaluate the rhythmogenic properties of the isolated lumbar spinal cord in the absence of sensory inflow. We report that, in the absence of descending modulatory inputs, the left–right alternating locomotor pattern was lost and could be restored after the activation of 5-HT_2 receptors.

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Materials and Methods

Animals. A total of 52 Wistar rats aged from postnatal day 0 (P0; defined as the first 24 hr after birth) to P7 were used. Each litter was divided into two groups: an experimental group of approximately seven pups whose spinal cord was transected on the day of birth and a sham group, which was operated, handled, and treated in the same way as spinal animals except for the spinal cord transection. All surgical and experimental procedures were made to minimize animal suffering and conformed to the guidelines from the French Ministry for Agriculture and Fisheries, Division of Animal Rights.

Surgical procedures. Rats were anesthetized by hypothermia until no reflexes could be evoked by pinching the tail or a limb. A laminectomy was made, the spinal cord was transected at the T8–T10 level with iridectomy scissors, and one or two segments of the cord were removed with fine forceps. The lesion cavity was then filled with sterile absorbable local hemostat Surgicoll (Medical Biomaterial Products, Neustadt-Glewe, Germany). Skin incisions were sutured using fine thread (PDSII 6.0, Ethicon; Johnson and Johnson, Brussels, Belgium) and covered by Steri-Strips (3M Health Care, St. Paul, MN). Animals were warmed and returned to the mother 1–2 hr after surgery. The completeness of spinal cord transection was verified postmortem by visual inspection of the lack of continuity between the spinal stumps.

In vivo *experiments.* The coordination between hindlimbs was investigated during airstepping in 19 spinal animals ($n = 3$ at P1, $n = 3$ at P2, $n = 2$ at P3, $n = 6$ at P6, and $n = 6$ at P7). Rats were held in a sling with the forelimbs and hindlimbs hanging on each side (see Fig. 1*A*) (Fady et al., 1998). Sequences of airstepping were triggered by pinching the tail with forceps. At least five recordings were obtained at 3 min intervals from each animal. Electromyographic (EMG) recordings were obtained by 100 μm silver wires (AM-Systems Inc., Carlsborg, WA) inserted into the left and right triceps surae muscles, parallel to the muscle fibers. A reference electrode was inserted through the skin of the back. Recordings were made after a 5–10 min recovery period. Signals were amplified, filtered (AC-coupled amplifiers; bandwidth, 70 Hz to 1 kHz), digitized, and stored on a hard disk (Digidata 1200 interface, pClamp 8 software; Axon Instruments, Foster City, CA; sampling frequency of 2 kHz).

A group of spinal animals ($n = 6$) was tested at P6–P7 with the serotonergic agonist 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI; Research Biochemicals, Natick, MA). DOI was dissolved in distilled water and injected in a volume of 1 ml/kg (0.15 mg/kg, i.p.) (Kim et al., 1999). Airstepping was tested before and after DOI injection.

In vitro *experiments.* The coordination between ventral root bursts was investigated during fictive locomotion in 33 animals: $n = 2$ at P2 (one spinal plus one sham), $n = 6$ at P3 (three spinal plus three sham), $n = 10$ at P4 (six spinal plus four sham), $n = 8$ at P5 (five spinal plus three sham), and $n = 7$ at P6 (five spinal plus two sham). Animals were anesthetized by hypothermia. They were then decerebrated at a postcollicular level, eviscerated, and pinned down onto a Petri dish. Dorsal craniotomy and laminectomy were performed; the brainstem, spinal cord, and lumbar ventral roots were removed. The preparation was then pinned down in the recording chamber with the ventral side up. All dissection and recording procedures were performed under continuous perfusion with saline solution containing (in mM): 130 NaCl, 4 KCl, 3.75 $CaCl_2$, 1.3 $MgSO_4$, 0.58 NaH_2PO_4, 25 $NaHCO_3$, and 10 glucose, oxygenated with 95% O_2 and 5% CO_2, pH adjusted to 7.4 and a temperature of 24–27°C. The concentration of KCl was raised to 6 mM in six experiments.

Monopolar stainless-steel electrodes were placed in contact with the roots and insulated with petroleum jelly for recording. Signals were amplified, filtered, digitized, and stored in a manner similar to that used for EMG recordings. Fictive locomotion was elicited by bath application of *N*-methyl-D, L-aspartate (NMA) (8–30 μM). Serotonin (0.1–5 μM) was added in some experiments. All compounds used in these *in vitro* experiments were purchased from Sigma (St. Louis, MO).

Statistical analysis. Subsequent analyses consisted of rectifying and integrating the recordings (time constant of 10 msec for EMG recordings and 50 msec for root recordings). A threshold function was used to determine the beginning and end of bursts of activity. The threshold was usually set to ~30% of the peak value. The duration of motor bursts was measured, and the middle of bursts was considered to calculate the period (defined as the time between the midpoint of two consecutive bursts) and the phase relationships between the left and right muscle activities (defined as the time between the cycle onset and the next burst in the contralateral muscle, divided by the period of the ongoing cycle). The overall interlimb coordination was evaluated by means of cross-correlation analysis (Statistica 4.5; StatSoft Inc., Tulsa, OK). The correlation coefficient between these signals was calculated and used to evaluate the degree of coactivation of contralateral muscles or ventral roots (Navarrete et al., 2002): the more positive this coefficient, the higher the degree of left–right cocontraction.

All results are given as means ± SEM. The test used for each statistical analysis is indicated in Results and figure legends. The Student's test and the Mann–Whitney test were used to compare two groups of data that followed Gaussian and non-Gaussian distributions, respectively (Prism 2; Graphpad Software Inc., San Diego, CA). One-way ANOVA with the Tukey post-test was used for statistical analysis between more than two groups. Phase data were multiplied by 360° to be analyzed by circular statistics (Oriana; Kovach Computing Services, Anglesey, UK). The statistical parameters used in the present study are based on the concept of the mean vector. A group of observations (or individual vectors) have a mean vector that can be calculated by combining each of the individual vectors. The mean vector has two properties; its direction (the mean angle) and its length (referred to using the letter *r*). The length ranges from 0 to 1; larger numbers indicate that the observations are clustered more closely around the mean than lower numbers. The SEM and thereby the 99% confidence interval are calculated based on the length of the mean vector (*r*). Rayleigh's uniformity test was used to determine whether the phase values were distributed in a uniform manner: a probability less than the chosen significance level of 0.05 indicates that the data are not distributed uniformly and that they show evidence of a preferred direction. The Watson's *F* test was used to compare two samples to determine whether their mean angles differed significantly.

Results

Increased excitability of the CPGs for locomotion after neonatal spinal cord transection

Mechanical stimulation of the tail in the animals with the spinal cord transected at birth elicited long-lasting sequences of airstepping (53.8 ± 3.8 sec; $n = 35$) (Fig. 1*A,B*). Three phases could be distinguished: (1) No clear pattern of motor activity was detectable in the first 2–3 sec after the sensory stimulation. (2) A rhythmic motor pattern was observed in the hindlimbs during the next ~20 sec (Fig. 1*C*). (3) Hindlimb movements were more variable at the end of each sequence, as illustrated by the fluctuations in the period (Fig. 1*C*, from about the 26th step onward). The period increased significantly with time within a given sequence of airstepping (589 ± 13 msec, $n = 336$ for the first 25 steps; 684 ± 16 msec, $n = 289$ beyond the 25th step; $p < 0.001$; Mann–Whitney test). The same stimulation applied to sham-operated neonates triggered only the initial phase mentioned above and a few steps (data not shown) (see also Robinson and Goldberger, 1986a, for data on neonatal cats). This is consistent with recent observations by Lev-Tov et al. (2000) that tail stimulation in hindlimb/tail–spinal cord preparations of the neonatal rat induces six to seven bursts in lumbar ventral roots with left–right alternation. The enhanced motor response after spinal cord section may be attributable to an increased excitability of the CPGs. *In vitro* experiments were performed to test this hypothesis.

The application of NMA (8–20 μM) (Fig. 2*A*) to the *in vitro* isolated spinal cord evoked a stable rhythmic motor pattern in both spinal (Fig. 2*B*) and sham (Fig. 2*C*) animals at P2–P3. Four experiments were performed simultaneously on both a sham and a spinal animal from the same litter. Both spinal cords were in the same chamber and superfused equally. This enabled us to compare the excitability of lumbar networks in the same experimental

conditions. In all of the experiments, the threshold NMA concentration to trigger motor bursts in lumbar ventral roots was lower (average difference, 3.5 ± 1 μM; $n = 4$), and the latency of these bursts relative to the perfusion onset was shorter (average difference, 77 ± 21 sec; $n = 9$ NMA concentrations tested) in spinal animals than in shams (Fig. 2*D*) ($p < 0.01$; paired *t* test). These results demonstrate that the excitability of the CPGs for locomotion is increased after removing the influence of supraspinal structures on the lumbar cord on the day of birth.

Evolution of the locomotor pattern after spinal cord section

We analyzed in spinal animals the pattern of airstepping between the 5th and the 24th steps to examine network operation in the most stable phase (see above). Figure 3 shows recordings from the left and right ankle extensor muscles in two rats, at P3 (Fig. 3A_1) and P6 (Fig. 3B_1). Activities were alternating in the former and in phase in the latter. As a result, the cross-correlogram computed from these activities showed that the peak near zero was negative at P3 (Fig. 3A_2) and positive at P6 (Fig. 3B_2). The distribution of the phase relationships of one EMG burst relative to the contralateral activity observed in all the spinal animals at P1–P3 confirmed the left–right alternating pattern, with an angle of the mean vector of 174.7 ± 5.1° (Fig. 3C_1) ($n = 294$ cycles in seven animals). The direction of the mean vector switched to 20.9 ± 10.1° at P6–P7 (Fig. 3C_2) ($n = 289$ cycles in seven animals). However, phase relationships were much more widely distributed in old than in young spinal rats, as revealed by the shortening of the mean vector with age ($r = 0.23$ at P6–P7 and 0.44 at P1–P3). The difference between the two distributions was highly significant ($p < 0.001$; Watson's *F* test).

The correlation coefficient between the two EMG activities, calculated during each episode of airstepping, was significantly higher at the end of the first postnatal week than in P1–P3 animals (Fig. 4*A*) ($p < 0.001$; *t* test). This indicates that the degree of coactivation of left and right ankle extensor muscles increased with age in spinal rats. The mean period was similar at all ages (Fig. 4*B*) ($p > 0.05$; Mann–Whitney test). The burst duration, normalized to cycle duration, increased significantly during this period (Fig. 4*B*) ($p < 0.001$; *t* test). Both the lengthening of EMG bursts and the change in the motor pattern may contribute to the improvement in the correlation between opposite sides.

At P4–P6, the NMA-induced motor rhythm observed *in vitro* in spinal animals was irregular, as shown by the recordings illustrated in Figure 5A_1 from a pair of lumbar ventral roots. Some bursts of activity in the left and right ventral roots were in phase, whereas in between them, some other bursts (usually of smaller amplitude) exhibited a left–right alternating pattern. The value at the center of symmetry (delay = 0) of the cross-correlogram computed from this activity was positive, indicating that the left and right ventral root activities are in phase overall (Fig. 5A_2).

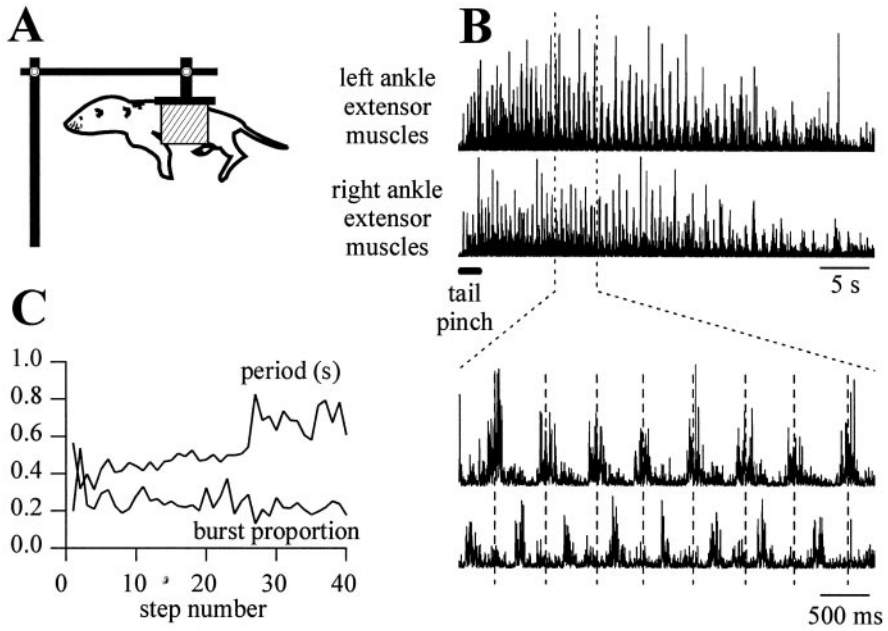

Figure 1. Tail stimulation triggers long sequences of airstepping in cord-transected neonates. *A*, Experimental device adapted from Fady et al. (1998). Animals were supported by means of an adjustable sling. The tail was pinched with a forceps. *B*, Rectified EMG activities from left and right ankle extensor muscles during airstepping induced by tail pinch (*horizontal bar*) in a 2-d-old rat that had been spinalized at birth. *Bottom traces* are shown at an extended time base to illustrate the pattern, consisting of left–right alternation. *C*, Evolution of the period and burst proportion (burst duration/period) in the left ankle extensor muscles during the episode of airstepping illustrated in *B*.

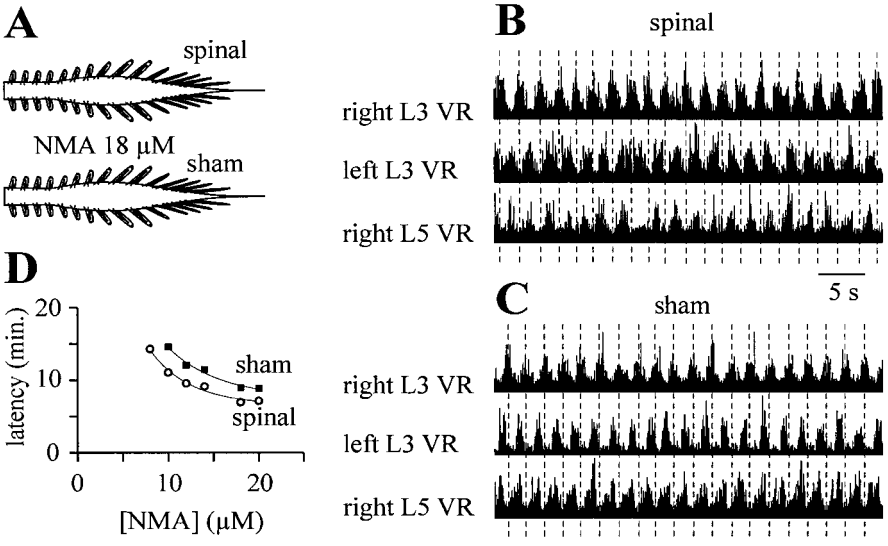

Figure 2. The excitability of CPGs is increased in neonates spinalized at birth. *A*, *In vitro* experiments on spinal cord preparations (T9–S4) isolated from two 2-d-old rats issued from the same litter. One animal had been spinalized at birth (*spinal*), and the other one had been operated in the same way except for the spinal cord transection (*sham*). *B*, *C*, Rectified ventral root (*VR*) activities from the spinal (*B*) and the sham (*C*) animals during fictive locomotion induced by NMA. Locomotor-like activity was characterized by alternation both between right and left ventral root activities and between L3 and L5 bursting on the same side. *Dashed lines* indicate the approximate peak of bursts occurring in the right L3 ventral root. *D*, Relationship between the latency of ventral root bursting (relative to the onset of NMA perfusion) and the NMA concentration in spinal (○) and sham (■) animals.

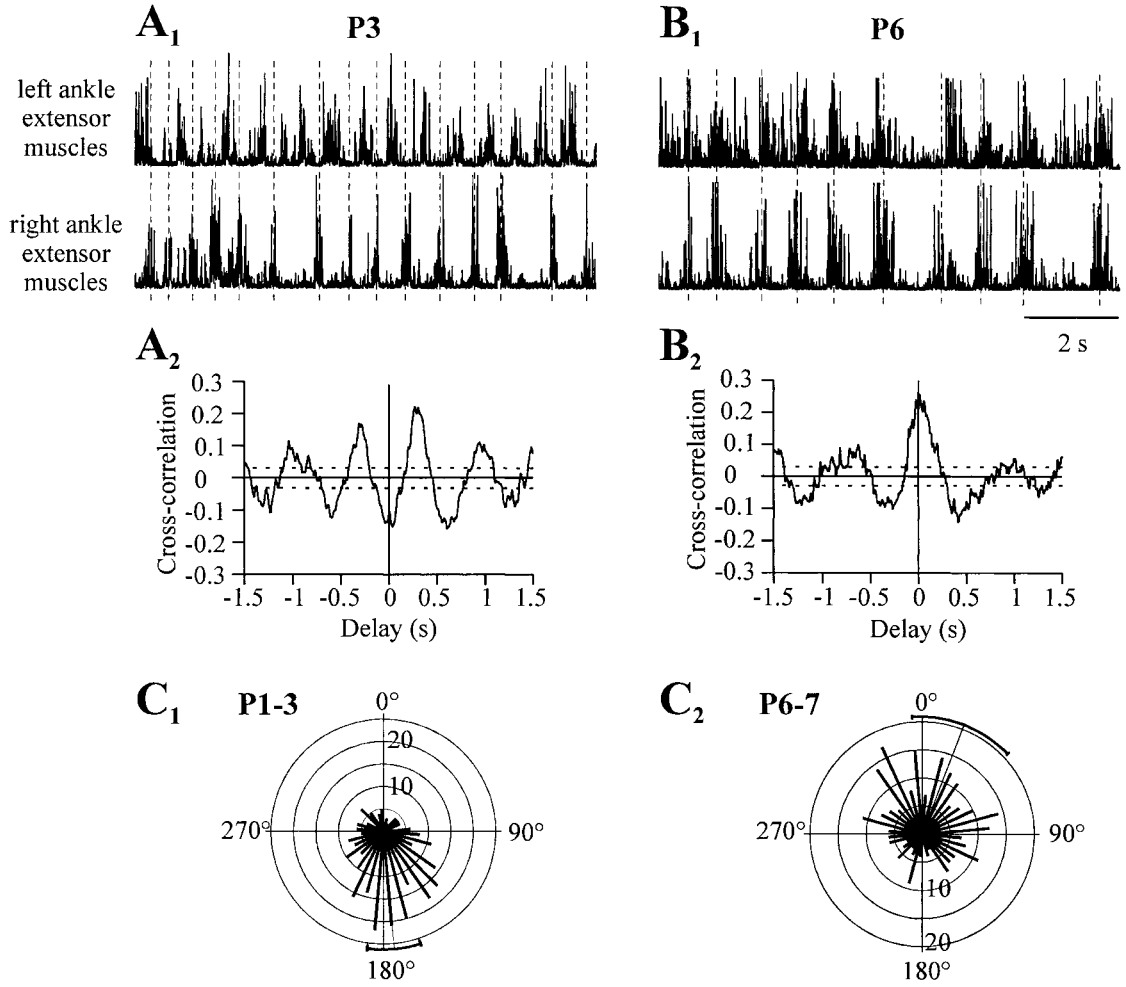

Figure 3. The left–right alternating pattern of airstepping is lost at the end of the first postnatal week in the absence of supraspinal inputs. A_1, B_1, Rectified EMG activity from left and right ankle extensor muscles in 3-d-old (A_1) and 6-d-old (B_1) spinal rats. Traces were selected 3–13 sec after the onset of the airstepping episode. *Dashed lines* indicate the approximate peak of bursts occurring in the right ankle extensor muscles. A_2, B_2, Cross-correlograms between the left and right EMG recordings illustrating the out-of-phase relationship at P3 (A_1) and a synchronization at P6 (B_1) between the bursts recorded in the two extensor muscles. Cross-correlation analysis was computed from 20 sec of airstepping activity. C_1, C_2, Circular histograms showing the distribution of phase relationships between left and right motor bursts at P1–P3 (C_1) and P6–P7 (C_2). *Bars* indicate the number of observations within each class range (width, 10°). Data from seven animals in each age group were pooled; ~40 steps in two episodes were selected for each animal. The mean vector angle was 174.7 ± 5.1° ($n = 294$) at P1–P3 and 20.9 ± 10.1° ($n = 289$) at P6–P7; the length of the mean vector was 0.44 and 0.23, respectively. The 99% confidence interval is illustrated.

Similar results were obtained in all of the spinal animals tested at P4–P6 ($n = 10$) (Fig. 6*A*,*C*), whatever the concentration of NMA used (10–25 μM). The mean correlation coefficient was positive in spinal animals (Fig. 5*B*) (29 applications of NMA in 10 experiments) and negative in shams (10 applications on four spinal cords). The difference between the two animal groups was highly significant ($p < 0.001$; Mann–Whitney test). Thus, the suppression of supraspinal influences on lumbar segments at birth leads to a disorganization of the left–right alternating locomotor pattern within 4–6 d.

Serotonin promotes the left–right alternating pattern

We tested serotonin on P4–P6 spinal animals *in vitro* because it is known to improve the NMA-induced locomotor rhythm when added to the bath (Cazalets et al., 1992). Spinal cord transection markedly reduces the 5-HT level in the lumbar enlargement within a few days (for review, see Schmidt and Jordan, 2000). Because the number of receptors or their binding affinity increases in the absence of 5-HT (Gao and Ziskind-Conhaim, 1993; Kim et al., 1999), the spinal cords of spinalized rats might be more sensitive to 5-HT than those of sham animals. Therefore, we examined the action of 5-HT at concentrations 10–100 times lower than those usually used with this preparation. Even at these low concentrations (~0.1 μM), 5-HT induced a marked increase in the ventral root activity (compare the baselines in Fig. 6*A*,*B*). Although a tonic activity was dominant in 7 of 10 preparations tested with 5-HT at low concentrations, a phasic fluctuation of activity was observed in the remaining three animals (Fig. 6*B*). In the latter experiments, left and right ventral root activities were alternating in the presence of 5-HT, whereas an overall synchronous pattern was visible in the presence of NMA alone (Fig. 6*A*). As a result, the peak near zero in the cross-correlograms was shifted from a positive to a negative value after 5-HT (Fig. 6*C*, *solid* and *dotted lines*, respectively, Fig. 7). The successive correlation peaks were decaying moderately (~25%) in the presence of 5-HT, whereas a substantial decline (~80%) was observed with NMA alone (Fig. 6*C*, *arrowheads*), indicating that 5-HT stabilized the period of the rhythm, in addition to shifting the overall pattern from left–right synchrony to alternation. In six experiments on P4–P6 spinal animals, NMA was applied before and after the concentration of extracellular potassium had been raised from 4 to 6 mM to test whether an increased network excitability could

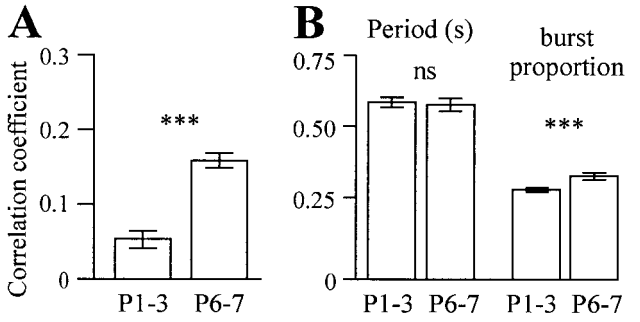

Figure 4. The degree of coactivation of left and right ankle extensor muscles during airstepping increased with age in spinal rats. *A*, Mean correlation coefficient between left and right EMG activities during 10–20 sec episodes of airstepping (40 episodes in 8 animals at P1–P3 and 71 episodes in 12 rats at P6–P7). ***$p < 0.001$; *t* test. *B*, Period of airstepping activity and burst proportion (burst duration/period) in the two age groups. Approximately 20 steps were considered (from the 5th to the 25th step) for analysis in each airstepping episode (314 steps taken into account). *ns*, Not significant; $p > 0.05$; Mann–Whitney test. ***$p < 0.001$; *t* test.

mimic the effect of 5-HT (Fig. 7). The correlation coefficient between left and right ventral root activities was not significantly different in both conditions (0.05 ± 0.09 in 4 mM potassium and 0.18 ± 0.06 in 6 mM; $p > 0.05$; paired *t* test). In three of the six preparations tested with increased potassium concentrations, left and right ventral root activities were alternating in the presence of 5-HT (4 mM potassium) (Fig. 7, *bottom*). This shifted the mean correlation coefficient toward negative values (−0.09 ± 0.09; $n = 6$; $p > 0.05$). Thus, the improvement of left–right alternation by 5-HT in spinal animals may not be related to an increased excitability of locomotor networks but rather to a specific action of 5-HT on left–right coordinating pathways.

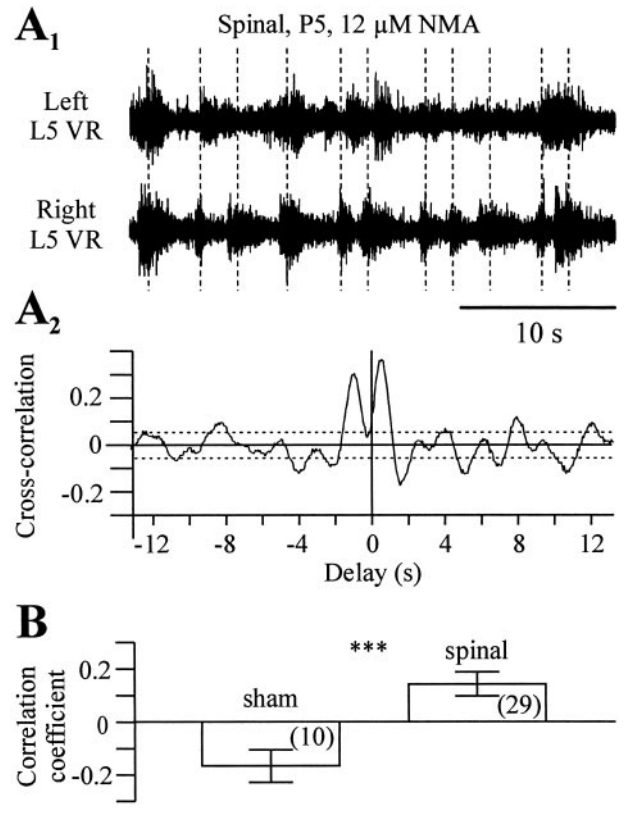

Figure 5. The *in vitro* NMA-induced locomotor-like rhythm is disorganized in spinal rats at P4–P6. A_1, Ventral root (*VR*) activities induced by NMA in a spinal rat at P5. A_2, Cross-correlogram between left and right L5 ventral root signals. The analysis was computed from 2 min of a locomotor-like activity similar to that illustrated in A_1. *B*, Mean correlation coefficient between left and right ventral root activities. Spinals, Twenty-nine applications of NMA in 10 experiments; shams, Ten applications on four spinal cords. ***$p < 0.001$; Mann–Whitney test.

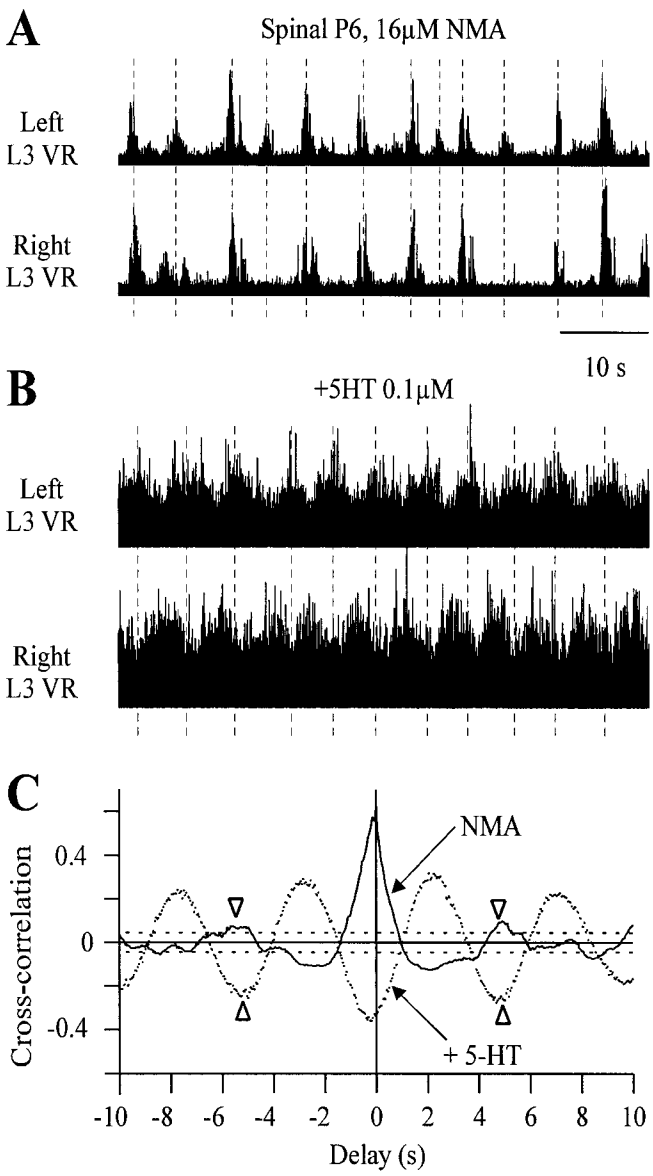

Figure 6. 5-HT *in vitro* switches the NMA-induced disorganized rhythm toward a left–right alternating pattern in spinal rats. *A, B*, Rectified ventral root (*VR*) recordings at P6 in the presence of NMA alone (*A*) or together with 5-HT (*B*). *Dashed lines* indicate the approximate peak of bursts occurring in the left L3 ventral root. *C*, Cross-correlograms between left and right L3 ventral root signals computed from 2 min of activity induced by NMA (*solid line*) or NMA plus 5-HT (*dotted line*). *Arrowheads* point to the successive peaks in the correlogram.

We tested the effects of DOI, an agonist acting directly at 5-$HT_{2A/2C}$ receptors, on the pattern of airstepping of spinal rats at P6–P7 ($n = 6$). The background activity in ankle extensor muscles increased within the first 3–5 min after the DOI injection (data not shown). Airstepping was tested every 3 min before (5–10 trials) and after (10–15 trials) DOI injection. Cross-correlation analysis was performed for each sequence on the first 10 sec of stable rhythm. Figure 8 A_1–A_4 shows the results from a single experiment. No clear peak was visible near zero in the control cross-correlation, and the value at zero was positive (Fig. 8 A_1); a downward peak appeared 5 min after DOI injection (Fig.

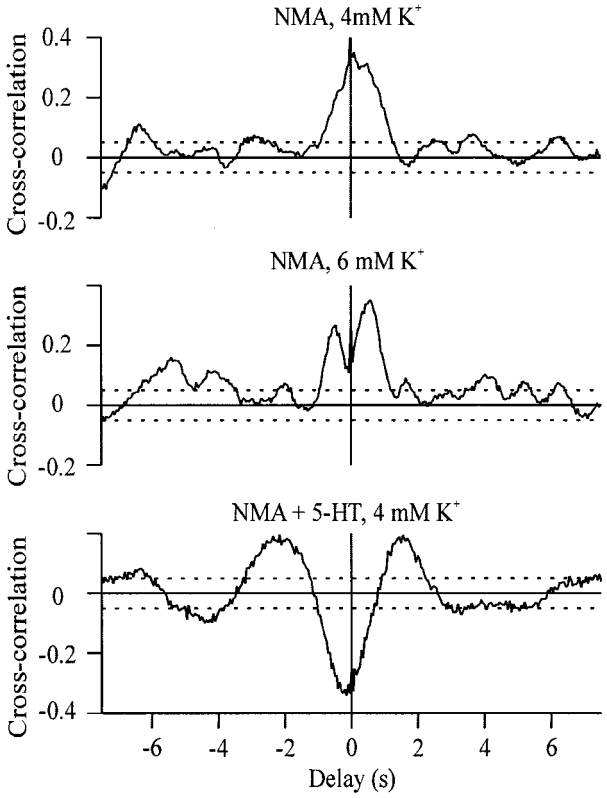

Figure 7. Increasing the excitability of the network does not mimic the effect of 5-HT. Cross-correlograms between left and right L3 ventral root signals computed from 2 min of activity induced by NMA before (*top*) and after (*middle*) increasing the extracellular concentration of potassium from 4 to 6 mM, or NMA plus 5-HT (*bottom*) are shown.

$8A_2$) and shifted to negative values 3 min later (Fig. $8A_3$). The whole cross-correlogram was centered on the *x*-axis 20 min after injection (Fig. $8A_4$). The emergence of a left–right alternating pattern is indicated by a negative peak appearing at the center of the cross-correlation with DOI. This is confirmed by comparing, in the same experiment, the distributions of phase relationships between contralateral EMG activities before (Fig. $8B_1$) and after (Fig. $8B_2$) the injection of the 5-HT_2 agonist.

Phase relationships were analyzed in four animals; they were always modified significantly by DOI ($p < 0.001$; Watson's F test). Before DOI injection, the angle of the mean vector was 89 ± 12.4° ($n = 292$ steps in four animals pooled); however, note that the mean vector was short ($r = 0.19$), indicating a relatively uniform distribution. After DOI, the mean vector direction switched to 177 ± 4° ($n = 442$ steps) and the phase relationships were clustered more closely around the mean angle than before DOI, as indicated by the increased length of the mean vector ($r = 0.42$). The period increased significantly after DOI in two animals (~20%; $p < 0.001$; Mann–Whitney test), whereas no effect was observed in the remaining two animals ($p > 0.05$; Mann–Whitney test).

The correlation coefficient between the two EMG activities was significantly reduced after DOI injection compared with before (Fig. 8*C*) (one-way ANOVA with Tukey post-test; 48 episodes examined after DOI in six animals; 71 episodes without DOI in 12 animals). The correlation coefficient after DOI injection was still larger than at P1–P3 (40 episodes in eight animals). These data show that DOI reduces the degree of cocontraction of the left and right ankle extensor muscles, which is consistent with the emergence of an alternating pattern.

Discussion

Our results demonstrate that the removal of the supraspinal influences on lumbar sensorimotor networks at birth increases their excitability. The left–right alternating locomotor pattern observed in young spinalized animals was gradually lost during the first postnatal week and reappeared after activating 5-HT receptors. These data provide new perspectives on the role of descending pathways in pattern generation.

Spinal cord transection releases locomotion in neonates

Stimulation of the tail triggered long-lasting sequences of airstepping in spinalized neonates (Fig. 1) and only a few steps in sham animals (Lev-Tov et al., 2000). Previous studies on kittens showed that some motor behaviors are suppressed by descending systems in newborns and are therefore released after spinal transection at birth (Robinson and Goldberger, 1986a). At least two mechanisms may account for this difference after spinal transection: (1) Monoamines depress several sensory pathways (for review, see Jankowska, 2001). Therefore, removal of this control by spinal transection may increase the motor effects of tail stimulation. (2) In addition, *in vitro* experiments indicated that the excitability of lumbar networks was increased in spinalized animals (Fig. 2). Spinal cord transection results in the removal of a number of inhibitory descending pathways (Holstege, 1991; for review, see Newman, 1995), thus leading to the disinhibition of spinal networks. The development of segmental inhibition may also be reduced in the absence of descending systems (Robinson and Goldberger, 1986b).

The presence of higher centers may be critical for the expression of a left–right alternating pattern in neonates

The overall pattern of airstepping switched in cord-transected animals from left–right alternation at P1–P3 to synchrony at P6–P7 (Figs. 3–5). This is consistent with a previous study, which showed synchronous airstepping during the first two postnatal weeks in kittens spinalized at birth (Bradley and Smith, 1988). Interestingly, these authors observed more alternation during treadmill stepping than during airstepping (~40 vs ~3% of alternating steps, respectively), suggesting that rhythmic ground contact may promote an alternating gait. Thus, airstepping may accurately represent "the natural capacity of the pattern-generating circuits to regulate stepping" (Bradley and Smith, 1988). The age-related switch observed in the present study in spinal animals toward a more synchronous gait could at initially imply a change in pace from walk to gallop. However, three arguments suggest that this is unlikely to be the case: (1) The period was similar in both age groups. (2) The burst duration relative to period in ankle extensor muscles was increased in older rats, instead of decreased as expected from gallop. (3) The pattern was rather well phase-locked on alternation in younger rats, as indicated by the low variability, whereas it was more variable in older rats. The increased variability at P6–P7 suggests that the overall synchrony is obtained by default and results more from the disorganization of the alternating pattern than from the emergence of a new pattern.

The alternation of muscle activities between the two hindlimbs relies on mutual inhibition of the networks on the two sides of the cord (Grillner et al., 1991). This inhibition involves both crossed reciprocal inhibitory interneurons and a disynaptic pathway with crossed excitatory interneurons (Kjaerulff and Kiehn, 1997). A left–right alternating pattern reappeared at P6–P7, when one of the modulatory inputs from the brainstem was re-

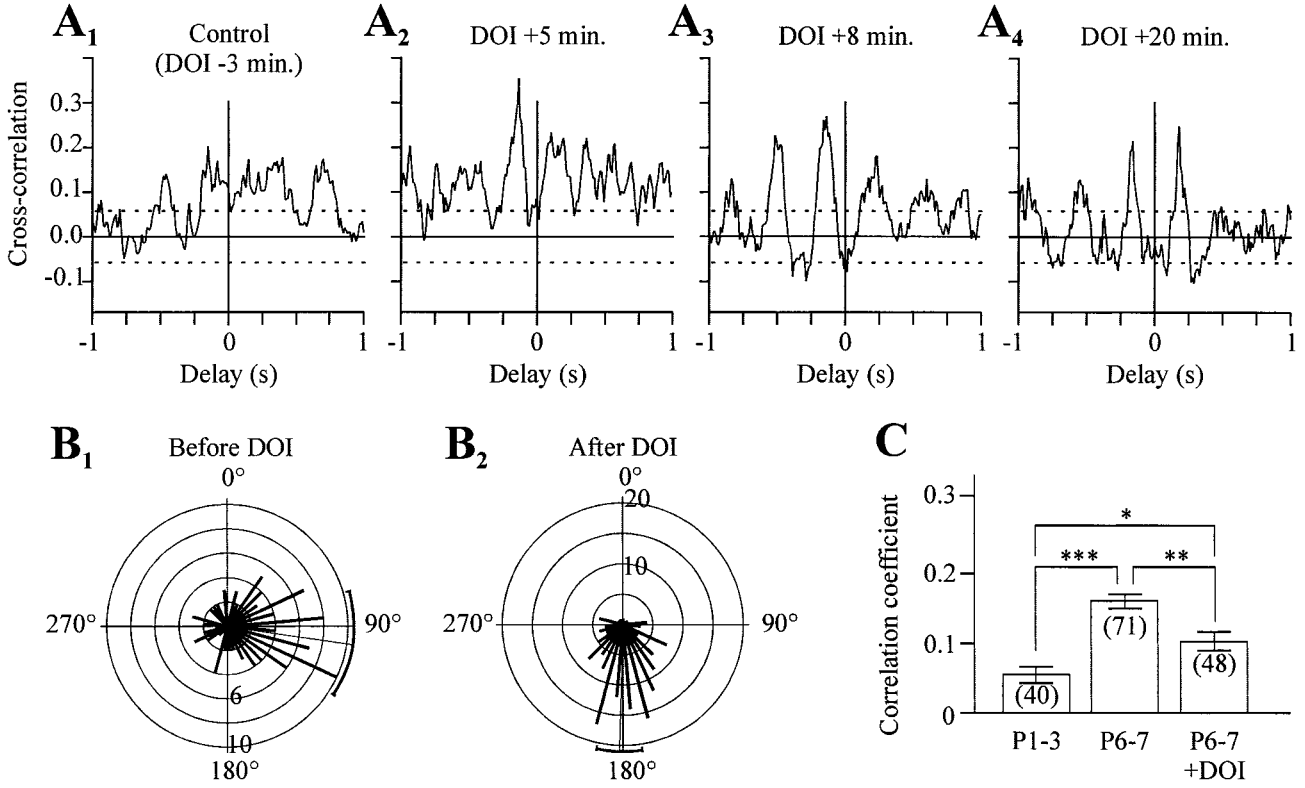

Figure 8. The left–right alternating pattern reappears *in vivo* after the activation of 5-HT_2 receptors. A_1–A_4, Cross-correlograms between left and right ankle extensor EMG signals during airstepping, before (A_1) and at different times (A_3, A_4) after the injection of DOI. The analysis was run on 10 sec of airstepping activity, starting 1 sec after the episode onset. $B_{1,2}$, Distribution of phase relationships between left and right motor bursts before (B_1) and after (B_2) the injection of DOI. Same experiment as in *A*. Mean vector angle: 98.4 ± 9.5°, $n = 107$ (length, 0.4) before DOI; 181.7 ± 4.1°, $n = 153$ (length, 0.68) after DOI. The 99% confidence interval is represented. *C*, Mean correlation coefficient at P1–P3, P6–P7 (identical to Fig. 4*A*), and P6–P7 after DOI injection (48 episodes analyzed in 6 animals). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; one-way ANOVA with Tukey post-test.

stored artificially (Figs. 6–8) (5-HT *in vitro* and DOI *in vivo*). This reversibility suggests that no structural change in the network underlies the disorganization of the pattern after spinalization; instead, descending pathways may control the strength of reciprocal inhibition (McDearmid et al., 1997). Higher centers use a vast array of signaling molecules to modulate spinal locomotor networks. Depending on the target neuron within the network on which these neuromodulators act, they may enable the emergence of distinct motor patterns, in a manner similar to what has been demonstrated in the stomatogastric ganglion (Combes et al., 1999; Nusbaum et al., 2001; Swensen and Marder, 2001; Thirumalai and Marder, 2002). The developmental acquisition of the alternating pattern occurs before birth (Ozaki et al., 1996), at a time when the first monoaminergic projections reach the lumbar enlargement (Rajaofetra et al., 1989). It is assumed that this switch from left–right synchrony to alternation is attributable to a change of glycine-evoked potentials from excitation to inhibition (Nishimaru and Kudo, 2000). In addition to this contribution, the present study suggests that the onset of modulation of left–right coordinating pathways by descending pathways may be an important step in the maturation of the locomotor pattern. Removing this modulation during a critical period after network formation may lead to a dedifferentiation or a disorganization of the pattern. The degree of disorganization may depend on the age at which the spinal cord is transected: hindlimb movements during airstepping are predominantly alternating in kittens spinalized at P14 and synchronous in kittens spinalized at birth (Bradley and Smith, 1988). However, the percentage of synchronous steps remains high, even 2 months after a spinalization at P14 (~40%; see below).

5-HT may regulate the left–right coordinating pathways

5-HT has been proposed to be critical for the selection between alternate reflex pathways in the cat (Aggelopoulos et al., 1996), enabling the crossed inhibition of contralateral motoneurons by group II muscle afferents, to the detriment of a crossed excitation, which is observed in the spinal animal. The present study suggests that 5-HT may strengthen the reciprocal inhibition via 5-$HT_{2A/2C}$ receptors (Fig. 8). Activation of these receptors enhances glycine and/or GABA responses in various spinal neurons in the rat (Xu et al., 1996, 1998; Li et al., 2000) and spontaneous IPSCs in pyramidal neurons (Zhou and Hablitz, 1999). Serotonin may act at a presynaptic level by causing a presynaptic facilitation of glycine release (Mintz and Korn, 1991) in the teleost Mauthner cell, or even by increasing the number of transmitter vesicles available for release (Wang and Zucker, 1998). It should be noted that 5-HT reduces the reciprocal inhibition in *Xenopus laevis* (McDearmid et al., 1997); these effects are likely mediated via presynaptic 5-HT_{1A} receptors (Wedderburn and Sillar, 1994). Whether these receptors mediate inhibitory effects on reciprocal inhibition in the neonatal rat remains to be tested. The net effect of 5-HT *in vitro* is excitatory and may mask an inhibition (Liu et al., 2000).

Serotonin-releasing neurons within the spinal cord are an integral part of the locomotor system in the adult lamprey (Zhang and Grillner, 2000). The spinal 5-HT level may also be critical for

the proper operation of networks in the neonatal rat. A depletion of 5-HT by daily injections of P-chlorophenylalanine from the day of birth in the rat leads, within a few days, to impaired interlimb coordination during locomotion (Myoga et al., 1995) and posture (Pflieger et al., 2002). The NMDA-induced locomotor activity observed in the neonatal rat spinal cord *in vitro* is abolished by 5-HT receptor antagonists, suggesting that an interplay between 5-HT and NMDA receptor actions is important for the production of rhythmic locomotor-like activity in this preparation (Maclean et al., 1998; Maclean and Schmidt, 2001). The 5-HT level is unchanged below the spinal cord transection in adult rabbits during the first few days after the operation; 5-HT then disappears suddenly to almost insignificant values (Andén et al., 1964). A similar time course for the disappearance of 5-HT in the rat spinal cord after neonatal transection would account for both observations made in the present study that the airstepping pattern is alternating at P1–P3 (Figs. 1, 3) and that the activity recorded *in vitro* at the same age after spinal cord transection was quite similar to that obtained in shams, except for the threshold NMA concentration (Fig. 2). In addition, 5-HT may facilitate the left–right coordinating pathways on a long-term basis.

Do descending modulatory inputs shape the locomotor pattern in adult mammals?

The descending modulatory input to spinal locomotor networks may be important not only during ontogeny, as suggested by the present study, but also in the adult. Although it is quite clear that the basic circuitry is restricted to the spinal cord, the importance of descending pathways in modulating synaptic interactions within the network, thereby shaping the locomotor pattern, has been mostly neglected. This is partially attributable to the fact that, after spinalization in adult cats, a hindlimb locomotor pattern close to normal can be obtained by training (Barbeau and Rossignol, 1987; De Leon et al., 1998; Edgerton et al., 2001) or pharmacological stimulation (Jankowska et al., 1967a,b; Grillner and Zangger, 1979) [for fictive locomotion, see Forssberg and Grillner (1973); for treadmill locomotion, see Rossignol (1996)]. However, most experiments used monoamines, and the contribution of these substances to shaping the pattern (i.e., not only to trigger activity in the spinal networks) may have been underestimated. Training may induce an activity-dependent tuning of the inhibitory synaptic strengths in the network (Soto-Trevino et al., 2001; Traub, 2001) that may compensate for the absence of monoamines.

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