Regional Distribution of the Locomotor Pattern-Generating Network in the Neonatal Rat Spinal Cord

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Cowley, K. C. and B. J. Schmidt. Regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord. J. Neurophysiol. 77: 247–259, 1997. The regional distribution of spinal cord networks producing locomotor-like activity, as well as non-locomotor-like activity, was studied with the use of an in vitro neonatal rat preparation. Rhythmic activity was induced by bath application of either serotonin (5-HT), acetylcholine (ACh), N-methyl-D,L-aspartate (NMA), or combined 5-HT/NMA, and was monitored via hindlimb flexor (peroneal) and extensor (tibial) electroneurograms (ENGs) or ventral root recordings. In some experiments, synchronous patterns were produced by the addition of inhibitory amino acid (IAA) receptor antagonists. Selective application of 5-HT to cervical and thoracic cord regions induced rhythmic activity in these segments but failed to evoke hindlimb ENG discharge. Exposure of the isolated lumbar region to 5-HT produced tonic activity only. Application of 5-HT to the whole cord produced locomotor-like activity in hindlimb ENGs that persisted after midsagittal section of the spinal cord from the conus to the thoracolumbar junction. In other experiments, transverse hemisection of the rostral lumbar cord during whole cord exposure to 5-HT abolished rhythmic activity in ipsilateral hindlimb ENGs, suggesting that under these conditions rhythmic activity on one side of the lumbar cord was insufficient to maintain rhythmic activity on the contralateral side. Selective application of NMA or ACh to cervical and/or thoracic cord regions evoked rhythmic activity in these supralumbar segments, as well as rhythmic, but non-locomotor-like, activity in the lumbar region. In contrast to the effect of 5-HT, both NMA and ACh evoked rhythmic activity when applied solely to the lumbar region, and the side-to-side alternation produced by whole cord ACh application was uncoupled by midsagittal lesions of the lumbar region. In the presence of IAA antagonists, the side-to-side coupling of bilaterally synchronous rhythms was maintained despite extensive midsagittal lesions leaving all but one or two segments of either cervical, thoracic, or lumbar cord bilaterally intact, and rhythmic activity could be maintained even in single isolated hemisegments. The effects of 5-HT/NMA were similar to those observed with the use of 5-HT alone, although 5-HT/NMA induced rhythmic activity in hindlimb ENGs when applied selectively to supralumbar regions. The results suggest that 1) a 5-HT-sensitive oscillatory network, capable of producing a locomotor-like pattern of activity, is distributed throughout the supralumbar region of the spinal cord and mediates descending rhythmic drive to lumbar motor centers; 2) NMA- and ACh-sensitive rhythmogenic elements are distributed throughout the spinal cord, including the lumbar region; and 3) the spinal cord contains an extensive propriospinal network of reciprocal inhibitory and excitatory connections characterized by redundantly organized side-to-side projections.

INTRODUCTION

It is well established that the mammalian spinal cord contains the neural circuitry required to generate a variety of rhythmic behaviors, including locomotion. Grillner (1981) proposed that the network producing rhythmic limb movements is composed of multiple “unit burst” generators, each driving a particular group of close synergists acting on a given joint. This concept implies that individual unit burst generators are distributed throughout the spinal cord close to the motoneuron populations they drive. Indeed, there is evidence of a distributed organization of unit generators in the locomotor systems of a number of experimental preparations including the dogfish (Grillner 1974), lamprey (Cohen and Wallen 1980), frog embryo (Khan and Roberts 1982), and embryonic chick (Ho and O’Donovan 1993). Similarly, the neural mechanism generating scratching is dispersed over multiple segments in the cat (Deliagina et al. 1983) and turtle (Mortin and Stein 1989). Some of these systems have also been characterized by a regional hierarchy wherein more rostral lumbar segments have a relatively greater capacity for rhythm generation compared with caudal segments (e.g., Deliagina et al. 1983; Ho and O’Donovan 1993; Mortin and Stein 1989).

Despite the evidence of distributed networks for locomotion in lower animals, and for scratching in the cat, it is unclear whether locomotor pattern-generating circuitry in mammals is also regionally dispersed. Several studies have documented that relatively caudal segments of the lumbar cord are capable of generating rhythmic locomotor activity. For instance, intravenous L-DOPA induces fictive alternation of ankle flexor and extensor activity in cats following acute isolation of the L₄–S₁ segments of lumbar cord, suggesting that at least some components of the locomotor pattern generator are located caudal to the L₅ level (Grillner and Zangger 1979). A small portion of chronically isolated cat lumbar hemiscord (hemisected at the L₅ level combined with midsagittal sectioning from L₃ to S₁) is capable of generating locomotor activity in the ipsilateral hindlimb, although rhythm generation in this preparation may depend, at least in part, on phasic afferent impulses (Kato 1990). In the presence of bath-applied N-methyl-D-aspartate (NMDA), alternating ankle flexor and extensor muscle activity has been documented following acute hemisection and isolation of the L₄–L₅ segment of the in vitro neonatal rat lumbar cord (Kudo and Yamada 1987). These studies, which focused on the rhythm-generating potential of middle and caudal lumbar segments, do not exclude the possibility of a distributed system that could include more rostral portions of the spinal cord. In contrast, however, recent investigations of the neonatal rat spinal cord preparation suggest that caudal lumbar segments participate in neither locomotor rhythm generation.
nor pattern organization; it was concluded these functions are localized in the L1 and L2 segments (Cazalets et al. 1995, 1996). This major disparity in the literature highlights the need for further investigation of the regional organization of mammalian locomotor pattern-generating networks.

In the present study we examine the effect of acute transverse and midsagittal lesions on locomotor rhythm generation in the isolated spinal cords of neonatal rats. In addition, because different neurochemicals preferentially induce specific patterns of rhythmic activity, not all of which are locomotor-like in nature (Cowley and Schmidt 1994b, 1995), we test the hypothesis that different forms of rhythmic behavior are mediated by networks with regionally distinct distributions. The evidence we provide in support of this hypothesis may account for some of the inconsistencies noted in the literature.

Preliminary findings of this work have appeared in abstract form (Cowley and Schmidt 1993; Harder and Schmidt 1992).

METHODS

Experiments were performed on 77 Sprague-Dawley rats (0–7 days). The in vitro bath system, artificial cerebrospinal fluid composition, and the method of isolating the intact spinal cord with hindlimb nerves attached have been described previously (Cowley and Schmidt 1994a,b, 1995). All preparations initially involved the use of a bilaterally strychnine (8–30 μM) bath in combination with either 5-HT, ACh/EDRO, or intact spinal cord from C1 to the conus medullaris (the cone-shaped terminal portion of the spinal cord). Specific segmental levels were identified by counting spinal roots starting with C1 and proceeding caudally. Peroneal nerve recordings were used to monitor ankle flexor activity. Ankle extensor activity was monitored with the use of the tibial nerve or one of the branches to the gastrocnemius, soleus, or posterior tibial muscles. In a few preparations, hip flexor (iliacus muscle) activity was monitored. In other experiments, ventral root recordings from cervical, thoracic, and/or lumbar segments were obtained.

Electroneurograms (ENGs) and ventral root recordings were obtained with the use of glass suction electrodes. Electromyograms were obtained with the use of 0.015-in. diam resin-coated wire. Records were digitized and stored at 5.5 kHz with the use of a Vetter pulse code modulator videocassette adaptor. Further analysis and display of selected segments of taped data was performed with the use of software developed for a Maccomp 5400 computer (sample rate 2 kHz per channel).

With the aid of a surgical dissecting microscope, fine insect pins were used to separate the left and right halves of spinal cord along the midsagittal plane. Transverse and hemitransverse cord lesions were made with iridectomy scissors. The completeness of the lesions was readily confirmed by separation of the sectioned tissue such that an unobstructed view of the black Sylgard base of the chamber was obtained. In some experiments the bath was partitioned by a barrier made of acetate film sealed at the chamber and cord contact edges with petroleum jelly. Absence of barrier leakage was confirmed at the beginning and end of experiments by draining one side of the barrier and using the dissecting microscope to look for fluid seepage across the partition. As an additional precaution, a higher fluid level (and therefore greater hydrostatic pressure) was maintained on the side of the partition not receiving the added neurochemicals.

All neurochemicals were initially dissolved in distilled water and stored as millimolar stock solutions. To obtain the selected final bath concentration of a neurochemical substance, stock solution was directly added in fractionated amounts, with the use of a micropipette, at the periphery of the chamber during vigorous bubbling. All concentrations, including ranges, specified in this report refer to the final bath concentration. Recordings were started once stable patterns of activity had emerged. If the discharge pattern was abolished or disturbed in response to a spinal cord lesion, the neurograms were then monitored for ≥30–60 min (and up to 3 h) to confirm that prelesion activity did not return; during this time repeated attempts were also made to induce rhythmic activity by reapplying the same neurochemical. With the use of a syringe, the bath solution was exchanged repeatedly with normal solution between tests of different substances. Rhythmic activity was induced with the use of either serotonin (5-HT, 10–125 μM), N-methyl-D,L-aspartate (NMA, 4–18 μM), or acetylcholine (ACh, 10–100 μM, in combination with the acetylcholinesterase inhibitor edrophonium EDRO, 100–300 μM). In some experiments, the effect of 5HT applied in combination with NMA was examined. Synchronous rhythms were obtained by adding strychnine (8–30 μM) or bicuculline (14–70 mM) to the bath in combination with either 5-HT, ACh/EDRO, or NMA, as detailed previously (Cowley and Schmidt 1995). All chemicals were obtained from Sigma.

RESULTS

Previously we documented that flexor and extensor hindlimb ENG activity generated by the in vitro neonatal rat spinal cord preparation was usually locomotor-like in response to bath-applied 5-HT, whereas non-locomotor-like patterns were more typical of NMDA- and ACh-induced rhythms (Cowley and Schmidt 1994b). In keeping with our earlier work, the use of the term “locomotor-like” in the present paper is restricted to ENG patterns characterized by alternation of ankle flexor-extensor activity in one hindlimb coupled with extensor-flexor alternation in the contralateral hindlimb (e.g., Fig. 1A). This pattern is similar to that reported for stepping in the adult rat in vivo (Gruner et al. 1980). However, it should be noted that alternation of ankle flexor-extensor activity alone, as monitored in these experiments, does not allow distinction between steppinglike and swimminglike patterns of locomotion (Gruner and Altman 1980). Furthermore, it remains to be shown whether or not the networks that generate other patterns of rhythmic discharge (such as side-to-side alternation of coactivated intra- limb flexor-extensor pairs, or rhythmic coactivation of all flexor and extensor ENGs bilaterally) share components of locomotor pattern-generating circuitry. However, for the purposes of this study, these other patterns of hindlimb flexor and extensor activation are referred to as “non-locomotor-like”.

Because different substances preferentially activate different patterns of activity (Cowley and Schmidt 1994b; Kiehn...
and Kjaerulff 1996), we first compared the effects of spinal cord lesions and bath partitioning on rhythms activated by single neurochemicals (5-HT, NMA, and ACh). As a means of examining the regional distribution of reciprocal excitatory connections within rhythmicogenic circuitry, we used similar lesioning and bath partition methods in other experiments, in which synchronous rhythms were evoked with the use of 5-HT, NMA, or ACh in the presence of inhibitory amino acid receptor antagonists (Cowley and Schmidt 1995). Finally, because 5-HT combined with NMA or the excitatory amino acid uptake inhibitor dihydrokainic acid (DHK) may produce more stable locomotor rhythms than obtained with either substance alone (Cowley and Schmidt 1994a; Kjaerulff et al. 1994; Sqalli-Houssaini et al. 1993), the bath.

Rostral-caudal distribution of the locomotor-like network activated by 5-HT

The minimal substrate required for 5-HT-induced locomotor-like patterns was examined first by completely transecting the spinal cord at several levels starting rostrally and proceeding caudally (n = 7 preparations). Application of 5-HT to the bath solution induced rhythmic locomotor-like discharge in the intact spinal cord. In the example shown in Fig. 1A, locomotor-like activity resumed 20 s after the spinal cord was transected between the T4 and T5 segments (A), indicating that the cervical enlargement and rostral thoracic cord were not essential for generating locomotor activity in the lumbar segments. Similarly, after transverse section between T12 and T13 (performed before the onset of the recording shown in Fig. 1B), rhythmic activity transiently ceased and then reappeared again within 4 min, as shown in Fig. 1B. However, after transection between T13 and L1 (Fig. 1B, B), 5-HT-mediated rhythmic hindlimb ENG activity terminated permanently (observed for up to 2.5 h in some preparations). Repeated attempts to establish rhythmic discharge with the use of progressively higher 5-HT concentrations (from 10 to 100 μM) produced only tonic activity. In contrast, ACh (Fig. 1D) and NMA were capable of eliciting rhythmic activity in the same isolated lumbar cords (see below), suggesting that the lack of 5-HT effect was unrelated to any nonspecific depression of neural activity that might result from an acute cord lesion. Similar results were obtained in all seven preparations. In two of the preparations, cervical ventral roots were also monitored. These roots showed continued rhythmic discharge after transection between T13 and L1, suggesting that the failure of 5-HT to induce rhythmic activity in the isolated lumbar cord cannot be accounted for by inadequate concentrations of 5-HT in the bath.

As illustrated in Fig. 1, A and B, the frequency of 5-HT-induced locomotion decreased during the serial transections. The frequency decreased by ~10% after lesioning between T4 and T5, and by 25% after transection between T13 and L1 (Fig. 1C, B). Similar decreases in frequency were observed in all seven preparations. However, it should be noted that 5-HT-induced rhythms in unlesioned control preparations also displayed a decline in frequency with time. For instance, the rhythm frequency in the control preparation shown in Fig. 1C decreased by 25% 8 min after the onset of 5-HT-induced locomotion, and continued at this frequency for the remainder of the 30-min observation period. In two other unlesioned preparations, 5-HT-induced locomotor rhythm frequency decreased by 20 and 40%, respectively, after 5 min of observation; the latter preparation showed a further decline to 25% of the original frequency at the end of 30 min. Because rhythm frequency spontaneously decreased in intact control preparations, we are unable to conclude that the decline in frequency observed during the lesioning experiments was specifically related to the effects of acute spinal cord transection and/or a reduction in the size of the rhythm-generating network.

In three other experiments a barrier was placed between...
T₁₃ and L₁ to partition the bath chamber into two compartments. Addition of 5-HT to the rostral side of the partition induced rhythmic activity in cervical segments but not in lumbar segments. Application of 5-HT in graded concentrations (from 10 to 100 μM) to the caudal compartment alone induced tonic discharge in hindlimb ENGs but no activity on the rostral side of the partition. The latter observation is consistent with the effect of 5-HT on the lumbar cord isolated by means of complete transection at the T₁₃/L₁ level (see above). Only when 5-HT was added to both the rostral and caudal compartments did rhythmic hindlimb ENG activity develop, in contrast to the effects of ACh and NMA (see below).

The above results suggested that supralumbar regions of the spinal cord may contain the rhythmogenic circuitry activated by 5-HT that is required for hindlimb locomotor output. Alternatively, the lumbosacral cord may also contain part of the 5-HT-sensitive circuitry, but a critical mass of intact network in this region is required for successful activation. In this case, residual network elements present in the isolated lumbosacral cord, after a T₁₃/L₁ transection, may be insufficient for rhythm generation. Two sets of experiments were performed to address this issue. First, the caudal portion of the spinal cord, and therefore any rhythmogenic neural substrate contained therein, was removed by transecting between L₂ and L₄ in one experiment and between L₄ and S₁ in a second preparation. Rostral transections were then started at the T₁₀ level and continued caudally one segment at a time. If the caudal transection removed critical network mass, then one would predict that the rostral limit of lumbar cord, which contained the minimal amount of network required for activation, should shift above the T₁₁/L₁ junction. However, as was the case in preparations with intact caudal lumbosacral cord, 5-HT-induced locomotor activity was still maintained in both preparations until the descending series of rostral transections reached the T₁₃/L₁ junction.

It is possible that the L₅/L₆ and L₆/S₁ transections had no effect on critical network mass in the lumbosacral cord simply because the caudal segments isolated by these transections did not contain 5-HT-sensitive rhythmic circuitry. Therefore in a second set of experiments we examined the rhythmic responsiveness of small portions of rostral lumbar cord to 5-HT. Motor axons to the iliacus muscle (hip flexor) exit the spinal cord via L₂ and L₃ ventral roots, whereas tibial and peroneal motor axons exit predominantly via the L₄ and L₅ ventral roots (Cowley and Schmidt 1994a). Therefore we used the iliacus electromyogram in combination with peroneal and tibial ENG recordings (Fig. 2A, prelesion) to monitor the effect of midlumbar transverse lesions in two experiments. In both preparations, transection between L₃ and L₄ terminated 5-HT-induced activity in the tibial and peroneal nerves, as expected, whereas rhythmic iliacus muscle activity continued. Rhythmic activity also persisted in the iliacus muscle after subsequent transection between T₁₂ and T₁₃ left only four cord segments intact (T₁₁−L₁ inclusive, as shown in Fig. 2B, left). Thus just a few segments of lumbar cord were capable of developing rhythmic activity provided continuity was maintained with the supralumbar region (T₁₃ segment in this case), even though 5-HT application to the entire lumbosacral cord, transected or partitioned at the T₁₃/L₁ junction, failed to generate rhythmic activity. After transection between T₁₃ and L₁ (Fig. 2B, right) rhythmic activity terminated permanently. Repeated applications of 5-HT also failed to produce rhythmic activity in the isolated L₁−L₅ segment. Therefore these observations support the concept of a distributed organization of 5-HT-sensitive rhythmogenic circuitry.
within the cervicothoracic cord, rather than a mechanism localized to the most caudal region of the thoracic cord or restricted to the rostral lumbar segments as suggested by Cazalets et al. (1995).

**Effect of midsagittal spinal cord lesions on 5-HT-induced locomotor-like activity**

To examine whether bilaterally distributed components are essential for generating and coordinating 5-HT-induced rhythms, the left and right sides of the spinal cord were separated along the midsagittal plane. The example in Fig. 3 shows that flexor-extensor and left-right relationships were maintained after midsagittal section from the conus to L1 inclusive. Left-right and intralimb flexor-extensor coordination was also maintained in eight other preparations after midsagittal section from the conus to the thoracolumbar junction region (L2/L3, n = 2; T13/L1, n = 3; and T12/T13, n = 3). Attempts to extend the midsagittal section more rostrally in these experiments resulted in an abrupt loss of 5-HT-induced rhythms. Repeated applications of 5-HT failed to reestablish rhythmic activity. Two of the preparations with midsagittal lesions of the lumbosacral spinal cord (extending to T13/L1) were also transected in the thoracic region, at the T4/T5 and T12/T13 junctions, respectively, without effect on flexor-extensor and left-right discharge in the hindlimb nerves. Thus even one bilaterally intact segment (T13) was capable of maintaining left-right coordination in the lumbar region. When midsagittal separation was started at C3 and extended caudally, the 5-HT-induced pattern, including the left-right phase relationship, was preserved until the lesion reached the T13/L1 junction (n = 2), at which point the rhythmic activity stopped.

The observation that 5-HT-induced rhythms were abolished by midsagittal lesions that extended into the thoracolumbar junction, regardless of whether the lesion originated from a rostral or caudal approach, suggested that cross projections in this region may be necessary to maintain locomotor network oscillatory activity. Therefore the effect of midsagittal section restricted to the thoracolumbar region (T10–L4, T8–L1, and T12–L1 inclusive) was examined in three preparations. These lesions disrupted neither hindlimb rhythm production nor coordination (Fig. 4). Attempts to extend the midsagittal lesion further rostrally, in the T10–L4 and T12–L1 preparations, resulted in the loss of ENG activity on one side. However, the remaining unilateral rhythmic activity (flexor-extensor alternating) continued until the midsagittal separation reached the T3 and C6 levels, respectively, in these two preparations. The midsagittal lesioning results are compatible with a distributed and redundant network organization, in which cross projections in the thoracolumbar region are not essential for rhythmogenesis, provided similar connections are preserved in the lumbosacral and cervicothoracic regions.

The results of the spinal cord transection (e.g., Figs. 1 and 2) and bath partition experiments suggest that 5-HT-induced locomotor rhythogenesis in the hindlimbs depends on network activation in the supralumbar region. However, the combined results of the midsagittal section experiments (e.g., Figs. 3 and 4) suggest that cross connections throughout the spinal cord, including the lumbar segments, contribute to locomotor network operation. That is, locomotion was obtained in the presence of midsagittal lesions in the thoracolumbar junction region only if spinal cord segments both rostral and caudal to the lesions remained bilaterally intact. However, the nature of the apparent permissive effect due to preserving cross connections in the lumbar cord was unclear. In particular, we examined whether 5-HT-induced rhythms were abolished by midsagittal sections restricted to the rostral lumbar segments as suggested by Cazalets et al. (1995).
side, whereas the capacity for alternating tibial and peroneal nerve activity was preserved on the contralateral side (Fig. 5). Once again, the observations are consistent with 5-HT activation of a predominately supralumbar network that generates descending (ipsilateral) drive to the lumbar cord. In contrast, any excitation that might be transmitted by segmental cross connections from the contralateral lumbar cord seems insufficient to activate or maintain rhythmic activity on the opposite side.

**Effect of spinal cord lesions and bath partition on ACh- and NMA-induced motor rhythms**

In contrast to 5-HT-evoked activity, rhythms activated by NMA or ACh are often non-locomotor-like in pattern (Cowley and Schmidt 1994b). Therefore it was of interest to examine whether the neural substrate(s) underlying NMA- and ACh-activated rhythms have the same, or a distinct, distribution compared with the network activated by 5-HT. After 5-HT-induced locomotor rhythms were abolished by complete spinal cord transections between the T₁₃/L₁ segments, and 5-HT was washed out from the bath, application of either ACh (in combination with EDRO) or NMA induced rhythmic hindlimb discharge in the isolated lumboSacral cord of all seven preparations tested. The pattern however, was not locomotor-like in quality (e.g., Fig. 1D). In two other preparations, with transverse hemissections at the L₁/L₂ level, endogenous excitatory amino acid transmission was enhanced by the bath application of the uptake inhibitor DHK. Consistent with the effect of NMA on the bilaterally intact lumbar cord, DHK restored rhythmic activity in ipsilateral hindlimb ENGs, despite the same transverse hemissection having previously abolished 5-HT-induced activity (as shown in Fig. 5). Complete transections were made at a more caudal level (L₄/L₅ junction) in eight other preparations. In each case, the transaction failed to abolish NMA-induced rhythmic activity, as monitored by lumbar ventral root recordings caudal to the transection (Fig. 6).

In all five preparations examined, selective NMA or ACh application to supralumbar cord regions (bath partitioned at C₈/T₁, n = 1; T₉/T₁₀, n = 1; and T₁₃/L₁, n = 3) elicited rhythmic activity in the cervicothoracic segments, as well as rhythmic, but non-locomotor-like, activity in the hindlimb ENGs. This observation contrasts with the failure to activate hindlimb rhythmicity by applying 5-HT to the supralumbar cord alone (see above). We also observed that application of either ACh or NMA to the lumbar cord alone (bath partitioned at the T₁₃/L₁ level, n = 3) induced rhythmic hindlimb ENG activity. Thus the lesioning and bath partition experiments both indicate that the isolated lumbar cord has the inherent capacity to generate rhythmic activity in response to NMA or ACh, but not 5-HT.

Left-right coordination of ACh-induced rhythmic hindlimb ENG activity was examined before and after isolation of the lumboSacral enlargement (transected at T₁₃/L₁, n = 3). In each instance the side-to-side relationship became uncoupled after the transection, suggesting that cross connections in supralumbar regions contribute to maintaining side-to-side relationships during ACh-induced activity. Compatible with the results obtained in these experiments, transsects at the T₁₃/L₁ transected preparations was another experiment in which rostrocaudal sansagittal section from C₁ to T₁₃ inclusive uncoupled the left-right coordination of ACh/EDRO-induced hindlimb rhythmic activity. We did not examine coupling between hindlimbs for NMA-induced rhythms because left-right phase relationships tended to be labile during trials of NMA application, as previously reported (Cowley and Schmidt 1994b).

ACh/EDRO-induced rhythmic hindlimb ENG activity persisted after separation of the left and right halves of the lumbar enlargement (midsagittal lesion from the conus to the T₁₂/T₁₃ junction) in seven of seven preparations. In four experiments side-to-side coordination was recorded both before and after the lesion. One of the four preparations showed bilateral rhythmic activity, but without a consistent side-to-side phase relationship, even before the lesion was made. Three of the four preparations demonstrated a left-right phase relationship before the lesion, but this activity became uncoupled after midsagittal sectioning. The unlesioned preparation shown in Fig. 7A generated phase-related left-right alternation, but intralimb flexor-extensor coactivation, typical of ACh-induced non-locomotor-like patterns (Cowley and Schmidt 1994b). The same unlesioned preparation also demonstrated a locomotor-like pattern in response to 5-HT (Fig. 7B). After midsagittal separation of left and right halves of the spinal cord, from the conus to T₁₃ inclusive, ACh/EDRO-induced rhythm generation was preserved, but at a much slower frequency (Fig. 7C). However, the left-right phase relationship was abolished. Before the midsagittal lesion was made the onset of left tibial ENG discharge regularly occurred after ~60% of the cycle period had elapsed (as measured from the onset of one right tibial ENG burst to the next, Fig. 7D). After the lesion, the onset
of rhythmic discharge in the left tibial nerve occurred with no consistent phase relationship to right tibial ENG discharge (Fig. 7D).

Subsequent transection of one side of the split lumbar-sacral cord, at the rostral lumbar level, completely isolated the corresponding hemisegment from the rest of the nervous system. ACh-induced rhythmic activity continued in lumbar hemisegment that remained in continuity with the supralumbar spinal cord, but ceased in the completely isolated contralateral hemisegment, in three of the four preparations tested. Thus, although the isolated bilaterally intact lumbar-sacral cord can regularly generate rhythmic activity in response to ACh application, the isolated lumbar-sacral hemisegment has less capacity for ACh-induced rhythmogenesis. Once interconnections with the contralateral lumbar cord are disrupted, descending drive may be required to support rhythm production in the lumbar-sacral hemisegment.

Further experiments demonstrated that midsagittal lesions limited to the thoracolumbar region (T13–L1 inclusive; n = 3) also uncoupled left-right hindlimb coordination during ACh-induced activity. In one of these experiments, 5-HT was applied after washout of ACh; phase-related side-to-side ENG discharge compatible with locomotion was produced. This observation was consistent with the effects of 5-HT in three other preparations with midsagittal sections limited to the thoracolumbar junction region (see above and Fig. 4). In summary, these findings suggest that cross connections located throughout the rostrocaudal axis of the spinal cord may be critical for maintaining left-right relationships within the ACh-sensitive network; in contrast, a relatively redundant system of cross projections appears to organize side-to-side coordination during 5-HT-induced rhythms.

**Effect of transverse and midsagittal lesions on synchronous motor rhythms**

Rhythmic patterns elicited by application of NMA, ACh, or 5-HT become synchronous during γ-aminobutyric acid (GABAA) or glycine receptor blockade (Cowley and Schmidt 1995). Strong and mutually excitatory links among functionally and regionally distinct motoneuron populations are likely to underlie this highly characteristic and reproducible pattern of neural rhythm activation. In an effort to learn more about the distribution of these excitatory interconnections, experiments involving the application of bicuculline and strychnine were performed.

We first examined whether synchronous rhythms could be generated within small portions of spinal cord that included the ankle flexor and extensor motoneurons in particular. Bilaterally synchronous hindlimb ENG activity, produced by the glycine receptor antagonist strychnine (n = 1) or the GABAA receptor antagonist bicuculline (n = 2) in combination with NMA, persisted after transection at the L3/L4 junction. Additional lesions in the lumbar regions demonstrated that just two segments of isolated tissue (L4–L6 inclusive) readily maintained synchronous hindlimb ENG discharge (e.g., Fig. 8A). Even single hemisegments of spinal tissue (L4 and L6) generated rhythmic discharge in the presence of inhibitory amino acid antagonists and NMA (Fig. 8B). Similarly, short lengths of cervical (C4–C6 inclusive), thoracic (T6–T10), and rostral lumbar (L1–L3) spinal cord were capable of producing synchronous rhythmic activity. Thus, during inhibitory amino acid receptor blockade, the synchronous rhythmic activity produced in the hindlimbs does not require contributions from supralumbar spinal tissue, in contrast to 5-HT-induced locomotor-like patterns.

We then examined the distribution of the cross connections that mediate side-to-side coupling of synchronous rhythms. In particular, we wished to determine whether any specific rostrocaudal level of the cord contained cross projections that were essential for maintaining left-right synchrony in the hindlimbs. Thus the effect of midsagittal lesions at various levels was examined in 20 preparations treated either with strychnine (n = 7), bicuculline (n = 12), or both strychnine and bicuculline (n = 1). Rhythmic activity was produced by either 5-HT (n = 5), ACh (n = 6), NMA (n = 8), or strychnine alone (n = 1). Bilateral synchrony
of hindlimb rhythmic activity was maintained in five of five preparations after mid sagittal lesions split the entire spinal cord except for one or two thoracic cord segments at various levels (e.g., Fig. 9, A1 and A2). After complete anatomic separation of the left and right sides of the spinal cord, rhythmic activity as well as intralimb flexor-extensor synchrony was maintained (Fig. 9B1), although side-to-side synchrony was abolished, as expected (Fig. 9B2).

We also determined whether progressive midsagittal separation of the cord, starting at the rostral or caudal end, was associated with a progressive decline in the ability of the network to maintain synchrony between left and right hindlimb motor nuclei (n = 13). A progressive out-of-phase shift of left-right discharge would imply the presence of a dominating or “leading” region within this rostrocaudally distributed system. However, as shown in Fig. 9C, no evidence of a shift in the left-right phase coupling of hindlimb ENGs was observed during rostral extension of cord separation, which started at the conus and ended when C1–C2 were the only segments still bilaterally intact (Fig. 9C). In two preparations, midsagittal lesions starting at C1 extended caudally into the lumbar segments. These lesions also failed to disrupt the left-right phase relationship of synchronous hindlimb activity (e.g., Fig. 9D).

Thus it appears that excitatory cross projections within just one or two segments at virtually any rostrocaudal level of the cord can mediate the synchronous coupling of sideto-side activity. These data, together with the results of the experiments in which isolated hemisegments were used, described above, suggest that the generation of rhythmic synchronous activity within each hemisegment can occur independent of any connections with the contralateral side of the network.

**Effect of spinal cord lesions and bath partition on combined 5-HT/NMA-induced rhythms**

Application of 5-HT combined with NMA (or DHK) has proven to be a useful means of establishing locomotor-like patterns in the in vitro neonatal rat whole spinal cord preparation (e.g., Cowley and Schmidt 1994a; Kjaerulff et al. 1994; Sqalli-Houssaini et al. 1993). Moreover, Cazalets et al. (1995) recently examined the effects of application of the 5-HT/NMA combination to specific segments of the lumbar cord in this preparation (see Discussion). However, selective application of these neurochemicals to supralumbar regions has not yet been reported. Therefore it was of interest to investigate the distribution of the rhythmogenic network activated by 5-HT/NMA in the present series, and to compare the results with observations obtained with the use of 5-HT alone.

Although NMA alone elicited rhythmic activity in caudal lumbar segments isolated by transection at the L3/L4 junction (Fig. 6), subsequent addition of 5-HT (10–125 μM) to the NMA-containing bath solution was associated with loss of rhythmicity caudal to the transection (n = 5). Instead, the NMA-induced rhythmic activity was replaced by tonic discharge, similar to the effect of 5-HT alone on the isolated lumbar cord. However, rostral to the transection, rhythmic activity monitored on cervical, thoracic, and rostral lumbar (e.g., L2) ventral roots continued. Further transection at T13/L1 in one preparation, and at T12/T13 in another, resulted in the loss of rhythmic activity on the L2 ventral root, but not on cervical and thoracic roots. In one preparation additional serial transections yielded short lengths of isolated cervical (C5–C6 inclusive) and thoracic (T5–T9 inclusive) cord that were still capable of generating rhythmic activity in the presence of 5-HT and NMA. Consistent with the effect of 5-HT alone, rhythmic lumbar activity evoked by the 5-HT/NMA combination persisted after midsagittal lesions through the lumbar enlargement (conus to T13, n = 1; or conus to L1, n = 1). In summary, comparison of these results with those obtained with the use of 5-HT or NMA alone suggests that the rhythmogenic network activated by the 5-HT/NMA combination shares more features in common with the 5-HT-sensitive network than with circuitry activated by NMA alone.

Application of 5-HT/NMA to the rostral side of baths partitioned at T8/T10 (n = 2) or C6/T1 (n = 2) produced rhythmic activity throughout the spinal cord as monitored via cervical, midthoracic, rostral, and caudal lumbar ventral root records (e.g., Fig. 10). The addition of 5-HT/NMA caudal to partitions established at the L3/L4 level produced only tonic activity, consistent with effect of 5-HT/NMA application to the transected (at L3/L4) spinal cord (see above). It appears that the network activated by the 5-HT/NMA combination is distributed throughout the supralumbar region, similar to the distribution of the network induced by 5-HT alone.
DISTRIBUTION OF THE LOCOMOTOR NETWORK

**DISCUSSION**

These results demonstrate the distributed nature of networks generating motor rhythms, including locomotion, in the mammalian spinal cord. In addition, the data suggest that different patterns of discharge, activated by specific neurochemicals, are mediated by neural circuits with heterogeneous regional distributions.

*Locomotor network is distributed in the supralumbar region of the spinal cord*

Previous work comparing the effects of bath-applied 5-HT, NMA, and ACh to the entire spinal cord indicated that a locomotor-like pattern of flexor and extensor activity is most commonly elicited by 5-HT (Cowley and Schmidt 1994b). In the present series, components of the 5-HT-sensitive network producing a locomotor-like pattern of hindlimb flexor and extensor activity were found to be distributed throughout the supralumbar region of the spinal cord. The lumbar cord itself displayed no inherent rhythmic response to 5-HT application. Supralumbar circuitry not only generated oscillatory drive, but also coordinated left-right interactions for more caudal (lumbar) spinal cord regions, as demonstrated by the results of midsagittal spinal cord sectioning experiments.

Our combined results, derived from a variety of transection and bath partition experiments (n = 14 total), suggest that the caudal boundary of the 5-HT-sensitive distributed network is located near the T12/L1 junction. Similarly, we found no evidence of rhythm production following application of the 5-HT/NMA combination to lumbar segments, although our 5-HT/NMA results are based on a smaller number of observations compared with the 5-HT data. It should be noted that Cazalets et al. (1995) obtained rhythmic activity in response to 5-HT/NMA application to the rostral lumbar segments, as did Kjaerulf and Kiehn (1994). Therefore, despite our negative results, we hesitate to exclude the possibility that rhythmicogenic circuitry responsive to the 5-HT/NMA combination may extend caudally into the rostral lumbar region. Possible reasons for the discrepancy between our results and those reported previously are discussed below.

Regardless of the exact spinal cord level containing the caudal limit of the distributed network, our observation that caudal lumbar segments fail to generate rhythmic activity on exposure to 5-HT/NMA is consistent with the results of...
Cazalets et al. (1995), who reported no evidence of inherent rhythm-generating capacity in segments caudal to the L2 level. Similarly, Kjaerulf and Kiehn (1994) found no rhythmic activity, or only slow, low-amplitude modulation, in isolated caudal lumbar segments exposed to the 5-HT/NMA combination. These combined observations are compatible with studies of locomotion in the chick embryo (Ho and O’Donovan 1993) and scratching in the cat (Deliagina et al. 1983) and turtle (Mortin and Stein 1989), which have shown dominance of the rhythmogenic capacity of rostral segments over more caudal regions.

The concept that the central pattern generator for locomotion is “not segmentally distributed but is restricted” to the L1 and L2 segments (Cazalets et al. 1995) is incompatible with the present results. In contrast to the study reported by Cazalets et al. (1995), we applied neurochemicals to supraspinal portions of the spinal cord, in addition to testing their direct effects on the lumbar cord. Thus rhythmic activity was induced in the cervical and thoracic spinal cord when 5-HT was applied to the isolated cervicothoracic region. Lumbar rhythmicity failed to occur after selective application of 5-HT to the lumbarosacral cord (which included the L1 and L2 segments), but was evoked in response to application of 5-HT to the entire spinal cord excluding the T10–L1 region. These observations not only suggest that the network is distributed in supraspinal regions, but also indicate that activation of rostral lumbar cord segments by the applied neurochemicals is not critical for rhythm generation. We also demonstrated combined 5-HT/NMA effects that are incompatible with a restricted L1/L2 localization for the locomotor network oscillator. As was observed in the presence of 5-HT alone, 5-HT/NMA elicited rhythmic activity in cervical and thoracic segments despite isolation from more caudal regions (including L1 and L2) by bath partition or cord transection. The development of rhythmic activity in the lumbar cord in response to selective application of 5-HT/NMA to the cervical or cervicothoracic segments further argues against a model characterized by hindlimb rhythm generators strictly localized to the L1/L2 segments. In addition, L2 ventral root rhythmic activity was abolished by transections at the T12/T13 or T13/L1 junctions despite continued exposure of the spinal cord below the lesion to 5-HT/NMA. Finally, midsagittal lesions from the conus through the L4 segment inclusive, or restricted to the thoracolumbar region in particular (e.g., T10–L4), had no effect on hindlimb rhythm generation or coordination. In summary, these observations strongly favor a system in which hindlimb locomotor output is under the influence of a distributed and predominantly supraspinal network. An anatomically dispersed organization of this type is compatible with the multiple “unit burst” concept of Grillner (1981) and is well suited to integrate forelimb and hindlimb rhythmic activity. In addition, this model can readily accommodate thoracic oscillatory mechanisms, as required for the generation of the rhythmic activity that occurs in axial muscles during locomotion (Ho and O’Donovan 1993; Koehler et al. 1984).

Although application of 5-HT to the whole cord produced locomotor-like activity in the hindlimb ENGs, addition of 5-HT to the cervicothoracic cord alone induced rhythmic activity only in supraspinal regions. Selective exposure of the lumbar region to 5-HT produced only tonic activity on hindlimb ENGs. Therefore relatively nonspecific background excitation of hindlimb motor centers, provided in this case by direct actions of 5-HT on the lumbar cord, may be required to bring lumbar circuitry above threshold for responding to the descending rhythmic drive (provided by the supraspinal oscillatory network). Similarly, Cazalets et al. (1995) reported that when 5-HT/NMA application to the L1/L2 segments failed to induce ventral root activity in caudal lumbar region, nonspecific electrical stimulation of the coccygeal spinal cord brought L1 motoneurons to threshold for rhythmic firing. Because exposure of the cervicothoracic spinal cord to a combination of 5-HT and NMA can induce hindlimb rhythmic activity in the absence of direct neurochemical excitation of the lumbar cord (e.g., Fig. 10), the 5-HT/NMA combination may be a more potent activator of supraspinal rhythmogenic circuitry, and its associated descending drive, than is 5-HT alone. This hypothesis is supported by the observation that locomotor-like rhythms induced by 5-HT, combined with NMA or DHK, are often better developed and more sustained than those induced with the use of either substance alone (Cowley and Schmidt 1994a; Sghalli-Houssaini et al. 1993).

Why 5-HT fails to induce rhythmic activity when applied directly to the lumbar spinal cord of the neonatal rat is unclear. Locomotor circuitry in Xenopus displays a rostrocaudal gradient of development and sensitivity to 5-HT in the early postembryonic stage, corresponding with the caudal growth of 5-HT-containing raphe projections (Sillar et al. 1992). In the rat, rhythmogenic circuitry is substantially reorganized by embryonic day 18 (Kudo et al. 1991) and 5-HT-induced patterns of locomotion remain stable in the immediate postnatal period (postnatal days 0–4) (Kiehn and Kjaerulf 1996). Immunohistochemical studies in rat indicate that descending 5-HT fibers enter the ventral and intermediate gray of the lumbar cord at approximately embryonic day 18 (Rajaofetra et al. 1989; Ziskind-Conhaim et al. 1993) and show close apposition to motoneurons by postnatal day 1 (Tanaka et al. 1992; Ziskind-Conhaim et al. 1993). However, the adult pattern of serotonergic innervation is not reached until 3 wk postnatally (Rajaofetra et al. 1989). During the first 2 wk of life, intact rats use mainly their forelimbs for pivoting and crawling; they do not develop sufficient hindlimb weight support for quadrupedal walking until day 12–13 (Altman and Sudarshan 1975). Although analysis of L-DOPA-induced air-stepping in suspended rats demonstrated coordinated gait, involving all four limbs on the day of birth, forelimb stepping predominated over quadrupedal patterns until after day 5 (McCrea et al. 1994; Stehouwer et al. 1994). Thus the possibility that the rostrocaudal gradient of spinal cord sensitivity to 5-HT observed in the present study corresponds, at least in part, with developmental factors, cannot be excluded. A further consideration is the distribution of intraspinal 5-HT-containing neurons. In addition to descending 5-HT projections, intraspinal 5-HT-containing neurons contribute 2–15% of the total 5-HT content in the rat spinal cord (Newton and Hamill 1988). Although these neurons may be anatomically related to the autonomic nervous system (Newton and Hamill 1988; Newton et al. 1989), clarification of their targets and functional role remains to be accomplished. In view of the present results, it is of interest that intraspinal 5-HT neurons...
are located primarily in the thoracic region and L₁ segment, whereas the L₂–L₆ segments contain none (Newton and Hamill 1988).

Intravenous injection of noradrenergic precursors (L-DOPA) or agonists (clonidine) induces locomotor activity in acutely spinalized cats (Barbeau and Rossignol 1991; Forssberg and Grillner 1973; Grillner and Zangger 1979). However, attempts to elicit locomotion in similar preparations with the use of serotoninergic drugs have been unsuccessful (Barbeau and Rossignol 1991; Grillner and Shik 1973). The present results, demonstrating a 5-HT-sensitive oscillatory network distributed rostral to the thoracolumbar junction, suggest that the failure of 5-HT to activate locomotion in previous cat experiments may have been related to the use of low (T₁₃) spinal preparations (Barbeau and Rossignol 1991; Grillner and Shik 1973). In support of this possibility is the observation that systemic administration of the serotoninergic precursor 5-HT evokes locomotion in spinalized rabbits in which some of the thoracic cord had been retained (Viala and Buser 1971). However, this explanation for the varied effects of 5-HT reported in the literature is based on the unproven assumption that a 5-HT-sensitive oscillatory network exists and has a similar regional distribution in these different species. Obviously, further experiments are necessary to clarify whether interspecies differences exist.

**Different neurochemicals activate different rhythmogenic substrates**

The present results support our earlier suggestion that different neurochemicals preferentially activate different rhythmogenic substrates (Cowley and Schmidt 1994b), and may also explain certain inconsistencies in the literature. For instance, although Cazalets et al. (1995) found no rhythmogenic properties caudal to the L₂ level, Kudo and Yamada (1987) observed that even isolated L₄–L₅ hemisegments generated alternating activity in the ipsilateral hindlimb. This discrepancy may be accounted for by the fact that Cazalets et al. (1995) used 5-HT/NMA in their study, whereas Kudo and Yamada (1987) applied NMA alone. In the present study, we showed that application of NMA alone to the lumbar region induces rhythmic activity in these segments, whereas exposure of the same lumbar tissue to 5-HT or 5-HT/NMA evokes tonic activity only. Why exposure of the lumbar cord to combined 5-HT/NMA should produce only tonic activity whereas NMA alone induces rhythmic activity is unclear. Possibly the discharge behavior of lumbar interneurons and/or motoneurons is dominated by tonic or excessive excitation during combined 5-HT/NMA exposure, in which case the successful induction of rhythmic network activity may require a careful balancing of the 5-HT and NMA concentrations. This may account for certain discrepancies in the literature, including, in the present study, the failure to induce rhythmic activity in rostral lumbar cord segments with the use of 5-HT/NMA, in contrast to the results of others (Cazalets et al. 1995; Kjaerulff and Kiehn 1994). Regardless of the exact explanation for conflicting observations in the literature, the present findings suggest that investigations of spinal cord rhythogenesis need to consider the particular activating substance(s) employed as well as the type of motor pattern examined.

The data suggest that the spinal cord has a greater inherent capacity to develop rhythmic activity in response to application of NMA than 5-HT, at least in the lumbar region. However, this may not be entirely unexpected. NMDA receptor activation is known to generate intrinsic oscillatory behavior in synaptically isolated spinal cord interneurons and motoneurons (Hochman et al. 1994a,b). Possibly, then, the requirement for NMDA receptor activation in spinal cord rhythmogenesis pertains mainly to the induction of membrane voltage bistability and/or oscillatory activity, whereas other neuromodulators or activators such as 5-HT have a greater role in organizing specific patterns of behavior, such as locomotion, at a network level. This may explain why the pattern of NMA-induced rhythmic activity we observed in the present and previous (Cowley and Schmidt 1994b) studies is often labile and non-locomotor-like in quality (although see Kudo and Yamada 1987), in contrast to the rhythms produced in the presence of 5-HT.

Although it was reported that ACh activates locomotor circuitry in the neonatal rat spinal cord (Smith et al. 1988), we rarely observe a locomotor-like sequence of ENG activity in response to this substance; more commonly side-to-side alternation of coactivated intralimb flexor-extensor pairs occurs (Cowley and Schmidt 1994b). The extent to which the distinct patterns of rhythmic activity evoked by 5-HT and ACh are mediated through differential modulation of common network components, as opposed to activation of separate neural substrates, is unknown. However, the present study demonstrates that ACh-sensitive rhythmogenic circuitry exists within the (bilaterally intact) lumbar cord, in contrast to the distribution of the 5-HT-sensitive network. Also in contrast to the results of 5-HT application, ACh-induced alternating left-right hindlimb phase relationships could be uncoupled by midsagittal lesions made at a variety of rostrocaudal levels of the spinal cord. The latter finding, in conjunction with the observation that ACh failed to induce rhythmic activity in the isolated lumbar cord hemihemisphere in three of four preparations, suggests that bilaterally distributed components are of particular importance for the activation and organization of the ACh-sensitive network. Similarly, evidence of an important contribution from contralateral spinal cord circuitry was recently found for the central pattern generator for scratching in the turtle (Stein et al. 1995). In summary, although a more complete description of 5-HT- and ACh-sensitive circuits is awaited, this study suggests that these networks can be characterized, at least in part, by regionally and anatomically distinct elements.

**Side-to-side phase relationships are mediated by distributed systems of cross connections**

Cazalets et al. (1995) demonstrated that separation of the left and right halves of the lumbar cord, up to the L₂/L₃ level, fails to disrupt the side-to-side relationship of 5-HT/NMA-induced rhythmic activity in the lumbar cord. Similar, chronic midsagittal separation of the cord between L₁ and S₁ had no effect on left-right hindlimb coordination during walking in cats (Kato 1988). In the present study we observed that midsagittal lesions extending from the conus to the thoracolumbar junction had no effect on 5-HT-induced locomotor-like patterns in the hindlimbs. Thus it appears
that in the presence of a bilaterally intact supraspinal lumbar spinal cord, reciprocal interconnections in the lumbar region are not essential for interlimb coordination. Hindlimb locomotor-like activity was also preserved in preparations with extensive midsagittal separation of the cervicothoracic spinal cord or more localized midsagittal lesions through the thoro-
columbar junction. Therefore it appears that no single region of the spinal cord is critical for the maintenance of 5-HT-induced reciprocal inhibitory interactions among the hind-
limbs, provided other regions of the cord are preserved bilater-
ally intact. These observations are compatible with a widely distributed and redundantly organized system of re-
ciprocal cross projections in the spinal cord. Presumably some of these interconnections are inhibitory in nature and help ensure an alternating pattern of left-right activation.

Similarly, the present results imply the existence of an extensively distributed and redundantly organized system of reciprocal excitatory cross projections. These pathways, which are unmasked by the blockade of inhibitory amino acid receptors, synchronize rhythmic activity among func-
tional antagonists (Cowley and Schmidt 1995), and therefore may be well suited to mediate coactivation of selected motor populations during locomotion and other behaviors. Although inter- and intralimb synchrony was generated by small portions of lumbar tissue, consistent with reciprocal excitatory connections at the segmental level, the present study also showed that synchronous activity was preserved despite almost complete midsagittal separation of the two halves of the spinal cord (i.e., sparing only a few segments of residual bilaterally intact cord). The location of the pre-
served residual cross connections along the rostrocaudal axis of the spinal cord was not important, compatible with a redundantly organized system of cross links.

Relevance to mammalian locomotion

We have described the regional distribution of circuitry activated in response to several neurochemicals applied to selected regions of the cord, or in the entire spinal cord in the presence of specific lesions. However, identification of the endogenous substances that activate and modulate locomotor networks in intact mammals, as well as the site and temporal pattern of release of those substances, awaits fur-
ther study. Neuromodulatory systems not yet examined or identified may elicit locomotor behavior through activation of networks with anatomic distributions that are distinct from those characterized in the present study. In addition, it may be discovered that different neuromodulators elicit specific behaviors through functional reconfiguration of the same anatomic network, as has been demonstrated in lower ani-
mals (for review see Harris-Warrick and Marder 1991). Further investigation of these issues is clearly needed.

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