Spinal Cholinergic Neurons Activated During Locomotion: Localization and Electrophysiological Characterization

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Huang, A., B. R. Noga, P. A. Carr, B. Fedirchuk, and L. M. Jordan. Spinal cholinergic neurons activated during locomotion: localization and electrophysiological characterization. J Neurophysiol 83: 3537–3547, 2000. The objective of the present study was to determine the location of the cholinergic neurons activated in the spinal cord of decerebrate cats during fictive locomotion. Locomotion was induced by stimulation of the mesencephalic locomotor region (MLR). After bouts of locomotion during a 7–9 h period, the animals were perfused and the L3–S1 spinal cord segments removed. Cats in the control group were subjected to the same surgical procedures but no locomotor task. The tissues were sectioned and then stained by immunohistochemical methods for detection of the c-fos protein and choline acetyltransferase (ChAT) enzyme. The resultant c-fos labeling in the lumbar spinal cord was similar to that induced by fictive locomotion in the cat. ChAT-positive cells also clearly exhibited fictive locomotion induced c-fos labeling. Double labeling with c-fos and ChAT was observed in cells within ventral lamina VII, VIII, and possibly IX. Most of them were concentrated in the medial portion of lamina VII close to lamina X, similar in location to the partition and central canal cells found by Barber and collaborators. The number of ChAT and c-fos–labeled neurons was increased following fictive locomotion and was greatest in the intermediate gray, compared with dorsal and ventral regions. The results are consistent with the suggestion that cholinergic interneurons in the lumbar spinal cord are involved in the production of fictive locomotion. Cells in the regions positive for double-labeled cells were targeted for electrophysiological study during locomotion, intracellular filling, and subsequent processing for ChAT immunohistochemistry. Three cells identified in this way were vigorously active during locomotion in phase with the spinal cord. Thus a new population of spinal cord cells can be defined: cholinergic partition cells with commissural projections that are active during the extension phase of locomotion.

INTRODUCTION

The spinal cord of vertebrates contains several types of cholinergic neurons, including motoneurons, preganglionic autonomic neurons, partition cells (lamina VII), central canal cluster cells (lamina X), and small dorsal horn cells scattered in lamina III–V (Barber et al. 1984; Borges and Iversen 1986; Houser et al. 1983; Phelps et al. 1984; Sherriff and Henderson 1994; for description of laminar location see Rexed 1954). There is consensus that these cholinergic cells form an extensive propriospinal system of interconnected neurons (see Sherriff and Henderson 1994) and may therefore play a role in activities requiring coordination among several spinal segments. Spinal motoneurons receive prominent cholinergic terminals (Arvidsson et al. 1997; Li et al. 1995; Nagy et al. 1993; Welton et al. 1999), and so it seems likely that some of the intrinsic spinal cholinergic cells are involved in the control of movement. Furthermore, the large cholinergic terminals, termed C terminals (Li et al. 1995; Nagy et al. 1993) have been implicated in spinal cord plasticity (Feng-Chen and Wolpaw 1996; Pullen and Sears 1983).

A role for acetylcholine in the initiation and control of locomotion in vertebrates is suggested on the basis of several lines of evidence. In the Xenopus frog embryo, Roberts and his colleagues (Panchin et al. 1991) observed that bath application of ACh evoked a burst of activity, followed by an increased frequency of spontaneous swimming episodes. The muscarinic antagonist atropine reversibly blocked the effects of ACh. Atropine also considerably shortened both spontaneous and evoked bursts of fictive swimming, while nicotinic antagonists appeared to have little effect. A portion of the drive to motoneurons during swimming in these animals is derived from a cholinergic input (Perrins and Roberts 1995a–c).

In the newborn rat, locomotion has been elicited by bath application of ACh and edrophonium, an anticholinesterase, to the spinal cord of an in vitro brain stem–spinal cord preparation (Cowley and Schmidt 1994; Smith and Feldman 1986, 1987; Smith et al. 1988). The effects appear to be mediated through the activation of muscarinic receptors at the spinal level since the muscarinic receptor antagonist atropine can completely suppress ACh-induced rhythmic activity in the spinal cord (Smith et al. 1988). Although a locomotor-like pattern of activity can be evoked by ACh, stimulation of cholinergic receptors in the neonatal rat cord often produces coactivation of flexors and extensors on one side that can alternate from side to side (Cowley and Schmidt 1994).

Using intracellular dye injection into physiologically identified neurons, coupled with immunohistochemical markers (Carr et al. 1994, 1995), we reported that a population of interneurons that were active during fictive locomotion produced by stimulation of the mesencephalic locomotor region (MLR) stain positive for the choline acetyltransferase (ChAT) enzyme and thus may be cholinergic. Details have not been provided, however, regarding the rostro-caudal and laminar distributions of locomotor-related cholinergic cells, nor has their pattern of activity during locomotion and the trajectory of their axons been described.

Expression of the c-fos transcription factor is now a well-established activity-dependent marker (see Herdegen and Leah 1998 for review). In the spinal cord, c-fos immunohistochemistry has been an effective means for revealing neurons acti-
FIG. 1. Pairs of photomicrographs from a locomotor animal showing the same field in sections double labeled by immunofluorescence for c-fos (A and C) and by immunoperoxidase for choline acetyltransferase (ChAT; B and D). Labeled neurons are indicated with an arrow, and unlabeled neurons are marked with an arrowhead. A and B: cells located in ventral lamina VII or lamina IX. C and D: cells located in medial lamina VII. All panels at ×400.

FIG. 2. Photomicrographs showing the differences in labeled neurons between Locomotor and Control sections. A and B: the field of spinal neurons in a locomotor cat. C and D: the same field of spinal neurons in the corresponding control cat (same spinal area as A and B). A and C: immunofluorescence for c-fos (arrows in left set of photomicrographs). B and D: immunoperoxidase for ChAT (arrows in right set of photomicrographs). All panels at ×200.
SPINAL CHOLINERGIC NEURONS ACTIVE DURING LOCOMOTION

TABLE 1. Total number of labeled cells in each animal

<table>
<thead>
<tr>
<th>Experiment Name</th>
<th>Labeled Cells</th>
<th>Number of Sections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Double</td>
<td>c-fos</td>
</tr>
<tr>
<td>FL-Cat1</td>
<td>196</td>
<td>805</td>
</tr>
<tr>
<td>FC-Cat1</td>
<td>49</td>
<td>192</td>
</tr>
<tr>
<td>FL-Cat2</td>
<td>147</td>
<td>927</td>
</tr>
<tr>
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<td>134</td>
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<tr>
<td>FL-Cat3</td>
<td>98</td>
<td>518</td>
</tr>
<tr>
<td>FC-Cat3</td>
<td>34</td>
<td>141</td>
</tr>
</tbody>
</table>

The total number of labeled neurons in the mesencephalic locomotor region–induced locomotion experiments is higher than in the controls. The number of sections used per segment is shown in the right column. ChAT, choline acetyltransferase; FL, fictive locomotion; FC, nonlocomoting control.

Methods

Six adult cats (1.8–2.6 kg) were used for c-fos combined with ChAT immunohistochemistry to map the segmental and laminar distributions of spinal cholinergic interneurons activated during locomotion, and we have used intracellular labeling combined with immunohistochemistry for ChAT to determine the activity pattern during locomotion and axonal trajectory of cholinergic interneurons.

Here, we have combined c-fos with ChAT immunohistochemistry to map the segmental and laminar distributions of spinal cholinergic interneurons activated during locomotion, and we have used intracellular labeling combined with immunohistochemistry for ChAT to determine the activity pattern during locomotion and axonal trajectory of cholinergic interneurons.

Methods

Six adult cats (1.8–2.6 kg) were used for c-fos combined with ChAT immunohistochemistry. All surgical procedures were carried out under halothane/nitrous oxide anesthesia. Cannulae were placed in the carotid artery to monitor blood pressure and in the jugular veins to infuse fluids. Peripheral nerves innervating the following muscles were dissected in both hindlimbs: posterior biceps and semitendinosus (PBST), semimembranosus and anterior biceps (SMAB) and mounted on stimulating/recording bipolar electrodes submerged in a mineral oil bath. The nerves to the sartorius (SA) muscle were cut and placed in buried cuff-style electrodes for stimulation. Following a craniotomy, the animal was decerebrated at the precollicular-postmammillary level by removing rostral portions of the brain. Anesthesia was then discontinued, and the animals were paralyzed with gallamine triethiodide (Flaxedil, Rhone-Poulenc: about 4 mg · kg⁻¹ · h⁻¹) and artificially ventilated. The end tidal CO₂ was maintained between 3.5 and 4.5%. Arterial blood pressure was maintained above 80 mmHg by infusion of small quantities of dextan. A bicarbonate solution (100 mM NaHCO₃ with 5% glucose) was infused to replace fluid loss and help to maintain a normal PH balance in the animals throughout the experiments.

Locomotion was induced by electrical stimulation (0.5–1.0 ms square-wave pulses, 15 Hz, 50–160 μA) of the MLR using insulated monopolar stimulating electrodes. Locomotion was monitored by electronograms. After at least a 1 h recovery period following the decerebration, animals were stimulated to produce bouts of locomotion. In all of the experiments reported here, there was at least an 8-h interval between decerebration and perfusion to eliminate c-fos expression by surgery. Control animals received the same surgical procedures as the locomotor cats, except that they were not subjected to the locomotor task.

After the 7 to 9 h locomotor trial periods, the cats were perfused via the left ventricle with a 0.9% saline prefixative followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Spinal segments L₁–S₁ were rapidly removed, postfixed for 5 h in the same fixative solution, followed by immersion for at least 4 days in a cold (4°C) cryoprotective medium consisting of 25% sucrose, 10% glycerol, and 0.001% sodium azide in 50 mM sodium phosphate buffer. Frozen tissue sections of 20–30 μm were cut on a sliding microtome and collected in PBS. A sequential fos–antibody (Cambridge Research Biochemicals, sheep polyclonal, 1:2,000) in 0.2 M sodium phosphate-buffered 0.9% saline with 0.3% Triton X-100 and 2% bovine serum albumin (BSA), where they were transferred to a primary antibody solution consisting of anti-cholocerebrovascularization (Barajon et al. 1992; Hunt et al. 1987), and walking on a rotating rod (Jasmin et al. 1994). In MLR-induced fictive locomotion in cats, this activity-dependent labeling has been applied successfully to locate the neurons activated in the spinal cord (Carr et al. 1995; Dai et al. 1990). Carr et al. (1995) reported that approximately one-third of the c-fos–positive cells in a limited sample were also immunoreactive for ChAT.

FIG. 3. Segmental distribution of labeled cells. The results from FL-Cat1 and FC-Cat1 are shown in A; FL-Cat2 and FC-Cat2 in B; and FL-Cat3 and FC-Cat3 in C. The distribution of double-labeled cells is presented in the left 3 histograms, that for c-fos–labeled cells is shown in the middle 3 histograms, and the distribution of ChAT-labeled cells is shown in the right 3 histograms. The calculation of averaged cells per section was done separately in each cat. This data presentation can correct for a potential bias induced by having an uneven number of tissue sections collected from different experiments. Note: the average number of double-labeled cells and c-fos–labeled cells in locomotor cats are higher than those in the control cats. Also, the averaged number of ChAT-labeled cells in most segments is higher in locomotor than control experiments.
The percent increase of ChAT-labeled cells in spinal dorsal horn, intermediate gray, and ventral horn areas of locomotor vs. control cats was obtained by dividing the total number of ChAT-labeled cells in each area by the total number of ChAT-labeled cells in the corresponding area of the control cat, and subtracting the former.

In three other experiments, intracellular recordings were obtained from neurons in the lumbar spinal cord during fictive locomotion. The neurons were labeled using glass microelectrodes filled with 2% tetramethylrhodamine dextran (TMRD), as previously described (Carr et al. 1994). In these experiments extensive hindlimb nerve dissections were done to allow recording of electroneurographic (ENG) activity and stimulation of a variety of peripheral afferents. The nerves dissected included PBST, SMAB, SA (as described above), as well as triceps surae (GS), plantaris (PL), superficial peroneal (SP), and sural (Sur) bilaterally. After recording the synaptic input to these cells and their activity pattern during fictive locomotion, the TMRD was injected, and 2–4 h was allowed to elapse for cytoplasmic filling of the processes of the injected cell. The animal was then perfused with fixative as described above, and the spinal cord removed and subsequently sectioned. The intracellularly recorded and dye-injected neurons were visualized, and selected sections were processed for ChAT immunohistochemistry (Carr et al. 1994).

Slides were examined and photographed with a Nikon Optiphot, labophot combination light/epifluorescence microscope. Double labeling of c-fos and ChAT-immunoreactivity in the same cell was determined by alternate observation of the same area of the sections using the appropriate filter cube for visualization of the CY3 fluorophore and using bright-field illumination for PAP. A Neurolucida image analysis system was employed to plot both c-fos and ChAT-positive cells. Only those neurons that obviously contained reaction product in the nucleus were considered to be labeled for c-fos, while ChAT-immunoreactivity is confined to the cytoplasm.

**RESULTS**

The quality of the labeling is depicted in the high-power (×400) photomicrographs shown in Fig. 1, A–D. Both pairs of photomicrographs show the same field in sections double-labeled by immunofluorescence for c-fos (Fig. 1, A and C) and by immunoperoxidase for ChAT (Fig. 1, B and D). They also illustrate the intensity difference between labeled and unlabeled cells. Double-labeled cells in Fig. 1, A and B, were located in ventral lamina VII or lamina IX; those in C and D were located in lamina VII. Another photomicrograph group shows the difference of c-fos- and ChAT-labeled cells between locomotor and control cats (Fig. 2).

**Comparisons between locomotor and control cats**

Three animals were used as controls for comparison to fictive locomotion animals. They were subjected to the same surgical procedures as the fictive locomotion animals, including the nerve dissection, paralysis with Flaxedil, and artificial ventilation following decerebration. Figure 2 shows an example of photomicrographs taken from the spinal cords of control (C and D) and locomotor task (A and B) animals. Both pairs of sections were processed for c-fos (A and C) by immunofluorescence and for ChAT (B and D) by immunoperoxidase. These low-power (×200) photomicrographs illustrate that the...
locomotor task produces clear c-fos nuclear labeling that is not evident in the control spinal cord.

In the three locomotor preparations, the total numbers of c-fos, ChAT, and double-labeled cells were increased compared with the numbers of similarly labeled cells in the three control preparations (Table 1). The number of c-fos and double-labeled cells in spinal segments (L₃–S₁) in locomotor cats is much higher than that in controls (Fig. 3). These differences are statistically significant (Student’s test, \( P < 0.01 \)). The difference ranged from 48% to 92% and from 39% to 78% for double-labeled and c-fos-labeled cells, respectively (see Fig. 3). These results confirm the previous report showing the location of interneurons in the lumbar spinal cord involved in the production of locomotion (Dai et al., unpublished observations). The increase in the number of double-labeled cells is most likely due to both increases of c-fos- and ChAT-labeled cells in sections from lumbar segments of animals subjected to locomotor trials.

The number of ChAT-labeled cells was also greater in locomotor cats compared with controls (Table 1). The difference ranged from 3% to 48%. In most segments the number of ChAT-labeled cells as a whole was increased compared with controls, but in two segments (S₁ in FL-cat2, L₅ in FL-cat3), ChAT-labeled cells decreased 5% and 9%, respectively. In two-thirds of the segments, the difference was significant (\( P < 0.01 \) or \( P < 0.05 \)). These changes between locomotor and control preparations can also be seen in Fig. 3. The increase was marked in the intermediate gray matter (lamina VII and VIII; Fig. 4), regions coextensive with the reported location of partition cells (Barber et al. 1984).

Distribution of labeled cells in the spinal cord

Bouts of fictive locomotion were evoked by MLR stimulation. Good locomotion (as illustrated in Fig. 5) was recorded in three fictive locomotion cats (FL-cat1, FL-cat2, and FL-cat3). The total time recorded was 2 h 55 min in FL-cat1, 3 h 5 min in FL-cat2, and 3 h 12 min in FL-cat3.

As previously reported (Dai, Douglas, Noga, and Jordan, unpublished observations), extended periods of fictive locomotion resulted in a characteristic distribution of c-fos-immunoreactive neurons among spinal lamina in the cat lumbar cord. Immunoreactive cells were most heavily concentrated in medial lamina VI and VII and in lamina VIII and X, while occasional c-fos-positive neurons were observed in lamina I–V of the dorsal horn and in the reticulate gray matter of lateral lamina V, VI, and VII (see Rexed 1954 and the inset of Fig. 8 for clarification of the location of spinal laminae).

The appearance and distribution of ChAT-positive neurons in transverse sections of lumbar spinal cord (Fig. 1, B and D) were very similar to those described in detail by Barber et al. (1984). Stained neurons were present throughout the spinal cord and were particularly concentrated in the ventral horn, central gray matter, and intermediate gray matter. Some of them were scattered in the dorsal horn (lamina III–V). The density of neuronal staining in different regions was not uniform; many cells in lamina VIII, IX, in medial part (lamina VII), and around the central canal were intensely stained, while cells in the dorsal horn were usually more lightly stained. When these sections from the lumbar spinal cord were incubated with both c-fos and ChAT antibodies, some c-fos–immunoreactive neurons were found to be ChAT positive. In FL-cat1, 24.4% of c-fos positive neurons were ChAT positive; in FL-cat2, 15.9% of c-fos–positive neurons were ChAT positive; and in FL-cat3, 18.9% of c-fos–positive neurons were ChAT positive (Fig. 6A). Figure 6B shows that of the total population of ChAT-positive neurons (including motoneurons), a relatively small proportion also expressed c-fos. The distributions of the double-labeled cells in each preparation is illustrated in Fig. 7. Maps of labeled cells were derived from Neurolucida drawings, as described in the methods. Data from the nonlocomoting control animals (FC) and the fictive locomotion animals (FL) are presented in pairs that had been processed simultaneously. Most of double-labeled neurons were distributed among the deeper lamina with the majority of...
c-fos/ChAT-positive cells situated in lamina VII and VIII. There was also a considerable number of double-labeled cells in the central part of the intermediate gray matter around the central canal in lamina X (Fig. 8). Little double labeling was observed in large neurons in the ventral horn, suggesting that motoneurons may not be labeled using our c-fos staining protocol. Differentiation between putative motoneurons and other spinal neurons was based on soma size and lamina location alone and was not considered unequivocal. In general, very few double-labeled cells were found in lamina I–III. The locations of double-labeled cells were similar in the three locomotor cats.

**Activity and axonal trajectory of ChAT-positive cells recorded during locomotion**

Intracellular recordings were obtained from five neurons that were active during locomotion and were subsequently found to be positive for ChAT immunoreactivity. Two of the cells were observed to project axons into the ventral white matter, in the direction of the ventral root exit zone, and were considered to be motoneurons. The other three neurons could clearly be distinguished from motoneurons because they were located in lamina VII of the L₅ spinal segment and did not project in the direction of the ventral root. Two of these cells are illustrated in Fig. 9, which shows the intracellular label (Fig. 9, A and B, left panels) and the ChAT label (A and B, right panels). Both cells were located in an area of the spinal cord that overlaps with an area containing cells double labeled for c-fos and ChAT (Fig. 2). The activity during locomotion of the cell illustrated in Fig. 9A is shown in C and D. The traces in C are the raw records of the intracellularly recorded cell along with simultaneous recordings of ENG activity in nerves located ipsilateral and contralateral to the cell body of this neuron. It is clear that this cell was active in phase with the ipsilateral
extensor (SMAB) ENG, and that its period of activity spanned the entire period of ipsilateral extension. It is striking that this neuron fired at a high-frequency throughout its period of activity, as illustrated in the frequency plot (Fig. 9D). The firing of the cell shown in B is also illustrated in the frequency plot (D). It was also active during ipsilateral extension, and displayed very high-frequency firing (approaching 300 Hz) in the early part of the extensor phase of the locomotor cycle. Interestingly, its activity seemed best related to the offset of flexor activity on the same side, beginning immediately prior to flexor offset. Its highest firing frequencies did not necessarily correlate to the largest extensor activity bursts; both of these cholinergic cells were very vigorously active during fictive locomotion.

These two cells were also similar in that they received excitatory group II afferent input from ipsilateral extensor nerves (central latencies of 1.2 and 2.2 ms for Fig. 9, A and B, respectively), and cutaneous input from ipsilateral cutaneous afferents (SP, 1.8 and 1.3 ms) and contralateral cutaneous afferents (SP and Sur, 1.9–11.5 ms). Both of these neurons also received di- to oligosynaptic excitation from the MLR sites (1.2–3.5 ms segmental latency). The axons of the two cells could be traced for a short distance (see Fig. 9A, left panel), and both projected to the contralateral side of the spinal cord and ascended in the contralateral medial funiculus to at least the T13 level where their axons could be antidromically activated. The sites of termination of these cells could not be determined, however. The third ChAT-positive TMRD-filled interneuron (not illustrated) received only inhibition from ipsilateral SA group II afferents, and from cutaneous afferents bilaterally. It was also spontaneously active during fictive locomotion, with the onset of its activity also most closely associated with the termination of activity in ipsilateral flexor ENGs.

An additional eight interneurons were filled that were also located in lamina VII and had contralaterally projecting axons that were ascending (not illustrated). Unfortunately, ChAT immunoreactivity could not be performed on these cells, so they were not confirmed to be cholinergic. Seven of these eight were spontaneously active during fictive locomotion, and of those seven, five had activity most closely related to ipsilateral flexion. The remaining two had activity most related to the termination of ipsilateral flexion. The similarity of the morphology and activity during fictive locomotion of these eight interneurons as compared with the three ChAT-positive interneurons suggests that despite the lack of immunocytochemical evidence, it is likely that some or all of these eight additional interneurons also belong to this class of cells.

**DISCUSSION**

Here we have shown a locomotor-related increase in the incidence of c-fos/ChAT double labeling that denotes cholinergic interneuron activity during MLR-evoked fictive locomotion in the cat. We have also shown an increase in the number of ChAT-positive neurons following fictive locomotion, consistent with a locomotion-related up-regulation of ChAT. We have provide a detailed map of the cholinergic interneurons involved in locomotion that can be detected with the c-fos activity label. Furthermore, we have provided the first recordings of identified cholinergic interneurons during locomotor activity.

As previously reported, extended periods of fictive locomotion in the decerebrate cat preparation result in a characteristic distribution of c-fos–positive neurons in the lumbar spinal cord (Dai et al., unpublished observations). ChAT-positive cells are also clearly active during locomotion. Double-labeled cells displayed a distribution extending to ventral laminae VII, VIII, and possibly IX. Most were located in the medial portion of lamina VII close to lamina X, where double-labeled cells were more numerous than in the lateral portion. These laminar locations are consistent with the idea that intermediate and deeper spinal laminae contain interneurons important in producing and organizing motor output.

Putative motoneurons were identified based on laminar location and somal size. Using these criteria, it appears that only
a small subpopulation of large, ventrally located motoneurons were c-fos positive. This is in agreement with previous reports, in which motoneurons proved difficult to label either with the c-fos or the 2-deoxyglucose method (Barajon et al. 1992; Dai et al. 1990; Viala et al. 1988).

Although our sample of identified cholinergic cells recorded during locomotion is limited, the results confirm that cholinergic cells in the region of partition cells are active during locomotion. Furthermore, it is possible from these results to define a new population of spinal neurons: cholinergic partition cells that are active during the extension phase of locomotion and project to the contralateral side of the spinal cord. The pattern of activity and high firing rates of the these cells suggests that they should make a significant contribution to the control of locomotion. The period of activity of all three cells started just prior to ipsilateral extension, spanned the entire extensor phase of the locomotor cycle, and had peak firing rates well in excess of 100 Hz.

Commissural interneurons in lamina VIII have been shown to project to contralateral motor nuclei by means of wheat germ agglutinin–horseradish peroxidase injections into a muscle nerve (Harrison et al. 1986). These cells may be involved in crossed extension reflexes (Grillner and Hongo 1972) or else mediate excitation or inhibition of contralateral motoneurons evoked by the vestibulo- and reticulospinal tracts (Hongo et al. 1975; Sasaki et al. 1962). Many commissural lamina VIII neurons receive a strong input from the descending locomotor command pathway. Their axons cross the midline and have collateral branches and terminals in the contralateral gray matter (Jankowska and Noga 1990; Noga et al. 1987b). Also, commissural cells appear to be involved in the first rhythmic activity that appears during development of the fetal rat (Kudo et al. 1991). Kjaerulff and Kiehn (1997) have described crossed inhibitory effects on motoneurons during fictive locomotion in the isolated neonatal rat spinal cord that are dependent on a crossed pathway involving glutamatergic transmission.

Investigations using various activity markers have suggested areas of importance for generating spinal locomotion. In the rabbit, the uptake of 2-deoxyglucose during 1-L,3,4-dihydroxyphenylalanine (L-DOPA)–induced fictive locomotion was found in cells in the intermediate gray (Viala et al. 1988). In decerebrate cat, c-fos–positive cells were localized in the intermediate gray and area X during MLR-induced locomotion (Dai et al., unpublished observations). Isopotential mapping studies in the cat have also implicated these areas as potential areas containing interneurons important in generating locomotor activity (Jordan 1991; Noga et al. 1995). Studies using optical imaging and ablation of neurons in the isolated spinal cord of the embryonic chick have revealed potential locomotor generators in the ventral and intermediate gray (O’Donovan et al. 1992). Recent experiments in the neonatal rat have detected sulfonohydramine-labeled cells throughout the lumbar spinal cord in the dorsomedial, the central, and the intermediate gray areas (Kjaerulff et al. 1994).

Previous studies regarding cholinergic elements in the spinal cord have identified partition and central canal cluster cells that are located in the intermediate gray and the area around the central canal (Barber et al. 1984; Borges and Iversen 1986). Partition neurons form a ChAT-positive network across the intermediate spinal region and may make propriospinal connections. Central canal cells in lamina X project to dorsal, intermediate, and ventral gray matter and have been proposed to provide integration between the sensory, motor, and autonomic elements of the spinal cord (Borges and Iversen 1986). Both partition and central canal neurons contribute to the intraspinal cholinergic circuitry that forms the main cholinergic innervation of the spinal cord and modulates the transmission of information in the spinal cord. It has been proposed that these widespread intersegmental connections may mediate central motor programs for motor functions such as walking (Woolf 1991).

The results obtained in this study confirm and extend the previous findings that lamina VII and lamina VIII cells are involved in locomotion. It is noted that many of the c-fos/ChAT–labeled cells are concentrated within the area of partition cells (see Fig. 7). In this area, the number of ChAT-positive cells showed a larger relative increase than dorsal horn or ventral horn cells, during fictive locomotion.

Many interneurons that mediate reflex effects are located in lamina VII. There are three types of identified interneurons within the ventral part of lamina VII: Ia inhibitory interneurons, Renshaw cells, and mid-lumbar interneurons activated by group II muscle afferents (Edgley and Jankowska 1987a,b; Edgley et al. 1988; Jankowska and Lindstrom 1971, 1972). Each of these cell types have been shown to be rhythmically active during MLR-induced locomotion (McCrea et al. 1980; Noga et al. 1987a; Pratt and Jordan 1987; Sheftchyk et al. 1990), and it seems likely that these populations contribute to the population of c-fos–labeled cells in this study.

Retrograde transport of fluorescent microspheres injected into selected motor nuclei has revealed that commissural cells that project to motoneurons have somal locations that overlap the area where partition cells and cluster cells are located (Hoover and Durkovic 1992). Partition cells send axons into the motoneuron pools (Phelps et al. 1984). Immunohistochemical and electron microscopic methods have shown the terminals of cholinergic fibers onto motoneurons (Li et al. 1995; Nagy et al. 1993). Thus the double-labeled cells in the present study may correspond to these cholinergic “partition cells” and

![Fig. 9. Intracellular recording and labeling of ChAT-positive interneurons. This figure shows 2 examples of interneurons that were intracellularly recorded and filled with TMRD. A and B: the photomicrographs of the tetramethylrhodamine dextran (TMRD) labeling (left) and the corresponding ChAT labeling (right) of the same field of view (×155). The position of the soma and axonal trajectory are shown to the left of the photomicrographs. C: sample of the recording obtained of the same interneuron as in A, during fictive locomotion. D: firing pattern and frequency of both of these neurons during the normalized fictive step cycle (neuron in A is plotted in black, B plotted in red). Ipsilateral semimembranous and anterior biceps (SMAB) activity was set to 75% of the step cycle (denoted by the bar) and the cycle divided into 30 bins in which the firing frequency was averaged. Both neurons were active during extension, and both exhibited firing rates >100 Hz. Both of these neurons also had axons that projected contralaterally to the medial funiculus (not illustrated), received di- to oligosynaptic excitation from the MLR sites (1.2–3.5 ms segmental latency), and received excitation from ipsilateral extensor Gr II afferents (1.2–2.2 ms) and from cutaneous afferents bilaterally (1.3–11.5 ms; segmental inputs not illustrated).](http://jn.physiology.org/ by 10.220.33.1 on October 9, 2016)
central canal “cluster cells.” Likewise, some of them may represent cholinergic commissural neurons.

A possible explanation of the increase in the number of ChAT cells during fictive locomotion is that neuronal activity alters ChAT labeling. It is known that neuronal depolarization can influence ChAT activity levels and therefore Ach production. Neurons treated with depolarizing agents or stimulated directly by electric current exhibit an increase in ChAT enzyme activity (Ishida and Deguchi 1983). Nishi and Berg (1981) reported that a high KCl concentration enhanced ChAT activity, protein synthesis, and other activities in neurons cultured from chick ciliary ganglion. These same phenomena were observed in mouse spinal cord (Ishida and Deguchi 1983). Bausero et al. (1993) proposed a possible functional relationship between c-fos and ChAT by studying the sequence of human ChAT gene promoter. They demonstrated a 4- to 13-fold increase in ChAT activity in cos-1 cells and NE-1-115 neuroblastoma cells following co-transfection with c-fos/c-jun expression vectors and suggested that the ChAT promoter region may be inducible by c-fos/c-jun and thus may be a target for the c-fos/c-jun proto-oncogene. This indicates that c-fos/c-jun may be capable of altering ChAT expression and/or activity. Therefore the degree of co-localization of c-fos and ChAT in cat spinal cord following locomotor trials may be a result of either c-fos expression in cholinergic neurons constitutively expressing ChAT, or ChAT induction in cells expressing c-fos. It is noteworthy that in this study there is an increased number of cholinergic neurons that could be detected with the ChAT immunohistochemistry after the fictive locomotor task. This change might reflect plasticity in the locomotor system such as that which occurs with locomotor training of spinalized animals (Barbeau and Rossignol 1987; Lovely et al. 1986).

Spinal cholinergic neurons are of considerable interest in the investigation of locomotion. Initial studies of locomotion using in vitro rat preparations suggested a possible role for cholinergic mechanisms in the induction of rhythmic spinal cord activity (Smith and Feldman 1987; Smith et al. 1988). A study of the Xenopus spinal cord activity also implicated cholinergic mechanisms in the operation of the central pattern generator (Panchin et al. 1991). Activation of cholinergic receptors in the neonatal rat cord can often produce ipsilateral synchronous motion (Panchin et al. 1991). Stimulation of cholinergic receptors in mechanisms in the operation of the central pattern generator of the in vitro rat preparations suggested a possible role for cholinergic mechanisms in the induction of rhythmic spinal cord activity (Ishida and Deguchi 1983). Barbeau and Rossignol (1987; Lovely et al. 1986). They demonstrated a 4- to 13-fold increase in ChAT activity by studying the sequence of human ChAT gene promoter. They showed that ChAT cells during fictive locomotion is that neuronal activation alters ChAT labeling. It is known that neuronal depolarization can influence ChAT activity levels and therefore Ach production. Neurons treated with depolarizing agents or stimulated directly by electric current exhibit an increase in ChAT enzyme activity (Ishida and Deguchi 1983). Nishi and Berg (1981) reported that a high KCl concentration enhanced ChAT activity, protein synthesis, and other activities in neurons cultured from chick ciliary ganglion. These same phenomena were observed in mouse spinal cord (Ishida and Deguchi 1983). Bausero et al. (1993) proposed a possible functional relationship between c-fos and ChAT by studying the sequence of human ChAT gene promoter. They demonstrated a 4- to 13-fold increase in ChAT activity in cos-1 cells and NE-1-115 neuroblastoma cells following co-transfection with c-fos/c-jun expression vectors and suggested that the ChAT promoter region may be inducible by c-fos/c-jun and thus may be a target for the c-fos/c-jun proto-oncogene. This indicates that c-fos/c-jun may be capable of altering ChAT expression and/or activity. Therefore the degree of co-localization of c-fos and ChAT in cat spinal cord following locomotor trials may be a result of either c-fos expression in cholinergic neurons constitutively expressing ChAT, or ChAT induction in cells expressing c-fos. It is noteworthy that in this study there is an increased number of cholinergic neurons that could be detected with the ChAT immunohistochemistry after the fictive locomotor task. This change might reflect plasticity in the locomotor system such as that which occurs with locomotor training of spinalized animals (Barbeau and Rossignol 1987; Lovely et al. 1986).

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