Localization of Spinal Neurons Activated During Locomotion Using the $c$-$f_{os}$ Immunohistochemical Method


Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

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Dai, X., B. R. Noga, J. R. Douglas, and L. M. Jordan. Localization of spinal neurons activated during locomotion using the $c$-$f_{os}$ immunohistochemical method. J. Neurophysiol. 93: 3442–3452, 2005. First published January 5, 2005; doi:10.1152/jn.00578.2004. The $c$-$f_{os}$ immunohistochemical method of activity-dependent labeling was used to localize locomotor-activated neurons in the adult cat spinal cord. In decerebrate cats, treadmill locomotion was evoked by electrical stimulation of the mesencephalic locomotor region (MLR). Spontaneous or MLR-evoked fictive locomotion was produced in decerebrate animals paralyzed with a neuromuscular blocking agent. After bouts of locomotion during a 7- to 9-h time period, the animals were perfused and the L3–S1 spinal cord segments removed for immunohistochemistry. Control animals were subjected to the same surgical procedures but no locomotor task. Labeled cells were concentrated in Rexed’s laminae III and IV of the dorsal horn and laminae VII, VIII, and X of the intermediate zone/ventral horn after treadmill locomotion. Cells in laminae VII, VIII, and X were labeled after fictive locomotion, but labeling in the dorsal horn was much reduced. In control animals, $c$-$f_{os}$ labeling was a small fraction of that observed in the locomotor animals. The results suggest that labeled cells in laminae VII, VIII, and X are premotor interneurons involved in the production of locomotion, whereas the laminae III and IV cells are those activated during locomotion due to afferent feedback from the moving limb. $c$-$f_{os}$-labeled cells were most numerous in the L6–S1 segments, consistent with the distribution of locomotor activated neurons detected through the use of MLR-evoked field potentials.

Introduction

The spinal cord of mammals is known to include a central pattern generator (CPG) for locomotion Grillner 1981; Jordan 1991; Rossignol 1996), and numerous attempts have been made to identify essential elements of the CPG using lesioning methods, intracellular and extracellular recording methods, and labeling of locomotor related cells with activity-dependent markers, as reviewed by Kiehn and co-workers (Kiehn and Butt 2003; Kiehn and Kjaerulff 1998).

The 2-deoxyglucose (2-DG) method was used to localize interneurons involved in locomotion (Viala et al. 1988) in acute low spinal rabbits injected with nialamide and 3,4-dihydroxy-1-phenylalanine (DOPA). An area within the intermediate part of the gray matter, corresponding to Rexed’s lamina VI and the dorsolateral portion of lamina VII, was labeled in the L4–S1 segments. Unfortunately, the resolution of this technique does not allow visualization of individual neurons active during locomotion, and the study excluded more rostral segments, which have been more recently implicated in the production of locomotion.

Last-order interneurons of the lumbar spinal cord, which project to specific motor nuclei and which are active during locomotion, have been labeled using retrograde trans-neuronal transport of wheat-germ agglutinin conjugated with horseradish peroxidase (Jankowska and Skoog 1986), but this approach does not reveal cells active during locomotion that do not project to the selected motoneurons.

Cells active during chemically induced locomotion in the neonatal rat spinal cord have also been mapped using sulforhodamine uptake into active terminals (Kjaerulff et al. 1994). In the isolated neonatal spinal cords examined in this study, labeled cells were “...concentrated in two columns lateral to the central canal in the medial-most part of the intermediate gray throughout the lumbar spinal cord.” A subsequent study using the same labeling method but an alternate chemical method of inducing locomotor activity has revealed a more diffuse pattern of labeling, however (Cina and Hochman 2000). This indicates that attempts to neurochemically activate locomotor neurons and subsequently label them with this technique are problematic due to the binding of these ligands to any cell (including cells unrelated to locomotion) possessing the appropriate postsynaptic receptor.

Localization of cells in functional pathways in the nervous system has been achieved through immunohistochemical detection of the cellular counterpart of the immediate early gene $c$-$f_{os}$. The development and use of this method has been reviewed (Armstrong and Montminy 1993; Munglani and Hunt 1995; Sheng et al. 1990). In the spinal cord, $c$-$f_{os}$ immunohistochemistry has been used to localize cells involved in the transmission of noxious stimuli (Bullitt 1989; Hunt et al. 1987; Menetrey et al. 1989), scratching (Barajon et al. 1992), walking on a rotating rod (Jasmin et al. 1994), and fictive locomotion (Carr et al. 1995; Huang et al. 2000). In the rotating rod study, the rostrocaudal distribution of cells related to the motor component of the rotating rod task was not determined, and the authors suggested that a substantial proportion of the labeled cells were active due to afferent input because the number of labeled cells diminished after dorsal rhizotomy. The degree to which the rotating rod task recruits the CPG for locomotion, rather than circuits involved in voluntary limb movements and/or balance control, is unknown. In the fictive locomotion studies, little detail regarding the distribution of $c$-$f_{os}$-labeled cells was provided.

Clearly, there is a need for detailed mapping of the lumbar interneurons that are active during a locomotor task, while...
avoiding the difficulties associated with possible nonspecific labeling due to the use of chemical agents to elicit locomotion. Experiments are also needed that clearly distinguish cells labeled due to sensory feedback from the moving limb and those responsible for the motor components of locomotion. Here, we have employed the c-fos immunohistochemical method to reveal the neurons in the lumbar spinal cord of adult cats that are activated due to treadmill and fictive locomotor tasks in decerebrate animals. Treadmill locomotion lead to labeling of cells responsive to sensory input from the moving limb as well as cells associated with the locomotor network, while fictive locomotion allowed detection of cells active in the absence of sensory input from the moving limb. To assure that labeling was not dependent on effects of brain stem stimulation not related to locomotion, both mesencephalic locomotor region (MLR)-evoked and spontaneous forms of fictive locomotion were used.

METHODS

Experiments were performed on 12 adult cats weighting 1.9–2.8 kg. The animals were anesthetized with halothane in a mixture of 70% nitrous oxide and 30% oxygen for all surgical procedures prior to and including the decerebration. Anesthetic was initially delivered through a face mask until the trachea was intubated. Both carotid arteries were ligated, and one was cannulated to allow continuous monitoring of blood pressure. The right jugular vein was cannulated to administer fluids and drugs. A bicarbonate solution (100 mM NaHCO3 with 5% glucose) was infused to replace fluid loss and help maintain a normal pH balance in the animals throughout the experiments. Each animal was given 2 mg dexamethasone (Hexadrol phosphate, Organon) intravenously to reduce tissue swelling. Animal body temperature was monitored with an esophageal temperature probe and maintained at 36–38°C using feedback-controlled heating lamps and pads.

The head of each animal was fixed in a stereotaxic headframe. In treadmill-locomotion (n = 2) and treadmill-control (n = 2) experiments, all four limbs were free to step on a treadmill belt, and the hindquarters were suspended by a sling under the abdomen. In the forelimb-only condition, all four limbs were free to step on a treadmill belt, and the hindlimbs were suspended by a sling under the abdomen. In the fictive-forelimb locomotion (n = 4) and fictive-control (n = 4) experiments, the animals were placed in a frame and supported by two vertebral clamps and pins attached to the iliac crests with legs pendant. Hindlimbs were disected bilaterally to monitor locomotion, including: anterior biceps, semitendinosus, medial and lateral gastrocnemius, tibialis anterior, and, in some cats, the sartorius. The nerves were mounted on bipolar electrodes submerged in a mineral oil bath contained in a plastic tray on both sides or placed in nerve tunnel electrodes. After a craniotomy, a mechanical precollicular-postmamillary decerebration was performed, and the anesthetic was discontinued. Blood loss during the decerebration was replaced with a perfluorocarbon blood substitute, Oxypherol-E.T. (Alpha Therapeutic, Los Angeles). Dextran (Travenol) was also administered intravenously, as necessary, to maintain blood pressure at or >80 mmHg. Two animals in the fictive-locomotion group were decerebrated at a precollicular-premamillary level to induce spontaneous locomotion.

In fictive-locomotion experiments, the animals were paralyzed with gallamine triethiodide (Flaxedil, Rhone-Poulenc; 80 mmHg. The ENGs were amplified, rectified and band-pass filtered (30–3,000 Hz) during each trial of locomotion before being sampled continuously at 200 Hz by a Concurrent 7100 computer. In most animals, MLR stimulation was begun 2–1 h after decerebration. Once an efficacious MLR site was located, the site was stimulated to produce bouts of locomotion throughout the next 7–9 h (until perfusion). The strength of stimulation was adjusted to a level that was suitable to maintain locomotion for prolonged periods. In the first animal tested (treadmill-locomotion cat 1), MLR stimulation was performed only during the last 2 h before perfusion. One of the precollicular-premamillary decerebrated cats did not locomote spontaneously but performed hindlimb locomotion when the forelimbs were moved rhythmically in the rostrocaudal plane. Control animals received identical treatment as the locomotion test animals except that they were not subjected to the locomotor task.

After the 7- to 9-h trial periods, the animals were re-anesthetized with sodium pentobarbital (30 mg/kg) and perfused intracardially with a perfusate (normal saline containing 0.1% NaNO3 and 100 units/ml heparin) in the amount of 0.3 ml/g of animal weight, followed by a fixative solution (4% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate buffer at pH 7.4) in the amount of 1 ml/g of animal weight. The areas of the lumbar enlargement chosen for study were those encompassing the areas of maximal activity evoked by MLR stimulation (Noga et al. 1995). Accordingly, spinal segments L3-S1 were removed, postfixed in the fixative solution for 5 h, and cryoprotected by washing in 0.1 phosphate buffer containing 25% sucrose and 10% glycerol for several days. Tissue sections of 20-μm thickness were sectioned coronally with a sliding microtome and collected in 0.1 M phosphate-buffered saline (PBS). Sixteen to 20 sections were collected from each segment and were chosen so that they were evenly distributed throughout the segment. The sections were prewashed in 0.1 M PBS containing 0.3% triton TX-100 overnight, incubated for 3 days in sheep primary antiserum to fos protein (Cambridge Research Biochemical) 1:2,000 in 0.2 M PBS containing 0.3% triton and 2% bovine serum albumin, and processed with an avidin-biotin complex (ABC) kit (Vector), using diaminobenzidine as the substrate.

Slides were examined under the light microscope, and gray matter outlines, as well as locations of labeled cells, were drawn using a camera lucida. Segmental laminae were identified according to the classification of Rexed (1954) and are illustrated in Fig. 2C.

RESULTS

Representative examples of photomicrographs taken from single sections of the spinal cords of locomotion and control animals are illustrated in Fig. 1. The locomotor task (treadmill locomotion in this case) produced clear c-fos nuclear labeling in numerous cells in the dorsal and ventral horns, including labeling in presumed motoneurons in the lateral region of the ventral horn. In contrast, labeled cells were completely lacking in these same areas of the section from a control animal. Sections such as these were used to produce complete maps (L3–S1) of the c-fos labeling produced in control animals (Fig. 2), in animals with MLR stimulation undergoing treadmill (Fig. 4) or fictive (Fig. 5) locomotion and in animals exhibiting spontaneous fictive locomotion (Fig. 6).

Note that labeling of the motor nuclei was sparse, indicating that many motoneurons do not exhibit marked c-fos expression in response to repetitive activation. A summary of the total numbers of labeled cells across all lumbar segments for each animal is given in Fig. 3.
Treadmill locomotion experiments

Treadmill locomotion (TL) was induced in two animals, and it consisted of vigorous locomotor movements of both fore- and hindlimbs and good weight support. Locomotion was repeatedly induced for periods of ~3- to 30-min duration, separated by periods in which no MLR stimulus was applied. For TL cat 2 (TL-2), locomotion occurred for 5 h and 22 min within a 9-h time period. For TL cat 1 (TL-1), a period of 8 h was allowed to elapse after the decerebration, and locomotion was induced and maintained throughout the last 2 h before perfusion. The procedure used for TL-2 may have been more effective for the production of c-fos labeling because a total of 2,737 cells were labeled in the spinal cord of this animal, whereas only 969 were labeled in TL-1 (Fig. 3).

The distribution of labeled cells (excluding presumed motoneurons) from L3 to S1 segments of TL-2 is illustrated in Fig. 4B. A bimodal distribution of labeled cells was found among Rexed’s laminae (Fig. 4D), such that labeled cells in the dorsal horn were concentrated mostly in laminae III and IV. A more ventrally located group of labeled cells was concentrated in laminae VII and VIII, with some extending into lamina VI. Labeled cells were also found in the part of lamina X which is adjacent to lamina VII.

The distribution of labeled cells in the spinal cord of TL-1 is illustrated in Fig. 4A and C. In addition to having a smaller total number of labeled cells, fewer labeled cells were observed in laminae VII and VIII in TL-1 than in TL-2. We speculate that the difference between the two treadmill animals was most likely due to the fact that insufficient time was allowed for the full expression of c-fos in the ventral horn neurons in TL-1 and the fact that the total time for locomotion for TL-1 was less than half of that for TL-2. Because of this, the protocol used for TL-2 was adopted for subsequent experiments.

The rostrocaudal distribution of labeled cells for treadmill locomotion experiments is summarized in Fig. 4, E and F. The number of labeled cells started to increase at L4 or L5, reaching its peak at L6 or L7. Large labeled cells in lamina IX, presumed to be motoneurons, were not counted to restrict the analysis to neurons that might be part of the CPG for locomotion and to other cells belonging to the premotoneuronal network responsible for the control of locomotion.

Fictive locomotion experiments

MLR STIMULATION-INDUCED LOCOMOTION. Bouts of fictive locomotion (FL) were evoked by MLR stimulation. Good locomotion was recorded in both fictive-locomotion cats (FL-1 and -2). Total time in which fictive locomotion was recorded was 2 h 37 min in FL-1 and 3.5 h in FL-2. The locations of labeled cells were similar in the two cats, as illustrated in the camera lucida drawings in Fig. 5.

Most of the cells were concentrated in laminae VII and VIII (Fig. 5, A--D). Interestingly, the labeling of dorsal horn cells...
that occurred in the treadmill-locomotion animals (Fig. 4, A and B) was tremendously reduced in fictive locomotion animals, and only a few labeled cells were found in the dorsal horn. The number of labeled motoneurons was much higher in FL-1 than in FL-2, although the total number of other labeled neurons was approximately the same (Fig. 3).

The rostrocaudal distribution of the cells labeled during MLR-evoked fictive locomotion is illustrated in Fig. 5, E and F. The number of labeled cells (excluding motoneurons) increased from the lowest number at L₃ to peak at L₆. This rostrocaudal distribution is similar to that observed in the animals subjected to treadmill locomotion (Fig. 4, E and F).

SPONTANEOUS FICTIVE LOCOMOTION. Spontaneous bouts of fictive locomotion appeared 7 h before perfusion in one animal (SFL), and good regular locomotion occurred that was maintained for the last 3 h prior to the perfusion. Most of labeled cells in this spontaneous fictive locomotion cat were located in...
laminae VII and VIII (Fig. 6, A and C). As was the case for the data obtained from the MLR-induced fictive-locomotion animals, only a few labeled cells were found in the dorsal horn laminae.

The rostrocaudal distribution of the labeled cells (excluding motoneurons) is illustrated in Fig. 6E, which shows that the number of labeled cells started to increase at the L4 segment, reaching a maximum at L6. Approximately half as many labeled cells were found in the spontaneous fictive locomotion animal as were seen in the two MLR-evoked fictive locomotion animals (Fig. 3).

FICTIVE LOCOMOTION WITH RHYTHMIC FORELIMB MOVEMENT. Locomotion was not initiated spontaneously in this animal. With swinging of the forelimbs, however, bouts of fictive locomotion were observed in the hindlimb ENG recordings. Constant forelimb swinging (FS) was necessary to maintain fictive locomotion. Locomotion was maintained for a total of 3 h during a 7-h period. The locations of labeled cells in spinal segments L3–S1 in this cat (FSFL) are shown in camera lucida drawings in Fig. 6, A and B, which were induced to walk on the treadmill with MLR stimulation. Each diagram includes all labeled cells in 19 (A) or 16 (B) sections of that segment; each dot represents 1 labeled cell. Labeled cells located in the dorsal horn were concentrated in Rexed’s laminae III and IV, whereas those in the ventral horn centered in laminae VII and VIII with some extending into lamina VI. Fewer labeled cells were observed in laminae VII and VIII in TL-1 than TL-2. The difference between the 2 treadmill animals was most likely due to the fact that insufficient time was allowed for the full expression of c-fos in the ventral horn neurons in TL-1 (see METHODS). The laminar distribution of labeled cells in each locomotor cat is illustrated in C and D for TL-1 and -2, respectively. The percentage of labeled cells (bar height) in each lamina in this and subsequent figures was obtained by dividing the total number of labeled cells in that lamina from L3 to S1 by the total number of labeled cells in the cat. The segmental distribution of labeled cells (excluding motoneurons) in each cat is illustrated in E and F for TL-1 and -2, respectively. The purpose is to correct the bias that is induced by uneven number of tissue sections collected during tissue processing. Note that the number of labeled interneurons starts to increase at L4, reaching its maximum at L6 or L7."

FIG. 4. Camera lucida drawings to show the distribution of c-fos immunoreactive neurons in the spinal segments L3–S1 in treadmill locomotion cats 1 (TL-1; A) and 2 (TL-2; B), which were induced to walk on the treadmill with MLR stimulation. Each diagram includes all labeled cells in 19 (A) or 16 (B) sections of that segment; each dot represents 1 labeled cell. Labeled cells located in the dorsal horn were concentrated in Rexed’s laminae III and IV, whereas those in the ventral horn centered in laminae VII and VIII with some extending into lamina VI. Fewer labeled cells were observed in laminae VII and VIII in TL-1 than TL-2. The difference between the 2 treadmill animals was most likely due to the fact that insufficient time was allowed for the full expression of c-fos in the ventral horn neurons in TL-1 (see METHODS). The laminar distribution of labeled cells in each locomotor cat is illustrated in C and D for TL-1 and -2, respectively. The percentage of labeled cells (bar height) in each lamina in this and subsequent figures was obtained by dividing the total number of labeled cells in that lamina from L3 to S1 by the total number of labeled cells in the cat. The segmental distribution of labeled cells (excluding motoneurons) in each cat is illustrated in E and F for TL-1 and -2, respectively. The purpose is to correct the bias that is induced by uneven number of tissue sections collected during tissue processing. Note that the number of labeled interneurons starts to increase at L4, reaching its maximum at L6 or L7.

Control experiments

TREADMILL-CONTROL EXPERIMENTS. Control animals were subjected to identical surgical procedures as the treadmill locomotion animals, placed in the stereotaxic apparatus, and suspended above the treadmill, but locomotion was not induced over the 7- to 9-h trial period prior to perfusion. Treadmill-control cat 1 (TC-1) was stimulated intermittently in the region...
of the MLR for ~1 h to assure that the preparation was comparable to those used for treadmill locomotion and capable of walking when the brain stem area was stimulated. Periods of locomotion were short, however, and the animal was then left unstimulated for the remaining period of 7 h. The locations of labeled neurons in spinal segments from this animal are shown in Fig. 2 (top). Treadmill-control cat 2 (TC-2) was not subjected to any electrical stimulation. The total number of labeled cells in each cat is shown in Fig. 3. It is obvious that the number of labeled cells in each of the treadmill control animals was extremely small compared with the treadmill- and fictive- locomotor animals. Furthermore, many of the cells labeled in these control experiments were located in the dorsal horn.

FICTIVE-CONTROL EXPERIMENTS. Four animals were used as controls for fictive locomotion. They were subjected to the same surgical procedures as the fictive-locomotion animals, including the nerve dissection, and they were paralyzed with Flaxedil and artificially ventilated after the decerebration. Only fictive-control cat 4 (FC-4) was not electrically stimulated. The others (FC-1 to FC-3) were simulated intermittently for ~1 h, but without any period of sustained locomotion, and then left unstimulated for the remaining time. As seen in Fig. 3, the numbers of labeled cells in the control animals were much lower than in the fictive locomotor animals.

Figure 2 (middle) illustrates the locations of labeled cells in a representative control animal (FC-4). The total number of labeled cells in this animal was the third largest among the six control animals (see Fig. 3). Labeled cells were scattered in the dorsal horn, with a few in medial lamina VII. Labeled cells in the remaining control animals had similar distributions.

The increased numbers of cells in the fictive-control animals as compared with the treadmill-control animals is most likely due to the additional surgery needed to expose the peripheral nerves, and the additional sensory input necessitated by the placement of the animals in the spinal frame (see METHODS). This is consistent with the fact that most of the c-fos positive cells in the fictive control animals were located in the dorsal horn (e.g., Fig. 2, FC-4).

DISCUSSION

These experiments confirm our previous suggestion (Carr et al. 1995) that it is possible, using the c-fos immunochemical method, to detect neurons in the lumbar spinal cord that are part of the locomotor CPG. This conclusion is based on the fact...
that c-fos labeling consistently occurs in discrete areas of the lumbar cord during all three types of locomotion used in these experiments (MLR-evoked treadmill, MLR-evoked fictive, and spontaneous fictive) and on the observation that labeling does not require sensory feedback from the moving limb. These experiments have also revealed the rostrocaudal and laminar distribution of c-fos labeling in the lumbar spinal cord of the decerebrate cat in which locomotion is induced either spontaneously or by MLR stimulation. Few labeled cells were observed in the spinal cords of nonlocomoting control animals, and most of this labeling can be attributed to either tonic sensory input resulting from placing the animal in the fixation apparatus necessary for the locomotion experiments or to sensory stimulation resulting from the surgical preparation.

Furthermore, these experiments demonstrate that certain dorsal horn neurons express the c-fos protein in response to proprioceptive input from the moving limb.

Previous studies have shown that neurons in certain regions such as the dorsal root ganglia (Hunt et al. 1987) and the substantia nigra (Dragunow and Faull 1989), despite being activated, do not show fos elevation. It was suggested that these neurons might lack the required biochemical messengers regulating fos activation (Dragunow and Faull 1989). Our own preliminary work using intracellular labeling of interneurons active during fictive locomotion in combination with c-fos immunohistochemistry revealed that many rhythmically active cells were not labeled for c-fos under the conditions of the experiments (Carr et al. 1995). This, and the fact that motoneurons and other cells that might be expected to be active during locomotion were not labeled, indicates that negative results (i.e., no fos induction) must be interpreted with caution. Both the rostrocaudal and laminar distributions of the cells labeled in this study correspond well with the field potentials recorded in the cat spinal cord during locomotion produced by MLR stimulation (Noga et al. 1995), suggesting that despite the possibility for false negatives, the overall distribution found here represents the distribution of neurons active during fictive locomotion. It is not possible to provide an estimate of the extent of false negative labeling in other cells involved in locomotion, but there are several studies showing interneuron activity during locomotion that would lead to an expectation of greater numbers of labeled neurons in the lateral portions of the ventral horn (reviewed in Kiehn and Kjaerulf 1998). False-positive labeling appears to be minimal in the present study.
because the mean number of c-fos-positive cells in the six control animals was 87, whereas the mean number of cells labeled in the fictive-locomotion animals with MLR stimulation was 942, and a mean of 537 cells were labeled in the animals with spontaneous and fore-limb swing assisted locomotion (see Fig. 3).

**Location of locomotor-related cells in the transverse plane**

**LABELED CELLS IN REXED'S LAMINAE I–VI.** In these laminae, much more extensive labeling was observed in the treadmill locomotor cats compared with fictive locomotor cats (Fig. 6), indicating that most labeled cells were labeled due to their activation by feedback from the moving limbs. Similarly, many of the cells labeled with c-fos in rats walking on a rotating rod were presumed to be activated by nonnociceptive afferents (Jasmin et al. 1994). Kiehn and co-workers (Kjaerulff et al. 1994) found that dorsal rhizotomy reduces labeling with sulforhodamine of dorsal horn cells in hindlimb locomoting prep-1994) found that dorsal rhizotomy reduces labeling with sulforhodamine of dorsal horn cells in hindlimb locomoting prep-

**LABELED CELLS IN LAMINA VII.** Labeled neurons were numerous in lamina VII in both treadmill- and fictive-locomotor cats, consistent with the distribution of MLR-evoked field potentials in the lumbar enlargement (Noga et al. 1995). Lamina VII contains interneurons have been shown to be rhythmically active during MLR-induced locomotion. These include Renshaw cells, Ia inhibitory interneurons, midlumbar group II interneurons (McCrea et al. 1980; Noga et al. 1987a; Pratt and Jordan 1987; Shefchyk et al. 1990) and others as reviewed by Kiehn and co-workers (Kiehn and Butt 2003; Kiehn and Kjaerulff 1998).

It is noteworthy that last-order interneurons thought to be involved in locomotion detected using the WGA-HRP retrograde transneuronal labeling technique were found primarily within laminae V–VIII of the L1–S1 spinal segments, especially the lateral portion of lamina VII (Jankowska and Skoog 1986). It is apparent from Figs. 4–6 that c-fos-labeled cells are more numerous in the medial portion of lamina VII close to lamina X than in the lateral portion of lamina VII. It is not clear whether this discrepancy is due to false negatives in our data or to the fact that the locomotor activity used in the WGA-HRP experiments may not have eliminated other behaviors not related to locomotion. Cells in the medial portion of lamina VII have been implicated in the control of mammalian spinal rhythmic activity in several other studies (Barajon et al. 1992; Butt et al. 2002a; Hochman et al. 1994; Jasmin et al. 1994; Kjaerulff and Kiehn 1996; Kjaerulff et al. 1994; Nakayama et al. 2002; Noga et al. 1995).

The medially located lamina VII cells were also observed in large numbers in locomotor cats without brain stem stimulation, ruling out the suggestion that they were activated nonspecifically by the electrical stimulation in the brain stem. Extracellular recordings have shown that neurons in the medial portion of lamina VII are rhythmically active during MLR-induced locomotion (Jordan and Noga 1991), and cells in this area possess N-methyl-D-aspartate (NMDA)-mediated oscillatory properties (Hochman et al. 1994). Oscillatory properties have also been observed in Hb9-positive interneurons in the nearby medial lamina VIII in response to NMDA, 5-hydroxytryptamine, and dopamine (Wilson and Brownstone 2004). Cells such as these are thus candidate neurons for mediating the locomotor promoting effects of NMDA receptor agonists in the cat spinal cord (Douglas et al. 1993).

The c-fos-labeled cells observed in laminae VII and X in this study overlap with the distribution of sulforhodamine-labeled neurons observed after fictive locomotion in the isolated neonatal rat spinal cord (Kjaerulff et al. 1994), where the majority of labeled cells were located in a bilateral cluster close to the central canal and in the medial intermediate gray matter.

Cells near the central canal were also observed in the adult rat spinal cord due to a rotating rod locomotor task (Jasmin et al. 1994), but the incidence of labeled cells in lamina VII appeared to be much lower under the conditions of their study. The rotating rod task has not yet been shown to be one that requires the recruitment of the CPG for locomotion, and so it is not possible to predict the degree of overlap expected when the results produced by this task are compared with those produced during fictive locomotion.

Scratching induces c-fos expression in the intermediate region of the lumbar cord (Barajon et al. 1992), with some labeling near the central canal. It is possible, therefore that certain interneurons in this region participate in both scratching and locomotion. Evidence for the possibility of shared neurons in the networks generating locomotion and scratching (Arshavsky et al. 1988) has recently been obtained (Perreault et al. 1999). The c-fos distribution produced in fictive scratch was only determined for the L2 and S1 segments (Barajon et al. 1992), however, and comparing this distribution (their Fig. 3) with our results in these same segments (Figs. 5 and 6) reveals that the locomotor-related neurons are concentrated in the medial areas of the ventral horn, whereas the scratch-related neurons were concentrated more laterally. Thus although it may be that some neurons participate in both types of rhythmic activity, there does not appear to be complete overlap. This would be consistent with the conclusion, based on the effects of scratching and locomotion on postsynaptic potentials in motoneurons, that the central pattern generators that produce fictive locomotion and scratching are organized differently (Degtyarenko et al. 1998).

Lesion studies to determine the laminar locations of interneurons necessary for production of rhythmic activity have been carried out in the chick (Ho and O’Donovan 1993) and the neonatal rat (Kjaerulff and Kiehn 1996) spinal cord. In the chick, normal alternation between flexor and extensor motorneuron groups remains if a region dorsomedial to the motoneurons (corresponding to lamina VII) is left intact. In the neonatal rat, this medial intermediate area is necessary for rhythmic activity. Previous work in slices of the lumbar enlargement from the neonatal rat has shown that rhythmic activity that persists in the presence of tetrodotoxin can be induced in interneurons near the central canal by agents that can induce fictive locomotion (Hochman et al. 1994).

Electrophysiological studies on the distribution of rhythmically active cells within the lumbar spinal cord have sampled only limited areas within the rostrocaudal extent of the lumbar enlargement. In a study on locomotion induced by stimulation of the MLR in decerebrate cats (Orlovsky and Feldman 1972), rhythmically active neurons were concentrated in the lateral and dorsolateral parts of the ventral horn. In thalamic cats with...
spontaneous fictive locomotion (Baev et al. 1979), interneurons in the lateral part of the intermediate zone and the ventral horn of the L6 and L7 segments participated in the locomotor rhythm. In the neonatal rat preparation, interneurons rhythmically active during fictive locomotion have been recorded in lamina VII throughout the lumbar enlargement (Butt et al. 2002a; Kiehn et al. 1996; MacLean et al. 1995).

Several ascending tracts (spinotectal, spinomesencephalic, spinoreticular) originate, in part, from cells in lamina VII (Burstein et al. 1990; Willis 1986; Willis et al. 2004), and such tract cells may have been labeled in this study. It is therefore possible that certain ascending neurons are activated by the locomotor CPG, but this issue was not directly assessed in this study.

**LABELED CELLS IN LAMINA VIII.** Cells in lamina VIII were labeled in both treadmill- and fictive-locomotor cats. A significant proportion of commissural interneurons of laminae VIII, secondarily labeled after WGA-HRP injection into a muscle nerve, have been shown to project to contralateral motor nuclei (Harrison et al. 1986). Noga and co-workers (Noga et al. 1987b) found that the contralaterally located last-order interneurons stained during fictive locomotion were restricted to lamina VIII after injection of the activity-dependent label WGA-HRP into the anterior biceps nerve. In the midlumbar segment (L3/L4), cells in lamina VIII have been suggested to have the same type of role in locomotion as ipsilateral neurons in lamina VII of the same segment which are activated by group II afferents (Jankowska and Noga 1990). These authors also demonstrated that lamina VIII cells projecting to contralateral motor nuclei are disynaptically activated from the cuneiform nucleus, the anatomical equivalent of the MLR. The results obtained in this study confirm the previous finding that lamina VIII cells are involved in locomotion. They are also consistent with the results of our field potential mapping study (Noga et al. 1995), in which negative field potentials produced by MLR stimulation were observed in lamina VIII, and with the recent findings showing that lamina VIII commissural neurons are rhythmically activated during MLR-evoked fictive locomotion in the cat (Matsuyama et al. 2004). Further evidence for cells in lamina VIII that are active during locomotion come from studies on transgenic mice. Visually identified cells in medial lamina VIII containing enhanced green fluorescent protein driven by the Hb9 promoter are rhythmically active during chemically evoked locomotion in the isolated mouse cord (Hinchley et al. 2004), and chemically induced locomotion in the isolated spinal cord of the fetal mouse upregulates c-fos expression in V0 cells of lamina VIII implicated in controlling left-right alternation (Lanuza et al. 2004). Extensive studies on commissural cells that are active during locomotion in the isolated spinal cord of neonatal rats have been recently carried out, and most of these cells are located in medial laminae VII and VIII (Butt and Kiehn 2003; Butt et al. 2002a,b; Nakayama et al. 2002).

**LABELED CELLS IN LAMINA IX.** Alpha-motoneurons can be distinguished from interneurons in the tissue sections with c-fos immunostaining on the basis of their large size. It was observed that some of the motoneurons were labeled (Figs. 1 and 4–6). However, the numbers of motoneurons labeled varied considerably. For instance, although locomotion in TL-2 was well developed, only a few motoneurons were labeled, whereas a large number of labeled interneurons in lamina IX were observed in the same animal (Fig. 4). Thus there is obviously false-negative labeling of many motoneurons.

**Rostrocaudal distribution of labeled cells**

The results described here suggest that in the cat spinal cord the interneurons active during locomotion are most numerous (per unit length) in the L5–L7 segments. Those related to MLR stimulation also appear to be most numerous in this same rostrocaudal area as revealed by evoked potential analysis (Noga et al. 1995). This is consistent with the observations (Grillner and Zangger 1979), showing that the ability to initiate locomotion in spinal animals is lost after spinal transections below caudal L5. In other studies, however, locomotion in spinal cats was absent after lesions at L4 (Afelt 1970; Rossignol et al. 2002). In spinal cats, microinjections of clonidine into a restricted area of the lumbar cord (the L1–L4 segments) also induces locomotion (Marcoux and Rossignol 2000), suggesting that this is the leading area for locomotion induced by activation of noradrenergic receptors in this species. For the fictive scratch reflex the L7–L5 segments have been proposed to be the “leading” (Deligianna et al. 1983) segments, although the rhythm can occur in more caudal segments. Viala and co-workers (1988) found that locomotion induced by DOPA increased metabolism of rabbit spinal cord cells in the intermediate gray matter from L6 to S1. This more caudal concentration of labeling in the rabbit cannot be explained on the basis of existing data, but it is plausible that it represents a species difference. Our data suggest that if the rhythm generating elements are concentrated in the rostral lumbar segments, many other cells that are active during locomotion, perhaps involved in the production of the locomotor pattern rather than in the generation of rhythmic activity, are located in more caudal segments.

Other evidence favoring concentration of rhythm generating elements in the rostral segments of the lumbarosacral enlargement comes from work on turtle (Mortin and Stein 1989), chick (Ho and O’Donovan 1993), and rodent (Bertrand and Cazalets 2002; Cazalets et al. 1995; Kiehn and Kjaerulff 1998). Cazalets and co-workers (Noga et al. 1995) have claimed that the interneurons required for locomotion are restricted to the L1–L2 region of the neonatal rat spinal cord. However, other work (Cowley and Schmidt 1997; Kjaerulff and Kiehn 1996; Kremer and Lev-Tov 1997) shows that the CPG is likely to be distributed throughout the lumbar enlargement in the neonatal rat as it appears to be in other species. More recent work (Bertrand and Cazalets 2002) has supported this basic conclusion although the authors indicate that the “rostral segments play a major functional role in locomotor genesis even if caudal segments could be involved in causal rhythogenesis.” This would be consistent with the observations that lesions of the intermediate gray matter at the L5 segmental level in the adult rat resulted in paraplegia without damage to white matter or motoneuronal pools (Hadi et al. 2000; Magnuson et al. 1999). A more rostral placement for interneurons related to locomotion might be expected in the rat than in the cat because in the former species, hindlimb motoneurons are contained within the L1–L6 segments (Nicolopoulos-Stournaras and Iles 1983), whereas in the cat the motoneurons to the hindlimb extend from caudal L4 to S1 (Vanderhorst and Holstege 1997). The distribution of locomotor-related cells revealed in this study is consistent with a
network of neurons distributed throughout the lumbar enlargement rather than concentrated in the rostral segments.

**Possible significance c-fos expression during locomotion**

Besides providing a means to map neurons that become active during a locomotor task, the expression of c-fos during locomotion may provide clues to the cellular processes for changes in gene expression required for plasticity in the locomotor system, such as occurs during training to restore locomotion after spinal cord injury (Barbeau and Rossignol 1987; Barbeau et al. 1993; de Leon et al. 1998, 1999). In addition, c-fos expression can be used to detect locomotor interneurons that respond to NMDA and/or express L-type calcium channels, both of which are implicated in the control of locomotion (Douglas et al. 1993; Kiehn et al. 1996) and are factors in the induction of c-fos expression in other cell types. We have shown that cells labeled with c-fos can be simultaneously labeled with antibodies to glutamate, aspartate, choline acetyltransferase, or with the NADPH-diaphorase reaction product (Carr et al. 1995; Huang et al. 2000). In preliminary studies (Johnson et al. 2002), we have also found that many locomotor-activated spinal neurons identified using c-fos immunohistochemistry are also innervated by descending monoaminergic fibers and express monoaminergic receptors implicated in the control of locomotion. Thus subpopulations of spinal neurons involved in the control of locomotion can potentially be distinguished on the basis of the neurotransmitters, receptors, channels, or by their expression of specific genes, such as the homeobox gene Dbx1 found in V0 interneurons (Lanuza et al. 2004).

The results presented in this study provide a firmer basis for experiments in the cat designed to restore locomotor function after spinal-injury preparation (Chau et al. 1998; de Leon et al. 1998, 1999; Lovely et al. 1986; Mushahwar and Horch 1998; Reier et al. 1992; Rossignol 1996; Rossignol et al. 1998). In particular, this data can be used to determine the most promising sites for intraspinal stimulation or intrathecal drug applications or the target sites for transplantation efforts using L-type calcium channels, both of which are implicated in the control of locomotion (Douglas et al. 1993; Kiehn et al. 1996) and are factors in the induction of c-fos expression in other cell types. We have shown that cells labeled with c-fos can be simultaneously labeled with antibodies to glutamate, aspartate, choline acetyltransferase, or with the NADPH-diaphorase reaction product (Carr et al. 1995; Huang et al. 2000). In preliminary studies (Johnson et al. 2002), we have also found that many locomotor-activated spinal neurons identified using c-fos immunohistochemistry are also innervated by descending monoaminergic fibers and express monoaminergic receptors implicated in the control of locomotion. Thus subpopulations of spinal neurons involved in the control of locomotion can potentially be distinguished on the basis of the neurotransmitters, receptors, channels, or by their expression of specific genes, such as the homeobox gene Dbx1 found in V0 interneurons (Lanuza et al. 2004).

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Present address of B. R. Noga: Miami Project to Cure Paralysis, University of Miami School of Medicine, P.O. Box 016990, R-48, Miami, FL 33101.

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