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Distributions of active spinal cord neurons during swimming and scratching motor patterns

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Abstract The spinal cord can generate motor patterns underlying several kinds of limb movements. Many spinal interneurons are multifunctional, contributing to multiple limb movements, but others are specialized. It is unclear whether anatomical distributions of activated neurons differ for different limb movements. We examined distributions of activated neurons for locomotion and scratching using an activity-dependent dye. Adult turtles were stimulated to generate repeatedly forward swimming, rostral scratching, pocket scratching, or caudal scratching motor patterns, while sulforhodamine 101 was applied to the spinal cord. Sulforhodamine-labeled neurons were widely distributed rostrocaudally, dorsoventrally, and mediolaterally after each motor pattern, concentrated bilaterally in the deep dorsal horn, the lateral intermediate zone, and the dorsal to middle ventral horn. Labeled neurons were common in all hindlimb enlargement segments and the preenlargement segment following swimming and scratching, but a significantly higher percentage were in the rostral segments following swimming than rostral scratching. These findings suggest that largely the same spinal regions are activated during swimming and scratching, but there are some differences that may indicate locations of behaviorally specialized neurons. Finally, the substantial inter-animal variability following a single kind of motor pattern may indicate that essentially the same motor output is generated by anatomically variable networks.

Keywords Locomotion · Central pattern generator · Interneuron · Activity-dependent labeling · Sulforhodamine

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Introduction

Distinct movements produced by the same muscles and motoneurons are often generated largely by a common network of multifunctional central nervous system neurons (Morton and Chiel 1994; Marder and Calabrese 1996; Kupfermann and Weiss 2001; Marder and Bucher 2001; Marder et al. 2005; Katz and Hooper 2007; Briggman and Kristan 2008; Berkowitz et al. 2010). Nonetheless, there are also behaviorally specialized central nervous system neurons, which are activated during just one kind of movement (Ramirez and Pearson 1988; Hennig 1990; Morton and Chiel 1994; Edwards et al. 1999; Kupfermann and Weiss 2001; Briggman and Kristan 2008; Berkowitz et al. 2010). Vertebrate rhythmic limb motor patterns underlying locomotion and scratching can be generated by the spinal cord alone, even without movement-related sensory feedback and input from the brain (Brown 1911; Goulding 2009; Grillner and Jessell 2009; Berkowitz et al. 2010; Kiehn 2011; Frigon 2012). How are spinal cord networks organized to produce multiple behaviors appropriately? Are the interneurons involved in different rhythmic limb motor patterns located in similar regions of the spinal cord or are there anatomically distinct clusters of behaviorally specialized interneurons?

The turtle spinal cord is a good model system to examine the network organization that selects and produces different vertebrate limb movements (Stein 2005;

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Berkowitz 2010). The turtle spinal cord can generate forward swimming, three forms of scratching (rostral, pocket, and caudal), and flexion reflex, even in the absence of movement-related sensory feedback and input from the brain (Lennard and Stein 1977; Stein and Grossman 1980; Stein et al. 1982; Mortin et al. 1985; Robertson et al. 1985; Juranek and Currie 2000). Single-neuron electrophysiological and morphological studies of the turtle spinal cord have identified both multifunctional and behaviorally specialized spinal interneurons (Berkowitz and Stein 1994a; Berkowitz 2001, 2002, 2005, 2007, 2008; Berkowitz et al. 2006). Multifunctional interneurons, including transverse interneurons, are activated during swimming, scratching, and flexion reflex motor patterns (Berkowitz 2002, 2008; Berkowitz et al. 2006). One set of behaviorally specialized interneurons has been described for scratching (Berkowitz 2002, 2008) and another set for flexion reflex (Berkowitz 2007). Flexion reflex-selective interneurons, which are often inhibited during swimming and scratching, have so far been found exclusively in the dorsal horn (Berkowitz 2007). Whether scratch-specialized interneurons, which are often inhibited during swimming, have a distinct anatomical distribution is not yet clear, due to the relatively low number of scratch-specialized neurons encountered in single-neuron studies.

We use an activity-dependent fluorescent dye, sulforhodamine 101, to examine a larger population of spinal cord neurons activated during each kind of motor pattern than is possible in single-neuron recording studies. We compare the anatomical distributions of spinal cord neurons activated during the motor patterns underlying forward swimming and the three forms of scratching. Sulforhodamine is taken up selectively by neurons at active synapses and transported retrogradely to neuronal cell bodies (Lichtman et al. 1985; Keifer et al. 1992; Teng et al. 1999); it has been utilized to visualize activated spinal cord or brainstem neurons in turtles (Keifer et al. 1992), frogs (Giszter et al. 1993), rats (Kjaerulff et al. 1994; Cina and Hochman 2000), and opossums (Lavallee and Pflieger 2009). Several studies have examined the anatomical distribution of activity-dependent labeling in the spinal cord during a single, rhythmic motor pattern (Viala et al. 1988; Barajon et al. 1992; Kjaerulff et al. 1994; Cina and Hochman 2000; Dai et al. 2005), but to our knowledge, no one has compared the anatomical distributions of active spinal cord neurons between different motor patterns such as locomotion and scratching. A comparison of the distributions of active neurons can potentially identify regions of multifunctional interneurons as well as regions containing behaviorally specialized neurons. Results have been previously reported in abstracts (Willis and Berkowitz 2008; Mui et al. 2009; Mui and Berkowitz 2010).

Methods

Turtle and surgery

Adult red-eared turtles ($n = 18$), *Trachemys scripta elegans*, weighing 300–1,300 g, were spinally transected under hypothermic analgesia between the dorsal 2 (D2) and D3 roots (Stein and Grossman 1980; Mortin et al. 1985; Robertson et al. 1985). Seven spinal cord segments, D6 through D10 and sacral (S) 1–2 were exposed. This includes the five hindlimb enlargement segments (D8–S2) and two pre-enlargement segments (D6–D7). Several right (ipsilateral) muscle nerves were dissected free for extracellular recording. These nerves included the hip flexor nerve, ventral puboischiofemoralis internus, pars anteroventralis, the hip extensor nerve, flexor cruris, pars flexor tibialis internus, and three knee extensor nerves, triceps femoris, pars iliotibialis, pars ambiens, and pars femorotibialis. The nerves were recorded via pairs of 100-μm silver wires in mineral oil (Robertson et al. 1985). Turtles were injected with the neuromuscular blocker, gallamine triethiodide (8 mg/kg, i.m.; Sigma-Aldrich, St. Louis, MO, USA) and artificially ventilated at room temperature. Procedures and protocols were approved by the University of Oklahoma Institutional Animal Care and Use Committee.

Stimulation sites and timing

Each animal (other than controls) was stimulated to produce one of the four motor patterns: forward swimming, rostral scratching, pocket scratching, or caudal scratching, ipsilateral to the right hindlimb nerve recordings (Fig. 1). Swimming motor patterns were evoked in nerves of the right (ipsilateral) hindlimb using a pair of 100-μm silver wires (California Fine Wire, Grover Beach, CA, USA), insulated except at the tip, delivering a 40-Hz train of electrical stimuli to the left (contralateral) D3 lateral funiculus (Lennard and Stein 1977; Juranek and Currie 2000; Berkowitz 2002). Each stimulus pulse was composed of 100-μs negative and 100-μs positive square waves of 350–450 μA each. Scratching motor patterns were evoked by continual gentle rubbing of the shell at one site using a glass rod with a fire-polished tip. For each form of scratching, stimulation was delivered on the right side, at the site that produced the strongest motor pattern for that form of scratching. Each turtle was stimulated to produce one kind of motor pattern for as long as the animal would produce it continuously (10–40 s, depending on the animal) every 2–5 min for 3–12 h while being exposed to sulforhodamine 101. In no-stimulation control animals, procedures were the same except that no stimulation was delivered.

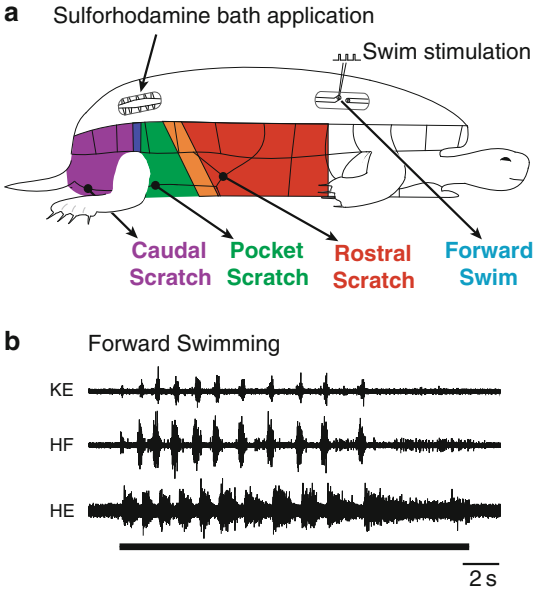

Fig. 1 Illustration of the experimental paradigm. **a** 0.04 % Sulforhodamine 101 was applied to the exposed spinal cord segments D6–S2. One kind of motor pattern was repeatedly evoked, either forward swimming, by electrical stimulation of the caudal cut surface of the transected D3 contralateral (*left*) lateral funiculus, or one form of scratching, by mechanical stimulation of a site in the appropriate region of the body surface [rostral scratch (*red*), pocket scratch (*green*), or caudal scratch (*purple*)]. **b** An example of a forward swimming motor pattern evoked in these experiments. *KE* knee extensor nerve, *HF* hip flexor nerve, *HE* hip extensor nerve

Sulforhodamine 101 bath application

The three layers of meninges were torn on the dorsal side of spinal cord segments D6–S2. A wax well was created over the exposed spinal cord to contain the solution. 0.04 % Sulforhodamine 101 (Invitrogen, Eugene, OR) in turtle saline was placed in the spinal cord well and the well was covered with aluminum foil due to light sensitivity of the dye. The sulforhodamine solution was left in the well for the duration of the stimulation. After stimulation, sulforhodamine was removed and the spinal cord was superfused with turtle saline for 1 h to remove excess dye. In the lidocaine-control animals, 2 % lidocaine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in turtle saline was applied to the spinal cord 30–90 min before the sulforhodamine application; 2 % lidocaine was also included in the sulforhodamine solution for these animals. The lack of nerve responses to scratch and flexion reflex stimulation was confirmed before the addition of sulforhodamine solution.

Perfusion and histology

Deep anesthesia was induced with sodium pentobarbital (390 mg, i.p). The turtle heart was exposed 1 h later and the turtle was perfused transcardially first with 800 ml turtle saline with 0.1 % sodium nitrite (Sigma-Aldrich, St. Louis, MO, USA), 10 units/ml heparin (Sigma-Aldrich), and 39 mg pentobarbital, then with 300 ml cold 4 % paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (PB; Fisher Scientific, Fair Lawn, NJ, USA), pH 7.4. The D6–S2 spinal cord was then removed and placed in the same fixative solution at 4 °C overnight. The spinal cord was then switched to a cold 20 % sucrose/PB solution for a minimum of 4 h prior to embedding. The spinal cord was embedded in a gelatin (Sigma-Aldrich)-albumin (Sigma-Aldrich)-glutaraldehyde (Fisher Scientific) medium and frozen sectioned at 50 µm either horizontally or transversely. For horizontally sectioned spinal cords, all sections were kept; for transverse sections, every 3rd or 4th section was kept for the analysis. Sections were mounted on gelatin-coated slides, air dried, and dehydrated in desiccant for a minimum of 2 days at 4 °C. Slides were then cleared with Histo-Clear (National Diagnostics, Atlanta, GA, USA) for at least 2 h. Slides were then cover-slipped with Entellan (Merck, Darmstadt, Germany) prior to photography and analysis.

Photography and analysis

Sections were viewed with rhodamine epifluorescence and photographed using a Nikon Optiphot-2 microscope (Melville, NY, USA) and an Olympus DP-70 camera (Center Valley, PA, USA); all labeled neurons were photographed. Whole horizontal sections were photographically assembled from photographs of each part of the section in Photoshop (Adobe Systems, San Jose, CA, USA). Cells were counted as active neurons if they had a clearly defined fluorescent cell body (approximately twice the fluorescence intensity of the background, or greater) and displayed the beginning of a process. Locations of labeled cells were counted by segment, side, and dorsoventral position in the gray matter. Since the turtle spinal cord gray matter does not show clear laminar arrangements (Kusuma et al. 1979), the gray matter was divided into the dorsal horn, intermediate zone, and ventral horn regions for quantitative analyses. This method of classifying regions of the turtle spinal gray matter has been used previously (Berkowitz and Stein 1994b; Berkowitz 2004; Nissen et al. 2008). Total numbers of labeled neurons and percentages of neurons in each spinal cord region (Fig. 4) were compared statistically between swimming ($n = 4$) and rostral scratching ($n = 4$) using InStat (GraphPad Software, La Jolla, CA, USA) and the 2-tailed Mann–Whitney test. Distributions of labeled cells in the transverse plane (Figs. 5, 6, 7) were created by superimposing each transverse section and its labeled cells onto a schematic cross section of the corresponding spinal cord segment; tracings were stretched or compressed as needed to match the schematic cross section.

Results

Sulforhodamine-labeled neurons were found in all animals. Labeled cells were typically much brighter than the background and could include one or more brightly fluorescing primary dendrites (Fig. 2). When sulforhodamine is applied to the spinal cord, it should only be taken up by neurons with axon terminals within the spinal cord, which mostly limits labeling to interneurons (Kjaerulff et al. 1994). Motoneurons with a central axon may also be labeled with these methods, but such motoneurons are probably few in turtles (Ruigrok et al. 1984).

Horizontal sections

A preliminary set of experiments ($n = 5$) was conducted in which the distributions of sulforhodamine-labeled cells were examined in horizontal sections. Each turtle received stimulation at one site to evoke forward swimming (henceforth, just "swimming"; Sw), rostral scratching (R Sc), pocket scratching (P Sc), or caudal scratching (C Sc) motor patterns repeatedly (see "Methods"), or received no stimulation (ns C). Animals that received stimulation to produce one of the four motor patterns had substantially more total labeled cells than the no-stimulation control animal, though total number of labeled cells varied in stimulated animals (Fig. 3a).

In each stimulated animal, labeled cells were distributed bilaterally; labeling ipsilateral to the motor pattern was usually greater, but there was much variability (Fig. 3b). Rostrocaudally, labeled cells were found distributed across all segments examined for all motor patterns, though segmental labeling patterns varied (Fig. 3c). In the transverse plane, following all motor patterns except for caudal scratching, labeled cells were primarily concentrated in the ventral horn, and to a lesser extent, the intermediate zone; following caudal scratching, there were comparable numbers of labeled cells in the intermediate zone and ventral horn (Fig. 3d). It is difficult, however, to assign labeled cells to a dorsoventral region accurately using horizontal sections.

Transverse sections

In a second set of experiments ($n = 11$), the turtle spinal cord was sectioned transversely to provide a more precise indication of labeling within the transverse plane. Labeling was quantified by spinal cord region (Fig. 4) and the locations of all labeled cells were also plotted on schematic cross sections to illustrate the patterns of labeling (Figs. 5, 6, 7). Although the total number of labeled cells varied

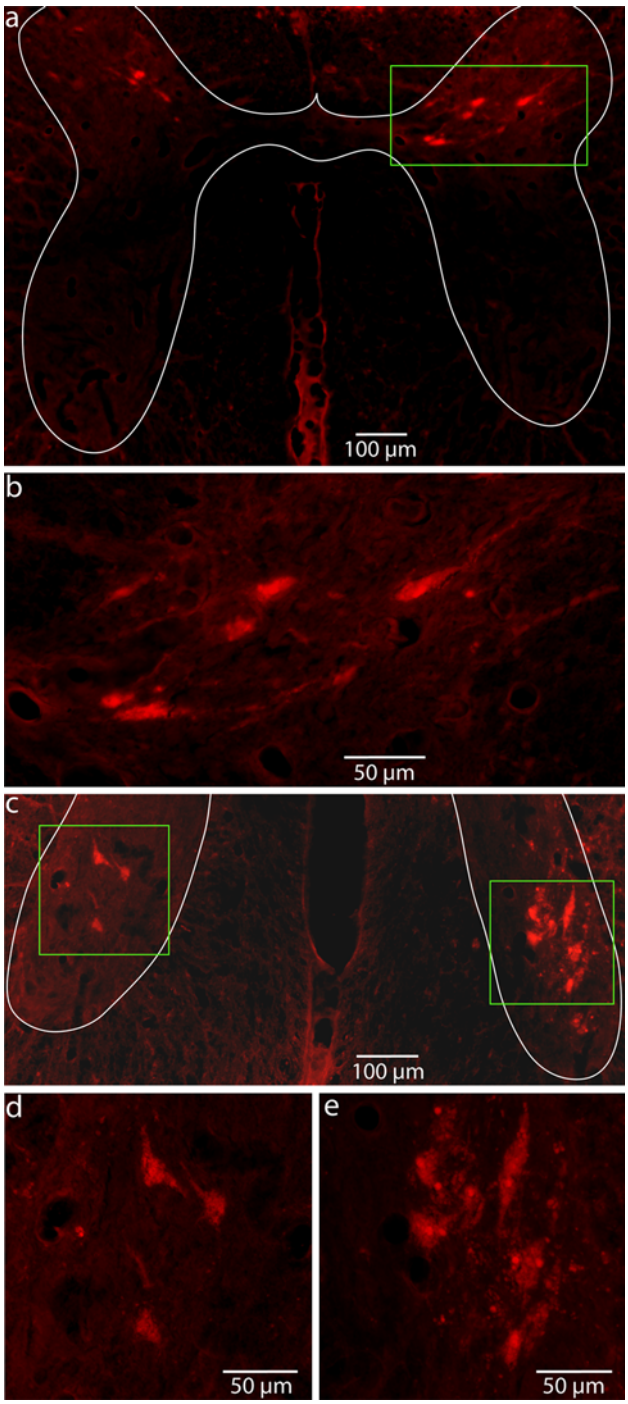

Fig. 2 Examples of sulforhodamine 101-labeled cells following swimming and scratching motor patterns. Labeled cells were typically much brighter than the background and could include one or more brightly fluorescing primary dendrites. **a** Transverse section showing D8 bilateral deep dorsal horn labeling following swimming motor patterns. *White tracing* outlines the gray matter. **b** Higher magnification view of the ipsilateral cells (*green box*) seen in (**a**). **c** Transverse section showing D10 bilateral ventral horn labeling following rostral scratching motor patterns. Higher magnification views of the labeled cells [*green boxes* seen in (**c**)] contralaterally (**d**) and ipsilaterally (**e**)

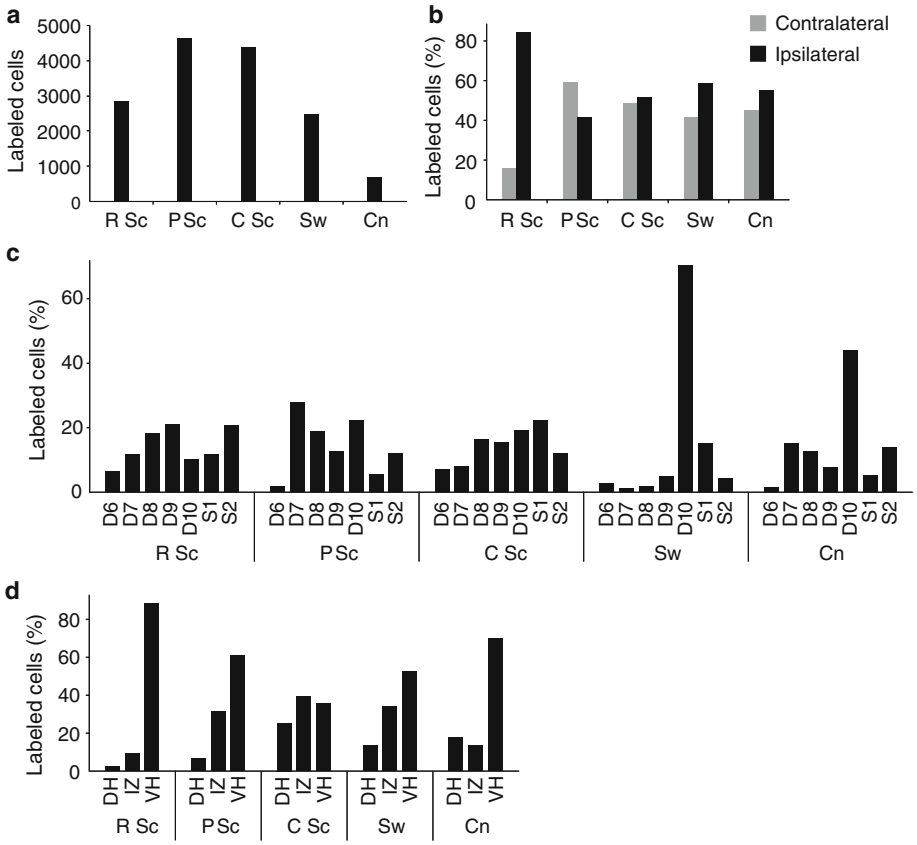

Fig. 3 Quantified distributions of sulforhodamine-labeled cells in horizontal sections following motor patterns. **a** Total number of labeled cells following forward swimming (*Sw*), rostral scratching (*R Sc*), pocket scratching (*P Sc*), caudal scratching (*C Sc*), and a no-stimulation control (*ns C*). One animal was used in each case for these preliminary experiments. **b** Percentage of labeled cells found contralateral (*gray*) and ipsilateral (*black*) to each motor pattern. **c** Percentage of labeled cells found in each spinal cord segment examined following each motor pattern. **d** Percentage of labeled cells found in the dorsal horn (*DH*), intermediate zone (*IZ*), and ventral horn (*VH*) following each motor pattern

between animals, all turtles that received stimulation to evoke swimming or scratching motor patterns had substantially more labeled cells than animals that received no stimulation (Fig. 4a). In two additional animals (li C), 2 % lidocaine hydrochloride was applied to the spinal cord before and during sulforhodamine (and no stimulation was delivered during sulforhodamine) application to further reduce neural activity. Following lidocaine application, no motor patterns were evoked by stimuli that previously evoked scratching and flexion reflex motor patterns, although one or a few very small hip flexor motoneurons displayed tonic spontaneous activity in one animal (not shown). The spinal cords of these two animals were sectioned horizontally, with all sections examined, to screen for labeled neurons in the entire tissue; substantially fewer neurons were labeled in these cases than even in the no-stimulation control animal without lidocaine (Fig. 4a), indicating that sulforhodamine neuronal labeling was indeed activity dependent.

Labeling was consistently bilateral following each motor pattern (Figs. 4b, 5, 6, 7) in the animals that had their spinal cords sectioned transversely. Although there were some differences in the left–right proportions among these animals, there was no consistent difference between the two sides following swimming ($n = 4$; left vs. right $p = 0.057$) or rostral scratching ($n = 4$; $p = 0.99$), for which multiple animals were obtained. Thus, there were remarkably high percentages of labeled cells contralateral to the unilaterally evoked motor patterns.

Rostrocaudally, labeled cells were found in all of the examined spinal cord segments (Figs. 4c, 5, 6, 7). A majority of labeled cells were in segments D7–D10 collectively for all four swimming animals, three of the four rostral scratching animals, and both the pocket scratching and the caudal scratching animals (Figs. 5, 6). The mean D7–D10 labeling was 69.8 + 13.1 % (SD) for swimming, 57.0 + 16.1 % for rostral scratching, 91.5 % for pocket scratching, and 63.9 % for caudal scratching. Comparing swimming and rostral scratching specifically (because multiple examples were obtained for each), a majority of labeled neurons were in the most rostral segments examined, D6–D9, collectively, in each of the four animals following swimming (Fig. 5), while a majority of labeled neurons were in the caudal segments (D10–S2, collectively) in each of the four animals following rostral scratching (Fig. 6). The mean percentage of labeling in the D6–D9 segments was significantly greater following swimming than following rostral scratching (swimming 75.8 ± 19.0 %; rostral scratching 39.0 ± 10.4 %; $p = 0.029$).

In the transverse plane, labeled cells were found in the dorsal horn, intermediate zone and ventral horn in all stimulated animals (Figs. 4d, 5, 6, 7). There tended to be more labeled cells in the dorsal horn following swimming

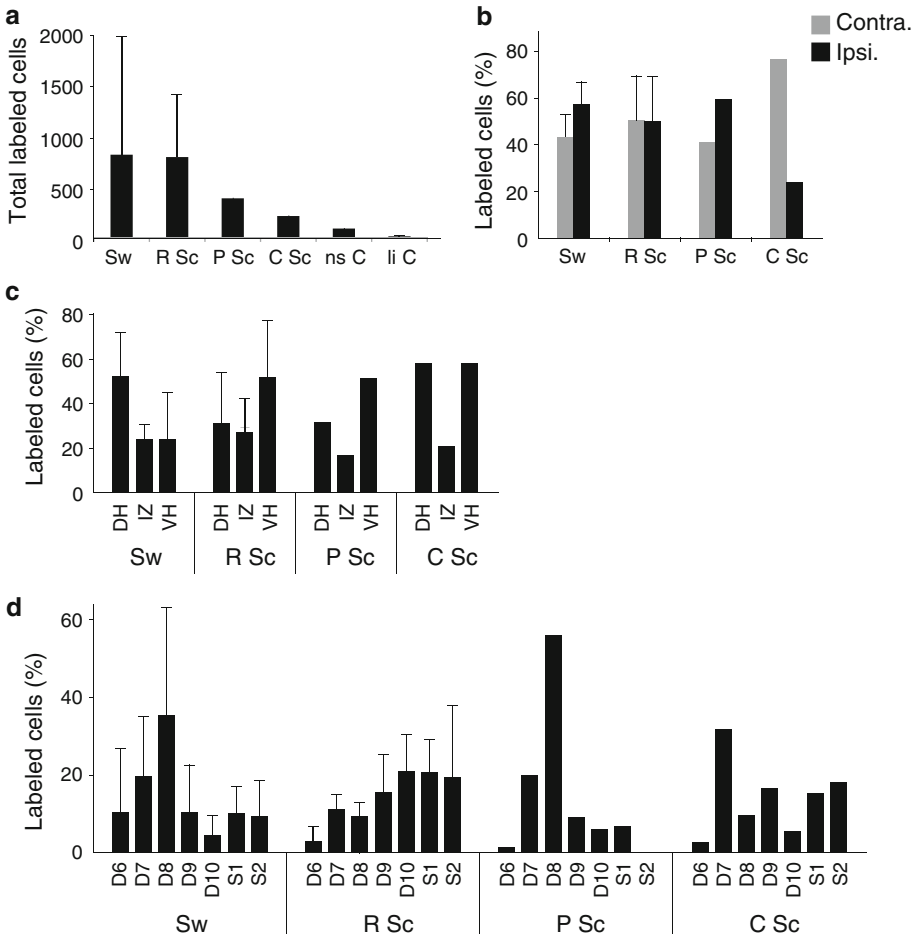

Fig. 4 Quantified distributions of sulforhodamine-labeled cells in transverse sections following motor patterns. **a** Total number of labeled cells following forward swimming (*Sw*; $n = 4$), rostral scratching (*R Sc*; $n = 4$), pocket scratching (*P Sc*), caudal scratching (*C Sc*), a no-stimulation control (*ns C*), and no-stimulation controls with lidocaine applied to the spinal cord before and during sulforhodamine application (*li C*; $n = 2$). For transverse sections (all examples in this figure except li C), every third or fourth section was kept and the numbers of labeled cells were multiplied by 3 or 4, respectively, to estimate the total number of labeled cells. **b** Percentage of labeled cells found contralateral (*gray*) and ipsilateral (*black*) to each motor pattern. **c** Percentage of labeled cells found in each spinal cord segment examined following each motor pattern. **d** Percentage of labeled cells found in the dorsal horn (*DH*), intermediate zone (*IZ*), and ventral horn (*VH*). For swimming, rostral scratching, and the lidocaine control in **a**, the numbers and percentages shown are mean ± SD; note that the *error bar* for *li C* is too small to be seen easily

than following rostral scratching and more labeled cells in the ventral horn following rostral scratching than following swimming. These differences, however, were not statistically significant (for swimming vs. rostral scratching, $p = 0.11$ for dorsal horn; $p = 0.69$ for intermediate zone; $p = 0.49$ for ventral horn).

Despite substantial variability, some common patterns of labeling in the transverse plane can be seen in Figs. 5, 6, and 7. Following swimming, labeled cells were mainly in the deep dorsal horn, the lateral intermediate zone, and the dorsal and middle portions of the ventral horn, bilaterally. Following rostral scratching, a similar pattern of labeling was seen, but there were substantial numbers of labeled cells in the deep ventral horn as well. The patterns of labeling following pocket and caudal scratching were broadly similar to those following forward swimming and rostral scratching, though they are harder to assess because there was just one animal for each. The pocket scratching animal had an especially large amount of labeling in D8, especially ipsilaterally. The caudal scratching animal had greater contralateral labeling. In general, both swimming and scratching animals had few labeled cells in the dorsalmost part of the dorsal horn and in the medial part of the intermediate zone (near the central canal).

Discussion

This is the first study, to our knowledge, to compare activity-dependent neuronal labeling distributions in the

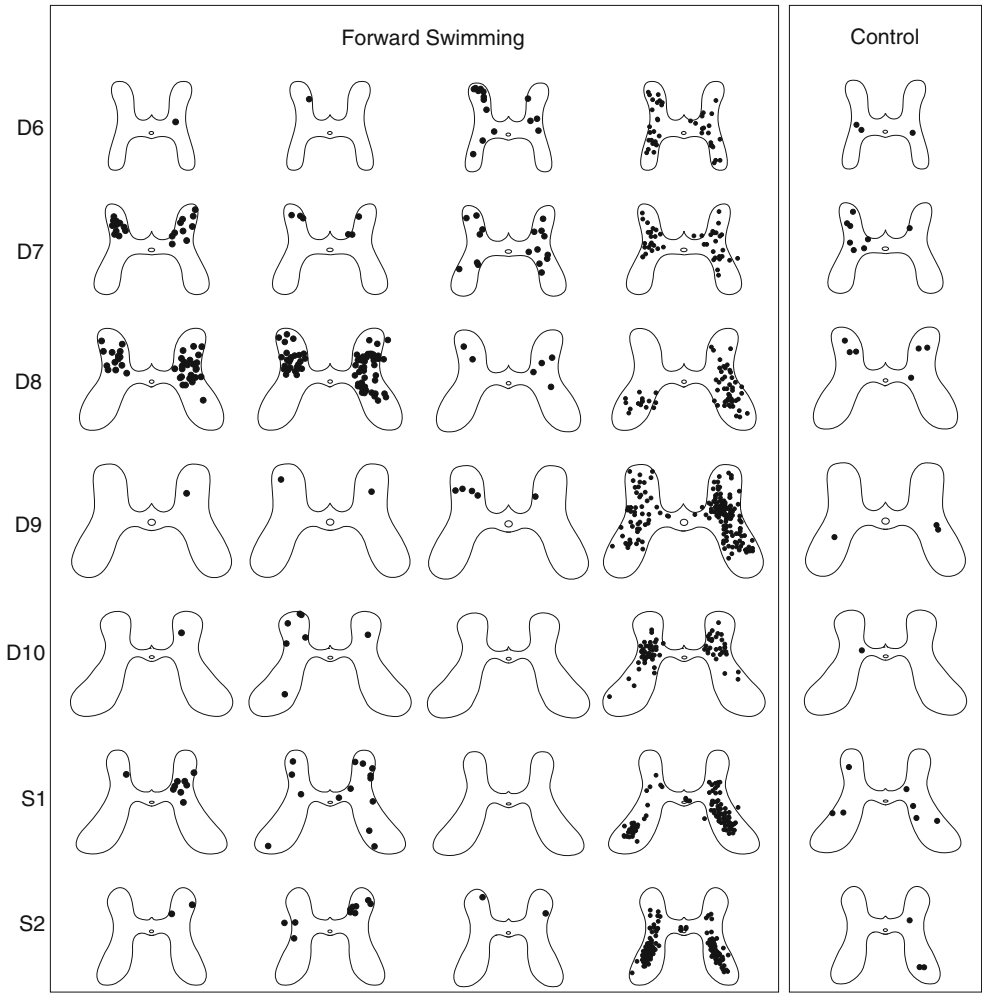

Fig. 5 Cross-sectional distributions of labeled neurons following forward swimming motor patterns and a no-stimulation control in all transversely sectioned cases. Each *filled circle* represents a single labeled neuron and each *column* represents one animal. For clarity, the size of each *circle* was reduced in one animal that had a larger number of cells labeled

spinal cord following different types of rhythmic limb motor patterns. The distributions of labeled neurons were remarkably wide following both forward swimming and each form of scratching, involving substantial labeling in all seven spinal cord segments examined, and in the dorsal horn, intermediate zone, and ventral horn, bilaterally. This finding is consistent with the hypothesis that locomotion and scratching are each produced by a network of neurons that are spread over a large area of the spinal cord. This finding is inconsistent with the hypothesis that locomotion and scratching each involve interneurons only within a well-defined region of the spinal cord. This finding is consistent with several previous studies in which individual swim- and/or scratch-activated turtle interneurons were recorded in all segments of the spinal cord hindlimb enlargement, in the dorsal horn, intermediate zone, and ventral horn, with many of the scratch-activated interneurons being activated when scratching was evoked on either the left or the right side (Berkowitz 2001, 2002, 2005, 2008; Berkowitz et al. 2006). Those previous studies, however, involved about 100 or fewer neurons each, while the present study involves thousands of neurons. Thus, the "big picture" captured in the present study is consistent with the results of the smaller samples obtained in the single-interneuron studies.

Bilateral labeling

The large percentage of labeled neurons found contralateral to the unilaterally evoked motor patterns may seem surprising, but is actually consistent with previous studies. It is consistent not only with single-interneuron recordings (see above), but also with additional studies that demonstrate the importance of the contralateral spinal cord for the generation of scratching. Lesion studies in turtles have shown that the contralateral spinal cord contributes to the hip extensor phase of the normal ipsilateral rostral scratch motor pattern (Stein et al. 1995) and to rostral scratch rhythmogenesis

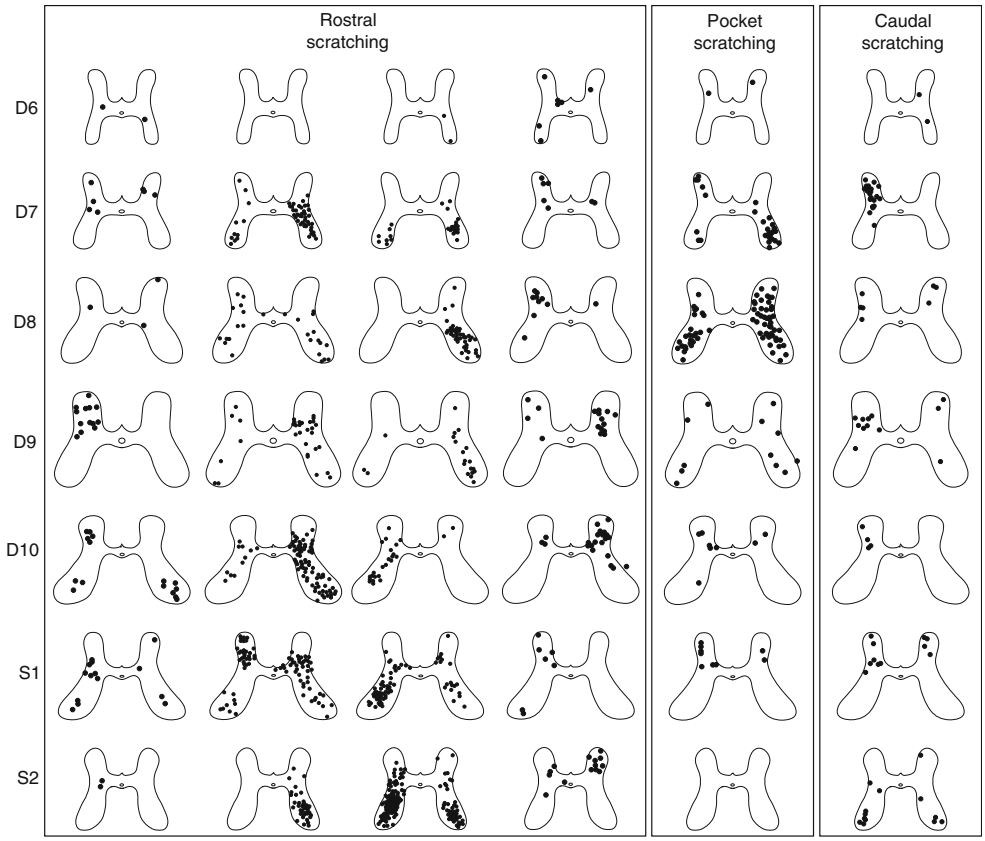

Fig. 6 Cross-sectional distributions of labeled neurons following scratching motor patterns in all transversely sectioned cases. Each *filled circle* represents a single labeled neuron and each *column* represents one animal. For clarity, the size of each *circle* was reduced in two rostral scratching animals that had a larger number of labeled cells

(Currie and Gonsalves 1997, 1999). The present study suggests that the contralateral spinal cord is also important for the generation of swimming, even when it is evoked in one limb via electrical stimulation of contralateral descending axons. It makes intuitive sense that contralateral interneurons would contribute to swimming because natural swimming is a bilateral behavior; the contralateral labeling common to swimming and scratching may indicate that much of the bilateral network used for swimming is also used for scratching. Contralateral labeling was particularly strong following caudal scratching, a behavior that often involves coordinated movements of the left and right hindlimbs when the sites scratched are near the midline (Field and Stein 1997). Bilateral distributions have also been found in other activity-dependent labeling studies evoking a unilateral behavior or motor pattern, such as scratching motor patterns in adult cats (Barajon et al. 1992) and unilateral spinal cord electrical stimulation in adult frogs (Giszter et al. 1993).

Segmental distributions

Labeled neurons were distributed across all spinal cord segments examined (D6–S2), but in most cases, a majority of labeled neurons were in segments D7–D10, which include the rostral portion of the hindlimb enlargement and the adjacent pre-enlargement segment. Other studies that examined the rostrocaudal distribution of activity-dependent labeling during rhythmic motor patterns also found a distributed pattern in the hindlimb enlargement in neonatal rats (Cina and Hochman 2000) and cats (Dai et al. 2005). Data from cooling and/or lesion studies of cat scratching (Deliagina et al. 1983), turtle scratching (Mortin and Stein 1989), chick embryo rhythmic motor patterns (Ho and O'Donovan 1993), and neonatal rat locomotor-like activity (Cazalets et al. 1995; Kjaerulff and Kiehn 1996) suggest that while the circuitry involved in producing rhythmic motor outputs is distributed throughout the hindlimb enlargement, the rostral segments of the hindlimb enlargement have a greater capacity for rhythmogenesis than the caudal segments. The pre-enlargement segments are also capable of rhythmogenesis in turtles (Currie and Gonsalves 1997). The proportion of labeled neurons in the rostral segments, D6–D9, was greater for swimming than for rostral scratching in the present study. This suggests that swimming network interneurons may be especially concentrated in the rostral enlargement and pre-enlargement

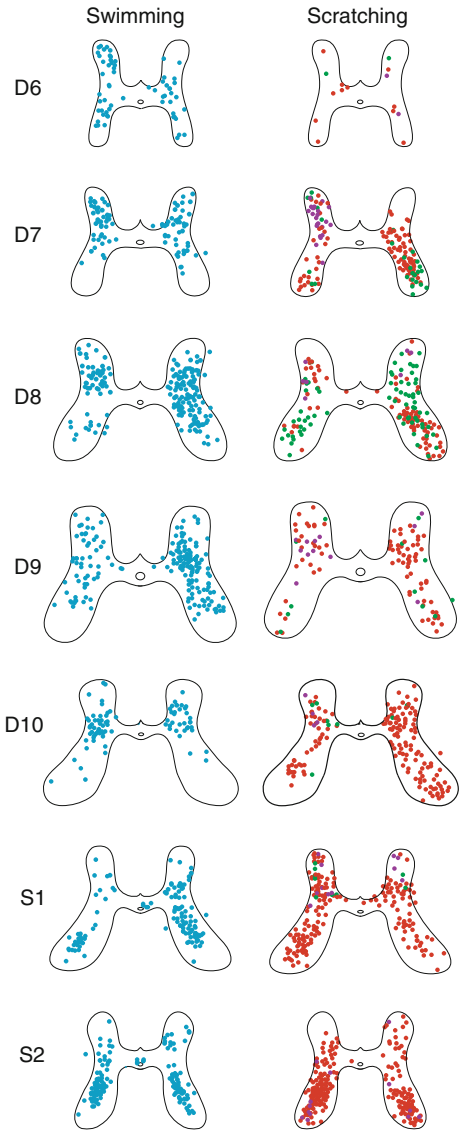

Fig. 7 Comparison of the swimming and scratching distributions of labeled cells. All labeled cells shown in Figs. 5 and 6 were superimposed for swimming animals (*left*; *blue circles*) and scratching animals (*right*). Rostral scratching, *red circles*; pocket scratching, *green circles*; caudal scratching, *purple circles*

segments and that these segments may be even more important for swimming than for rostral scratching. Segmental lesion studies during turtle swimming and scratching motor patterns may help resolve this issue.

Transverse plane distributions

The concentration of activated neurons within the intermediate zone and dorsal part of the ventral horn seen here is similar to findings from activity-dependent labeling studies in other vertebrates following locomotor and scratching motor patterns. Distributions of sulforhodamine labeling seen following drug-induced locomotor-like activity in dorsal-rhizotomized (Kjaerulff et al. 1994) and isolated (Cina and Hochman 2000) neonatal rat spinal cord preparations were predominantly in laminae VI–VII and X, and in lamina VII, respectively. In cats, c-fos staining following locomotor patterns (Carr et al. 1995; Dai et al. 2005) was primarily in laminae VII–VIII and X, while following scratching motor patterns (Barajon et al. 1992), labeled cells were mainly in the lateral intermediate zone and dorsolateral part of the ventral horn. 2-Deoxyglucose uptake in rabbits following locomotor patterns showed increased metabolism primarily in and around the intermediate zone (Viala et al. 1988).

Many neurons activated in the dorsal horn during locomotion or scratching movements may have been activated by sensory input and may not be necessary for generating rhythmic motor output (Ho and O'Donovan 1993; Kjaerulff et al. 1994; Kjaerulff and Kiehn 1996; Dai et al. 2005; Antri et al. 2011). In activity-dependent labeling studies, a reduction of labeling was seen in laminae I–VI in neonatal rats (Kjaerulff et al. 1994) and cats (Carr et al. 1995; Dai et al. 2005) when limb sensory feedback was removed. Other studies showed that when the dorsal horn was removed, rhythmic output could still be seen in chick embryos (Ho and O'Donovan 1993) and in neonatal rats (Kjaerulff and Kiehn 1996; Antri et al. 2011). Together, these previous studies suggest that neurons in the dorsal horn are not required for rhythm generation. In the present study, however, there was no movement-related sensory feedback for any motor patterns (because the animals were chemically immobilized) and the swimming motor patterns were evoked by electrical stimulation of descending axons, bypassing sensory activation. Furthermore, sensory innervation of the caudal scratch site stimulated was outside the segments examined; sensory innervation of the rostral scratch sites stimulated may have been provided by D6 in some animals, but little or no dorsal horn labeling occurred in D6 of these animals. Thus, it is likely that at least some of the interneurons labeled in the deep dorsal horn in this study, following either swimming or scratching motor patterns, actually contribute to motor pattern generation, rather than just to sensory processing. This conclusion is consistent with a recent finding that there are deep dorsal horn interneurons in neonatal mice that are rhythmically active during locomotor-like activity and that project directly to motoneurons (Wilson et al. 2010).

Concentration of active neurons in the intermediate zone and dorsal part of the ventral horn in activity-dependent labeling studies following rhythmic motor patterns is consistent with single-interneuron studies. Electrophysiological data from cat (McCrea et al. 1980), rodent (MacLean et al. 1995; Tresch and Kiehn 1999; Butt et al. 2002; Butt and Kiehn 2003; Hinckley et al. 2005), and

turtle studies (Berkowitz 2001, 2005, 2008; Berkowitz et al. 2006) have indicated that many interneurons that may be important for rhythmic motor pattern generation reside in the intermediate zone and the ventral horn. These include Ia inhibitory interneurons, Renshaw cells, group II interneurons, Hb9 interneurons, commissural interneurons, and other rhythmic interneurons.

In the present study, few labeled neurons were found in the medial intermediate zone around the central canal, unlike studies involving adult cat mesencephalic locomotor region electrically evoked spontaneous locomotion (Dai et al. 2005) and neonatal rat drug-induced locomotor-like activity (Kjaerulff et al. 1994; Cina and Hochman 2000), where many activated neurons were found in lamina X. One possible reason for this difference is that these previous studies examined the distributions of activated neurons following bilateral locomotor patterns, while in the present study, locomotor and scratching motor patterns were presumably unilateral. Thus, lamina X, the medial aspect of the intermediate zone, might represent a region of the spinal cord that is important for left–right limb coordination rather than for rhythmogenesis. Additional support for this idea can be found in the difference in the distributions of activated neurons in the intermediate zone in adult cats, where lamina X labeling was notably absent following motor patterns for scratching (Barajon et al. 1992), a unilateral limb behavior, but was present following bilateral locomotor patterns (Dai et al. 2005). Other studies also suggest that lamina X (along with laminae VII–VIII) contains commissural interneurons that may play a significant role in left–right limb coordination during bilateral rhythmic motor patterns (Butt et al. 2002).

While the distributions we found were generally similar to the key regions identified in other studies, there were some important methodological differences that could account for some differences in labeling distributions. First, for the present experiments, adult animals were used, unlike previous experiments on chicks and rodents, which used neonatal animals (Ho and O'Donovan 1993; Kjaerulff et al. 1994; Cazalets et al. 1995; Cina and Hochman 2000). This is important not only because rats and mice do not display free walking with weight support until about 10 days postnatally (Westerga and Gramsbergen 1990; Jiang et al. 1999), but also because the underlying spinal cord organization may change substantially during development. For example, Lavallee and Pflieger (2009) showed that, in postnatal opossums, the total number and segmental distribution of sulforhodamine-labeled spinal cord neurons following spontaneous rhythmic activity depend importantly on the developmental stage. Relative locations of cells within the gray matter may change as neurons migrate during development (Leber and Sanes 1995; McLean and Fetcho 2009).

Second, we evoked locomotor and scratching motor patterns using naturalistic stimulation (for scratching) and electrical stimulation (for locomotion), while most studies of locomotor-like activity in neonatal rodents have used pharmacologically evoked motor patterns. Kjaerulff et al. (1994) and Cina and Hochman (2000) found different distributions of neurons activated during locomotor-like activity within the intermediate zone; the difference was attributed to the drug cocktails used to evoke the motor patterns (Kjaerulff et al. 1994; Cina and Hochman 2000). Kjaerulff et al. (1994) used a combination of NMDA and 5-HT and found a medial bias in the intermediate zone distribution, while Cina and Hochman (2000) used only 5-HT and found labeled cells dispersed mediolaterally throughout the intermediate zone. Each drug cocktail used to evoke locomotor-like activity may activate somewhat different sets of neurons (Kiehn and Kjaerulff 1996) and these may differ from those activated during natural locomotion.

Comparison of swimming and scratching distributions

The distributions of activated neurons following swimming and scratching motor patterns were generally very similar to each other (Fig. 7). Overlap in the scratching and swimming distributions was extensive in the central and deep dorsal horn, the lateral intermediate zone, and the dorsal to middle ventral horn, bilaterally. This is consistent with the hypothesis that a multifunctional network of interneurons is largely responsible for both swimming and scratching, though, of course, the activity-dependent labeling seen in different animals here does not demonstrate that any of the labeled neurons actually contributes to both behaviors. The present findings are also consistent with single-interneuron electrophysiological and morphological studies in turtles that have identified many locomotion- and scratch-activated neurons in these same areas of the spinal cord (Berkowitz 2001, 2005, 2008; Berkowitz et al. 2006). These are also largely the same areas in which the cell bodies of descending and ascending propriospinal neurons are found in turtles (Berkowitz and Stein 1994b; Berkowitz 2004).

Despite the similarities in distributions of sulforhodamine-labeled neurons following scratching versus swimming, some differences were seen. More neurons were labeled in the dorsal horn following swimming than scratching and more were labeled in the deep ventral horn following scratching than swimming. Also, significantly more labeling was found in the D6–D9 segments following swimming and more in the D10–S2 segments following rostral scratching. The apparently greater number of labeled neurons in the deep ventral horn during scratching compared to swimming might indicate that there are

scratch-specialized interneurons located in close proximity to motor neurons. Differences in distributions of active neurons can also be seen by comparing activity-dependent labeling following cat scratching (Barajon et al. 1992) and cat locomotion (Dai et al. 2005), though these were assessed in separate studies. The distributions of activated neurons showed overlap primarily in the intermediate zone and the dorsal part of the ventral horn; there were also differences in the mediolateral distributions, with scratch-activated cells located more laterally and locomotion-activated cells more medially. These results together with the present findings suggest that many neurons activated during different rhythmic motor patterns may reside in the same areas, but some regions of the gray matter may contain more neurons specialized for particular motor patterns.

The large increase in the number of labeled cells in D8 following pocket scratching but not rostral scratching or caudal scratching, especially in the dorsal horn, may be due in part to the activation of sensory interneurons by the stimulation itself, because D8 provides sensory innervation of the pocket scratch site stimulated (Mortin and Stein 1990). The majority of segments in which rostral and caudal scratch sensory input enters the spinal cord, D3–D6 and S2-caudal segments, respectively, were mostly not examined, so, similar segmental peaks in labeling for processing of rostral scratching and caudal scratching sensory inputs were not likely to be seen here.

Inter-animal variability

The distributions of labeled neurons in this study following both swimming and rostral scratching motor patterns showed substantial variability between animals. One possibility, of course, is that these differences were caused by unidentified methodological differences between experiments. However, because we used the same procedures for all these animals and because the no-stimulation control animals had substantially fewer labeled neurons, it seems likely that the inter-animal variability seen was due to genuine differences in the distributions of activated neurons when essentially the same motor pattern was evoked in different animals. Even if the whole network of activated neurons was largely the same in the different animals, because we used conservative criteria to count neurons as labeled (see "Methods"), it still might be that we counted only the "tip of the iceberg" in each animal and that this "tip" (though not the "iceberg") varied between animals.

We should consider the possibility, however, that the set of neurons activated during each type of motor pattern was actually substantially different in different animals. It is known that there is variability between animals in the details of each type of motor pattern (Earhart and Stein 2000), so it may be that partly different networks were used in different animals because these animals actually produced somewhat different versions of the "same" motor patterns. Alternatively, even if the same types of interneurons and synaptic connections comprise the pattern-generating networks in different animals, these interneurons may be born in similar locations initially (McLean and Fetcho 2009) but migrate to diverse locations during development, obscuring their common characteristics. Finally, it is also possible that the networks of interneurons used in different animals to generate the same motor output actually differ anatomically and functionally. The possibility that pattern-generating networks for each kind of motor pattern vary between animals has received increasing support in recent years from the findings, in invertebrates, that identified neurons and connections can actually vary substantially in their ion channel compositions and strengths of synaptic connections without substantially altering the motor outputs they collectively generate (Prinz et al. 2004; Calabrese et al. 2011; Marder 2011; Roffman et al. 2012). Thus, it may also be true in the spinal cord that different animals use somewhat different neuronal networks to produce essentially the same motor outputs.

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Conflict of interest The authors declare no conflicts of interest.

Ethical standards The experiments described here comply with the current laws of the USA.

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