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Physiology and Morphology of Shared and Specialized Spinal Interneurons for Locomotion and Scratching

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Berkowitz A. Physiology and morphology of shared and specialized spinal interneurons for locomotion and scratching. *J Neurophysiol* 99: 2887–2901, 2008. First published April 2, 2008; doi:10.1152/jn.90235.2008. Distinct types of rhythmic movements that use the same muscles are typically generated largely by shared multifunctional neurons in invertebrates, but less is known for vertebrates. Evidence suggests that locomotion and scratching are produced partly by shared spinal cord interneuronal circuitry, although direct evidence with intracellular recording has been lacking. Here, spinal interneurons were recorded intracellularly during fictive swimming and fictive scratching *in vivo* and filled with Neurobiotin. Some interneurons that were rhythmically activated during both swimming and scratching had axon terminal arborizations in the ventral horn of the hindlimb enlargement, indicating their likely contribution to hindlimb motor outputs during both behaviors. We previously described a morphological group of spinal interneurons (“transverse interneurons” or T neurons) that were rhythmically activated during all forms of fictive scratching at higher peak firing rates and with larger membrane potential oscillations than scratch-activated spinal interneurons with different dendritic orientations. The current study demonstrates that T neurons are activated during both swimming and scratching and thus are components of the shared circuitry. Many spinal interneurons activated during fictive scratching are also activated during fictive swimming (scratch/swim neurons), but others are suppressed during swimming (scratch-specialized neurons). The current study demonstrates that some scratch-specialized neurons receive strong and long-lasting hyperpolarizing inhibition during fictive swimming and are also morphologically distinct from T neurons. Thus this study indicates that locomotion and scratching are produced by a combination of shared and dedicated interneurons whose physiological and morphological properties are beginning to be revealed.

INTRODUCTION

How do neural circuits produce a variety of movements using the same motoneurons and muscles? Much of what we know at the cellular level is from a few intensively studied invertebrate circuits that generate multiple rhythmic motor patterns, largely via a common set of multifunctional neurons (Marder and Calabrese 1996; Marder et al. 2005). However, there are also examples in invertebrates of interneurons that participate in only one behavior (Briggman and Kristan Jr 2006; Edwards et al. 1999; Huang and Satterlie 1990; Inoue et al. 1996; Jing and Gillette 2000, 2003; Jing and Weiss 2001, 2002; Jing et al. 2004; Kovac and Davis 1980; Krasne and Lee 1988; Kristan Jr and Shaw 1997; Norekian and Satterlie 1996; Shaw and Kristan Jr 1997). Distinct behaviors may thus be generated using a combination of shared and specialized neurons in invertebrates.

Vertebrate pattern-generating neurons generally appear to be multifunctional, but the evidence is circumstantial for most neural circuits and behaviors. Only in studies of tadpole (Li et al. 2007; Soffe 1993) and fish (Kimura et al. 2006) axial body movements, turtle hindlimb scratching and withdrawal (Berkowitz 2005; Berkowitz et al. 2006), and mammalian eupneic breathing, sighing, coughing, and sneezing (Lieske et al. 2000; Shiba et al. 2007) have investigators recorded from individual interneurons while eliciting multiple types of naturalistic motor patterns and also assessed the axonal projections or synaptic targets of these cells. The issue of whether interneurons contribute to one or multiple types of movement is conveniently addressed using the turtle spinal cord, which can produce hindlimb motor patterns underlying forward swimming, three forms of scratching, and withdrawal without input from the brain and movement-related feedback (Stein 2005). Many extracellularly recorded spinal interneurons are rhythmically activated during all forms of scratching (Berkowitz 2001b; Berkowitz and Stein 1994), and often during swimming as well (scratch/swim neurons; Berkowitz 2002), whereas others are activated during scratching but not swimming (scratch-specialized neurons; Berkowitz 2002).

From extracellular recording studies alone, one cannot be certain that multifunctional interneurons affect hindlimb motor output at all because they may have outputs only to the brain. Thus we recently recorded scratch-activated interneurons intracellularly, filled them with Neurobiotin, and traced their axons (Berkowitz 2005). Some interneurons activated during all three forms of scratching had axon terminals in the hindlimb enlargement ventral horn, indicating their likely contribution to hindlimb motor output. The current experiments used a similar approach, but investigated fictive swimming along with fictive scratching, to determine whether any scratch/swim neurons have axon terminals in the hindlimb enlargement, as well as to assess potential morphological differences between scratch/swim and scratch-specialized neurons.

To understand how the spinal cord produces multiple behaviors, it will also be necessary to elucidate the cellular properties and synaptic connections of spinal circuit components. Spinal cord neurons are not individually identifiable, but neuronal types have been described based on morphology, physiology, pharmacology, and/or gene expression (Gordon and Whelan 2006; Grillner 2006; Kiehn and Kullander 2004). The contributions of some spinal interneuron types have been determined for multiple, distinct axial movements. For embryonic tadpole struggling and swimming and for larval zebrafish escape and swimming, most spinal interneuron types are used

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for both behaviors, but some are used for just one (Kimura et al. 2006; Li et al. 2007; Ritter et al. 2001; Soffe 1993).

Less is known about types of spinal interneurons contributing to distinct limb movements. Recently, we defined a morphological group, transverse interneurons (T neurons), that are activated during all forms of scratching. They tend to have higher peak firing rates than those of other scratch-activated interneurons and can have axon terminals in the hindlimb enlargement ventral horn (Berkowitz et al. 2006). It is not known, however, whether T neurons are activated during swimming. Thus another goal of this study was to determine whether T neurons are scratch/swim neurons or scratch-specialized neurons or can be either. Preliminary results have been reported in abstracts (Berkowitz 2007a,b).

METHODS

Animal preparation

Adult red-eared turtles (*Trachemys scripta elegans*, 400–1000 g, both sexes, $n = 19$) were prepared for recording as previously described (Berkowitz 2001a; Robertson et al. 1985). Briefly, animals were anesthetized and surgically dissected to 1) transect the spinal cord between the dorsal 2 (D2) and D3 postcervical segments, 2) expose the spinal cord between the D6 and sacral 2 (S2) segments, and 3) prepare several right hindlimb motor nerves for electroneurographic recordings (ENGs; Fig. 1A): the hip flexor, ventral puboischiofemoralis internus, pars anteroventralis (HF), the hip extensor, flexor cruris, pars flexor tibialis internus (HE), and one or more of the knee extensors (KE): triceps femoralis, pars iliotibialis (IT), pars ambiens (AM), or pars femorotibialis (FT). Unless otherwise stated, the monoarticular knee extensor, FT, is shown in illustrations. Following surgery, the animal was immobilized with gallamine triethiodide (8 mg/kg, administered intramuscularly; Sigma-Aldrich, St. Louis, MO) and artificially respiration at room temperature for the duration of recording. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma.

Electrophysiology

Intracellular recordings ($n = 30$ interneurons activated during fictive scratching and/or fictive swimming with soma-dendritic morphology recovered) were obtained from the gray matter on the right side of one or more of the five segments of the hindlimb enlargement

(D8, D9, D10, S1, and S2), using sharp microelectrodes filled with 4% Neurobiotin (Vector Laboratories, Burlingame, CA), as previously described (Berkowitz 2005). Fictive scratching was evoked using mechanical stimulation with a fire-polished glass probe attached to a force transducer (Fig. 1A). Fictive forward swimming was evoked by electrical stimulation of the contralateral D3 lateral funiculus (0.1-ms,

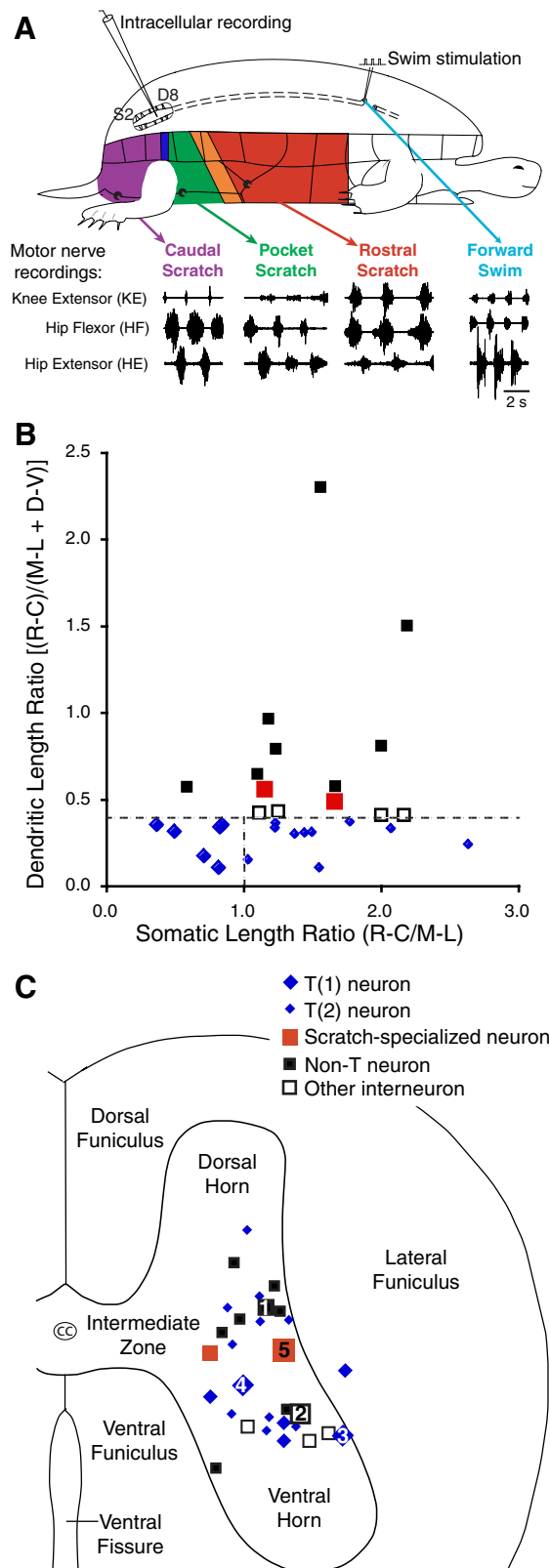


FIG. 1. Recording paradigm and summary of neurons studied. *A*: schematic illustration of the experiment, in which interneurons were intracellularly recorded from the hindlimb enlargement spinal cord [segments dorsal 8 through sacral 2 (D8–S2)], while evoking fictive scratching by continually rubbing a location on the ipsilateral body surface with a smooth glass probe, or evoking fictive forward swimming by electrical stimulation of D3 contralateral descending axons, and monitoring motor output via ipsilateral knee extensor, hip flexor, and hip extensor motor nerves. Colored regions of the body surface indicate the receptive fields for rostral scratch (red), pocket scratch (green), and caudal scratch (purple), as well as the transition zones (orange and blue) between them (Mortin et al. 1985). Examples of each form of fictive scratching and of fictive forward swimming from one animal are shown below. *B*: morphological definitions of T neurons, as cells that meet a criterion of relative dendritic and somatic lengths [T(1)] or meet only the dendritic criterion [T(2)], as well as of non-T neurons and other interneurons. Morphological measurements are also shown for scratch-specialized neurons (one a non-T neuron and one an “other” interneuron), which are defined functionally rather than morphologically. *C*: schematic illustration of the soma locations in the transverse plane for all recorded interneurons, by morphological group. The locations of scratch-specialized neurons are also shown. Numbers on some symbols indicate cells illustrated in Figs. 2–7; corresponding numbers are used for those cells in those figures. D–V, dorsoventral; M–L, mediolateral; R–C, rostrocaudal; cc, central canal.

200- to 400- μ A pulses at 40 Hz). Following characterization, each cell was injected with net depolarizing current (0.6- to 3.0-nA, 4-Hz sinusoidal current for 1–38 min) to eject Neurobiotin. No more than one interneuron fill was attempted in each spinal cord segment, to ensure unambiguous identification. ENGs were obtained using pairs of 100- μ m silver wires in a pool of mineral oil. Recordings were stored on digital audio tape (TEAC America, Montebello, CA) and redigitized and analyzed off-line using Datapac software (Run Technologies, Laguna Hills, CA).

Histology

Immediately following the recording session, the animal was deeply anesthetized and transcardially perfused with saline followed by 4% paraformaldehyde, as described previously (Berkowitz 2005). The spinal cord was postfixed overnight, cryoprotected, embedded in a gelatin-albumin-glutaraldehyde medium, and frozen-sectioned at 100 μ m horizontally. Sections were rinsed, incubated in 0.3% H_2O_2 , rinsed, incubated overnight at 4°C in 1:100 ABC solution (Vector)/0.3% Triton X-100, rinsed, and reacted 4–10 min in 0.5 $\times$ Vector SG. Sections were mounted, air-dried, counterstained with Fast Nuclear Red (Vector), dehydrated, cleared, and mounted. Reconstructions were made via a camera lucida; digital photomicrographs were obtained with a DP-70 camera (Olympus America, Melville, NY). Photomontages were assembled using Photoshop (Adobe Systems, San Jose, CA); only brightness and contrast were manipulated.

Physiological analysis

FIRING RATES. Dual-referent phase histograms of interneuron mean firing rates were calculated with respect to the integrated and smoothed ipsilateral hip flexor nerve activity cycles, using Datapac, for fictive forward swimming and for each available form of ipsilateral fictive scratching, provided that at least two cycles of the rhythm occurred and the interneuron fired at least ten action potentials, as described previously (Berkowitz 2001b, 2002; Berkowitz and Stein 1994). Dual-referent phase histograms assess firing rates as a function of phase within the burst and the interburst interval independently and thus are useful for analysis of motor patterns that have a variable duty cycle (Berkowitz and Stein 1994). The phase histogram data were then used to calculate the mean vector (Batschelet 1981; Berkowitz and Stein 1994; Drew and Doucet 1991; Mardia 1972). The mean vector length (MVL) indicates the degree of rhythmic modulation; an MVL of 0 would indicate no phase preference with respect to the hip flexor cycle, whereas an MVL of 1 would indicate that all action potentials occurred in the same phase (one tenth) of each cycle. The mean vector angle (MVA) indicates the neuron's phase preference within the hip flexor cycle; an MVA of 0–180° (or 0–0.5) would indicate a phase preference during hip flexor activity and an MVA of 180–360° (or 0.5–1.0) would indicate a phase preference within the hip flexor interburst intervals. The null hypothesis that the interneuron's firing occurred at random with respect to the hip flexor cycle was evaluated using the Rayleigh test (Batschelet 1981; Berkowitz and Stein 1994; Drew and Doucet 1991; Mardia 1972). MVAs were used in analyses only if the phase histogram passed the Rayleigh test with $P < 0.01$ (Berkowitz 2001b, 2002; Berkowitz and Stein 1994). The scratch MVL used for comparison to the swim MVL (Fig. 8A) was the average of the MVLs for all available forms of scratching. The scratch MVA used for comparison to the swim MVA (Fig. 8B) was for the form of scratching that passed the Rayleigh test with the lowest P value, unless phase histograms for multiple forms of scratching had $P < 0.001$, in which case the one among these with the highest MVL was used (Berkowitz 2001b, 2002; Berkowitz and Stein 1994). For each cell, the two values of peak firing rate graphed (Fig. 10A) were the highest mean firing rate of the ten values (ten bins) of the scratch phase histogram used for MVA analysis ("High MVL"

peak firing rate) and the highest mean firing rate of the ten bins of any of the scratch phase histograms ("Overall" peak firing rate).

MEMBRANE POTENTIAL OSCILLATIONS. Dual-referent phase-averaged membrane potential oscillations were calculated after action potential deletion and signal smoothing, using Datapac, as described previously (Berkowitz 2005). The scratch oscillation used for each cell for comparison to the swim oscillation (Figs. 9 and 10, B and C) was for the form of scratching used for its phase histogram (see earlier text), unless the cell fired fewer than ten action potentials during ipsilateral scratching or failed the Rayleigh test for all forms of scratching, in which case the form of scratching with the largest phase-averaged oscillation was used.

Morphological analysis

Soma mean diameter was calculated as the average of the major- and minor-axis diameters in horizontal sections. The group of T neurons (Berkowitz et al. 2006) was defined (Fig. 1B) using two criteria, which were ratios of somatic and dendritic lengths along the three axes (rostrocaudal, R-C; mediolateral, M-L; dorsoventral, D-V). There was a dendritic criterion [dendritic R-C/(M-L + D-V) < 0.4] and a somatic criterion (somatic R-C/M-L < 1). Cells meeting both criteria were termed T(1) neurons; cells meeting only the dendritic criterion were termed T(2) neurons. Non-T neurons were defined as cells with dendritic R-C/(M-L + D-V) > 0.5 . Other interneurons were defined as those with dendritic R-C/(M-L + D-V) of 0.4–0.5.

Statistics

Statistical comparisons of mean peak firing rates and mean oscillation amplitudes were made using the Mann-Whitney test; in general, two-tailed tests were used, but for comparisons of firing rates and oscillation amplitudes between T neurons and non-T neurons, one-tailed tests were used because there was prior evidence that T neurons have higher peak firing rates and larger oscillations than those of non-T neurons (Berkowitz et al. 2006). To test for correlations of individual neuron parameters between scratching and swimming, Pearson's correlation coefficient (r) was used for linear parameters (MVL and oscillation amplitude), whereas the circular-circular correlation coefficient (r_c) (Batschelet 1981) was used for cyclical parameters (MVA and oscillation phase and trough). Significance level was set at $P < 0.05$ for each of these tests.

RESULTS

General features of recorded interneurons

Thirty interneurons activated during fictive scratching and/or fictive forward swimming were successfully recorded and filled (Fig. 1). All 30 cells were activated during scratching. All but 3 were also activated during swimming. One of those 3, however, displayed subthreshold depolarizations during most episodes of both swimming and scratching (and also occasionally fired action potentials during caudal scratching), so was not considered a scratch-specialized neuron. The remaining 2 cells showed increased firing during scratching but reduced firing during swimming and thus were scratch-specialized. Cells were generally activated throughout the period of fictive scratching and/or fictive swimming, even when the motor pattern continued beyond the period of stimulation. Cells generally ended their activity and oscillations at approximately the same time as the motor pattern ended. During mechanical or electrical stimulation of the ipsilateral dorsal foot that evoked a fictive flexion reflex, 21 of 28 scratch/swim interneurons were depolarized and fired one or more action potentials,

2 were hyperpolarized, and 5 showed no response. One of the 2 scratch-specialized interneurons was depolarized and fired during fictive flexion reflex; the other showed no response.

The recorded cells included (Fig. 1*B*) 6 T neurons meeting both dendritic and somatic criteria [T(1); see METHODS], 10 additional T neurons that met only the dendritic criterion [T(2)], 9 non-T neurons (including one scratch-specialized neuron), and 5 other interneurons (including one scratch-specialized neuron). The somata of neurons in each of these categories were scattered in the transverse plane (Fig. 1*C*) within the deep dorsal horn, the intermediate zone, and the dorsal half of the ventral horn. Somata were also scattered rostrocaudally within the hindlimb enlargement, except for the 2 scratch-specialized neurons, which were both in the D8 intermediate zone (in different animals).

The soma diameters of the cells studied (Table 1) were similar to those of turtle spinal interneurons previously studied during fictive scratching alone (Berkowitz et al. 2006). The soma diameters were slightly larger than those of cells studied using patch electrodes in turtle spinal cord slices (Smith and Perrier 2006), but smaller than the measurements those authors made from the small-scale whole cell reconstructions available in the published figures for 12 of the 47 cells studied by Berkowitz et al. (2006). The different morphological groups of interneurons in this study had similar mean soma diameters (Table 1).

Axon pathways and terminals

If spinal interneurons contribute to hindlimb motor output (rather than just sending axons to the brain) for both scratching and forward swimming, they would be expected to have axon terminals within the spinal cord hindlimb enlargement, most likely in the ventral horn (Berkowitz 2005). Thus all recorded interneurons were examined for axons and axon terminals. Figures 2–4 show two interneurons that were activated during both scratching and swimming (Figs. 2*A* and 4*A*) and had axons that descended in the ipsilateral lateral funiculus and gave off axon collaterals with terminal arborizations in the ipsilateral intermediate zone (Fig. 3*A*) and ventral horn (Figs. 3, *B* and *C* and 4*B*). Both cells were rhythmically activated during both scratching and swimming, as shown by phase histograms (Figs. 2*D* and 4*C*) and phase-averaged membrane potential oscillations (Figs. 2*E* and 4*D*). Interneuron 1 (in Figs. 2 and 3) was a non-T neuron in the intermediate zone and interneuron 2 (in Fig. 4) was an “other interneuron” in the ventral horn (Fig. 1*C*).

Axons could be unambiguously identified and followed for 13 of the 30 recorded interneurons (Table 2). Most either bifurcated within the ipsilateral lateral funiculus, descended within the ipsilateral ventral funiculus, or bifurcated within the contralateral ventral funiculus. Four had identifiable axon terminal arborizations. One of the scratch-specialized neurons was a non-T neuron that bifurcated within the ipsilateral lateral funiculus and had axon terminal arborizations within the ipsilateral ventral horn.

T neuron activity during forward swimming

T neurons, defined by their morphology (Fig. 1*B*), were previously shown to be robustly activated during fictive scratching (Berkowitz et al. 2006), but it was not known whether they were also activated during fictive forward swimming. Figures 5 and 6 show two T(1) neurons that were strongly activated during both scratching and swimming (Figs. 5*A* and 6*A*) and had the mediolateral elongation of soma, and especially dendrites, typical of T neurons (Figs. 5*B* and 6*B*). Both of these cells had rhythmic phase histograms of mean firing rate (i.e., a mean vector length, or MVL, that passed the Rayleigh test with $P < 0.01$) with relatively high peak firing rates, during both scratching and swimming (Figs. 5*C* and 6*C*), except interneuron 4 during caudal scratching. The timing of interneuron 4's bursts varied within the hip flexor cycle during caudal scratching, leading to a nonrhythmic caudal scratch phase histogram (Fig. 6*C*); most likely, interneuron 4's bursts were phase-locked to bursts of a different motor nerve that was not recorded, perhaps a knee flexor. Interneuron 4 nonetheless had a clear phase-averaged membrane potential oscillation for caudal scratching (Fig. 6*D*) because during another episode of caudal scratching (not shown), it had strong membrane potential oscillations that peaked during hip flexor interburst intervals, although it fired few action potentials. Both of these cells had clear membrane potential oscillations that were phase-locked to both the scratch and the swim rhythms (Figs. 5*D* and 6*D*); the amplitudes of these oscillations were larger for scratching than for swimming for both of these cells. The preferred phases of firing (the mean vector angles, or MVAs) and the peaks of the membrane potential oscillations were similar for swimming and most forms of scratching for both cells, but different for caudal scratching for interneuron 4 in Fig. 6 (see earlier text). All 6 T(1) neurons and all 10 T(2) neurons were activated during both scratching and swimming.

TABLE 1. Mean soma diameters of spinal interneurons studied

	Mean Diameter		Major Axis		Minor Axis		n
	Mean	Range	Mean	Range	Mean	Range	
T(1)	20.6 ± 3.3	14.8–25.0	28.0 ± 6.2	21.3–38.9	13.1 ± 3.9	8.3–18.5	6
T(2)	19.4 ± 3.4	14.3–23.8	25.1 ± 6.4	17.9–34.5	13.8 ± 2.8	10.7–18.5	10
NT	24.0 ± 8.9	13.7–42.9	31.0 ± 14.2	15.5–61.9	17.1 ± 4.9	13.0–23.8	9
Other	19.1 ± 3.1	16.1–22.7	26.3 ± 6.4	17.9–35.2	11.8 ± 4.3	7.1–17.9	5
Total	21.0 ± 5.7	13.7–42.9	27.7 ± 9.3	15.5–61.9	14.3 ± 4.2	7.1–23.8	30
2006 study	20.4 ± 5.0	10.6–33.8	26.1 ± 7.2	14.1–40.8	14.6 ± 4.5	4.5–31.0	47

Values are means ± SD, expressed in μm . T(1), transverse interneurons meeting both dendritic and somatic criteria (see METHODS); T(2), transverse interneurons meeting only the dendritic criterion; NT, non-T interneurons; Other, other interneurons; Total, all the interneurons in this study; 2006 study, all the interneurons from Berkowitz et al. (2006).

Scratch-specialized neurons

It was previously shown that some scratch-activated spinal interneurons were not activated and usually were suppressed during fictive forward swimming (Berkowitz 2002). However, it was not known whether such scratch-specialized neurons are

directly inhibited during swimming and nothing was known of their morphologies. In this study, these two questions were addressed. Figure 7 shows one of the two scratch-specialized neurons recorded here, interneuron 5. It was rhythmically activated during scratching (Fig. 7, *A* and *B*). During swimming, its spontaneous activity was dramatically reduced (Fig. 7*C*), via strong hyperpolarization of its membrane potential that continued for several seconds after the end of the swim stimulation (Fig. 7*D*). Its firing during scratching was strongly rhythmic (Fig. 7*F*) and its scratch membrane potential oscillations were large (Fig. 7*G*). In contrast, it rarely fired during swimming (Fig. 7*F*). It did, however, have a small phase-averaged membrane potential oscillation during swimming, indicating that the input interneuron 5 received during swimming had both tonic (Fig. 7*D*) and rhythmic (Fig. 7*G*) components. Morphologically, interneuron 5 was an “other” interneuron in the intermediate zone (Figs. 1*C* and 7). Both its soma and its dendrites were rostrocaudally elongated, in contrast to T neurons; its dendritic tree also included more secondary and tertiary branching (Fig. 7*E*) than is typical of T neurons (cf. Figs. 5 and 6 and Berkowitz et al. 2006). The other scratch-specialized neuron was a non-T neuron in the intermediate zone (Fig. 1*C*), with a bifurcating axon in the ipsilateral lateral funiculus and axon terminals in the ipsilateral ventral horn (not shown).

Quantitative analyses of physiology for all cells

Phase-averaged firing rates (i.e., phase histograms) were compared between forward swimming and scratching across the population of recorded interneurons. Figure 8*A* compares the degree of rhythmic modulation (MVL) of each interneuron during swimming and scratching (scratch average MVL; see METHODS). The two were clearly correlated (Pearson's $r = 0.56$; $P < 0.01$; $n = 26$), indicating that interneurons that are highly rhythmic during either swimming or scratching tend to be highly rhythmic during both, whereas interneurons that are relatively nonrhythmic during one are also relatively nonrhythmic during the other. Correlations between the MVL for swimming and the MVL for an individual form of scratching (not shown) were also significant for rostral scratching ($r = 0.43$; $P < 0.05$; $n = 24$) and pocket scratching ($r = 0.61$; $P < 0.01$; $n = 24$), although not for caudal scratching ($r = 0.38$; $P > 0.05$; $n = 17$).

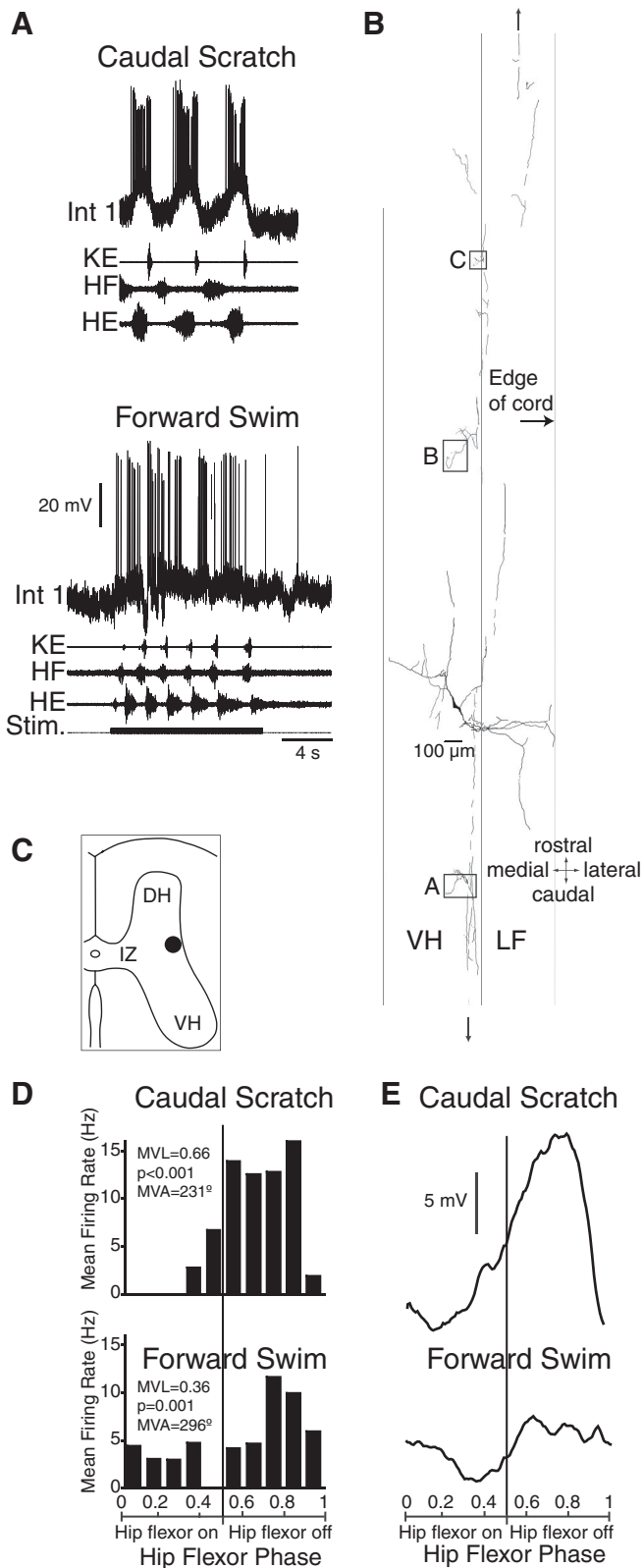


FIG. 2. Example of a rhythmic scratch/swim neuron with axon terminal arborizations in the hindlimb enlargement spinal cord. *A*: rhythmic activation of interneuron 1 during both fictive caudal scratching and fictive forward swimming. *B*: morphological reconstruction of interneuron 1 from horizontal sections, including axon terminal arborizations in the hindlimb enlargement intermediate zone and ventral horn. Photomicrographs of boxed regions (*A*–*C*) are shown in Fig. 3 by corresponding letters. Vertical gray lines in this and other reconstructions indicate borders of gray matter and white matter regions. *C*: cell body location of this neuron in the transverse plane. *D*: phase histograms of mean firing rate of interneuron 1 during caudal scratching and swimming. Probability indicated is from the Rayleigh test (see METHODS). *E*: phase-averaged membrane potentials of interneuron 1 during caudal scratching and swimming. On this and all other figures, the phase histograms and phase-averaged membrane potentials shown were calculated from all available episodes of scratching or swimming, not just the episode shown in the figure. LF, lateral funiculus; VH, ventral horn; DH, dorsal horn; IZ, intermediate zone; HE, hip extensor; HF, hip flexor; Int, interneuron; KE, knee extensor; MVA, mean vector angle; MVL, mean vector length (see METHODS); Stim., stimulation.

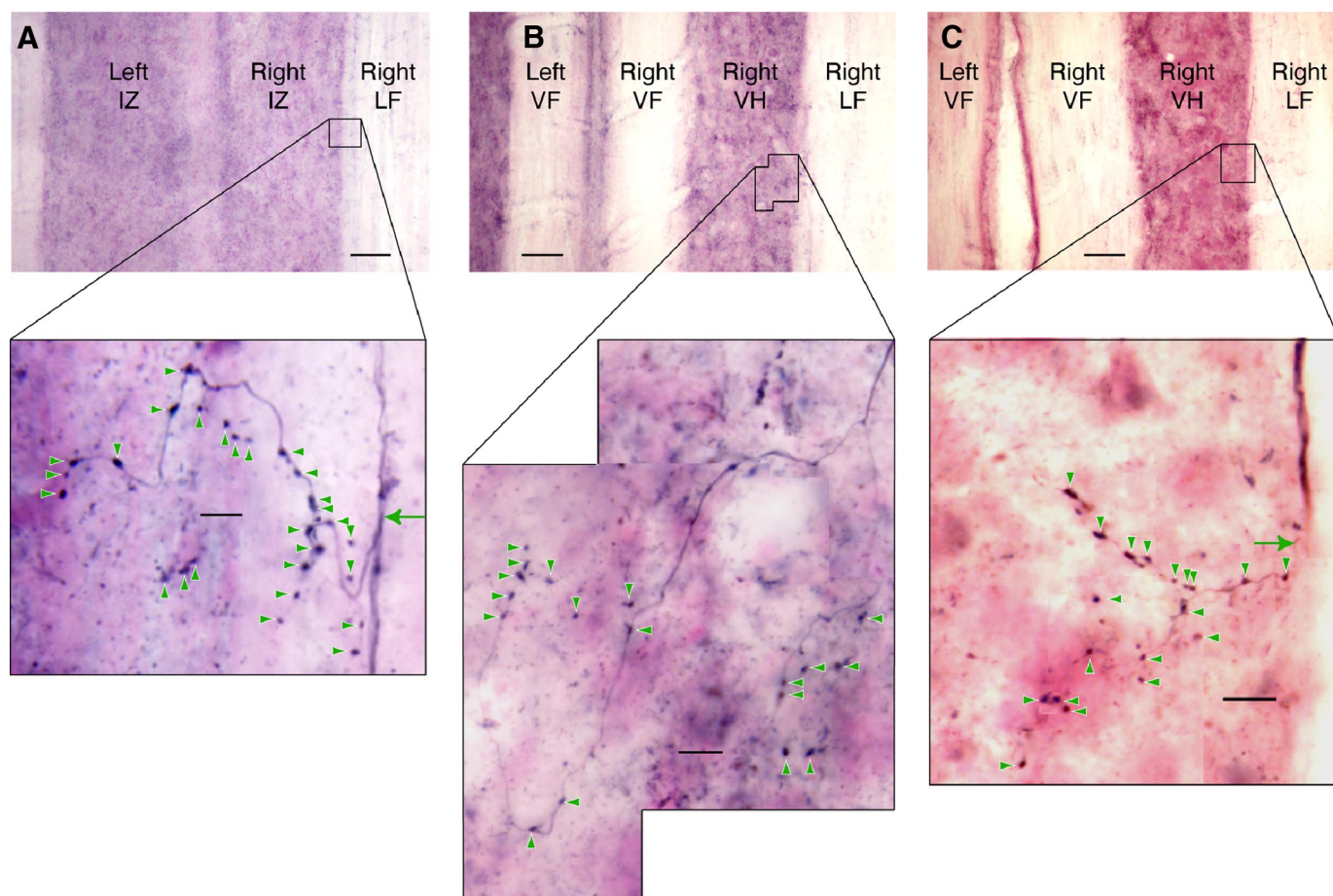


FIG. 3. Hindlimb enlargement axon terminal arborizations of the scratch/swim interneuron shown in Fig. 2. *A*: terminals in the ipsilateral intermediate zone. *B* and *C*: terminals in the ipsilateral ventral horn. In each case, a high-magnification single-section photomontage is shown at the bottom, with a low-magnification photomicrograph of the section from which it comes shown above it. Axon collaterals branching off the parent axon are indicated with arrows; axon terminal swellings are indicated with arrowheads. Scale bars: top row, 100 μ m; bottom row, 10 μ m. VF, ventral funiculus; other abbreviations as in Fig. 2.

Figure 8*B* compares the phase preference of each neuron within the hip flexor activity cycle (MVA) during swimming and the most rhythmic form of scratching. The two were highly correlated (circular–circular correlation coefficient, $r_c = 0.64$; $P < 0.01$; $n = 14$), indicating that interneurons tend to fire in the same phase of the hip flexor cycle during swimming and scratching. Taken separately (not shown), MVAs during rostral scratching and pocket scratching were correlated with those during swimming (rostral scratch vs. swim $r_c = 0.72$; $P < 0.01$; $n = 11$; pocket scratch vs. swim $r_c = 0.68$; $P < 0.01$; $n = 12$), but for the smaller number of cells for which MVAs could be calculated for both caudal scratching and swimming, the correlation was not significant ($r_c = 0.27$; $P > 0.1$; $n = 5$). The correlations of both MVL and MVA between swimming and scratching appeared to occur both within the population as a whole as well as within morphological groups (Fig. 8). Phase preferences were scattered through most of the hip flexor activity cycle, as seen previously for scratch- and swim-related interneurons (Berkinblit et al. 1978a; Berkowitz 2001b, 2002; Berkowitz and Stein 1994).

Phase-averaged membrane potential oscillations were also compared across the population of recorded interneurons. For both scratching (Fig. 9*A*) and swimming (Fig. 9*B*), the peak phase and trough phase were correlated (scratch $r_c = 0.61$; $P < 0.01$; $n = 28$; swim $r_c = 0.56$; $P < 0.01$; $n = 25$) and tended

to differ by about half a cycle (0.5 phase). This appeared to be true both within the population as a whole and within morphological subsets (Fig. 9, *A* and *B*). Both the peak phases and the trough phases of the recorded population were scattered throughout the hip flexor activity cycle. The peak phase of each cell's oscillation during swimming appeared similar to its peak phase during scratching (Fig. 9*C*), but the two were not statistically correlated ($r_c = 0.29$; $P > 0.1$; $n = 25$). In contrast, the trough phase of each cell's oscillation during swimming was highly correlated with its trough phase during scratching (Fig. 9*D*; $r_c = 0.60$; $P < 0.01$; $n = 25$). Taken separately (not shown), the oscillation trough phases during rostral scratching and swimming were correlated ($r_c = 0.52$; $P < 0.01$; $n = 22$), but there were no other significant correlations between swimming and a particular form of scratching in either peak phase or trough phase (peak phases: rostral scratch vs. swim, $r_c = 0.35$; $P > 0.1$; $n = 22$; pocket scratch vs. swim, $r_c = 0.18$; $P > 0.1$; $n = 24$; caudal scratch vs. swim, $r_c = 0.20$; $P > 0.1$; $n = 19$; trough phases: pocket scratch vs. swim, $r_c = 0.29$; $P > 0.1$; $n = 24$; caudal scratch vs. swim, $r_c = 0.30$; $P > 0.1$; $n = 19$).

Peak firing rates from phase histograms were compared across morphological groups of cells, as well as between swimming and scratching for the same group of cells (Fig. 10*A*). For each group of cells, two different values were used for the

scratch peak firing rate (Berkowitz et al. 2006): one for the form of scratching that was most rhythmic ("High MVL"; see METHODS) and the other the highest peak firing rate for any form of scratching ("Overall"). T(1) neurons had significantly higher peak firing rates than those of non-T neurons during scratching,

TABLE 2. Identified axon projections and terminations of spinal interneurons studied

Axon Projection	Morphological Cell Type				Totals
	T(1)	T(2)	NT	Other	
Ascending					
Contralateral ventral funiculus	1				1
Descending					
Ipsilateral ventral funiculus		2		1 (1)	3
Contralateral ventral funiculus		1			1
Bifurcating					
Ipsilateral lateral funiculus		1	5 (2)		6
Contralateral ventral funiculus		2 (1)			2
Totals	1	6	5	1	13

Abbreviations as in Table 1. Numbers in parentheses indicate numbers of cells with ventral horn axon terminal arborizations.

irrespective of whether the high-MVL ($P = 0.044$; Mann-Whitney one-tailed test) or overall high scratch values ($P = 0.026$) were used. T(1) neurons and T(2) neurons taken together ("T neurons") had significantly higher peak firing rates than those of non-T neurons using the overall high scratch values ($P = 0.029$), but the difference was not quite significant using the high-MVL values ($P = 0.060$). Differences between T neuron and non-T neuron peak firing rates were not significant during swimming [T(1) vs. non-T, $P = 0.090$; T vs. non-T, $P = 0.157$]. Differences in peak firing rates between scratching and swimming for all cells taken together were also not significant (high MVL scratch vs. swim $P = 0.889$; overall scratch vs. swim $P = 0.254$; Mann-Whitney two-tailed tests).

The peak-to-trough amplitudes of phase-averaged membrane potential oscillations of the recorded cells as a whole were correlated between swimming and scratching (Fig. 10B), just as the MVLs were correlated (Fig. 8A). Thus cells that had large oscillations during scratching also tended to have large oscillations during swimming. Nonetheless, it is apparent from Fig. 10B that most cells (20/25) had larger oscillations during scratching than during swimming. Indeed, the mean oscillation amplitude during scratching (for the most rhythmic form of scratching) was significantly greater than the mean oscillation amplitude during swimming (Fig. 10C; $P = 0.002$, Mann-Whitney two-tailed test). However, this comparison used whichever form of scratching gave the most rhythmic cell activity for each cell; this may have biased the measure toward larger values for scratching. Thus the mean oscillation amplitude during each individual form of scratching was also compared with the amplitude during swimming (Fig. 10C). Although the mean amplitude was similar for the three forms of scratching, the rostral scratch mean oscillation amplitude was significantly greater than the swim oscillation amplitude ($P = 0.012$), whereas the pocket scratch ($P = 0.156$) and caudal scratch ($P = 0.180$) oscillation amplitudes were not because

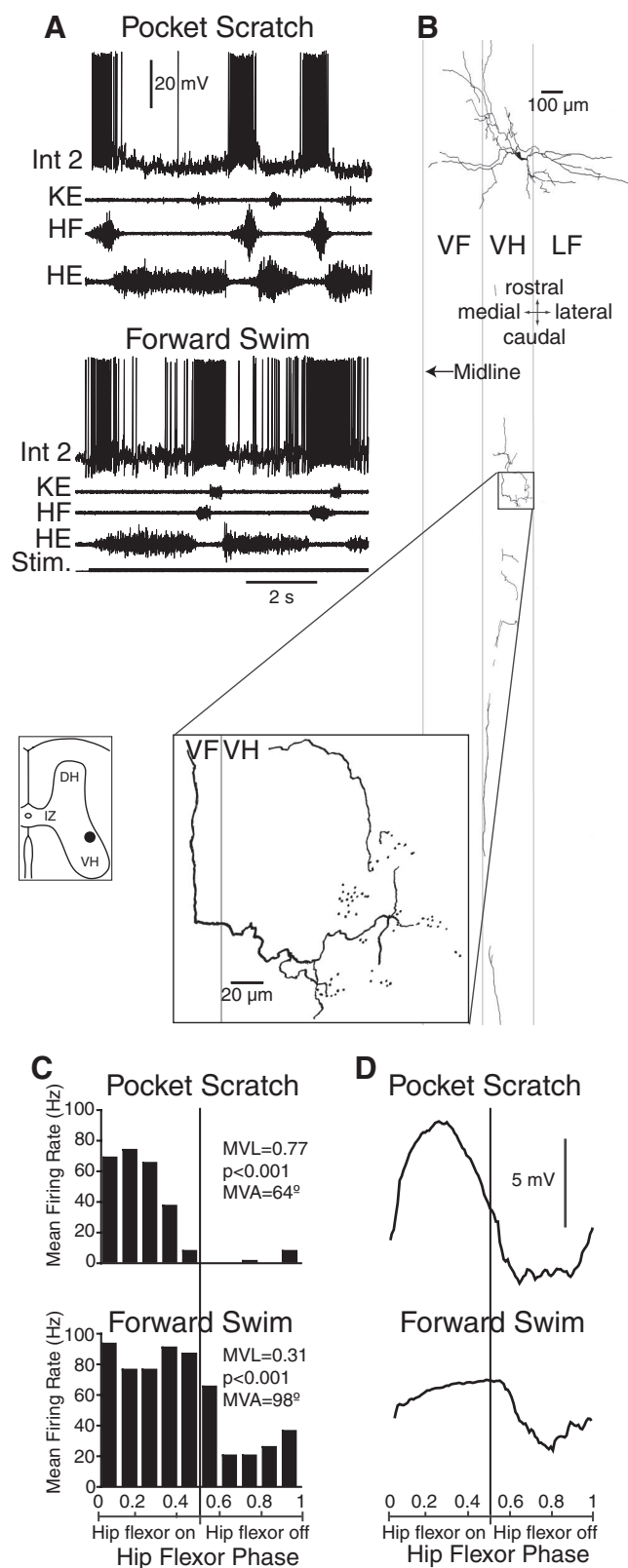
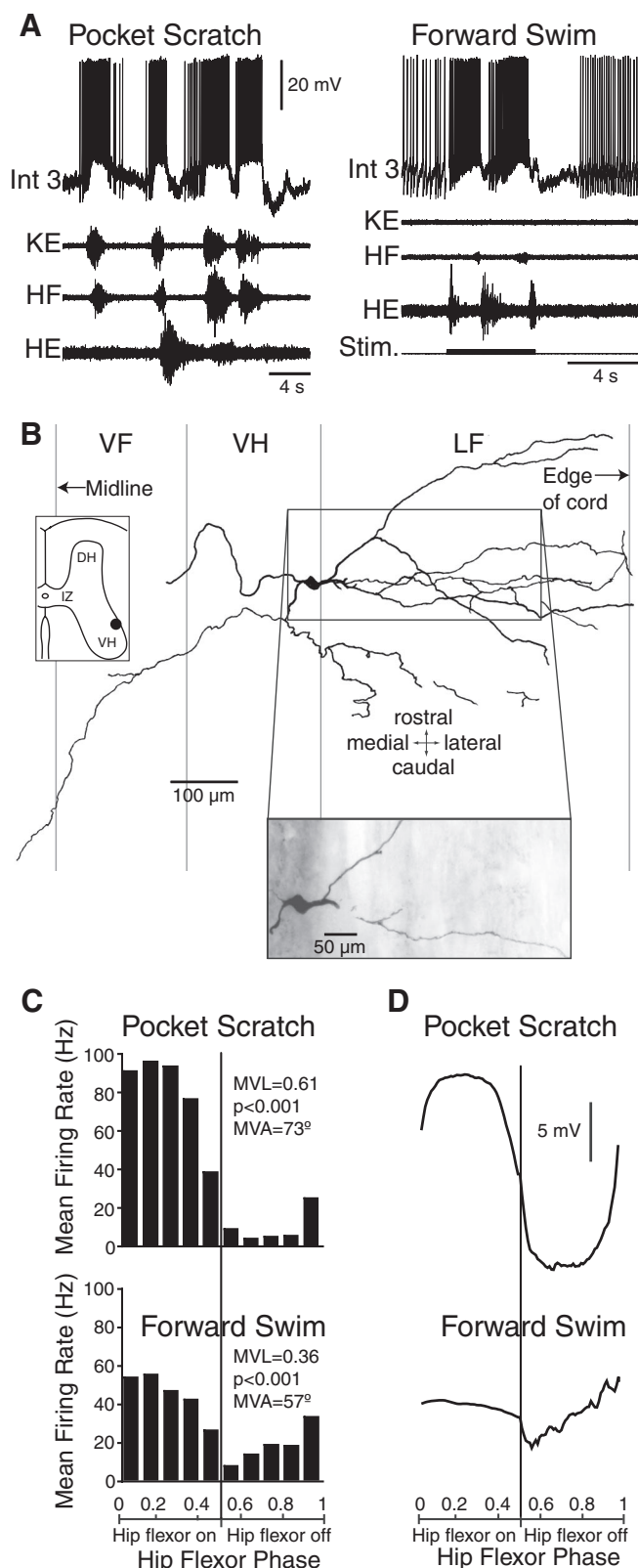


FIG. 4. A second, rhythmic scratch/swim neuron with axon terminal arborizations. **A**: rhythmic activation of interneuron 2 during both fictive pocket scratching and fictive forward swimming. **B**: morphological reconstruction of interneuron 2, including high-magnification axon terminal arborizations in the hindlimb enlargement ventral horn and soma location in the transverse plane (insets). **C**: phase histograms of mean firing rate of interneuron 2 during pocket scratching and swimming. **D**: phase-averaged membrane potentials of interneuron 2 during pocket scratching and swimming.

there was less variability in the amplitude during rostral scratching. Anatomical groups of cells did not differ significantly in their mean oscillation amplitude [T(1): 6.77 ± 2.23 mV; T: 7.46 ± 1.11 mV; non-T: 7.27 ± 1.77 mV].



Scratch and swim oscillation amplitudes were also measured separately for “hip flexor on” (0.00–0.49 oscillation peak phase) and “hip flexor off” (0.50–0.99 peak phase) groups of interneurons, based on their oscillation peak phases during the high-MVL form of scratching (Fig. 10C). Oscillation amplitudes were not significantly different between these two groups of interneurons during either scratching or swimming. Scratch oscillation amplitudes were significantly greater than swim oscillation amplitudes for hip flexor on cells ($P = 0.0036$), but not for hip flexor off cells ($P = 0.53$).

DISCUSSION

Rhythmic firing and membrane potential oscillations during swimming and scratching

The demonstration here that some spinal interneurons that are rhythmically activated during both fictive forward swimming and fictive scratching (i.e., scratch/swim neurons) have axon terminal arborizations in the ventral horn of the hindlimb enlargement provides the most direct evidence to date that swimming and scratching have some shared interneuronal circuitry. The firing rates of scratch/swim neurons were usually rhythmically modulated to a similar degree during fictive swimming and fictive scratching. They also tended to fire in the same phase of the hip flexor activity cycle during swimming and scratching, suggesting that they may play similar roles in the two behaviors, as suggested previously (Berkowitz 2002).

This study is the first to record intracellularly from spinal interneurons during both fictive forward swimming and fictive scratching. Scratch/swim neurons generally displayed oscillations of their phase-averaged membrane potentials during both swimming and scratching. On average, these oscillations were larger during scratching than during swimming, although this difference should be interpreted cautiously for two reasons: 1) fictive scratching was evoked using naturalistic mechanical stimulation of the body surface, whereas fictive swimming was evoked using electrical stimulation in the spinal cord; and 2) the difference in oscillation amplitudes was significant for the most rhythmic form of scratching for each cell and for rostral scratching, but not for pocket scratching or caudal scratching. A possible reason for this difference in scratch forms is that hip flexor bursts are strongest during rostral scratching, whereas forward swimming has much weaker hip flexor bursts. Thus the greater amplitude of oscillations during rostral scratching compared with forward swimming may relate to the required inputs to hip flexor motoneurons during these two behaviors. Consistent with this idea, interneurons with oscillation peaks during the hip flexor-on phase had significantly larger oscillations during scratching than swimming (whereas interneurons with peaks during the hip flexor-off phase did not). Thus the difference in oscillation amplitudes

FIG. 5. Rhythmic activity of a transverse interneuron (T neuron) during both fictive scratching and fictive swimming. **A**: rhythmic activation of interneuron 3 during both fictive pocket scratching and fictive forward swimming. [Note: KE is here pars ambiens (AM), not pars femorotibialis (FT) (see METHODS); FT in this animal burst during HE in fictive pocket scratching, but the FT recording was not available at this time.] **B**: morphological reconstruction of interneuron 3. Note the mediolateral elongation of soma and especially dendrites that is typical of T neurons. **C**: phase histograms of mean firing rate of interneuron 3 during pocket scratching and swimming. **D**: phase-averaged membrane potentials of interneuron 3 during pocket scratching and swimming.

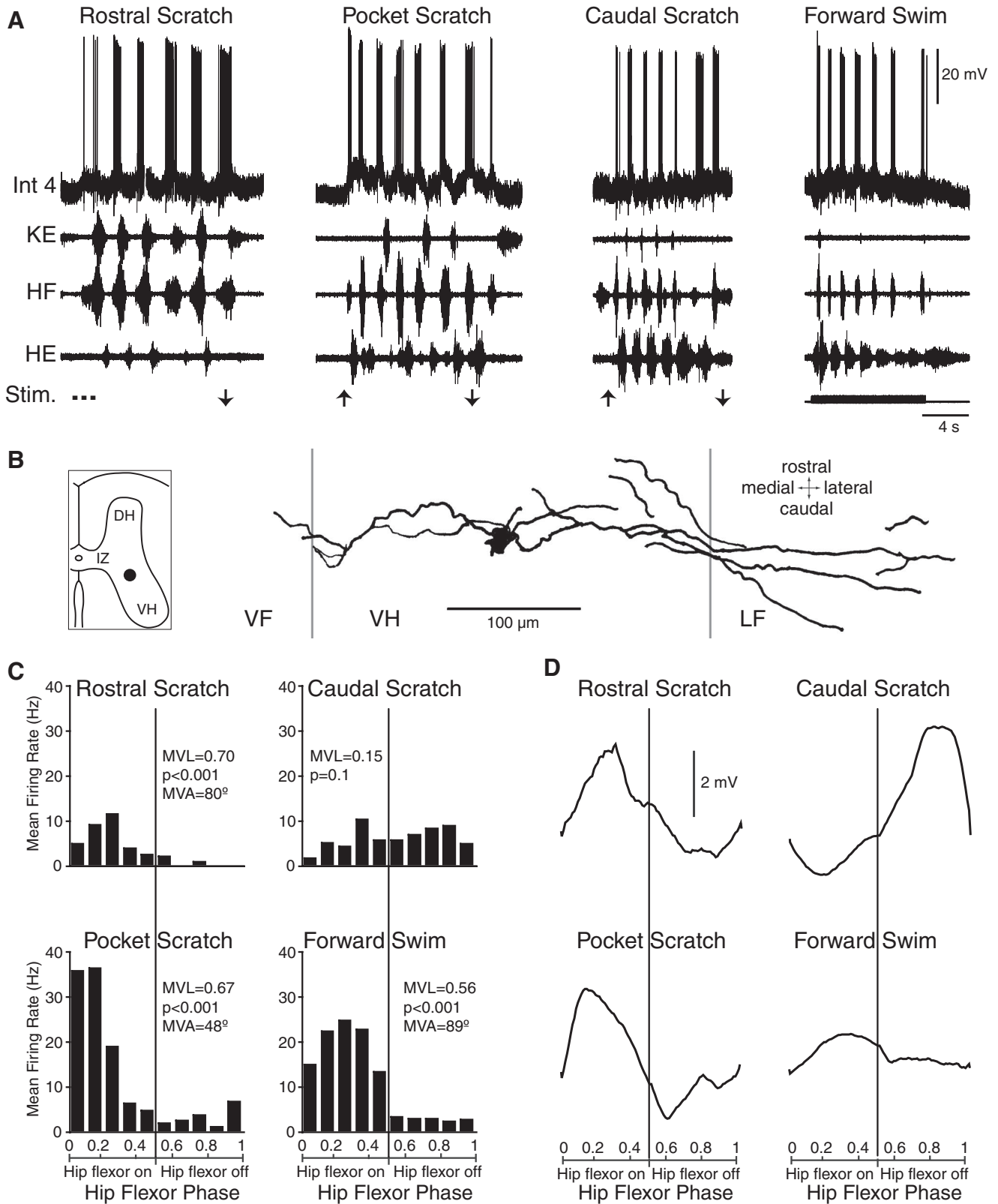


FIG. 6. Rhythmic activity of a second T neuron during both fictive scratching and fictive swimming. *A*: rhythmic activation of interneuron 4 during all 3 forms of fictive scratching, plus fictive forward swimming. [Note: KE is here AM, not FT (see METHODS).] *B*: morphological reconstruction of interneuron 4. Note the mediolateral elongation of soma and especially dendrites that is typical of T neurons. *C*: phase histograms of mean firing rate of interneuron 4 during all 3 forms of scratching, plus swimming. *D*: phase-averaged membrane potentials of interneuron 4 during all 3 forms of scratching, plus swimming.

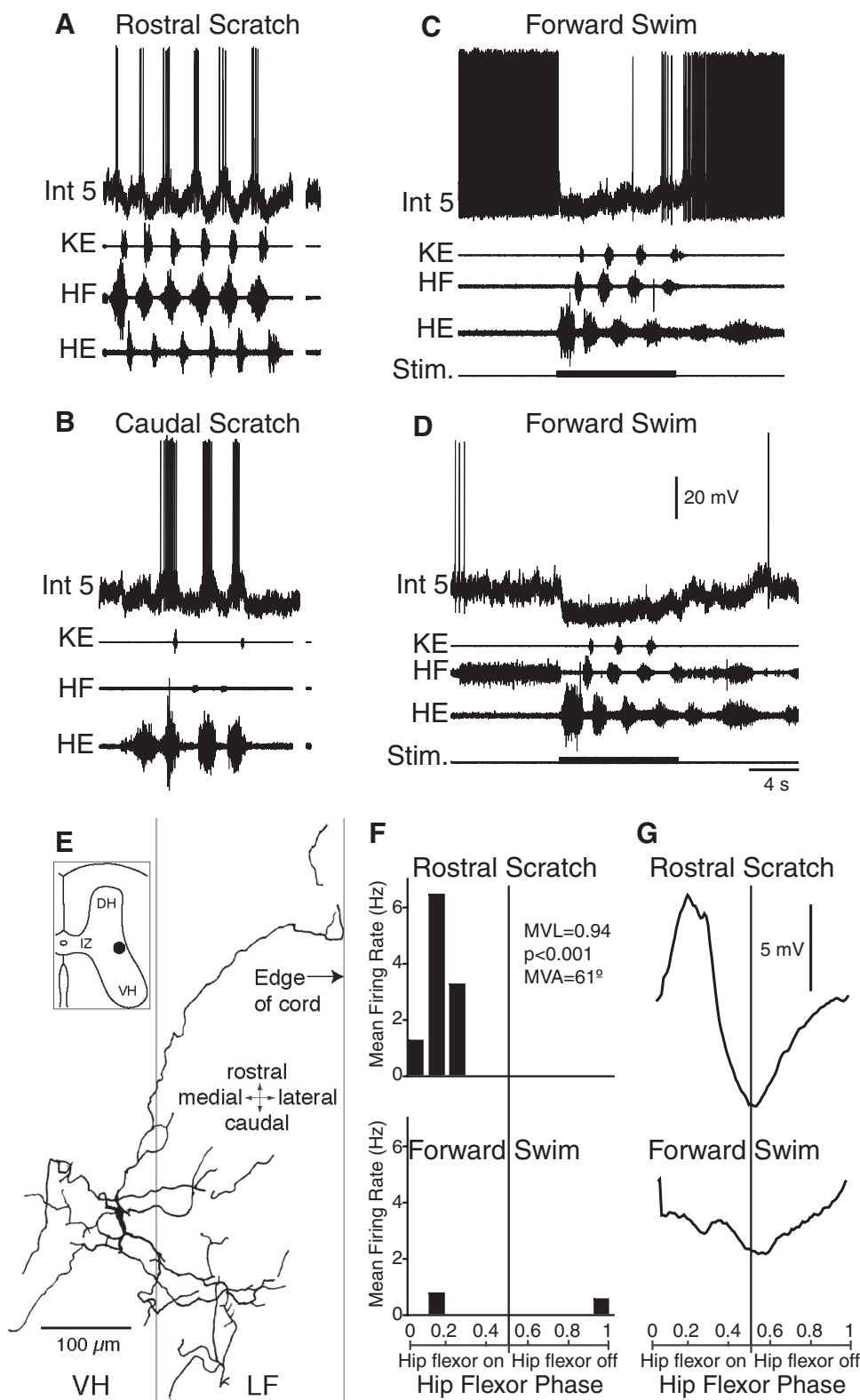


FIG. 7. Physiology and morphology of a scratch-specialized neuron. Interneuron 5 was rhythmically activated during fictive rostral scratching (A) and fictive caudal scratching (B), but was inhibited during fictive swimming (C, D). Note that the hyperpolarizing inhibition during fictive swimming could outlast the swim stimulation by several seconds. E: morphological reconstruction of interneuron 5. Note the rostrocaudal elongation of soma and dendrites, in contrast to T neurons (Figs. 5 and 6). F: phase histograms of mean firing rate of interneuron 5 during rostral scratching and swimming. G: phase-averaged membrane potentials of interneuron 5 during rostral scratching and swimming.

between scratching and swimming appears to be due to the effects of hip flexor-on interneurons. This group of interneurons may help make hip flexor motoneuron bursts stronger in rostral scratching than in forward swimming.

The trough phases of interneuron membrane potential oscillations were significantly correlated between swimming and

scratching, even though the peak phases were not. This suggests that the trough phases may play a more consistent and perhaps more important role than the peak phases in shaping the cells' rhythmic activity. If so, synaptic inhibition during the trough phase may contribute importantly to rhythmic modulation of these interneurons, as previously suggested for mo-

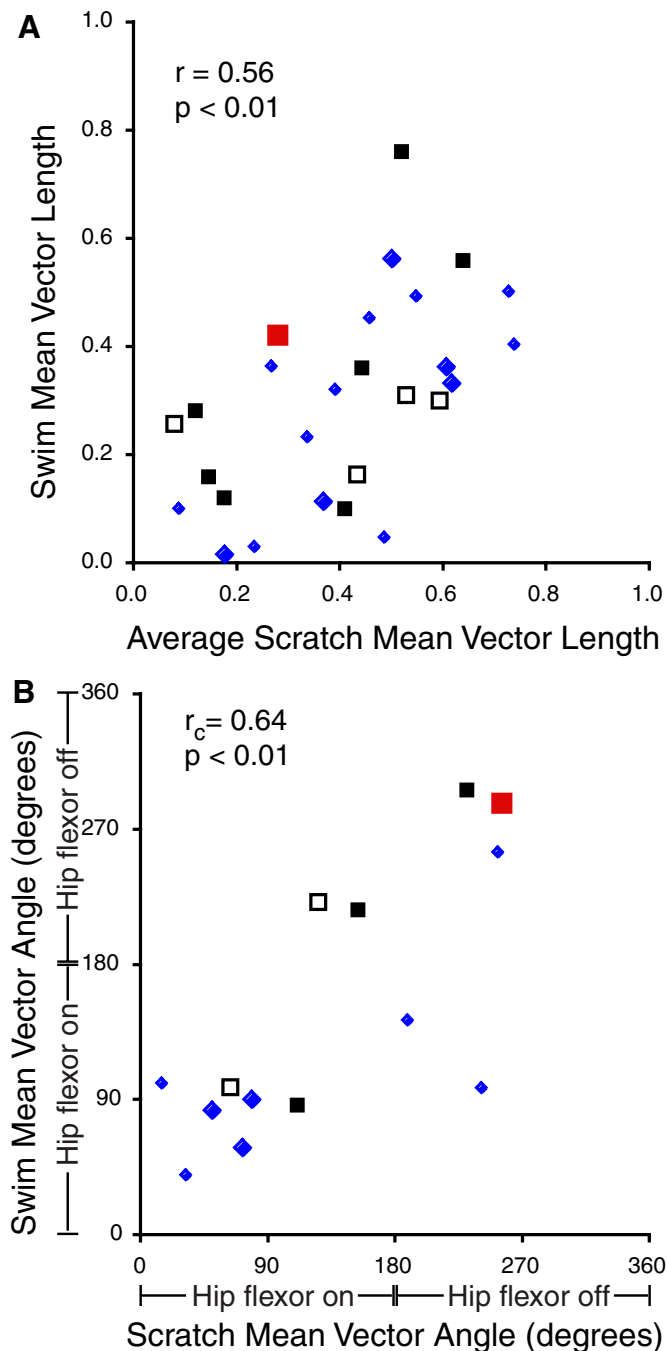


FIG. 8. Comparisons of the rhythmic firing of all cells during fictive forward swimming and fictive scratching. *A*: mean vector length (a measure of degree of rhythmic modulation; see METHODS) during swimming vs. scratching, for all cells and by category (see Fig. 1 for meaning of symbols); r is Pearson's correlation coefficient. *B*: mean vector angle (a measure of phase preference of firing within the hip flexor activity cycle; see METHODS) during swimming vs. scratching, for all cells and by category; r_c is the circular-circular correlation coefficient (Batschelet 1981).

toneurons (Robertson and Stein 1988; but see Berg et al. 2007), even if synaptic inhibition occurs in all phases of the scratch cycle (Berg et al. 2007; Robertson and Stein 1988). The current finding that trough-phase inhibition likely plays an important role, based on intracellular recordings from rhythmic interneurons, contrasts with the suggestion from Berg et al. (2007) that inhibition occurs mainly during the depolarizing phase of

scratching, which was inferred from calculations from intracellular motoneuron recordings. The current finding is consistent, however, with a previous finding from extracellular recordings of scratch-activated interneurons that the cells' MVLs were negatively correlated with their firing rates, i.e., the interneurons were more rhythmic when they fired at lower rates, which also suggested the relative importance of inhibition in making spinal interneurons rhythmic (Berkowitz and Stein 1994).

For forms of scratching examined individually, trough phases were significantly correlated between swimming and rostral scratching, but not between swimming and either pocket or caudal scratching. The greater correlation with rostral scratching may be due to the fact that rostral scratching and forward swimming share a common knee-hip synergy that is not shared by the other two forms of scratching (Earhart and Stein 2000a,b; Juranek and Currie 2000).

T neurons are a subset of scratch/swim neurons

An earlier study defined a morphological group of spinal interneurons, transverse interneurons, or T neurons, that are strongly and rhythmically activated during all forms of ipsilateral fictive scratching (and usually activated during fictive limb withdrawal), and on average have higher peak firing rates, narrower action potentials, briefer afterhyperpolarizations, and larger scratch phase-averaged membrane potential oscillations than those of scratch-activated interneurons with distinct dendritic tree orientations (Berkowitz et al. 2006). At least some T neurons have axon terminals in the hindlimb enlargement ventral horn and thus very likely affect motor output for all three forms of scratching (Berkowitz et al. 2006). T neurons recorded in the current study were all activated during forward swimming as well as scratching, demonstrating that T neurons are a subset of the shared, scratch/swim neurons (Berkowitz 2002). This finding helps reveal the role of a particular group of spinal interneurons in the control of two kinds of limb movements, a process that will be critical to elucidating the central control of multiple vertebrate behaviors that use the same muscles. T neurons in the current study had higher peak firing rates than those of non-T neurons during scratching, as shown previously (Berkowitz et al. 2006), but differences between T and non-T neurons in other physiological parameters examined were not significant here, probably because this study had a smaller sample of T neurons.

Scratch-specialized interneurons can be inhibited during swimming

This study also demonstrates for the first time that scratch-specialized spinal interneurons can receive hyperpolarizing inhibition during fictive forward swimming. Evidence from extracellular single-neuron recording had previously shown that scratch-specialized neurons were often suppressed during fictive swimming (Berkowitz 2002), but this suppression could have been due to either reduced excitatory drive or to direct inhibition of scratch-specialized neurons (or both). The current study shows (albeit with a very small sample size) that scratch-specialized neurons can be directly inhibited during swimming. Moreover, the fact that this inhibition could outlast the swim-triggering electrical pulses for several seconds suggests that the

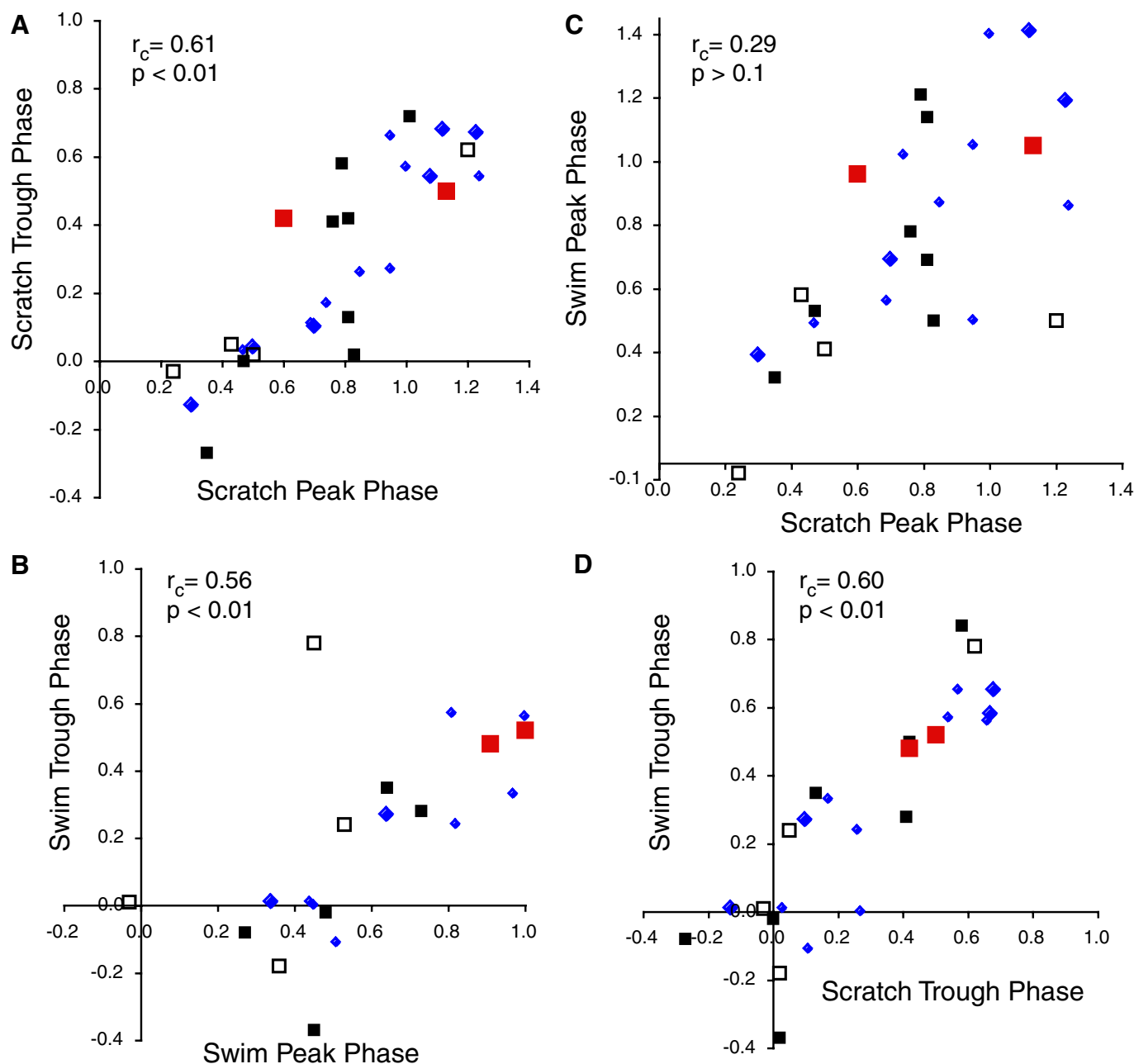


FIG. 9. Comparisons of peak and trough phases of phase-averaged membrane potential oscillations during fictive forward swimming and fictive scratching, for all cells and by category. *A*: scratch trough phase vs. scratch peak phase. *B*: swim trough phase vs. swim peak phase. *C*: swim peak phase vs. scratch peak phase. *D*: swim trough phase vs. scratch trough phase. Note that correlations are significant for *A*, *B*, and *D*, but not *C*.

inhibition derives from the swim pattern-generating circuit, rather than directly from some of the descending axons that were electrically stimulated. The neurons and neurotransmitter(s) underlying this swim-related inhibition will be an important topic for future research.

The scratch-specialized neurons studied here were also morphologically distinct from T neurons, suggesting that interneuronal roles in swimming and scratching might be predictable based on cell morphology, although this suggestion must be very tentative, given the small sample of scratch-specialized cells studied here. The fact that scratch-specialized neurons made up a much smaller fraction of the interneurons studied here than in the previous extracellular single-neuron recording

study (Berkowitz 2002) suggests that extracellular and intracellular recording biases may be quite different relative to the morphologies of scratch-specialized neurons. The finding that one of the scratch-specialized cells had axon terminal arborizations in the hindlimb enlargement ventral horn suggests that some scratch-specialized neurons affect hindlimb motor output.

Shared interneurons for vertebrate rhythmic motor patterns

Previous evidence also suggested that locomotion and scratching share spinal circuitry (Berkinblit et al. 1978b; Berkowitz 2002; Deliagina and Feldman 1981; Earhart and Stein 2000a,b; Gelfand et al. 1988; Juranek and Currie 2000;

Perreault et al. 1999; Pratt and Jordan 1987). There is also evidence for sharing of some spinal interneuronal circuitry between walking and paw-shaking in cats (Carter and Smith 1986), walking and hatching in chicks (Bekoff et al. 1987, 1989), swimming and struggling in tadpoles (Green and Soffe

1996; Li et al. 2007; Soffe 1993, 1996), swimming and escape movements in fish (Kimura et al. 2006; Ritter et al. 2001; Svoboda and Fetcho 1996), limb withdrawal and scratching in turtles (Berkowitz et al. 2006; Currie and Stein 1989), and the three forms of scratching in turtles (Berkowitz 2001b, 2005; Berkowitz and Stein 1994; Mortin and Stein 1989; Robertson et al. 1985; Stein et al. 1986). Evidence also suggests there is some shared central circuitry for vertebrate respiratory behaviors such as eupneic breathing, sighing, gasping, coughing, and sneezing, as well as swallowing and vomiting (Gestreau et al. 1996; Grelot et al. 1993; Lieske et al. 2000; Oku et al. 1994; Shiba et al. 2007; Yajima and Larson 1993).

Shared and specialized interneuronal circuitry

The finding of both shared and specialized spinal interneurons for fictive swimming and fictive scratching (here and in Berkowitz 2002) suggests that swimming and scratching in turtles are produced by a combination of shared and dedicated circuitry, as are swimming and struggling in embryonic tadpoles (Green and Soffe 1996; Li et al. 2007; Soffe 1993, 1996), swimming and escape movements in fish (Kimura et al. 2006; Ritter et al. 2001; Svoboda and Fetcho 1996), swimming and crawling in leeches (Briggman and Kristan Jr 2006; Briggman et al. 2005; Esch et al. 2002; Kristan Jr et al. 2005), and swimming and shortening in leeches (Shaw and Kristan Jr 1997). In several of these cases, the dedicated neurons appear to be functionally asymmetric, with more cells specialized for (leech) crawling, (tadpole) struggling, and (turtle) scratching than for swimming. What this means is not clear, but one possibility is that swimming developed more recently over evolutionary time in each of those cases and coopted some but not all of the preexisting circuitry for rhythmic control of the same muscles (Briggman and Kristan Jr 2006). A combination of shared and specialized circuitry also controls scratching and limb withdrawal in turtles (Berkowitz 2007c; Berkowitz et al. 2006), dorsal and ventral local bending in leeches (Lockery et al. 1989), escape swimming and turning in *Pleurobranchia* (Jing and Gillette 2003), and ingestion and egestion in *Aplysia* (Jing and Weiss 2001, 2002; Jing et al. 2004). The inhibition of some scratch-specialized neurons during fictive swimming suggests the possibility that mutual inhibition between members of competing networks plays a role in vertebrate behavioral choice, as has been shown for several invertebrate behav-

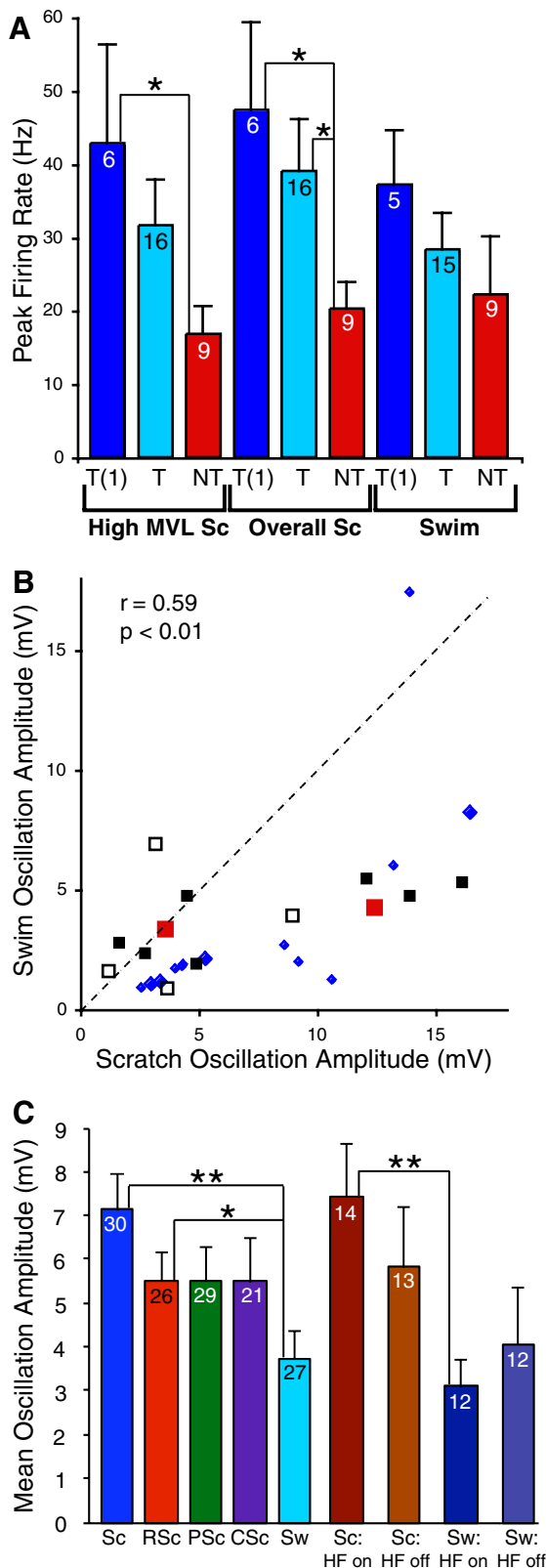


FIG. 10. Peak firing rates and oscillation amplitudes during fictive swimming and fictive scratching. **A:** means of cells' peak firing rates during scratching and swimming for T neurons meeting both dendritic and somatic criteria [T(1)], for all T neurons meeting the dendritic criterion (T), and for non-T neurons [(NT)]. Error bars indicate SE; the number inside each bar is *n*. Asterisks indicate statistical significance at $P < 0.05$ by the Mann-Whitney test; all other differences between cell groups within a motor pattern group or between motor patterns for the same cell group were not statistically significant. **B:** comparison of membrane potential oscillation amplitudes during swimming and scratching, for all cells and by group. Note that amplitude was correlated between swimming and scratching, but the vast majority of cells had larger oscillations during scratching than during swimming. **C:** mean oscillation amplitudes of all cells during the form of fictive scratching for which they were most rhythmic (Sc), during rostral (RSc), pocket (PSc), and caudal (CSc) fictive scratching, and during fictive forward swimming (Sw). Mean oscillation amplitudes are also given for scratching (Sc) and swimming (Sw) for interneurons grouped according to their peak phase during high-MVL scratching: hip flexor (HF) on or HF off. Error bars indicate SE; the number inside each bar is *n*. * $P < 0.05$; ** $P < 0.01$ by the Mann-Whitney test.

iors, especially in mollusks and crustaceans (Edwards et al. 1999; Huang and Satterlie 1990; Inoue et al. 1996; Jing and Gillette 2000; Kovac and Davis 1980; Krasne and Lee 1988; Kristan and Shaw Jr 1997; Norekian and Satterlie 1996).

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GRANTS

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