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Physiology and Morphology Indicate That Individual Spinal Interneurons Contribute to Diverse Limb Movements

Ari Berkowitz

Department of Zoology, University of Oklahoma, Norman, Oklahoma

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Berkowitz, Ari. Physiology and morphology indicate that individual spinal interneurons contribute to diverse limb movements. *J Neurophysiol* 94: 4455–4470, 2005. First published September 7, 2005; doi:10.1152/jn.00229.2005. Overlapping neuronal networks have been shown to generate a variety of behaviors or motor patterns in invertebrates, but the evidence for this is more circumstantial in vertebrates. The turtle spinal cord can produce multiple forms of hindlimb scratching movements as well as hindlimb withdrawal, but it is still uncertain whether individual spinal cord interneurons contribute to the motor output for more than one type of limb motor pattern. In this study, individual spinal cord interneurons were recorded intracellularly in vivo in spinal immobilized turtles, and, after characterization, were filled with Neurobiotin. Interneurons that were rhythmically activated during multiple forms of ipsilateral fictive hindlimb scratching often had axon-terminal arborizations in the ventral horn of the spinal cord hindlimb enlargement. This provides some of the strongest evidence to date that interneurons involved in multiple forms of scratching contribute directly to hindlimb motor output. Moreover, most of these interneurons were also active during contralateral fictive scratching and during ipsilateral fictive hindlimb withdrawal, suggesting that they contribute to motor output for these additional behaviors as well. Such interneurons may provide the cellular basis for the contralateral contributions to ipsilateral scratching that have been demonstrated previously. Taken together, these findings suggest that diverse vertebrate limb movements are produced by spinal cord interneuronal networks that include some shared components.

INTRODUCTION

The same muscles are used for a wide variety of behaviors. Are distinct behaviors or motor patterns involving the same muscles generated by separate or by partly shared neuronal networks? In invertebrates, it is clear that neuronal networks for multiple rhythmic behaviors often include shared pattern-generating neurons (Marder and Calabrese 1996; Pearson 1993). In vertebrates, the situation is less clear, and the evidence is largely circumstantial, especially for the neuronal networks controlling limb movements (Bekoff et al. 1987; Berkinblit et al. 1978; Berkowitz 2001b, 2002a; Berkowitz and Stein 1994b; Carter and Smith 1986; Currie and Stein 1989; Earhart and Stein 2000a,b; Juranek and Currie 2000; Mortin and Stein 1989; Perreault et al. 1999; Smith et al. 1986).

The turtle spinal cord is a convenient model system with which to address this question. It can generate multiple forms of hindlimb scratching, hindlimb swimming, and hindlimb withdrawal (flexion reflex) motor patterns, even in the absence of input from the brain and movement-related sensory feed-

back (Currie and Stein 1989; Juranek and Currie 2000; Lenard and Stein 1977; Mortin et al. 1985; Robertson et al. 1985; Stein et al. 1982). Previous research has shown that many individual spinal cord interneurons are rhythmically active during all three forms of ipsilateral fictive scratching (Berkowitz 2001b, 2002a; Berkowitz and Stein 1994b). In addition, many spinal interneurons are rhythmically active during both ipsilateral and contralateral fictive scratching (Berkowitz 2001b, 2002a; Berkowitz and Stein 1994b). Moreover, many scratch-activated spinal interneurons are also activated during fictive forward swimming and fictive hindlimb withdrawal (Berkowitz 2002a). These studies suggest that at least some spinal interneurons contribute to the generation of motor patterns for multiple, diverse hindlimb behaviors.

One concern, however, has been that correlations between single-neuron activity and motor output do not demonstrate that any of these neurons actually affect motor output for any behavior. For ascending spinal interneurons, a reasonable alternative hypothesis is that these neurons provide feedback signals to higher motor control centers in the brain as has been shown for cat ventral spinocerebellar tract neurons (Arshavsky et al. 1972, 1978, 1984) but have no direct effect on spinal motor output. Some rhythmically active spinal interneurons may mediate primary afferent depolarization (Rudomin and Schmidt 1999) and have no direct effects on motor output. If, however, some spinal interneurons do directly affect motor output for multiple hindlimb behaviors, they should have axon terminals within the ventral horn of the spinal cord hindlimb enlargement. Several physiological types of turtle spinal cord interneurons have previously been studied using a combination of intracellular recording and staining (Fernandez et al. 1996; Hounsgaard and Kjaerulff 1992; McDonagh et al. 1998, 2002; Russo and Hounsgaard 1996a,b), but those experiments were conducted using slice preparations that do not produce scratch rhythms. To test the prediction that interneurons active during multiple forms of scratching and withdrawal have axon terminals within the ventral horn of the spinal cord hindlimb enlargement, I recorded intracellularly from interneurons that were active during multiple, diverse hindlimb motor patterns in vivo and stained each with Neurobiotin to reveal its morphology. Preliminary results have been reported previously in an abstract (Berkowitz 2003).

Address for reprint requests and other correspondence: A. Berkowitz, Dept. of Zoology, University of Oklahoma, Norman, OK 73019 (E-mail: ari@ou.edu).

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METHODS

Animal preparation

Adult red-eared turtles (*Trachemys scripta elegans*, 500–1,200 g, both sexes, $n = 29$) were prepared for recording as previously described (Berkowitz 2001a; Robertson et al. 1985). Briefly, animals were anesthetized and surgically dissected to transect the spinal cord between the dorsal 2 (D_2) and D_3 postcervical segments, expose the spinal cord between the D_6 and sacral 2 (S_2) segments, and prepare several hindlimb motor nerves for electroneurographic recordings (ENGs), including the right and left hip flexor nerves, ventral puboischiofemoralis internus, pars anteroventralis (HF) and, in some experiments, the right knee extensor nerve, triceps femoralis, pars femorotibialis (Fig. 1). After surgery, the animal was immobilized with gallamine triethiodide (8 mg/kg im, 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 ($n = 47$ scratch-activated interneurons 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 (D_8 , D_9 , D_{10} , S_1 , and S_2), using sharp microelectrodes made from thin-walled borosilicate glass with filament (Sutter Instrument Company, Novato, CA), fabricated with a P-97 puller (Sutter) and filled with 4% Neurobiotin (Vector Laboratories, Burlingame, CA) dissolved in 1 M KCl, with resistances of 60–120 M Ω . The meninges were torn on the dorsal surface of the spinal cord at the site of each intracellular microelectrode penetration. The electrode was advanced using an Inchworm piezoelectric motorized microdrive (Burleigh Instruments, Fishers, NY); impalements were often facilitated using 5-ms negative capacitance “tickle” pulses delivered by the intracellular amplifier (World Precision Instruments, Sarasota, FL), triggered by a Master-8 stimulator (A.M.P.I., Jerusalem, Israel). Membrane potentials and spike heights were each typically within the range of 40–70 mV. In some cases, 0.1- to 0.2-nA constant hyperpolarizing current was injected to stabilize the recording. After each successful intracellular recording, sinusoidal (4 Hz) current with a peak depolarization of 0.1–3.0 nA was injected for 1–30 min to eject Neurobiotin. No more than one interneuron fill was attempted in each spinal cord segment to ensure unambiguous identification. ENG were obtained using pairs of 100- μ m silver wires in a pool of mineral oil and were amplified (1,000 $\times$) and filtered (100–1,000 Hz) using differential AC amplifiers (A-M Systems, Carlsborg, WA). Fictive

scratching and fictive withdrawal were elicited by mechanical stimulation of the shell or skin with a fire-polished glass probe attached to a force transducer (Grass-Telefactor, West Warwick, RI). All recordings were stored on an RD-145 digital audio tape recorder (TEAC America, Montebello, CA), displayed on a DL 716 Scopecorder (Yokagawa Corporation of America, Newnan, GA), and redigitized and analyzed off-line using Datapac 2000 software (Run Technologies, Laguna Hills, CA).

Histology

Immediately after the recording session, the animal was killed with pentobarbital (390 mg ip); after ≥ 1 h, the heart was exposed and perfused with 800 ml turtle saline containing 0.1% sodium nitrite, 10 units/ml heparin, and 39 mg pentobarbital, followed by 300 ml chilled 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The spinal cord was postfixed overnight at 4°C, cryoprotected in 20% sucrose/PB, embedded in a gelatin-albumin-glutaraldehyde-PB medium, and frozen-sectioned at 100 μ m horizontally. Sections were rinsed 3×5 min in phosphate-buffered saline (PBS), incubated 30 min in 0.3% H_2O_2 /PBS, rinsed 4×10 min in PBS, incubated overnight at 4°C in 1:100 ABC solution (Vector)/0.3% Triton X-100/PBS, rinsed 4×10 min in PBS, reacted 4–7 min in 0.5 $\times$ Vector SG solution/PBS, and rinsed 3×10 min in PBS; all steps were done on a rotator. Sections were mounted, air-dried, counterstained in Fast Nuclear Red (Vector), dehydrated in graded ethanols, cleared in HistoClear (National Diagnostics, Atlanta, GA), and mounted with Permount (Fisher Chemicals, Fairlawn, NJ).

Morphological analysis

Neurons were included in this study only if they were activated or rhythmically modulated during fictive scratching, one and only one stained soma with attached stained processes was recovered at the expected site, and, for ventral horn neurons, the processes were darkly stained and yet no stained process was seen in a ventral root (to eliminate motoneurons from the study). Reconstructions were made via a camera lucida attached to an Optiphot-2 microscope (Nikon Instruments, Melville, NY); digital photomicrographs were obtained with a DP-70 camera (Olympus America, Melville NY) mounted on this microscope. Photomontages were assembled using Photoshop (Adobe Systems, San Jose, CA).

Physiological analysis

The modulation of each interneuron's firing rate was quantitatively analyzed separately for each form of ipsilateral fictive scratching in

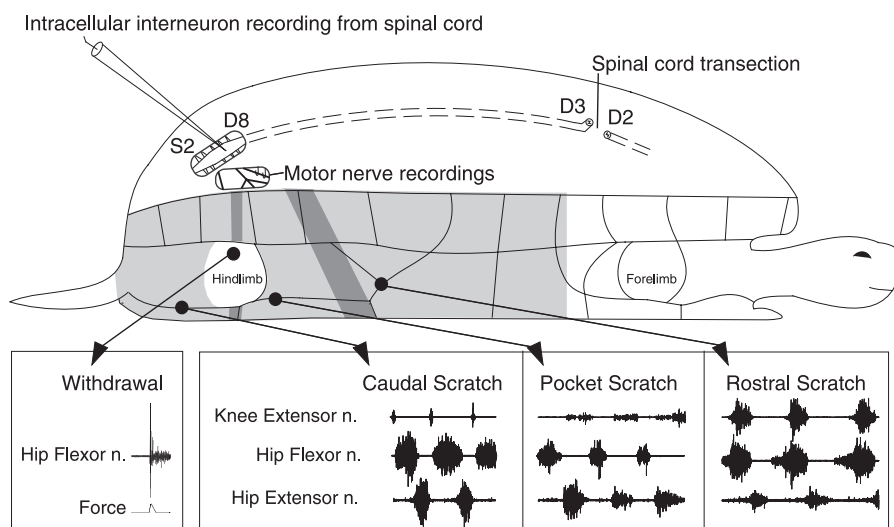


FIG. 1. Sketch of the experimental preparation. Spinal interneurons were recorded from intracellularly on the right side of the hindlimb enlargement spinal cord, along with (right and left) hindlimb motor nerves, during *in vivo* fictive scratching and fictive withdrawal, in immobilized, spinal turtles. D, dorsal segment; n, nerve; S, sacral segment.

which there were clear, alternating bursts and silent periods of the ipsilateral hip flexor motor nerve, at least two such cycles of fictive scratching occurred, and the interneuron fired ≥ 10 action potentials during these cycles. In each such case, a dual-referent phase histogram (with 5 bins for the hip flexor burst and 5 bins for the hip flexor interburst interval) was calculated with respect to the rectified and smoothed bursts and interburst intervals of the ipsilateral hip flexor nerve, using Datapac 2000 (Berkowitz 2001b, 2002a; Berkowitz and Stein 1994b). The phase histogram data were then used to calculate the mean vector (Batschelet 1981; Berkowitz and Stein 1994b; Drew and Doucet 1991; Mardia 1972). The mean vector length is a measure of the degree of rhythmic modulation of a neuron's activity with respect to a cycle; it can vary from 0 to 1 with 0 indicating minimal rhythmicity and 1 indicating maximal rhythmicity. The mean vector angle is a measure of the phase preference of a neuron within the hip flexor cycle; it can vary from 0 to 1 (or 0–360°) with 0 to 0.5 (or 0–180°) indicating a phase preference during hip flexor activity and 0.5 to 1 (or 180–360°) indicating a phase preference within the hip flexor interburst intervals (Berkowitz 2001b, 2002a; Berkowitz and Stein 1994b). 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 1994b; Drew and Doucet 1991; Mardia 1972). Mean vector angles were used in analyses only if the phase histogram passed the Rayleigh test with $P < 0.01$ (Berkowitz 2001b, 2002a; Berkowitz and Stein 1994b). Most cells were active during multiple forms of fictive scratching, so multiple phase histograms were constructed for each cell; the phase histogram used for measurements of mean vector length, mean vector angle, and peak firing rate (Figs. 8, A–C, and 9D) was the one that passed the Rayleigh test with the lowest P value, unless multiple phase histograms had $P < 0.001$, in which case the one among these having the highest mean vector length was used (Berkowitz 2001b, 2002a; Berkowitz and Stein 1994b). For each cell, the peak firing rate used (Fig. 8A) was the highest mean firing rate of the 10 values (10 bins) in this phase histogram.

Quantitative analyses of membrane potential oscillations were conducted for all interneurons during all forms of ipsilateral fictive scratching that met the first two criteria in the preceding text. In each such case, using Datapac 2000, action potentials were deleted from the intracellular recording (and the missing voltage values were then interpolated from the values just before and just after each action potential), the recording was linearly smoothed with a time constant of 200 ms, and the signal was dual-referent phase-normalized and averaged for all cycles of fictive scratching at that site of stimulation. The phase-normalized and averaged oscillation used in further analysis for each cell was from the site used for its phase histogram (see preceding text), unless the cell fired < 10 action potentials during ipsilateral scratching in which case the ipsilateral site with the largest phase-averaged oscillation was used. The peak phase, trough phase, and oscillation amplitude were measured from this phase-averaged oscillation (see Fig. 9A). Comparisons of each cell's peak and trough phases were conducted using two approaches. In the first approach, the oscillation peak was arbitrarily defined as the first event and its phase compared with that of the ensuing trough (Fig. 9C); in some cases, this meant adding 1.0 to the trough phase value (e.g., if the peak phase was 0.7 and the trough phase was 0.3, the trough phase was represented as 1.3). In the second approach, the phase difference between the peak and trough was calculated as the minimum phase difference, no matter whether the peak or the trough occurred earlier in the hip flexor cycle (e.g., if the peak phase was 0.7 and the trough phase was 0.3, the minimum phase difference was 0.4); thus phase differences could vary only from 0 (in phase) to 0.5 (out of phase).

RESULTS

Forty-seven scratch-activated spinal interneurons were recorded during multiple forms of fictive scratching plus fictive

withdrawal and had adequate morphology recovered (METHODS). The somata of these interneurons were mainly in the deep dorsal horn, the intermediate zone, and the dorsal half of the ventral horn; most were in the central to lateral parts of the gray matter (Fig. 2).

Physiology of scratch-activated interneurons

All 47 cells were active during at least two of the three forms of ipsilateral fictive scratching and most were active during all three forms of scratching (Figs. 3A, 5A, and 6A and Table 1). Each interneuron showed some degree of rhythmic modulation of its spike rate and/or its membrane potential, phase-locked to each scratch rhythm with which it was active (Figs. 3A, 4A, 5A, 6A, and 7A). The strength of rhythmic modulation during fictive scratching, as assessed for firing rate by the mean vector length (Fig. 8B) and for membrane potential by the amplitude of the averaged membrane potential oscillation (Fig. 9B), varied substantially among neurons, from extremely rhythmic (e.g., Fig. 6A) to only slightly rhythmic (e.g., Fig. 7A).

The membrane potential oscillations usually involved both depolarizing and hyperpolarizing waves that were phase-locked to the scratch rhythm (Figs. 5A, 1–3, 6A, 1, 2, and 4, and 7A3). These depolarizing and hyperpolarizing waves were probably caused by alternating synaptic excitation and inhibition, because in many cases, the membrane potential alternately went above and below the prescratch resting potential (Figs. 5A, 1–3, 6A, 1, 2, and 4, 7A3, and 10A); in very rhythmic neurons, the hyperpolarizing waves were often larger than the depolarizing waves (Figs. 5A, 1–3, and 6A, 1 and 2). Moreover, when constant-current pulses were injected into such an inter-

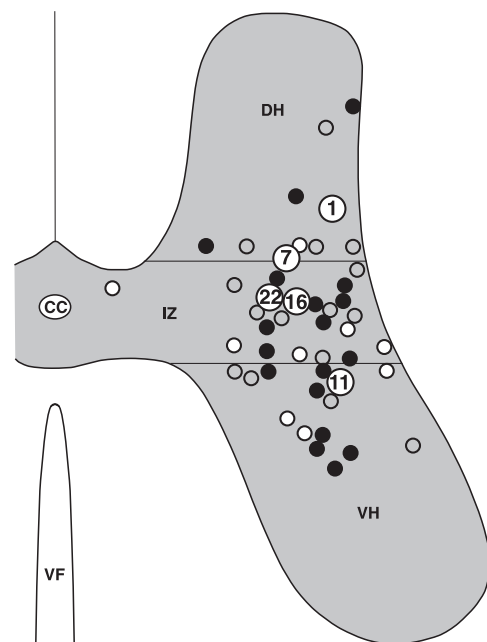
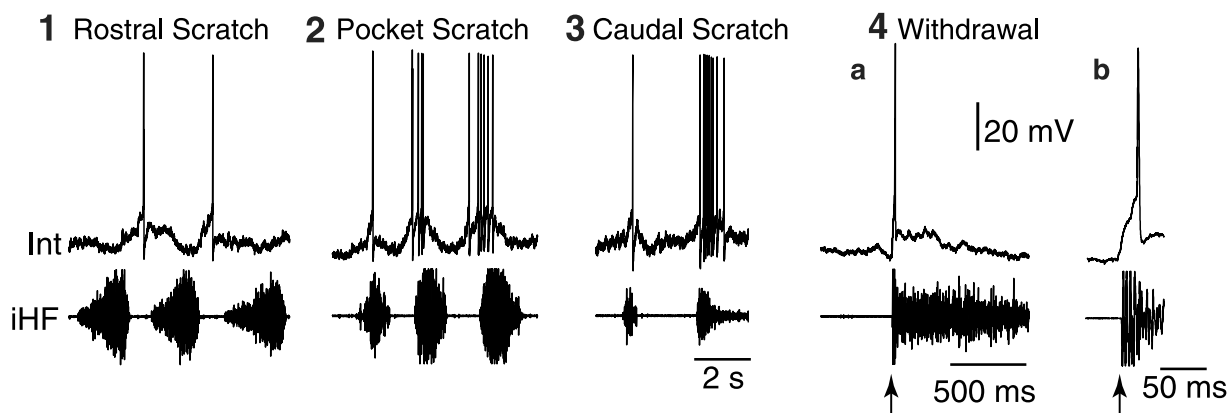


FIG. 2. Transverse-plane soma locations for all scratch-activated interneurons the morphology of which was recovered ($n = 47$). Soma locations are marked on a schematic cross section, relative to the borders of the dorsal horn (DH), intermediate zone (IZ), and ventral horn (VH). Numbered circles indicate cells illustrated in Figs. 3–7. ● other cells with axon terminal arborizations recovered; ○, cells with axon trajectories but no terminal arborizations recovered; ⊙, cells with neither axon trajectory nor terminal arborizations recovered. cc, central canal; VF, ventral fissure.

A Physiology



B Morphology

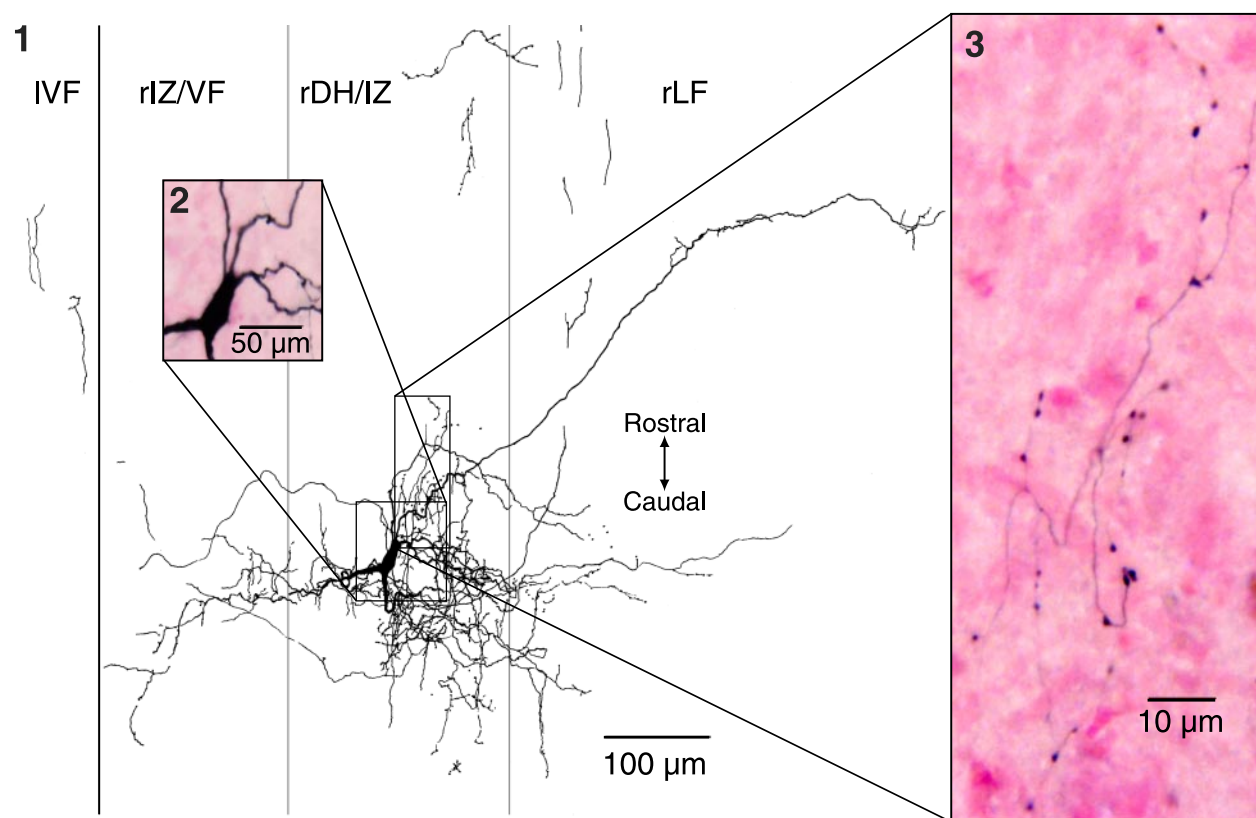


FIG. 3. Physiology and morphology of an interneuron (*cell 1* in Fig. 2 and Table 1) that was rhythmically activated during all 3 forms of ipsilateral fictive scratching and was activated during ipsilateral fictive hindlimb withdrawal. *A*: physiology: 1–3, ipsilateral scratching; 4, ipsilateral withdrawal; 4*b* is an expansion of the early part of the response in 4*a*. Arrows in 4 indicate the onset of a tap to the dorsal foot. Int, interneuron; iHF, ipsilateral (right) hip flexor motor nerve. *B*: morphology: 1, camera lucida reconstruction from horizontal sections; 2, single-section photomontage of the soma and proximal dendrites, in the right S2 deep dorsal horn; 3, single-section photomontage of axon-terminal arborizations in the ipsilateral intermediate zone. LF, lateral funiculus; l, left; r, right. This cell had a mean vector length of 0.81 and a membrane potential oscillation amplitude of 7.6 mV (during ipsilateral pocket scratching).

neuron, the cell's input resistance declined during all phases of the scratch rhythm as compared with just before and just after the scratch episode (Fig. 10), indicating that there were conductance increases associated with both the depolarizing and the hyperpolarizing phases. These conductance increases were dramatic in some neurons, such as the one shown in Fig. 10A

(note especially Fig. 10A2, ↑), which is the same neuron as is illustrated in Fig. 5. The conductance increases were more subtle in other neurons, such as the one shown in Fig. 10B, which is the same neuron as is illustrated in Fig. 7. While conductance increases were evident in all phases of fictive scratching, the amplitude of the conductance increases could

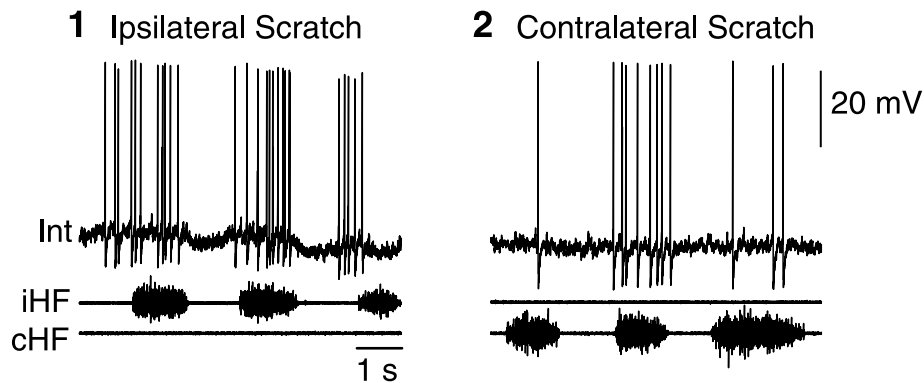
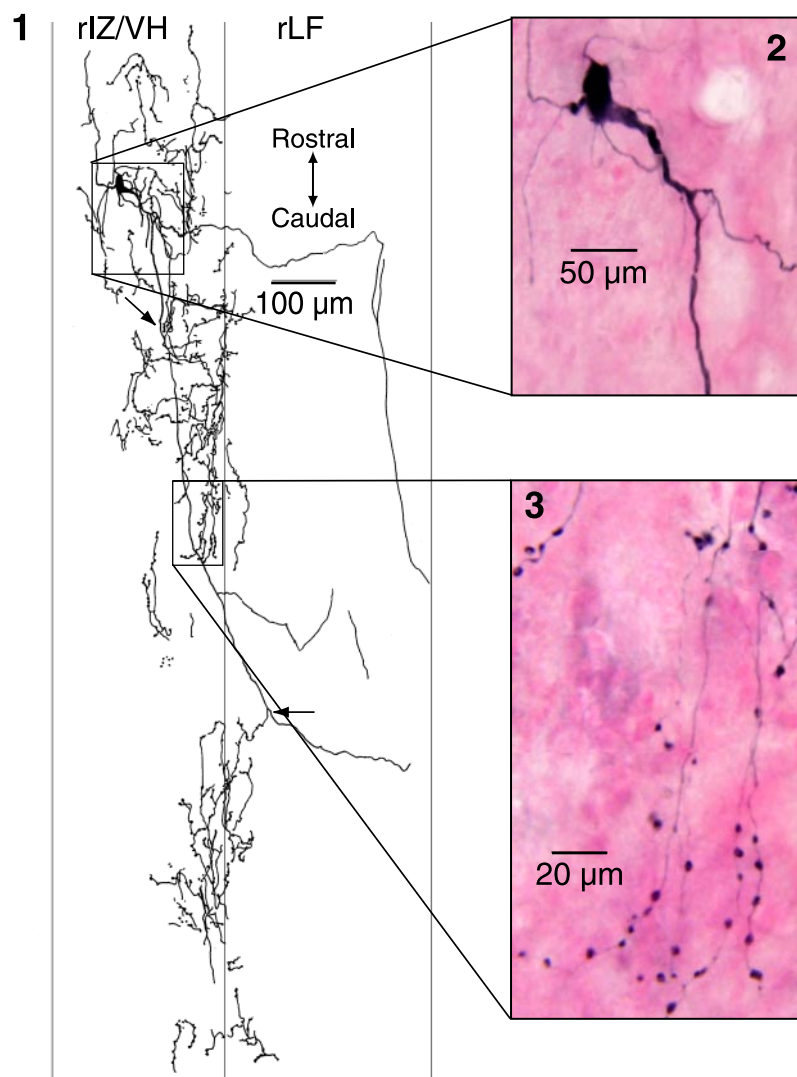
A Physiology**B Morphology**

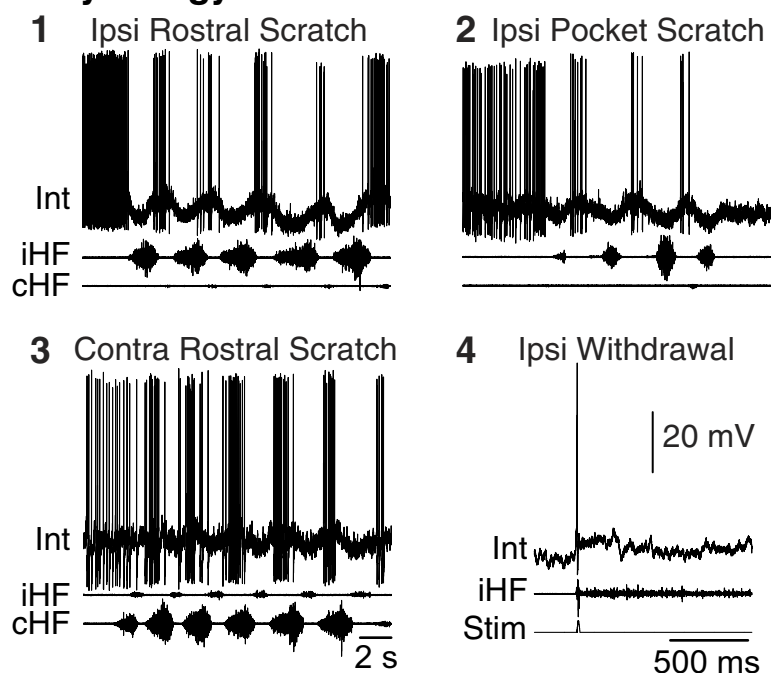
FIG. 4. Physiology and morphology of an interneuron (*cell 16*) that was rhythmically active during all forms of both ipsi- and contralateral fictive scratching and was activated during ipsilateral fictive withdrawal. **A**: physiology during ipsilateral fictive caudal scratching (**1**) and contralateral fictive caudal scratching (**2**). cHF, contralateral hip flexor motor nerve. **B**: morphology: **1**, camera lucida reconstruction; arrows indicate axon collaterals with terminal arborizations; **2**, single-section photomontage of the soma and proximal dendrites, in the right D₁₀ intermediate zone; **3**, single-section photomontage of axon-terminal arborizations in the ipsilateral ventral horn. This cell had a mean vector length of 0.27 and an oscillation amplitude of 2.4 mV (during ipsilateral caudal scratching).

vary with the phase of the scratch rhythm (indicated by the bursts and quiescent intervals of the ipsilateral hip flexor motor nerve, iHF). For example, for the neuron shown in Figs. 5 and 10A, the conductance increases were smallest during the tran-

sitions from hyperpolarization to depolarization and back (Fig. 10A3, ↑).

Different interneurons exhibited peak firing rates and peak depolarizations in different phases of the scratch rhythm. Most

A Physiology



B Morphology

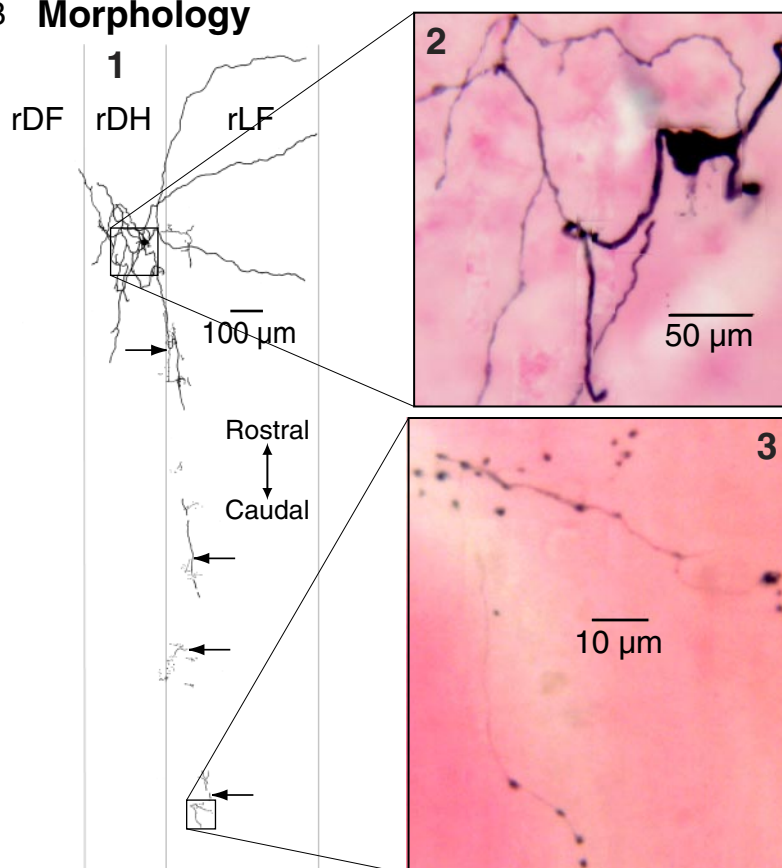


FIG. 5. Physiology and morphology of an interneuron (*cell 7*) that was rhythmically active during all forms of ipsi- and contralateral fictive scratching and was activated during both ipsi- and contralateral fictive withdrawal. *A*: physiology during ipsilateral fictive scratching (*1* and *2*), contralateral fictive scratching (*3*), and ipsilateral fictive withdrawal (*4*). Stim, force transducer trace during a tap to the dorsal foot. *B*: morphology: *1*, camera lucida reconstruction; arrows indicate axon collaterals with terminal arborizations; *2*, single-section photomontage of the soma and proximal dendrites, in the right D_6 deep dorsal horn and adjacent intermediate zone; *3*, single-section photomontage of axon-terminal arborizations in the ipsilateral ventral horn. This cell had a mean vector length of 0.85 and an oscillation amplitude of 3.6 mV (during ipsilateral pocket scratching).

interneurons, however, displayed similar phases of peak firing, relative to the iHF rhythm, during all forms of ipsilateral scratching for which they were active (Figs. 5*A*, *1* and *2*, 6*A*, *1*

and *2* and *4* and *5*). Thus mean vector angles (preferred phases) of cells were highly correlated in paired comparisons of the three forms of ipsilateral scratching (Fig. 8, *D–F*), as has been

found previously using extracellular recording of spinal interneurons with descending axons (Berkowitz and Stein 1994b) and extracellular recording of spinal interneurons with D₈–S₂ somata (Berkowitz 2001b). Nonetheless, a few neurons exhibited depolarization and spiking in substantially different phases of the iHF rhythm during different forms of scratching. For example, the neuron shown in Fig. 3 depolarized and fired action potentials during iHF quiescence in rostral scratching (Fig. 3A1), during late iHF quiescence and early iHF activity in pocket scratching (Fig. 3A2), and during late iHF activity in caudal scratching (Fig. 3A3).

The peak phases (and the trough phases) of the membrane potential also varied considerably among neurons. The trough phase occurred about half a cycle after the peak phase on average (Fig. 9C). However, the mean phase difference between the peak and the trough (a measure that can vary only from 0 to 0.5; see METHODS) was 0.38 ± 0.01 (SE); thus the peak and trough occurred in substantially different phases of the hip flexor cycle, but were not entirely out of phase in all cells. The preferred phase of firing was similar to the phase of peak depolarization in most cells (Fig. 9D); the difference between these phases was on average 0.12 ± 0.02 (SE).

In addition to being active during multiple forms of ipsilateral fictive scratching, 43 (91%) of these interneurons were rhythmically active during both ipsilateral and contralateral fictive scratching (Figs. 4A, 5A, 6A, and 7A and Table 1). During unilateral fictive scratching, bursts of the iHF and the contralateral hip flexor nerve (cHF) typically alternate with each other, with much larger iHF bursts during ipsilateral fictive scratching and much larger cHF bursts during contralateral fictive scratching (e.g., Fig. 5A, 1–3, and 6A, 4 and 5) (see also Berkowitz and Stein 1994b; Currie and Stein 1989; Stein et al. 1995). Some interneurons were depolarized and fired action potentials in the same phase of the iHF rhythm during ipsilateral and contralateral scratching (Figs. 5A and 6A), while other interneurons depolarized and fired in different phases during ipsilateral and contralateral scratching (Fig. 4A).

Thirty-two (68%) of these same interneurons also fired during ipsilateral fictive hindlimb withdrawal (flexion reflex; Figs. 3A4, 5A4, and 7A2 and Table 1). This was true even for several neurons that were active in fictive scratching mainly when the iHF was quiescent (e.g., Fig. 5A). An additional six cells (13%) displayed subthreshold depolarization and seven others (15%) displayed hyperpolarization alone during ipsilateral fictive withdrawal (Fig. 6A3 and Table 1). In contrast, only 14 cells (30%) fired or were depolarized during contralateral fictive withdrawal and four others (9%) displayed hyperpolarization alone during contralateral fictive withdrawal (Table 1).

Intracellular staining

The soma and processes of the scratch-activated interneurons included in this study were stained black, typically with the dendritic processes contiguously stained (Figs. 3B, 4B, 5B, 6B, and 7B). The somata of scratch-activated interneurons were found in the dorsal horn, usually deeply (Figs. 2 and 3), in the intermediate zone, usually in its lateral half (Figs. 2, 4, 5, and 7), and in the ventral horn, usually in its dorsal half (Figs. 2 and 6). The dendrites often extended laterally completely across the ipsilateral lateral funiculus to the edge of the spinal cord (Figs. 3B1, 4B1, 5B1, and 6B1), as has been seen previously for

turtle spinal motoneurons (Berkowitz and Stein 1994c; McDonagh et al. 1998, 2002; Ruigrok et al. 1984; Skydsgaard and Hounsgaard 1994) and interneurons (Berkowitz and Stein 1994c; Fernandez et al. 1996; McDonagh et al. 1998, 2002); in several cases, stained dendrites even appeared to penetrate and extend beyond the pia (not shown). In contrast, axons often showed patchy staining, in which black or gray axonal segments alternated with unstained segments along the visible length of the axon, as has been observed previously with intracellular injection of biotinylated compounds (McDonagh et al. 2002). In the best-stained cases, stained funicular axons could be followed 1–2 mm from the soma. Axons were usually so thin ($\ll 1 \mu\text{m}$) along most of their visible length that the axon diameter could not be measured precisely.

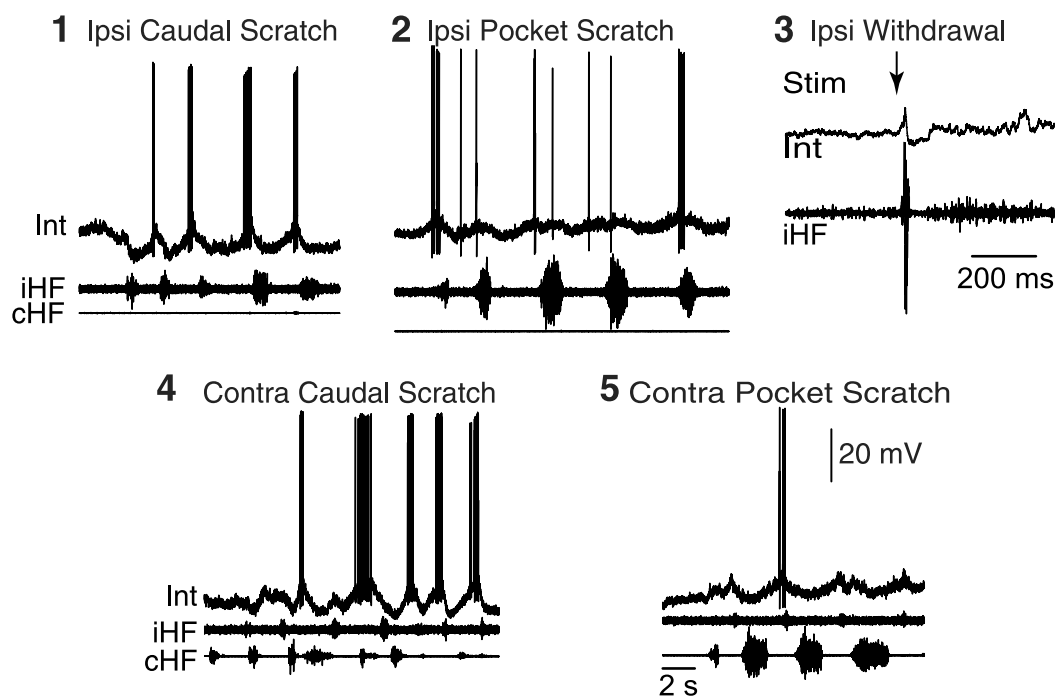
This report focuses on the axonal projections and terminations of these interneurons; correlations between soma-dendritic parameters and physiological parameters (Berkowitz et al. 2004, 2005) will be described separately. Some spinal neurons with endogenous biotin staining (Berkowitz 2002b) were also observed in these sections, but such neurons were always stained gray instead of black and never had more than the soma and the most proximal dendrites stained. In contrast, Neurobiotin-stained cells were always much more darkly stained with numerous distant and thin processes stained and occurred only at the expected locations; thus it was never difficult to distinguish Neurobiotin-stained neurons from endogenously stained cells.

Intracellularly stained axons were distinguishable from stained dendrites using one or more of the following criteria: axons ascended or descended rostrocaudally for a long distance, usually within a funiculus; axons either did not branch or gave off individual, thin collaterals at approximately right angles (Figs. 4B1, 5B1, 6B1, and 7B, 1 and 2), in contrast to dendrites, which branched repeatedly and at a variety of oblique angles (Figs. 3B1, 5B1, 6B1, and 7B1); and axon collaterals terminated, almost always within the gray matter, by branching into extremely thin processes, near the limits of visibility, with clusters of black, ovoid varicosities along their lengths and at their tips, like grapes on a vine (Figs. 3B3, 4B3, 5B3, 6B3, and 7B3). If a process could not be unambiguously identified as an axon by these criteria, the process was not assumed to be an axon. The somata of cells with axon terminals stained, cells with funicular axons stained but no detectable terminals, and cells with neither funicular axons nor terminals stained were interspersed in the same regions of the gray matter (Fig. 2); thus it seems likely that these groups of cells simply differed in how completely their axons were stained and do not represent distinct morphological or functional populations of interneurons.

Axon trajectories and terminals of scratch-activated interneurons

For 33 (70%) of the scratch-activated interneurons studied here, either a stained axon could be seen entering and traveling rostrocaudally within one or more funiculi, or axon-terminal arborizations could be identified within the gray matter, or both (Fig. 2 and Table 1). (Some axon-terminal arborizations were also seen in the white matter, usually adjacent to the gray matter, but such terminals were not studied systematically.) Interneurons with identifiable axon terminal arborizations

A Physiology



B Morphology

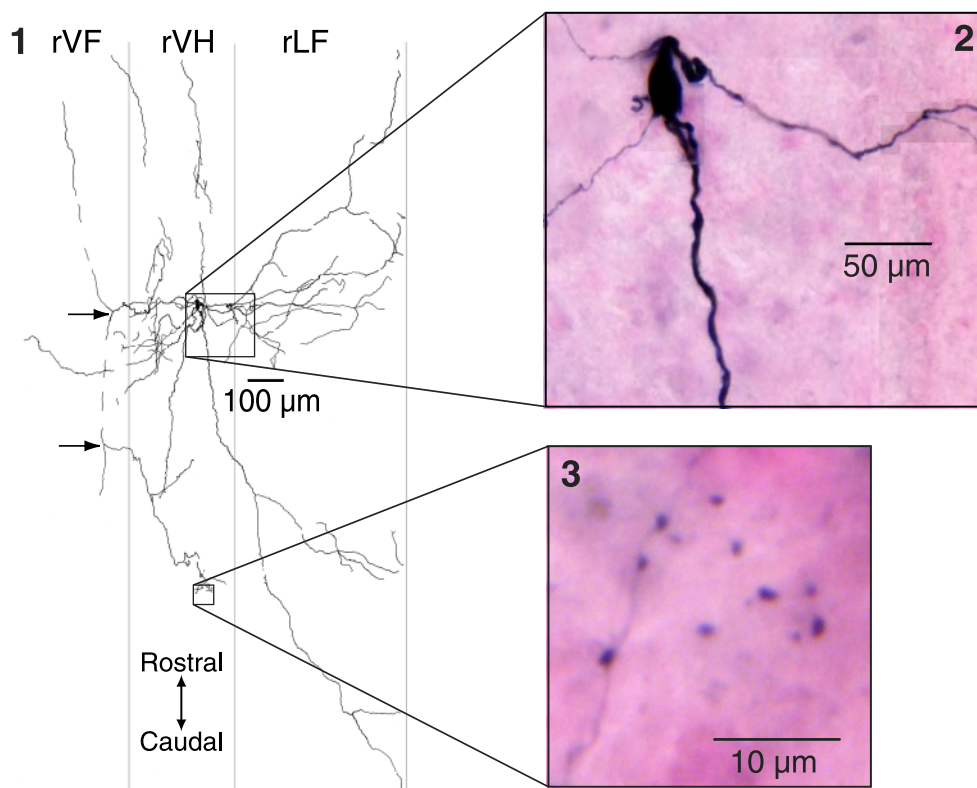


FIG. 6. Physiology and morphology of an interneuron (cell 11) that was active with strong rhythmic modulation during multiple forms of ipsi- and contralateral fictive scratching and displayed subthreshold depolarization during ipsi- and contralateral fictive withdrawal. *A*: physiology during ipsilateral fictive scratching (1 and 2), ipsilateral fictive withdrawal (3), and contralateral fictive scratching (4 and 5). *B*: morphology: 1, camera lucida reconstruction; upper and lower arrows indicate, respectively, the axon bifurcating into ascending and descending branches and an axon collateral with terminal arborizations; 2, single-section photomontage of the soma and proximal dendrites, in the dorsal part of the right D₁₀ ventral horn; 3, single-section photomontage of axon-terminal arborizations in the ipsilateral ventral horn. This cell had a mean vector length of 0.96 and an oscillation amplitude of 7.6 mV (during ipsilateral caudal scratching).

TABLE 1. Summary of physiological activities, axon trajectories, and axon-terminal arborizations for all scratch-activated interneurons with axonal morphologies recovered

Cell	Soma Location		Physiology								Axon Trajectory					Axon Terminals					
	Segment	Region	iR	iP	iC	cR	cP	cC	iW	cW	iLF	iVF	iGM	cVF	cGM	iDH	iIZ	iVH	cDH	cIZ	cVH
1	S2	DH	+	+	+	E	E	E	+	—	A			A		+	+				
2	D8	dVH	+	+	+	E	+	+	+	E	A							+			
3	D10	vDH	+	+	+	+	E	E	E	—	B						+	+			
4	D10	dVH	+	+	+	+	+	+	+	—	B							+			
5	D8	dVH	+	+	+	+	+	+	E	+	B										
6	D10	IZ	+	+	+	+	+	+	+	—	D							+			
7	D9	DH/IZ	+	+	+	+	+	+	+	+	D							+			
8	D10	dVH	+	+	+	+	+	+	+	—		A						+			
9	S1	dVH	+	+	+		+	+	+			B						+			
10	D8	dVH	+	+	+	+	+	+	I	I		B									
11	D10	dVH	+	+	+	E	+	+	E	E		B						+			
12	S1	IZ	+	+	+	+	+	+	I	—		B									
13	S1	IZ	I	+	+	+	+	+	I	I		D						+			
14	D9	IZ	+	+	I	E	E	E	E	—		D						+			
15	S1	dDH	+	+	+	+	+	+	E	—			A			+					
16	D10	IZ	+	+	+	+	+	+	+	—			B				+	+			
17	S1	IZ	+	+	+	+	+	+	+	—			B	A			+	+			
18	D8	VH	+	+	+	+	+	+	+	—			B	D				+			
19	D10	IZ	+	+	E	I	I	I	+	+				B				+			
20	D8	dVH	+	+	+	+	+	+	+	E				B							+
21	D8	VH	+	+	+	+	+	E	+	—				B							+
22	D8	IZ	+	+	+	+	+	+	+	—				B							+
23	D9	IZ	+	+	+	+	+	+	+	+				B							
24	D9	IZ	+	+	+		+		+	+				D							+
25	D8	IZ	+	+	+	+	+	+	—	—				D							+
26	S2	IZ	+	+	+	+	+	+	+	—				D							
27	S1	vDH	+	+	+	+	+	+	+	—				A							
28	D10	dVH	+	+	+	+	+	+	I	I				A							
29	D9	IZ	+	+	+	+	+	+	+	—				A							
30	S1	IZ	+	+	+	+	+	+	+	—					B						
31	D8	IZ	+	+	+	+	+	+	—	—						+	+	+			
32	D9	vDH	+	+	+	+	+	+	+	—						+	+				
33	D8	VH	+	+	+	+	+	+	+	—								+			

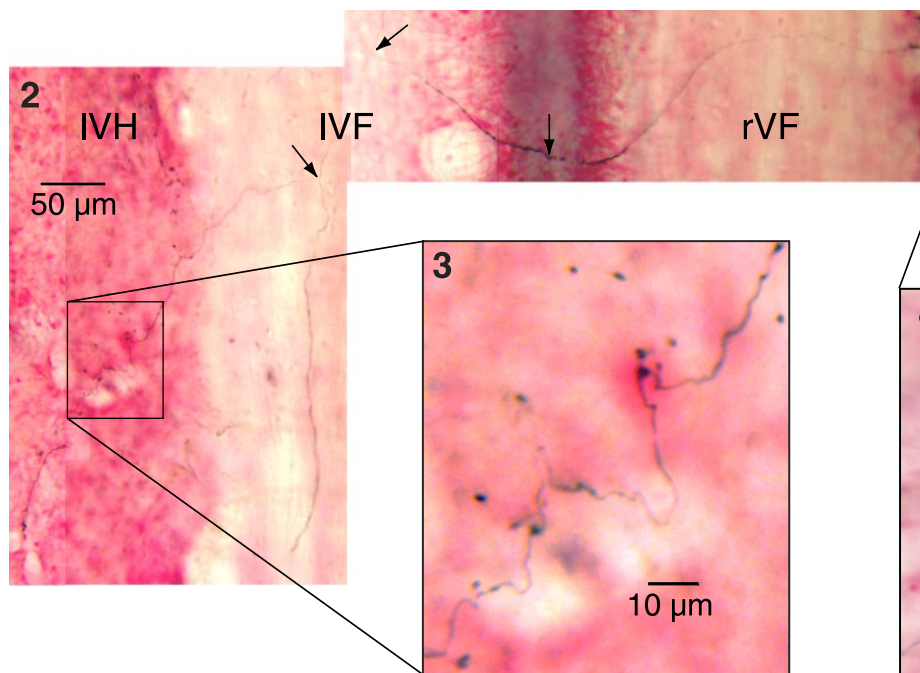
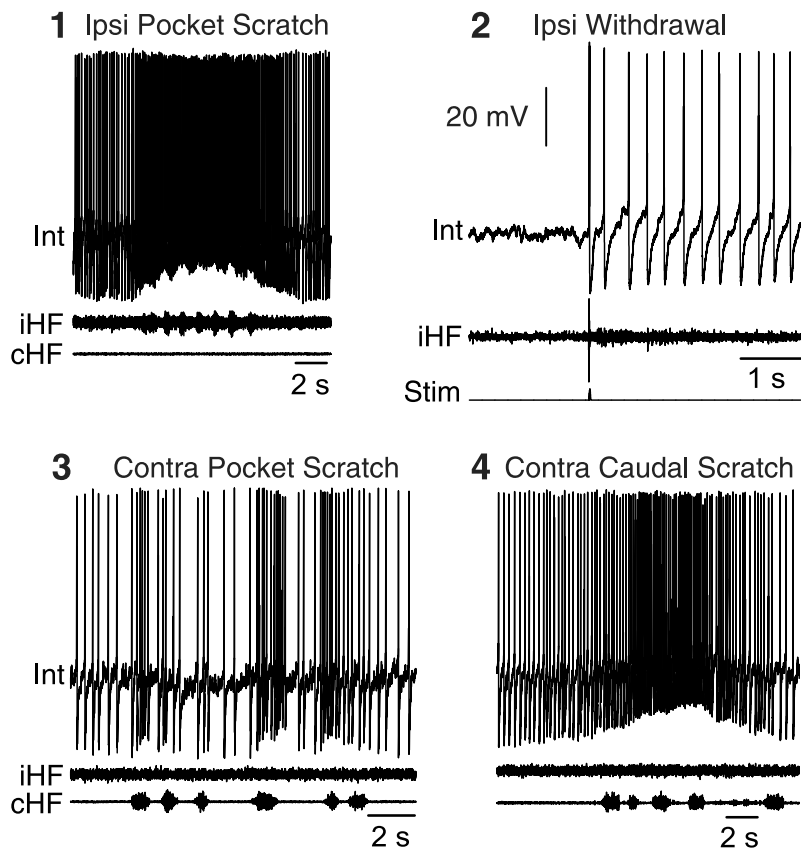
Rows are arranged to highlight repeated patterns of axon trajectories and terminations. This table only includes those cells for which an axon trajectory and/or axon-terminal arborizations were recovered. DH, dorsal horn; IZ, intermediate zone; VH, ventral horn; d, dorsal; v, ventral; i, ipsilateral; c, contralateral; R, rostral scratching; P, pocket scratching; C, caudal scratching; W, hindlimb withdrawal; +, increased firing rate or rhythmic modulation for scratching; and increased firing for withdrawal; E, subthreshold depolarization; I, hyperpolarization only; —, no response; DF, dorsal funiculus; LF, lateral funiculus; VF, ventral funiculus; GM, gray matter; A, ascending axon; D, descending axon; B, bifurcating (ascending and descending) axon. Under Axon Terminals: +, gray matter locations of stained axon terminals.

within the gray matter appeared to be representative of the recorded population of scratch-activated interneurons with respect to soma location (Fig. 2), peak firing rate during fictive scratching (Fig. 8A), degree of rhythmic modulation (mean vector length) during fictive scratching (Fig. 8B), firing rate phase preference within the hip flexor activity cycle during fictive scratching (Fig. 8C), and amplitude of phase-normalized membrane potential oscillations (Fig. 9B).

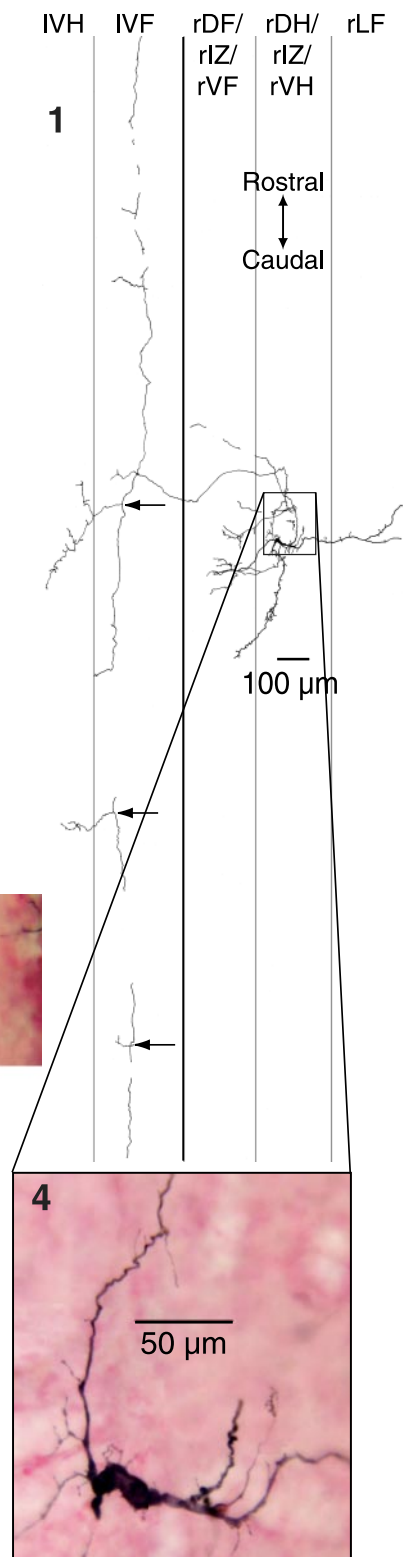
Figures 3–7 illustrate the range and diversity of morphological properties of typical scratch-related interneurons. Figure 3 shows an interneuron that was rhythmically activated during all three forms of ipsilateral scratching as well as ipsilateral withdrawal (Fig. 3A); it had axon branches ascending in both the ipsilateral lateral funiculus and the contralateral ventral funiculus (Fig. 3B1) as well as axon-terminal arborizations in the local ipsilateral dorsal horn and intermediate zone (Fig. 3B3). Figure 4 shows an interneuron that was rhythmically active during both ipsilateral and contralateral scratching (Fig. 4A) as well as ipsilateral withdrawal (not shown); its axon ascended and descended within the ipsilateral gray matter (and

perhaps the lateral funiculus, further caudally; Fig. 4B1), giving off collaterals with abundant terminal arborizations in the ipsilateral intermediate zone and ventral horn (Fig. 4B, 1 and 3). Figure 5 shows an interneuron that was rhythmically active during multiple forms of ipsilateral scratching (Fig. 5A, 1 and 2) and contralateral scratching (Fig. 5A3), as well as being excited to spike during hindlimb withdrawal, both ipsilaterally (Fig. 5A4) and contralaterally (not shown); its axon descended in the ipsilateral lateral funiculus and gave off short collaterals with terminal arborizations in the ipsilateral ventral horn and the immediately adjacent lateral funiculus (Fig. 5B, 1 and 3). Figure 6 shows an interneuron that was activated in brief, high-frequency bursts during multiple forms of ipsilateral (Fig. 6A, 1 and 2) and contralateral (Fig. 6A, 4 and 5) scratching as well as being depolarized during withdrawal, both ipsilaterally (Fig. 6A3) and contralaterally (not shown); its axon bifurcated into ascending and descending branches within the ipsilateral ventral funiculus (Fig. 6B1) and had local axon-terminal arborizations in the ipsilateral ventral horn (Fig. 6B3). Figure 7 shows an interneuron that was strongly activated but with weak

A Physiology



B Morphology



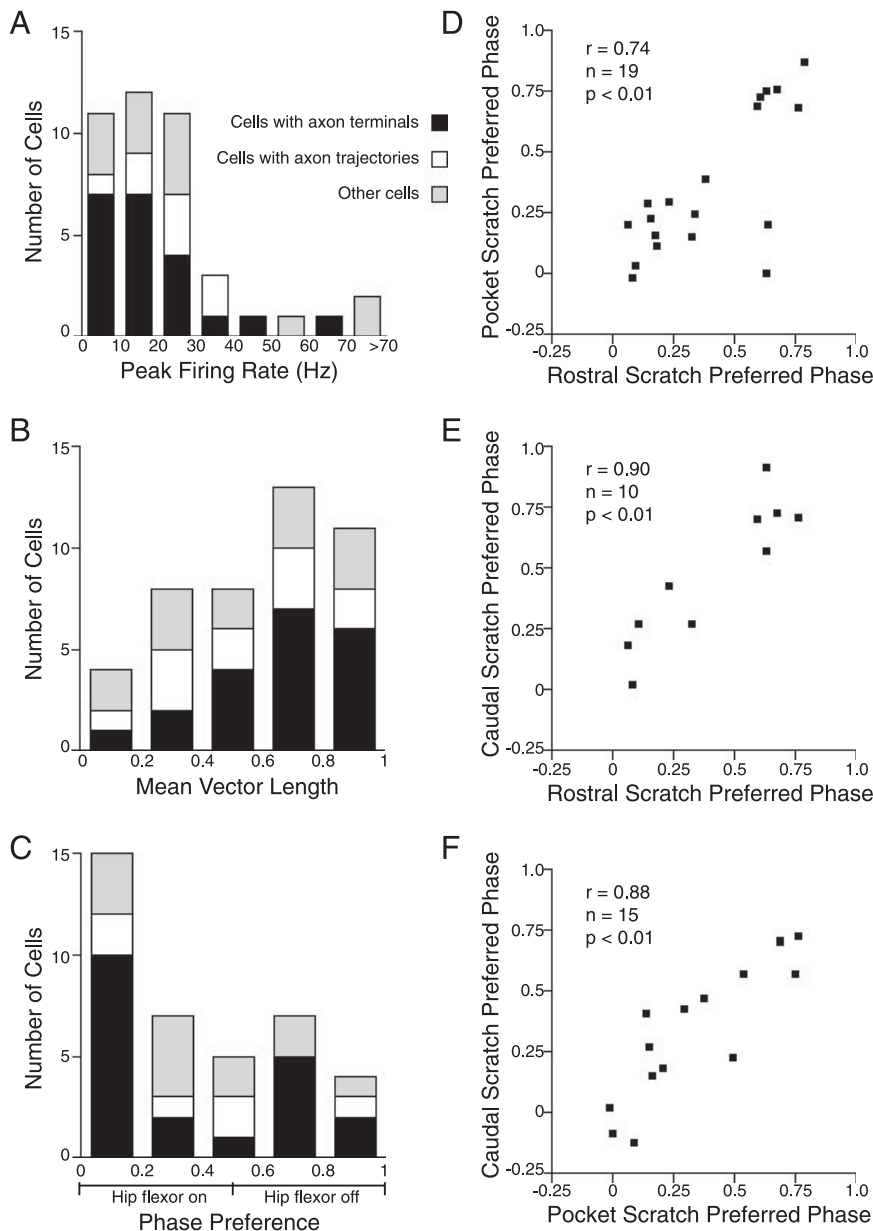


FIG. 8. Quantitative summary of interneurons' firing rate modulation during fictive scratching. *A*: histogram of cells' peak firing rates during fictive scratching. The bar on the far right indicates all values >70 Hz. *B*: histogram of cells' mean vector lengths (a measure of the degree of rhythmic modulation of activity) with respect to the hip flexor cycle during fictive scratching (see METHODS). *C*: histogram of cells' phase preferences (mean vector angles) with respect to the hip flexor cycle during fictive scratching (see METHODS). Note that the cells with identifiable axon terminal arborizations were representative of the whole population of scratch-activated interneurons recorded with respect to these three physiological parameters. *D–F*: 2-way comparisons of cells' preferred phases for the 3 forms of ipsilateral fictive scratching. Note that cells tended to have the same phase preference within the hip flexor cycle for all forms of scratching.

rhythmic modulation, during ipsilateral scratching (Fig. 7*A1*) as well as being activated during contralateral scratching (Fig. 7*A*, 3 and 4) and ipsilateral withdrawal (Fig. 7*A2*); its axon crossed into the contralateral ventral funiculus, where it bifurcated into ascending and descending branches and gave off axon collaterals with terminal arborizations in the contralateral ventral horn (Fig. 7*B*, 1–3).

Despite the great morphological diversity of scratch-activated interneurons, there were several repeated patterns of axonal trajectories (Table 1). Of the 30 interneurons the axon trajectories of which could be seen, 7 (23%) had an axon in the

ipsilateral lateral funiculus, 7 (23%) had an axon in the ipsilateral ventral funiculus, and 14 (47%) had an axon that crossed and traveled in the contralateral ventral funiculus; in addition, 4 (13%) only had an axon that remained within the ipsilateral gray matter and 1 only had an axon within the contralateral gray matter. None of the scratch-activated interneurons stained had an axon in the dorsal funiculus, either ipsilaterally or contralaterally, and no axons traveled in the contralateral lateral funiculus. Sixteen (53%) of the 30 axons with identifiable trajectories bifurcated into ascending and descending branches; this included 3 of the 7 ipsilateral lateral

FIG. 7. Physiology and morphology of an interneuron (*cell 22*) that was activated with weak rhythmic modulation during all forms of ipsi- and contralateral fictive scratching and was activated during ipsilateral fictive withdrawal. *A*: physiology during ipsilateral fictive scratching (1), ipsilateral fictive withdrawal (2), and contralateral fictive scratching (3 and 4). *B*: morphology: 1, camera lucida reconstruction; arrows indicate axon collaterals with terminal arborizations contralaterally; 2, photomontage of axon trajectory; arrows (from right to left) indicate the axon crossing the midline, the axon bifurcating, and an axon collateral that gives rise to terminal arborizations, respectively; 3, photomontage of a portion of the axon collateral in 2, showing axon-terminal arborizations in the contralateral ventral horn at higher magnification; 4, single-section photomontage of the soma and proximal dendrites, in the right D_8 intermediate zone. This cell had a mean vector length of 0.08 and an oscillation amplitude of 2.2 mV (during ipsilateral caudal scratching).

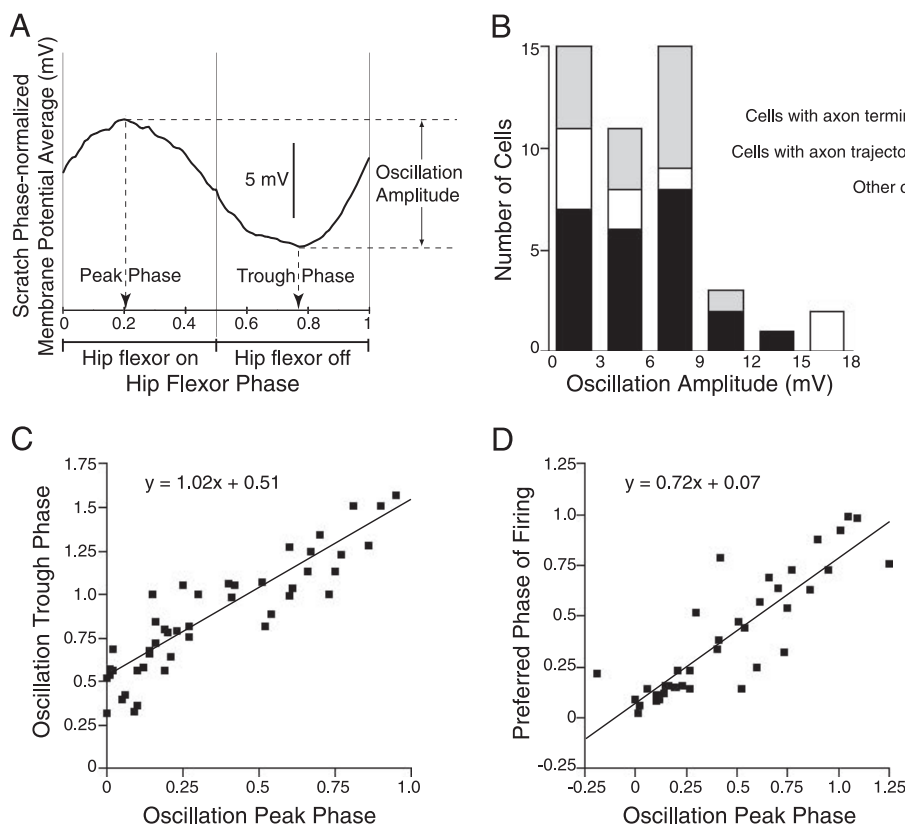


FIG. 9. Quantitative summary of interneurons' membrane potential oscillations during fictive scratching. *A*: example of 1 cell's dual-referenced scratch phase-normalized membrane potential average and the measurement of peak phase, trough phase, and oscillation amplitude (see METHODS). *B*: histogram of cells' average membrane potential oscillation amplitudes. Note that the cells with identifiable axon terminal arborizations were representative of the whole population of scratch-activated interneurons. *C*: relationship between cells' peak phases and the following trough phases for all cells studied. Note that the trough usually followed the peak by about half a cycle. *D*: relationship between the oscillation peak phase and the preferred phase of firing for all cells studied. Equations in *C* and *D* are for best-fit linear regression lines.

funiculus axons, 4 of the 7 ipsilateral ventral funiculus axons, 3 of the 4 ipsilateral gray matter axons, 5 of the 14 contralateral ventral funiculus axons, and the 1 contralateral gray matter axon.

Of the 24 scratch-activated interneurons with identifiable axon-terminal arborizations, 21 (88%) terminated in the ventral horn, including 16 (84%) of the 19 cells with ipsilateral terminals and all 5 (100%) of the cells with contralateral terminals (Table 1). Axon-terminal arborizations in the ipsilateral ventral horn were seen for 12 (71%) of the 17 neurons with identifiable ipsilateral axon trajectories and arborizations in the contralateral ventral horn were seen for 5 (33%) of the 15 neurons with identifiable contralateral axon trajectories.

All 16 (100%) of the scratch-activated interneurons with ipsilateral ventral horn axon terminals were activated during multiple forms of ipsilateral fictive scratching (Table 1). Fourteen (88%) of these 16 cells were also active during contralateral fictive scratching. During ipsilateral fictive withdrawal, 11 (69%) of these 16 cells fired, an additional 3 cells displayed subthreshold depolarization, and 1 displayed hyperpolarization alone. In contrast, during contralateral withdrawal, only two of these cells fired, two additional cells displayed subthreshold depolarization, and one displayed hyperpolarization alone.

All five scratch-activated interneurons with contralateral ventral horn axon terminals were activated during multiple forms of ipsilateral fictive scratching and multiple forms of contralateral fictive scratching. Four of these five cells also fired during ipsilateral withdrawal. During contralateral withdrawal, one of these cells fired and an additional one displayed subthreshold depolarization.

DISCUSSION

Interneurons contribute to multiple forms of scratching

The primary finding of this study was that many spinal interneurons that are rhythmically active during multiple forms of fictive scratching have axon-terminal arborizations in the ventral horn of the spinal cord hindlimb enlargement and thus are not simply relaying corollary discharge signals to the brain. This finding strongly suggests that many scratch-activated spinal interneurons have direct effects on hindlimb motoneurons and/or hindlimb motor pattern-generating circuitry for multiple forms of scratching. This finding provides some of the strongest evidence to date that individual interneurons contribute to more than one type of rhythmic limb movement in vertebrates. Some of these interneurons may themselves contribute to pattern generation for multiple behaviors, while others may relay the outputs of pattern-generating circuitry to motoneurons.

This evidence strengthens the conclusions of a large set of studies that relied on more circumstantial evidence, such as extracellular single-unit recordings correlated with fictive motor patterns, to suggest that some interneurons are shared components of the interneuronal networks underlying distinct types of limb movements, in turtles (Berkowitz 2001b, 2002a; Berkowitz and Stein 1994b; Currie and Stein 1989; Earhart and Stein 2000a,b; Juranek and Currie 2000; Mortin and Stein 1989), chicks (Bekoff et al. 1987), and cats (Arshavsky et al. 1972, 1978, 1984; Berkinblit et al. 1978; Carter and Smith 1986; Deliagina and Feldman 1981; Deliagina and Orlovsky 1980; Feldman and Orlovsky 1975; Perreault et al. 1999; Pratt and Jordan 1987; Smith et al. 1986). Similar types of studies have also suggested that vertebrate interneuronal networks controlling multiple forms of breathing, vocal-

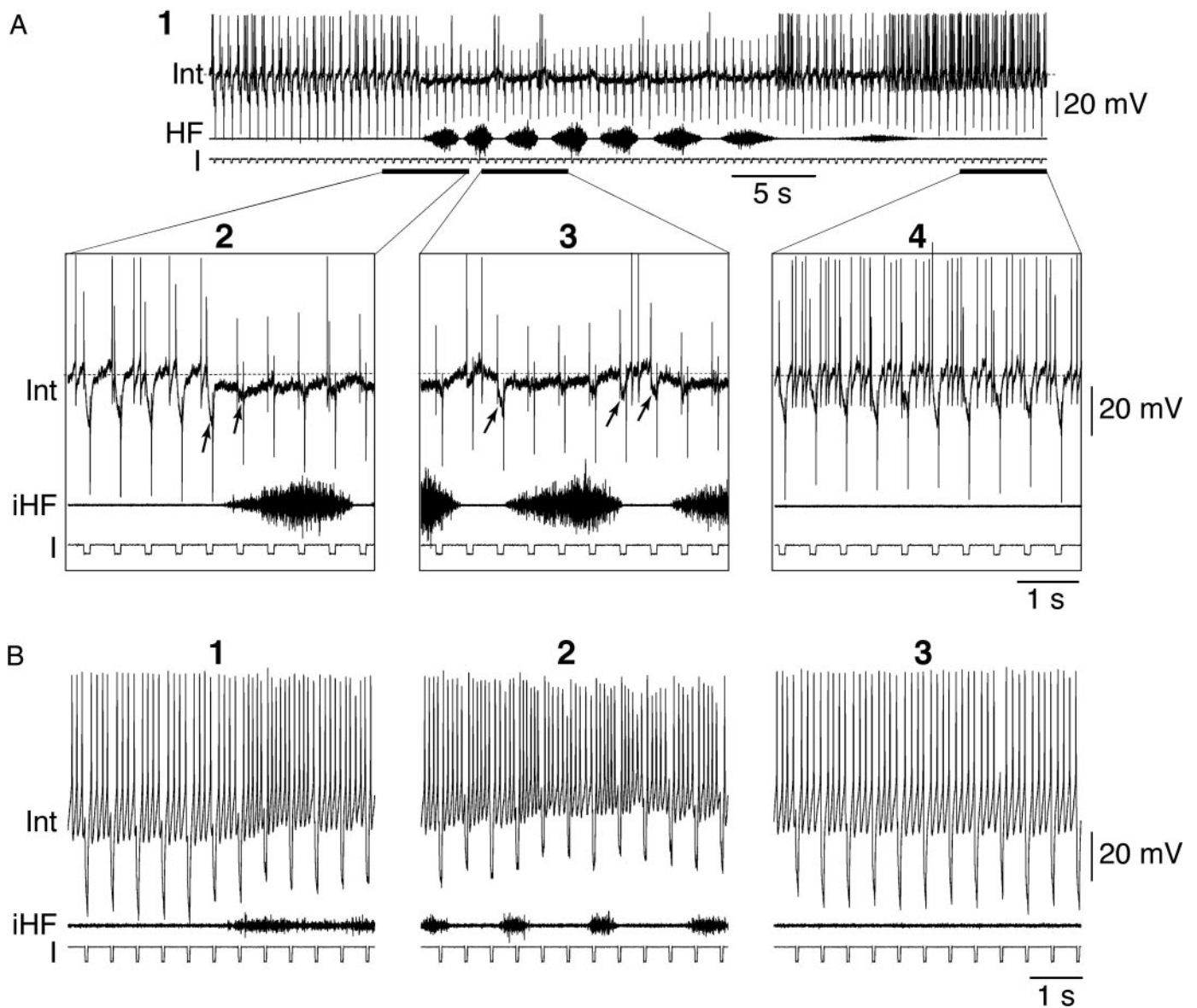


FIG. 10. Interneuronal conductance increases occur during all phases of fictive scratching. *A*: 0.06-nA, 100-ms hyperpolarizing current pulses were delivered to cell 6 (the same neuron as illustrated in Fig. 5) at 2 Hz. 2–4: expansions of the traces in *I* at scratch onset (2), during scratching (3), and after scratching (4). ---, prescratching resting membrane potential. Note the large conductance increases associated with all phases of scratching. ↑, immediate conductance increase at the onset of scratching (in 2) and the phases of scratching that show the smallest conductance increases (in 3). *B*: 0.35-nA, 100-ms hyperpolarizing current pulses were delivered to cell 21 (the same neuron as illustrated in Fig. 7) at 2 Hz, at scratch onset (1), during scratching (2), and after scratching (3). *I*, intracellular current monitor.

ization, mastication, and swallowing in mammals (Grelot et al. 1993; Larson et al. 1994; Lieske et al. 2000; Oku et al. 1994; Westberg et al. 1998; Yajima and Larson 1993) and axial swimming and escape movements in fish (Svoboda and Fetcho 1996) involve some shared interneurons. Moreover, paired intracellular recordings of interneurons and motoneurons have directly demonstrated that individual spinal interneurons contribute to generating multiple forms of axial locomotion in embryonic tadpoles (Soffe 1993; Soffe et al. 1984). Taken together, these studies strongly suggest that there is some sharing among the vertebrate neuronal networks that generate diverse behaviors involving the same muscles, as has been demonstrated most clearly for invertebrates (Marder and Calabrese 1996; Pearson 1993).

Interneurons contribute to scratching bilaterally

A second finding of this study was that many spinal interneurons that are rhythmically active during both ipsi- and contralateral fictive scratching have axon-terminal arborizations in the ventral horn of the spinal cord hindlimb enlargement. This finding strongly suggests that many scratch-activated interneurons have direct effects on motor output for both right and left hindlimb scratching. Some have axon terminals in the ipsilateral ventral horn and others in the contralateral ventral horn. Previous work has shown that although scratching is essentially a unilateral behavior, important contributions to scratch motor pattern generation

nonetheless come from the contralateral spinal cord (Berkowitz and Stein 1994b; Stein et al. 1995, 1998). Interneurons with the activity and morphology seen in this study may mediate contralateral contributions to ipsilateral scratching.

Interneurons contribute to scratching and withdrawal

A third finding was that spinal interneurons that are activated during both fictive scratching and fictive withdrawal have ventral horn axon terminals and thus are likely to have direct effects on hindlimb motor output. It was much more common for such cells to be activated during ipsilateral withdrawal than contralateral withdrawal. Previous research showed that spinal interneurons active during both fictive scratching and fictive swimming ("scratch/swim" neurons) have a high probability of also being activated during ipsilateral withdrawal, while interneurons active during scratching but suppressed during swimming ("scratch-specialized" neurons) are less likely to be activated during withdrawal (Berkowitz 2002a). Taken together with the current results, it seems likely that at least some scratch/swim neurons have axon terminals in the spinal cord ventral horn, but further experiments will be required to test this hypothesis.

Interneuronal synaptic inputs

A fourth finding was that scratch-activated interneurons typically exhibited alternating de- and hyperpolarizing waves and substantial conductance increases throughout fictive scratching, suggesting that they receive alternating excitatory and inhibitory scratch-related synaptic inputs. The peaks and troughs of these membrane potential oscillations were nearly half a cycle apart in most cells. Similar de- and hyperpolarizing waves and conductance increases have been described in turtle motoneurons during in vivo fictive scratching (Robertson and Stein 1988) and in turtle spinal motoneurons and interneurons during in vitro turtle rostral scratch motor patterns (Alaburda et al. 2005). In the current study, cells with very rhythmic activity often had hyperpolarizing waves that were larger than the depolarizing waves. This result adds to the previous finding from single-unit recording that a cell's mean firing rate and its degree of rhythmic modulation were negatively correlated (Berkowitz and Stein 1994b). Together, these findings suggest that rhythmic inhibition may play a large role in shaping the rhythmic activity of turtle spinal interneurons.

Axon trajectories

Finally, there was great diversity in the axon trajectories of scratch-activated interneurons. About half could be seen to bifurcate into ascending and descending branches, and the total numbers of ascending and descending axons were approximately equal. A recent retrograde labeling study (Nissen et al. 2004) indicates that a minority of turtle D₉ segment interneurons have bifurcating axons, so scratch-activated interneurons may be more likely than other interneurons to have bifurcating axons. Because many of the interneurons in the current study had a descending axon, this set of interneurons should be largely overlapping with the descending propriospinal neurons whose single-unit activities and axon projections have been studied previously

(Berkowitz and Stein 1994a–c; Stein and Daniels-McQueen 2002, 2003); moreover, the soma locations of the scratch-activated interneurons studied here (Fig. 2) were quite similar to the soma locations of descending propriospinal neurons (Berkowitz and Stein 1994c). The axons of the scratch-related interneurons studied here traveled in either the lateral funiculus or the ventral funiculus ipsilaterally but exclusively in the ventral funiculus contralaterally. Within this group of scratch-activated interneurons, there were no obvious correlations between a neuron's physiological behavior and its axon trajectory. The axon trajectories of turtle scratch-related spinal interneurons are generally consistent with the axon trajectories of spinal interneurons involved in swimming in the lamprey (Buchanan 1996; Grillner et al. 1991), zebrafish (Fetcho 1991; Lewis and Eisen 2003), and embryonic tadpole (Li et al. 2001; Roberts and Clarke 1982), but there are apparently fewer morphological types in those cases, and the morphologies seen are more clearly correlated with particular physiological roles. These differences may be due to the greater complexity of the spinal cord in adult, limbed vertebrates. Correlations between the soma-dendritic morphologies of the turtle scratch-related spinal interneurons and their physiological behavior during fictive scratching (Berkowitz et al. 2004, 2005) will be examined in more detail in the near future.

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