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Multifunctional and specialized spinal interneurons for turtle limb movements

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The turtle spinal cord can help reveal how vertebrate central nervous system (CNS) circuits select and generate an appropriate limb movement in each circumstance. Both multifunctional and specialized spinal interneurons contribute to the motor patterns for the three forms of scratching, forward swimming, and flexion reflex. Multifunctional interneurons, activated during all of these motor patterns, can have axon terminal arborizations in the ventral horn, where they likely contribute to limb motor output. Specialized interneurons can be specialized for a behavior, as opposed to a phase or motor synergy. Interneurons specialized for scratching can be hyperpolarized throughout swimming. Interneurons specialized for flexion reflex can be hyperpolarized throughout scratching and swimming. Some structure–function correlations have been revealed: flexion reflex-selective interneurons had somata exclusively in the dorsal horn, in contrast to scratch-activated interneurons. Transverse interneurons, defined by quantitative morphological criteria, had higher peak firing rates, narrower action potentials, briefer afterhyperpolarizations, and larger membrane potential oscillations than scratch-activated interneurons with different dendritic morphologies. Future investigations will focus on how multifunctional and specialized spinal interneurons interact to generate each motor output.

Keywords: locomotion; scratching; swimming; flexion reflex; dedicated; central pattern generator

Introduction

How does the central nervous system (CNS) select and generate an appropriate behavior for each circumstance an animal faces? This question has received increasing experimental attention in diverse contexts. Foci have included how the primate forebrain mediates perceptual decision making,[1,2] how diverse animals make behavioral choices,[2,3] and how CNS networks generate multiple rhythmic motor patterns.[4–6]

Understanding mechanisms underlying behavioral choice or motor pattern selection ultimately requires cellular analysis. Thus, there are advantages to studying smaller and more accessible nervous systems or components to test circuit-level hypotheses.[5,7–9] One simple hypothesis is that a separate set of dedicated CNS neurons produces each type of movement. An opposing hypothesis is that a single set of multifunctional CNS

neurons produces all movements of a given body part.

There is experimental support for both dedicated and multifunctional circuits, especially from invertebrates. Pioneering work in crayfish and mollusks revealed inhibition between specialized neurons or circuits for competing behaviors.[3,10] Also, separate groups of specialized interneurons are rhythmically activated during walking and flying in locusts[11] and during stridulation and flying in crickets.[12] However, different forms of rhythmic crustacean digestive movements are generated largely by multifunctional neurons that are reconfigured by neuromodulators.[5,6,13–15] Distinct rhythmic behaviors of leeches are produced by a combination of multifunctional and specialized neurons.[8,16]

Studies of vertebrate brainstem and spinal cord have also addressed these issues. For example, multifunctional brainstem interneurons are rhythmically activated during different forms of mammalian

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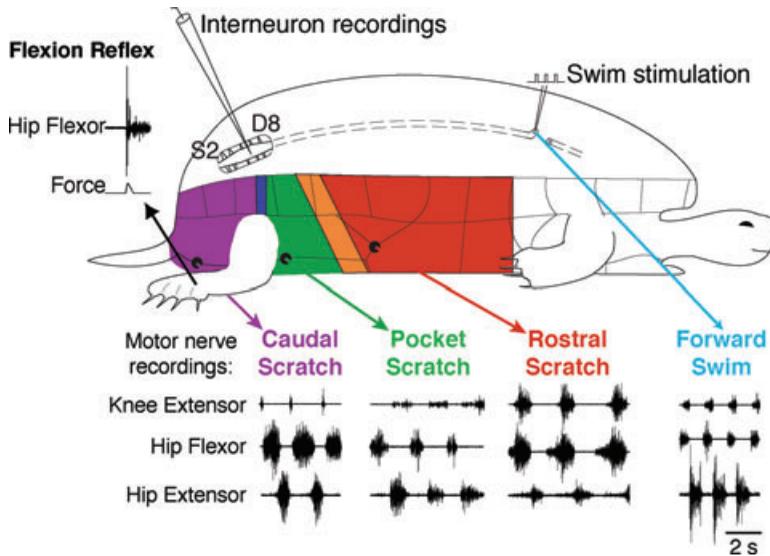
Figure 1. Diagram of the spinal turtle preparation, showing fictive motor patterns from one animal. Scratching is evoked by mechanical stimulation of the body surface; color-coded regions indicate the three scratch form receptive fields and transition zones between forms. Flexion reflex (limb withdrawal) is evoked by a tap to the dorsal foot. Forward swimming is evoked by electrical stimulation in the contralateral dorsal funiculus rostrally. Interneurons are recorded in the hindlimb enlargement segments, dorsal (D) 8–10 and sacral (S) 1–2.

respiration and oromotor movements.[17–24] Different forms of axial locomotion in larval fish and tadpoles are produced by a combination of multifunctional and specialized neurons.[25–30]

We have focused on spinal cord interneurons activated during limb motor patterns in the adult turtle spinal cord *in vivo*. We can thus investigate at a cellular level how an adult CNS mediates selection and generation of distinct naturalistic motor patterns for a multi-jointed limb. The adult turtle spinal cord, without input from the brain and movement-related sensory feedback, can appropriately generate the hindlimb motor patterns for forward swimming, three forms of scratching, and withdrawal or flexion reflex[31] (Fig. 1).

In vivo studies of limb movements are more feasible in adult turtles than in adult mammals, partly because turtles (being diving animals) resist hypoxia much better than mammals.[32] Also, groundwork has been laid for these studies by detailed descriptions of muscle and nerve activity patterns underlying several behaviors.[33–37] Findings from turtle spinal cord likely have counterparts in mammals because spinal cord mechanisms are largely conserved.[38–40] Moreover, spinal interneurons are involved not only in rhythmic limb movements and

reflexive movements, but also in voluntary movements.[41] In this review, I will highlight recent evidence that a combination of multifunctional and specialized turtle spinal interneurons contributes to scratching, swimming, and flexion reflex.

Results and discussion

Spinal interneurons and the three forms of scratching

Mechanical stimulation of a site on a spinalized turtle's body surface evokes scratching, in which the ipsilateral hindlimb rubs repeatedly and rhythmically against the site.[34] Three forms of scratching—rostral, pocket, and caudal—are used to reach three regions of the body surface (Fig. 1). Sites within narrow transition zones can elicit either of two scratch forms or a blend of the two. The three forms differ in the part of the limb that rubs against the body and the relative timing of knee and hip movements. Fictive scratching motor patterns in immobilized animals are very similar to muscle motor patterns in moving animals.[35]

In principle, each form of scratching could be produced by a separate group of dedicated spinal interneurons. Such neurons would be activated

whenever one form of scratching occurs and not at all during the other two. We searched for such interneurons using extracellular single-neuron and intracellular recordings.[42–44] Out of approximately 200 scratch-activated interneurons studied, only two had excitatory receptive fields similar to a scratch form receptive field (both for caudal scratching).[43] This was true using white matter recordings of descending axons[42] as well as gray matter somatic recordings,[43] so it is unlikely to be due to the recording method. Thus, spinal interneurons dedicated to one form of scratching appear to be rare.

Instead, most scratch-activated spinal interneurons were activated during all three forms. Their firing rates during scratching typically varied systematically according to the site stimulated, with a peak at one rostrocaudal region (Fig. 2).[42,43] In other words, each neuron was coarsely or broadly tuned to a region of the body surface. Different interneurons in the same animal were broadly tuned to different regions. This suggests the hypothesis that scratch form selection is mediated by a large population of multifunctional and broadly tuned neurons (rather than dedicated neurons), as has been shown in several other systems.[45,46]

Most scratch-activated interneurons were also rhythmically modulated with scratching[44,47–49] (Figs. 3A and B). Thus, some interneurons may contribute to both selection and generation of a scratch form. The degree of rhythmic modulation a neuron displayed for different sites was often negatively correlated with its mean firing rate.[47] This suggests that rhythmic inhibition plays an important role in sculpting scratch motor patterns. Firing tends to occur in a particular phase of the hip flexor activity cycle for each interneuron, regardless of which form of scratching is produced (e.g., Fig. 3A), although a small number do shift phase depending on the site stimulated.[44,47,48]

Scratch-activated interneurons were often multifunctional in additional ways. Many were activated during ipsilateral fictive flexion reflex[44] (Fig. 3A). Moreover, many were rhythmically activated during fictive scratching of either hindlimb[42–44,47,48] (Fig. 3B). This surprising finding suggested that contralateral interneurons contribute to ipsilateral scratching. This suggestion was strongly supported by studies in which the hindlimb enlargement spinal cord was ablated on one side.[50] This lesion elim-

inated the hip extensor phase of rostral scratching on the intact side, even though the hip flexor rhythm remained.

Of course, a temporal correlation between an interneuron's activity and motor output cannot demonstrate a causal role for the interneuron. An alternative hypothesis is that these interneurons send corollary discharge signals to the brain but have no direct effect on motor output. This is unlikely for many interneurons studied, however, for two reasons. First, such interneurons recorded via their spinal descending axons[47] almost certainly terminate within the spinal cord. Second, intracellular recordings and dye injections of such interneurons revealed axon terminal arborizations in the ventral horn of the hindlimb enlargement (e.g., Fig. 3C), suggesting that they affect hindlimb motor output relatively directly.[44]

Spinal interneurons during scratching and swimming

Given that scratch-activated interneurons are often multifunctional, do they also contribute to swimming? Hindlimb swimming movements[33] and fictive motor patterns[37] can be evoked in a spinalized turtle by electrical stimulation of descending axons in the contralateral lateral funiculus (Fig. 1). We recorded from spinal interneurons extracellularly and intracellularly during fictive scratching and fictive forward swimming.

Most scratch-activated interneurons were also activated during swimming; they often showed a similar degree of rhythmic modulation and a similar phase preference within the hip cycle (Fig. 4).[51,52] The phases of their membrane potential oscillation troughs were significantly correlated between scratching and swimming, although the phases of their oscillation peaks were not, which again suggests the importance of rhythmic inhibition in sculpting scratching and swimming motor patterns.[52] Another subset of cells was rhythmically activated during scratching but tonically activated during swimming.[51]

Surprisingly, though, a subset of rhythmic, scratch-activated interneurons was not activated during swimming.[51,52] Some had their activity suppressed during swimming.[51] These scratch-specialized interneurons could receive hyperpolarizing inhibition during swimming[52] (Fig. 5). This hyperpolarization could outlast by several seconds
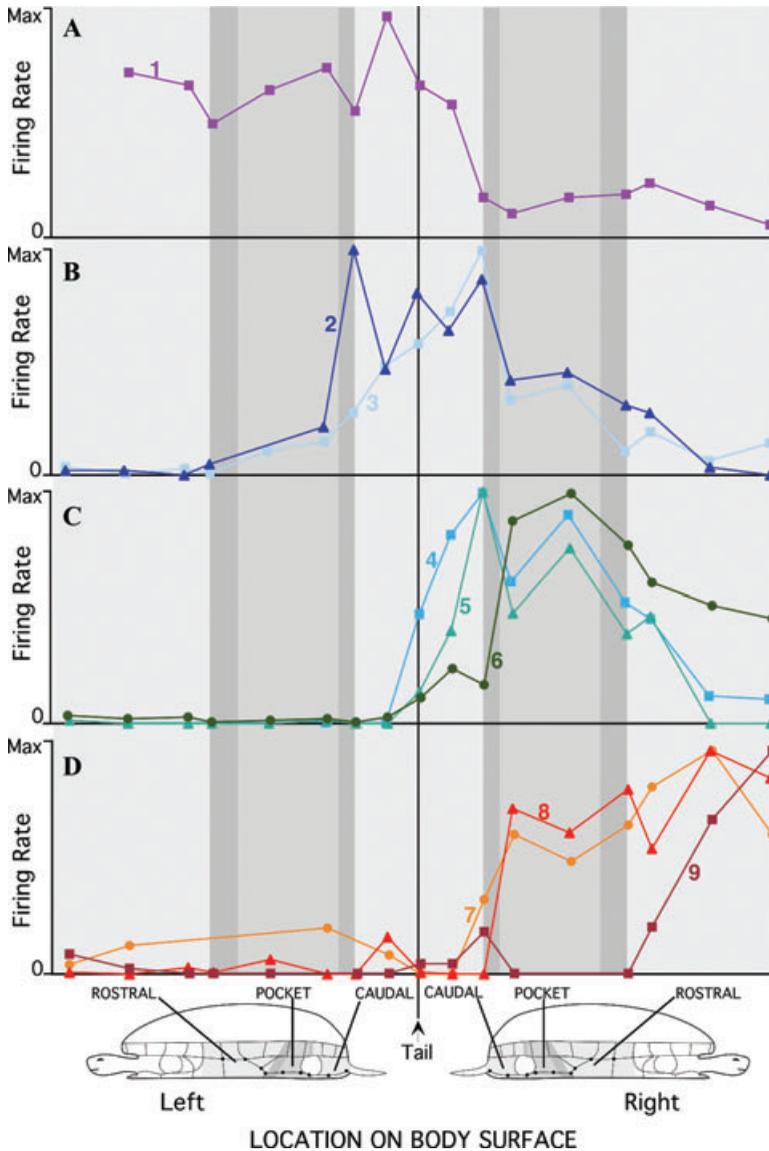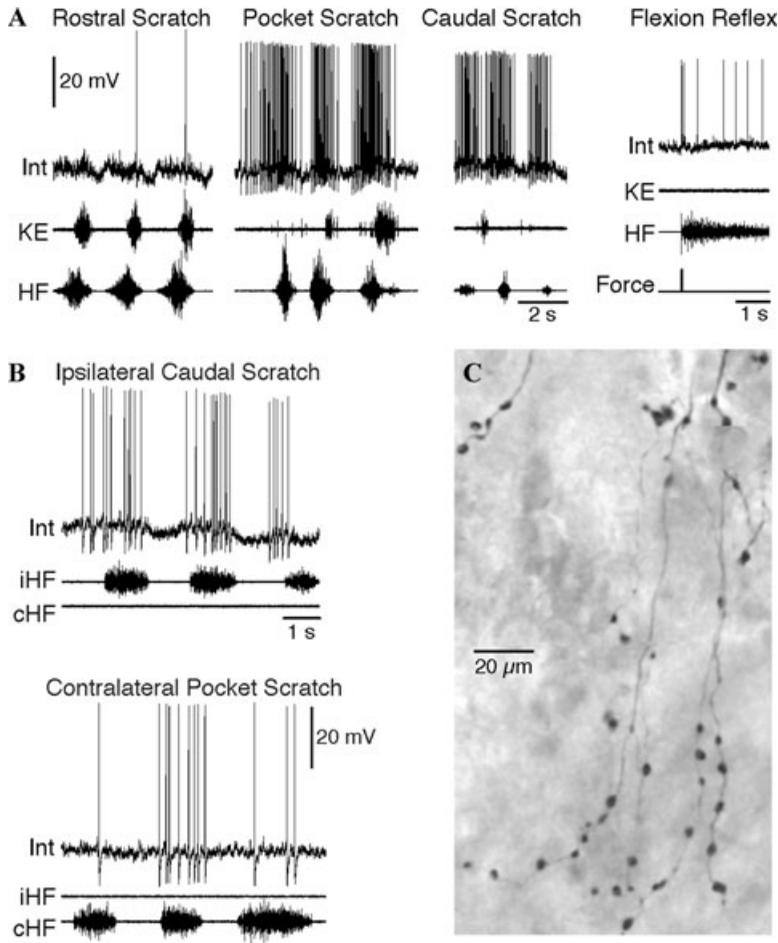
Figure 2. Scratch-activated spinal interneurons are often broadly tuned to a region of the body surface. Tuning curves are shown for nine interneurons recorded on the right side of one animal (A–D). (From *J. Neurophysiol.* **86:** 1017–1025, 2001; used with permission of the American Physiological Society.)

the electrical stimulation that evoked swimming, indicating that the inhibition was closely associated with the swimming motor pattern itself, not simply with the electrical stimulation used to evoke it.

One might hypothesize that specialized, rhythmic interneurons would be specialized for a particular motor synergy. This appears not to be the case, however, at least for scratch-activated interneurons.

Forward swimming and rostral scratching share a knee-hip synergy in which the monoarticular knee extensor bursts during the latter portion of each hip flexor burst;[37,53–55] the knee-hip synergies for pocket scratching and caudal scratching differ.[53–55] Nonetheless, rhythmic, scratch-activated interneurons tend to be activated during all three forms of scratching (which have different knee-hip synergies), while scratch-specialized interneurons are

Figure 3. Intracellular recordings from multifunctional scratch-activated spinal interneurons. (A) An interneuron that was rhythmically activated during all forms of ipsilateral fictive scratching, plus fictive flexion reflex. (B) An interneuron that was rhythmically activated during ipsilateral and contralateral fictive scratching. (C) Ventral horn axon terminal arborizations for the interneuron shown in B. Int, interneuron; KE, knee extensor; HF, hip flexor; i, ipsilateral; c, contralateral. (B-C from *J. Neurophysiol.* 94: 4455–4470, 2005; used with permission of the American Physiological Society.)

activated during rostral scratching but not forward swimming (which share a knee-hip synergy).[51] Thus, scratch-specialized interneurons are specialized for a particular behavior, not for a particular synergy.

The existence of multifunctional, scratch/swim interneurons is consistent with the hypothesis that a single central pattern generator (CPG) produces scratching and locomotion, as first proposed in a pioneering study of the cat spinal cord.[56] The existence of scratch-specialized interneurons, however, suggests additional complexity. Scratching and

swimming may be produced by a combination of multifunctional and specialized interneurons, as has been shown for feeding, withdrawal, and swimming in *Pleurobranchea*,[57] for crawling and swimming in leeches,[8,16] for feeding behaviors in *Aplysia*,[58] and for different forms of axial locomotion in larval zebrafish and tadpoles.[25–28,30,59] In at least some of these cases, it may be that distinct, rhythmic behaviors evolved at different times, and that the later-evolving behavior utilized some, but not all, of the existing CPG components.[8,16,51] This could give rise to an asymmetric
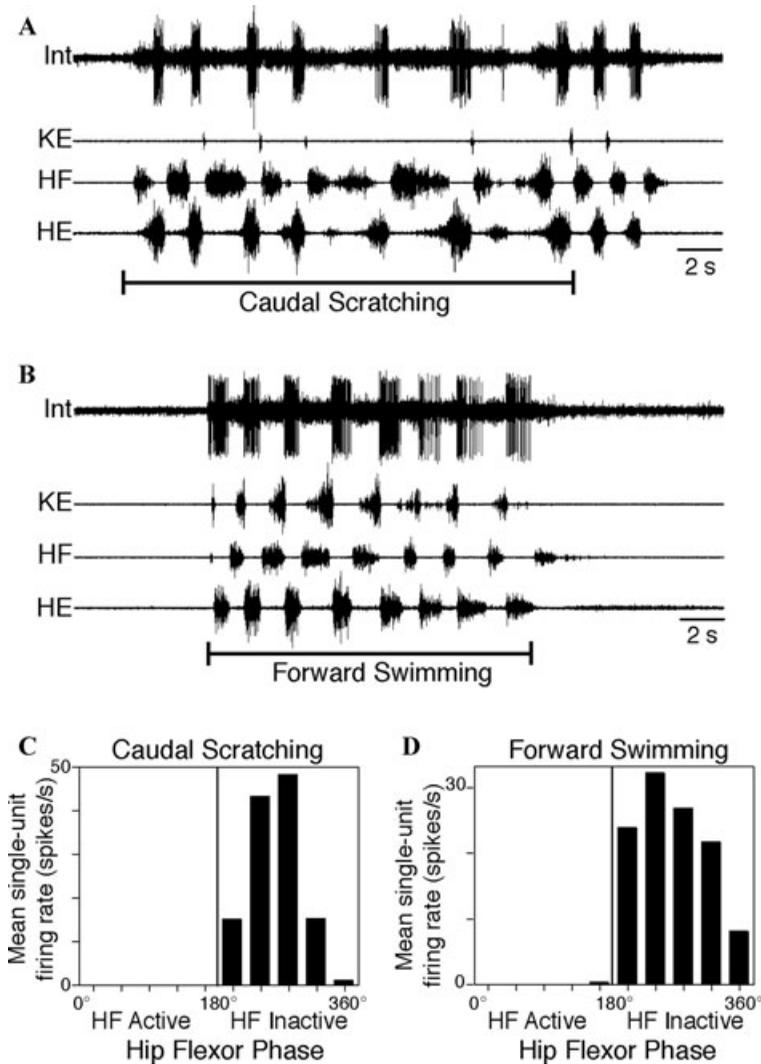
Figure 4. Example of a multifunctional scratch/swim spinal interneuron. Activity of the interneuron during (**A**) caudal scratching and (**B**) forward swimming. (**C, D**) Dual-referent phase histograms of the interneuron's firing rate during each motor pattern. Note that the neuron fired rhythmically in the same phase of the hip flexor activity cycle during scratching and swimming. (With kind permission from Springer Science + Business Media: *J. Comp. Physiol. A*, "Both shared and specialized spinal circuitry for scratching and swimming in turtles," Vol. 188, 2002, pp. 225–234, Ari Berkowitz, Figure 1.)

CPG organization, in which one behavior relies on a larger fraction of specialized neurons than the other behavior.

Correlations between morphology and physiology of interneurons

Are there structure–function correlations among scratch-activated interneurons? Using intracellular interneuron recording and Neurobiotin injection, we identified a morphological grouping of spinal interneurons that were strongly activated during limb motor patterns.[49] These transverse interneurons, or T neurons, had dendrites that were extensive in the transverse plane, but short rostrocaudally; they also tended to have mediolaterally elongated somata (Figs. 6 and 7A). T neurons were strongly activated during all forms of fictive scratching, forward swimming, and usually ipsilateral flexion
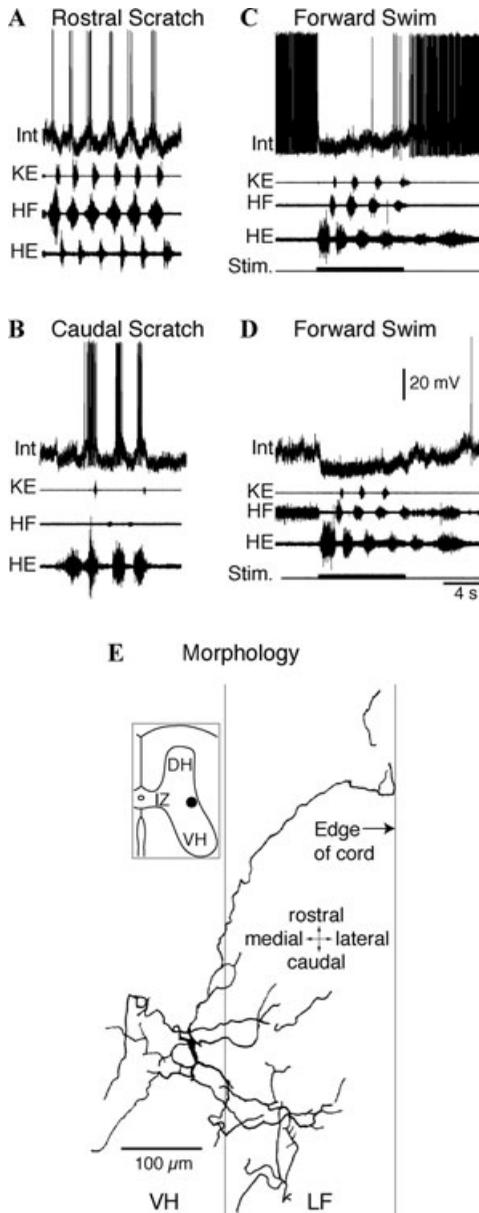
Figure 5. Example of a scratch-specialized spinal interneuron recorded intracellularly. Activity of the neuron during (**A**) rostral scratching, (**B**) caudal scratching, and (**C, D**) forward swimming. Note that the interneuron received hyperpolarizing inhibition during swimming. Int, interneuron; HE, hip extensor, Stim., stimulus. (**E**) Reconstruction of this neuron's morphology from horizontal sections; *inset* indicates soma location in cross section. DH, dorsal horn; IZ, intermediate zone; VH, ventral horn; LF, lateral funiculus. (From *J. Neurophysiol.* **99:** 2887–2901, 2008; used with permission of the American Physiological Society.)

reflex.[49,52] T neurons are thus multifunctional and are a subset of scratch/swim neurons.

T neurons differed physiologically in several respects from scratch-activated interneurons with different dendritic morphologies ("non-T neurons").[49] T neurons on average displayed significantly higher peak firing rates during scratching than non-T neurons (Fig. 7B). We explored possible mechanisms for these higher firing rates using measurements of averaged action potentials (APs) (Figs. 7C–E) and of scratch phase-averaged membrane potential oscillations (with action potentials deleted; Fig. 7F). T neurons on average had significantly narrower APs (Fig. 7D) with briefer afterhyperpolarizations (AHPs) (Fig. 7E) than non-T neurons. T neurons also had significantly larger scratch membrane potential oscillations (Fig. 7F).

The strong and rhythmic activity of T neurons during scratching makes them good candidates to be pattern-generating neurons and/or last-order premotor interneurons. In favorable cases, we could follow T neuron axons to axon terminal arborizations, which could be found in the ventral horn of the hindlimb enlargement (Fig. 6D), consistent with their having relatively direct effects on limb motoneurons.[49]

Flexion reflex-selective interneurons

In addition to scratch-specialized neurons, we found a group of spinal interneurons specialized for flexion reflex.[60] These interneurons were strongly activated during a mechanically evoked fictive flexion reflex (Fig. 8A). Electrical stimulation of the dorsal foot skin evoked interneuron firing within 20 ms (fast for a turtle!), earlier than the onset of the hip flexor burst (Fig. 8B). One might hypothesize that such interneurons would also be activated during the hip flexor bursts of scratching and swimming. However, these interneurons were not activated during scratching or swimming. In fact, most received hyperpolarizing inhibition during scratching (Fig. 8C) and swimming (Fig. 8D). The hyperpolarization typically included a rhythmic component. The maximal hyperpolarization (i.e., the trough of the membrane potential) could occur during the hip flexor bursts (Fig. 8E). Thus, flexion reflex-selective interneurons (like scratch-specialized interneurons) are apparently specialized for a particular behavior, not a particular muscle or hip phase.
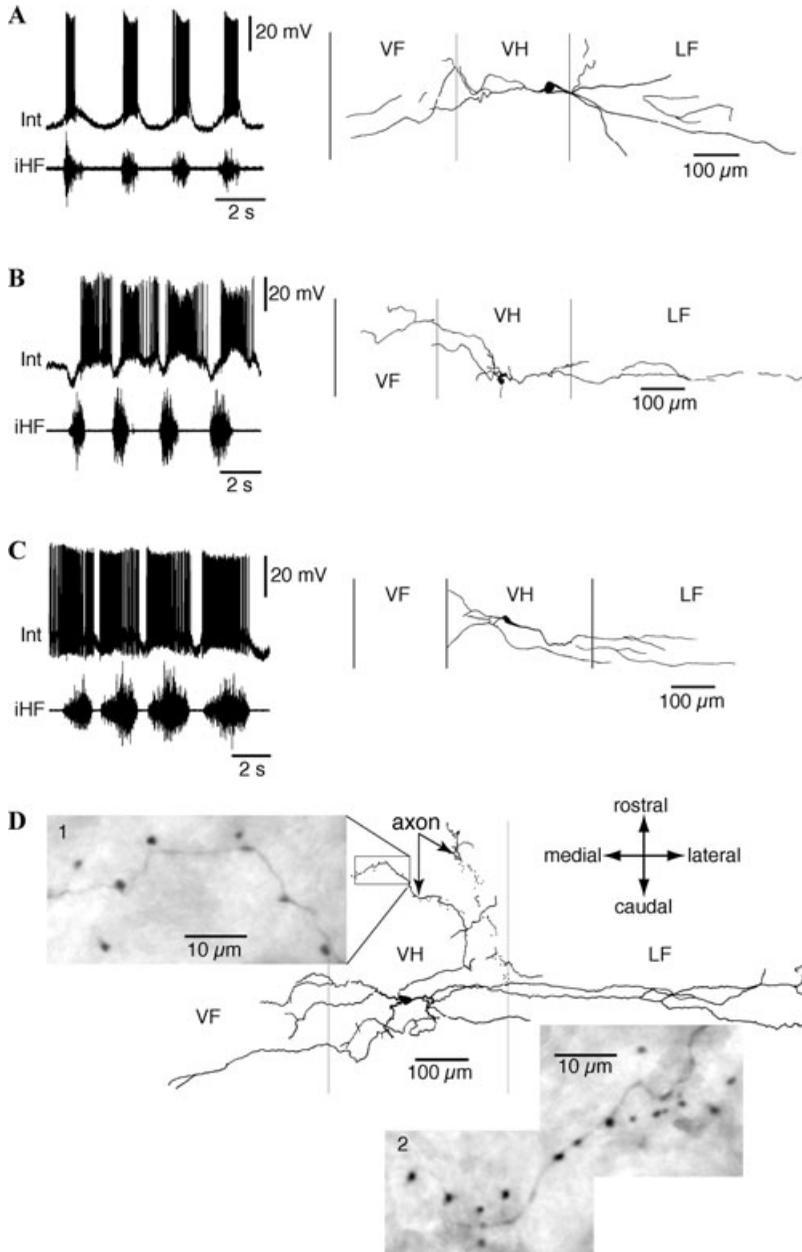
Figure 6. Examples of transverse interneurons (T neurons). (**A–C**) Three T neurons: *left*, activity during (**A, B**) caudal scratching and (**C**) rostral scratching; *right*, morphological reconstructions. (**D**) Morphological reconstruction of another T neuron (caudal axon truncated); *insets* show example ventral horn axon terminal arborizations (1) rostral and (2) caudal to the soma. VF, ventral funiculus. (B, C (right), and D from *J. Neurophysiol.* 95: 2821–2831, 2006; used with permission of the American Physiological Society.)

Flexion reflex-selective interneurons had somata in the dorsal horn, unlike most T neurons and other scratch-activated interneurons[60] (Fig. 8F). Thus, to some extent, there is anatomical segre-

gation of this functional group of interneurons. Flexion reflex-selective interneurons also displayed a variety of somato-dendritic morphologies, which could include complex higher-order branching and
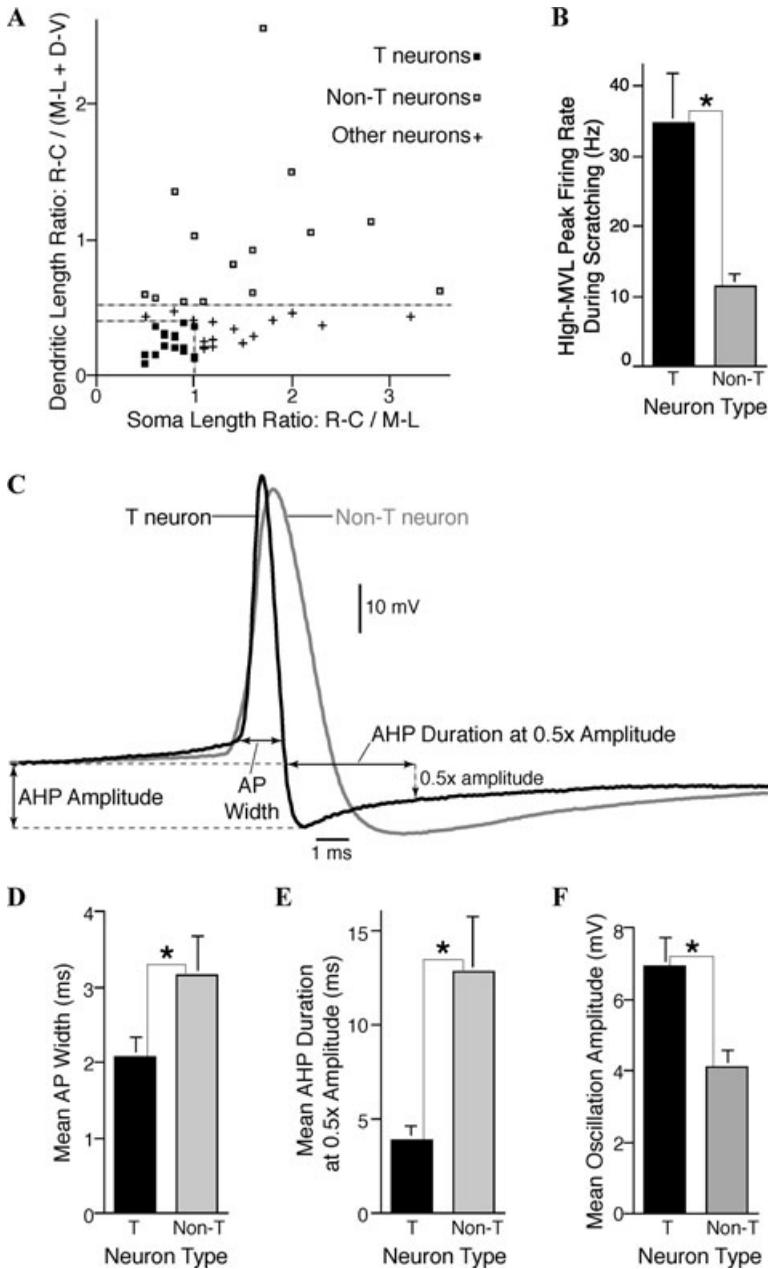
Figure 7. Physiological differences between T neurons and other scratch-activated interneurons. (**A**) T neurons were defined by quantitative somato-dendritic features. (**B**) T neurons fired at significantly higher peak rates during scratching than scratch-activated interneurons with different dendritic morphologies ("non-T neurons"). (**C**) Illustration of AP parameters measured from the averaged AP of each interneuron, with example T neuron and non-T neurons shown. (**D–F**) Significant differences between T neurons and non-T neurons in (**D**) mean AP width, (**E**) mean AHP duration, and (**F**) scratch phase-normalized membrane potential oscillation amplitude. (From *J. Neurophysiol.* **95**: 2821–2831, 2006; used with permission of the American Physiological Society.)
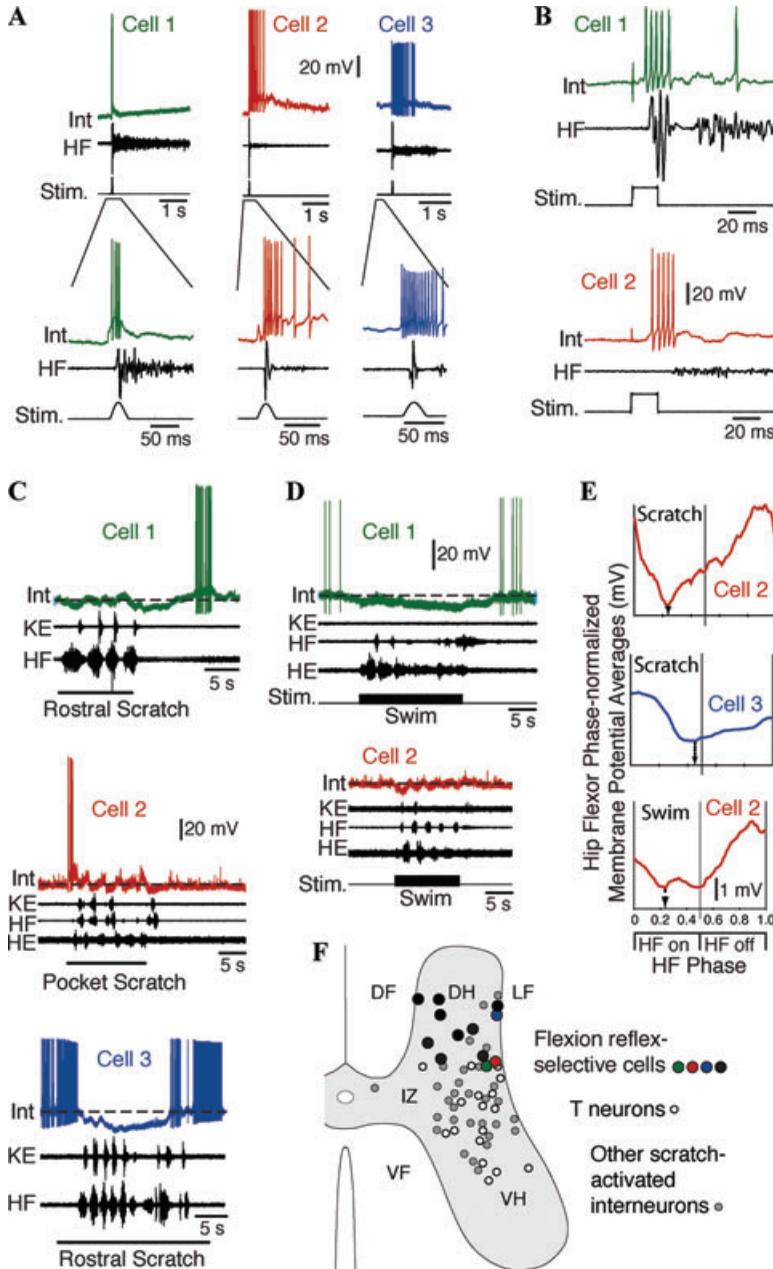
Figure 8. Examples of flexion reflex-selective interneurons. (**A**) Responses of three flexion reflex-selective interneurons to a dorsal foot tap; records below expand initial portions of records above. Note that each interneuron responded quickly and strongly, beginning before the hip flexor burst. (**B**) Responses of two of these cells to a dorsal foot skin electrical pulse. Note that the interneurons began firing within 20 ms, before the hip flexor burst. (**C, D**) Responses of the same interneurons during (**C**) scratching and (**D**) swimming. Note that these cells were hyperpolarized during scratching and swimming. (**E**) Phase-averaged membrane potentials for two of these cells. Note that maximal hyperpolarization could occur during the hip flexor burst of scratching and swimming even though these cells were activated during the hip flexor burst of flexion reflex. (**F**) Soma locations of all flexion reflex-selective interneurons recorded were in the dorsal horn, in contrast to T neurons. DF, dorsal funiculus. (From *J. Neurosci.* 27:4634–4641, 2007; used with permission of the Society for Neuroscience.)

rostrocaudally oriented dendrites, in contrast to T neurons.[60]

Conclusions

A combination of multifunctional and specialized spinal interneurons appears to mediate selection and generation of limbed locomotion, scratching, and withdrawal in turtles. Future research will aim to decipher how each type of interneuron contributes to motor pattern selection and generation. One possibility is that multifunctional neurons are components of a rhythm- and/or pattern-generator, while specialized neurons are sensory interneurons that differentially trigger or modify the operation of a shared CPG. This would be consistent with the relatively dorsal location of flexion reflex-specialized and scratch-specialized neurons studied to date. This would also be consistent with recent findings that distinct classes of spinal interneurons provide the excitatory drive to the CPG during swimming and struggling in hatchling tadpoles.[27] Alternatively, some specialized neurons may be unshared CPG components. To distinguish these possibilities, it will be important to obtain physiological and/or morphological evidence of the postsynaptic targets of multifunctional and specialized interneurons. In particular, which, if any, of these types of interneurons are last-order premotor neurons? T neurons are multifunctional neurons that are good candidates to be premotor neurons, based on their large membrane potential oscillations and high peak firing rates.[49,52] It will also be important to determine whether each type of multifunctional and specialized interneuron is excitatory or inhibitory. Pharmacological and/or immunocytochemical approaches should allow us to address this issue.

Until recently, the idea that the vertebrate spinal cord and medulla use largely shared CPGs to generate distinct rhythmic motor patterns in common sets of muscles has been emphasized.[17–23,37,47,48,54,56,61–73] For example, it was hypothesized that cat locomotion and scratching use the same CPG[56] and that swimming and struggling in tadpoles use the same interneuron types (though more cells are recruited from each type during struggling).[68–70] However, during the past decade, new studies of larval zebrafish spinal cord,[25,26,29,30] hatchling tadpole spinal cord,[27] adult turtle spinal cord,[51,52,60] and cat medulla[24] have all identified behaviorally specialized interneurons, potentially including CPG components, that may play important roles selectively in one or another of these rhythmic motor patterns. These new findings necessitate a more complex and nuanced view of how distinct rhythmic movements of shared muscles are generated in vertebrates, involving combinations of multifunctional and specialized neurons.

The earlier emphasis on shared CPGs in vertebrates may have been strongly influenced by data from several intensively studied invertebrate systems, especially the crustacean stomatogastric nervous system (STNS), in which (unlike vertebrate CPGs) it is possible to obtain a complete circuit diagram of the CPG for each motor pattern. In the STNS, a variety of neuromodulators can reconfigure a single, or largely shared CPG, to produce distinct outputs for each situation.[5,6,13–15] There are reasons to think, however, that additional mechanisms of motor pattern selection may operate in the spinal cord. First, fast (electrical and chemical) synaptic inputs from distinct projection neurons contribute to behavior-specific circuit reconfiguration (in addition to neuromodulation), even in the STNS.[8] Thus, there are behaviorally specialized neurons associated with the STNS, even if they are not CPG components. Second, there are entirely separate CPGs for the control of shared muscles for some invertebrate behaviors, such as locust flight and walking[11] and cricket flight and stridulation.[12] Third, there is now evidence that combinations of multifunctional and specialized neurons select and generate behaviors for feeding, withdrawal, and swimming in *Pleurobranchea*,[57] for crawling and swimming in leeches,[8,16] and for feeding behaviors in *Aplysia*,[58] in addition to the vertebrate examples mentioned above. Fourth, reconfiguration via neuromodulators may occur too slowly to be the exclusive mechanism of motor pattern selection for behaviors that must be initiated quickly, such as those commonly used to escape from predators. In such cases, recruitment of specialized interneurons, perhaps in addition to differential secretion of neuromodulators, may allow the necessary behavior to be initiated in time to escape predation.

Conflicts of interest

The author declares no conflicts of interest.

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