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THE CENTRAL PATTERN GENERATORS FOR SWIMMING AND SCRATCHING ARE PARTLY SHARED OR INTERACT AT THE INTERNEURONAL LEVEL IN ADULT TURTLES

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A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BIOLOGY

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Abstract

Distinct rhythmic behaviors involving a common set of motoneurons and muscles can be generated by separate central nervous system networks, a single network, or partly overlapping networks in invertebrates. Less is known for vertebrates. Does the spinal cord use a single network to generate distinct rhythms or two separate networks? The turtle spinal cord contains networks that generate swimming as well as three forms of scratching (rostral, pocket, and caudal) with three different knee-hip synergies.

First, I directly compared the adequacy of rostral segments in generating swimming and scratching. I separated caudal segments from immobilized, low-spinal turtles by sequential spinal cord transections. After the separation of the caudal four segments of the five-segment hindlimb enlargement, the remaining enlargement segment and five pre-enlargement segments still produced rhythms for forward swimming and both rostral and pocket scratching. The swimming rhythm frequency was usually maintained. Some animals continued to generate swimming and scratching rhythms even with no enlargement segments remaining, using only pre-enlargement segments. The pre-enlargement segments and rostral-most enlargement segment were also sufficient to maintain hip flexor (HF) motoneuron quiescence between HF bursts, which normally occurs during each hip extensor (HE) phase, during swimming. In contrast, the HF-quiescent phase was increasingly absent (i.e., HE-phase deletions) during rostral and pocket scratching. Moreover, respiratory motoneurons that normally burst during HE bursts continued to burst during the HF quiescence of swimming even with the caudal segments separated. Thus, the same segments are sufficient to generate the basic

rhythms for both locomotion and scratching. These segments are also sufficient to produce a reliable HE phase during locomotion but not during rostral or pocket scratching. I hypothesize that the rostral HE-phase interneurons that rhythmically inhibit HF motoneurons and interneurons are sufficient to generate HF quiescence during HE-biased swimming, but not during the more HF-biased rostral and pocket scratching.

I then simultaneously activated two networks to reveal overlap or interactions between them. The simultaneous stimulation for forward swimming and each form of scratching could 1) increase the rhythm frequency; 2) evoke switches, hybrids, and intermediate motor patterns; 3) reconstruct a swim motor pattern when the swim stimulation was subthreshold, and 4) disrupt rhythm generation entirely. The strength of swim stimulation could influence which effect was obtained. Thus, even pocket scratching and caudal scratching, which do not share a knee-hip synergy with forward swimming, can interact with swim stimulation to alter both rhythm and pattern generation.

Collectively, these findings suggest that the spinal cord networks that generate locomotion and scratching have important shared components or strong interactions between them. If the latter, interactions could occur in motoneurons themselves or earlier, in spinal interneurons. I thus recorded intracellularly from hindlimb motoneurons during dual stimulation. Motoneuron membrane potentials displayed a regular oscillation at a higher frequency than during swim or scratch alone. In contrast, arithmetic addition of the oscillations during swimming alone and scratching alone with

various delays always generated irregular oscillations. Also, the standard deviation of the dual-referent phase-normalized membrane potential was similar during dual stimulation and swimming or scratching alone. In contrast, the standard deviation was greater when pooling cycles of swimming alone and scratching alone. This shows that dual stimulation generates a single rhythm prior to motoneurons.

In conclusion, either swimming and scratching share a rhythm generator or the two rhythms are integrated into one by interneurons.

Key words: spinal cord, locomotion, rhythm generation, lesion, motor pattern interaction.

Abbreviations: AM-KE, nerve triceps femoralis, pars ambiens; cIN, commissural interneuron; CNS, central nervous system; CoBL, commissural bifurcating longitudinal interneuron; CPG, central pattern generator; C Sc, caudal scratch; dIN, desending interneuron; dINr, repetitive-firing dIN; FT-KE, nerve triceps femoralis, pars femorotibialis; HE, hip extensor; HF, hip flexor; IT-KE, nerve triceps femoralis, pars iliotibialis; KE, knee extensor; MCoD, multipolar commissural descending interneuron; MN, motoneuron; OA, oblique abdominus; P Sc, pocket scratch; R Sc, rostral scratch; SD, standard deviation; SE, standard error; stim., stimulation; TA, transverse abdominus.

Chapter 1: Introduction

Animals are able to perform a variety of adaptive behaviors when facing changing situations. When sitting alone on a summer night, a male cricket either needs to move around to avoid predators or to sing for a mate (Hennig, 1990). A larval zebrafish either needs to swim away when it touches an obstacle or to struggle vigorously when it is grabbed (Ritter et al., 2001). After inhaling, a mouse may exhale effortlessly or sneeze (Moore et al., 2014). A fruit fly may fly away from a high-CO_2 environment when the odor means danger or fly towards the environment when the odor means ripe fruit (Turner and Ray, 2009). When a shadow appears on the sky, a turkey may call alarmingly or ignore the shadow (Schleidt et al., 2011). A bat needs to change its calling frequency when chasing a mosquito, depending on the distance of its prey (Griffin, 1953; Griffin et al., 1960). A male fruit fly needs to court when encountering a potential mate and to attack when encountering an opponent (Fernandez and Kravitz, 2013). And to compete for resources and establish social status, a crayfish needs to display either a surrender or a fight gesture to an intruder (Bovbjerg, 1953).

The possible examples are endless. It is interesting and inspiring to understand the neuronal mechanisms that produce the right behavior at the right time.

Different external cues may trigger different responses. In the presence of a female's pheromone, a male *Drosophila* will likely attempt courting instead of aggression (Fernandez and Kravitz, 2013). When the environment contains fruit-related chemicals, a *Drosophila* will likely move towards a high-CO_2 environment instead of avoiding it

(Turner and Ray, 2009). When the water level is high, a leech will likely swim, but when the water level is low, it will likely crawl (Esch et al., 2002).

The inner state of an animal may also affect the behavior the animal performs. For example, a crayfish or a mollusk is more likely to feed rather than to escape when it is starving (Siegler et al., 1974; Bellman and Krasne, 1983; Gillette et al., 2000).

Central pattern generators

Besides differences in external and internal cues, it is also crucial to understand the neuronal circuits within the "black boxes" that produce these behaviors. Animals have the ability to generate many complex behaviors that are crucial for their survival. For example, a female graylag goose displays and finishes a fixed action pattern to roll any egg-like object back to its nest even if the object was removed halfway (Lorenz and Tinbergen, 1957). Many male insects and birds can display complicated courtship dances in the presence of females (Sturtevant, 1915; Fisher, 1930; Fusani, 2008; Fernandez and Kravitz, 2013). These highly stereotyped behaviors suggest that built-in networks generate the behaviors.

To understand the neural circuits that generate these fixed action patterns, many researchers have chosen to work with basic rhythmic behaviors, such as respiration, scratching and locomotion, given their highly stereotyped and simple patterns.

The pioneering studies on rhythmic behaviors were focused on locomotion. Debates took place about whether locomotion is generated by a reflex chain that requires feedback from muscle contractions (Sherrington, 1910; Gray and Lissmann, 1946; Grillner, 2011) or by a central program that generates the rhythm without any feedback (Weiss, 1936; Von Holst, 1954; Grillner, 2011). In 1911, Brown published a smoked-drum recording showing that a decerebrate cat could generate rhythmic alternations between two hindlimb antagonist muscles, even after all movement-related sensory feedback from the leg was eliminated by cutting the dorsal roots (Brown, 1911a).

Despite this clear evidence, however, the concept of reflex chain was dominant until the central generation of rhythmic behaviors was confirmed by Wilson's studies with locusts. A locust could generate the flight rhythm even after its wings were removed (Wilson, 1961, 1966). This research and many similar studies of deafferented animals thereafter (Jankowska et al., 1967; Grillner, 2011) demonstrated again the sufficiency of the central nervous system to generate basic rhythmic behaviors. Further, when muscle contractions were completely removed or abolished by neuromuscular-junction blockers, central nervous systems could still generate "fictive motor patterns" that were very similar to real behaviors (Grillner, 2011), such as the leech heartbeat rhythm (Calabrese and Peterson, 1983), crustacean feeding (Marder and Calabrese, 1996), cat and rodent respiration (Lieske et al., 2000; Moore et al., 2014), turtle and cat locomotion (Lennard and Stein, 1977; Grillner and Zangger, 1979; Earhart and Stein, 2000b; Kiehn and Butt, 2003), and turtle and cat scratching (Deliagina et al., 1975; Mortin et al., 1985).

These findings led to the concept of a central pattern generator (CPG)-that the central nervous system contains sufficient circuitry to generate appropriate motor output without movement-related sensory feedback (Engberg and Lundberg, 1969; Marder and Bucher, 2001; Grillner, 2011).

Forebrain inputs to the CPG are not necessary to generate many rhythmic behaviors either. In invertebrates, an isolated ganglion is sufficient to generate rhythmic motor patterns that are similar to moving animal behaviors (Peterson, 1983; Stevenson and Kutsch, 1987; Marder and Calabrese, 1996). In vertebrates, spinal cord-transected tadpoles and larval zebrafish can generate swimming behavior (Roberts, 1990; Saint-Amant and Drapeau, 1998) and motor patterns (Saint-Amant and Drapeau, 2000; Li et al., 2006; Roberts et al., 2010). Decerebrate cats and spinal turtles can generate locomotion (Lennard and Stein, 1977; Deliagina et al., 1983; Juranek and Currie, 2000; Grillner, 2011). Spinalized animals can still generate scratch behaviors (Sherrington, 1906; Deliagina et al., 1975; Mortin et al., 1985; Grillner, 2011) and motor patterns (Deliagina et al., 1975; Robertson et al., 1985).

The concept of a CPG is further supported by studies of several *in vitro* preparations showing that only a fraction of the central nervous system without any muscles attached can still generate rhythmic motor outputs (Calabrese and Peterson, 1983; Currie and Lee, 1996; Marder and Bucher, 2001; Kiehn, 2006; Bianchi and Gestreau, 2009; Moore et al., 2014).

The ability of a ganglion, the brain stem, or the spinal cord to generate multiple rhythmic behaviors that involve the same motoneurons and muscles raises the question: are these basic rhythmic motor patterns generated by a single, shared CPG or by multiple specialized CPGs that are each dedicated to one motor pattern?

Mapping the CPGs

To start with, we may ask one simple question: do different CPGs contain the same or different neurons? Initially, investigators thought this question could be answered by studying each neuron's synaptic connections, its firing properties, and its contribution to the CPGs (Marder and Calabrese, 1996), at least in some simple invertebrate systems with a small number of identifiable neurons.

One of the best-studied systems is the crustacean stomatogastric ganglion (STG), which contains 30 identifiable neurons and can generate multiple rhythmic stomach motor patterns. An *in vitro* STG is still able to generate several rhythmic motor patterns, including the pyloric rhythm, which controls the dilation and constriction of the pylorus (Hooper and Marder, 1987; Marder and Calabrese, 1996), and the gastric mill rhythm, which controls chewing in the stomach (Mulloney and Selverston, 1972; Li et al., 2007). Initially, detailed investigation of each neuron's properties and connections seemed to provide a clear map of rhythm generation: the pyloric rhythm was generated by a pacemaker cell, the anterior burster (AB). Pyloric dilator (PD) cells, which were electrically coupled with AB and four other cells [lateral pyloric (LP), inferior cardiac (IC), pyloric (PY), and ventricular dilator (VD)] generated bursts that followed the AB

rhythm and occurred sequentially to generate the pyloric pattern (Miller and Selverston, 1982; Marder and Calabrese, 1996). The gastric mill rhythm was generated by another set of neurons with interneuron 1 playing the key role in rhythm generation (Mulloney and Selverston, 1972; Marder and Calabrese, 1996).

However, "each set of neurons is dedicated to one rhythmic motor pattern" turned out not to be the final answer. The 30 neurons within the STG are under various neural modulations from the brain and are densely interconnected. The modulations could alter the connection strengths and the cell properties, which could alter the output dramatically. Adding red pigment concentrating hormone, a neuromodulatory peptide, could potentiate some synaptic connections and synchronize two motor patterns into one new motor pattern (Dickinson et al., 1990). Further, activating the pyloric suppressor neuron (PS) could selectively recruit neurons from the two CPGs to build a new network for a different motor pattern (Meyrand et al., 1991, 1994). Now, the STG network is viewed as one single group of neurons that is able to generate multiple motor patterns by network reconfiguration (Marder et al., 2005).

Even in a slightly larger network, such as a leech segmental ganglion, which contains about 300 neurons (Kristan et al., 2005), the task of intracellularly recording from each neuron and elucidating its synaptic connections and contributions seemed unfeasible. Still, intracellular recordings provided useful information. Four interneurons, named 123, 28, 33, 27, were determined to be the key swimming CPG neurons by their phase-locked depolarization and their ability to shift the rhythm when they were

hyperpolarized by intracellular current injection (Friesen et al., 1978; Brodfuehrer et al., 1995). However, the CPG neurons generating another rhythmic form of locomotion, crawling, have barely been studied to date even though this motor pattern can be generated by an isolated ganglion *in vitro* (Kristan et al., 2005; Puhl and Mesce, 2008).

As an alternative to intracellular recording, voltage-sensitive dyes provided a very powerful tool for monitoring more than 100 neurons simultaneously. The majority, but not all, of the imaged neurons in the ganglion were activated during both swimming and crawling (Briggman et al., 2005). This result supported largely but not completely shared components between swimming and crawling CPGs. However, the final conclusive answer will require detailed study of each neuron belonging to the swimming and crawling CPGs.

Specialized vs. multifunctional neurons

In vertebrates, detailed studies of each CPG-related neuron and mapping of a whole CPG circuit are even harder. Still, investigating single-neuron properties provided useful information, especially in some young and simple vertebrates.

The spinal cords of two limbless young vertebrates, hatchling *Xenopus* tadpoles and larval zebrafish, were studied intensively (McLean and Fetcho, 2008; Berkowitz et al., 2010; Roberts et al., 2010; Fetcho, 2012). Despite their early developmental stage and their relatively simple nervous systems, tadpoles and larval zebrafish can perform a variety of behaviors, such as escaping, swimming and struggling. Both swimming and

struggling feature rhythmic bending between the left and right side of the body. Swimming happens spontaneously or is evoked by a gentle touch on the skin, with the wave of body bending propagating from head to tail (Roberts et al., 1981; Kahn et al., 1982; Masino and Fetcho, 2005). Struggling is evoked by gripping or constant touching of the body. Compared with swimming, the amplitude of the body movement during struggling is stronger at a lower frequency, with the wave of body bending propagating from tail to head (Kahn and Roberts, 1982; Liao and Fetcho, 2008).

Horseradish peroxidase staining of tadpole spinal cord neurons revealed only 9 types of neurons based on the location of the soma and the projection of the dendrites and axons (Roberts and Clarke, 1982). Whole-cell patch-electrode recording combined with neurobiotin staining revealed 2 additional types of neurons based on their firing pattern during swimming and struggling (Li et al., 2007). The simplicity of these spinal cords makes the tadpole and the larval zebrafish very powerful model systems for understanding the fundamental organization of the spinal cord.

For example, commissural interneurons (cINs) are the interneurons most commonly encountered in the tadpole spinal cord. The cINs project to the contralateral side of the spinal cord (Roberts and Clarke, 1982) and provide reciprocal inhibition during both swimming and struggling (Li et al., 2007). Similar commissural inhibitory interneurons, commissural bifurcating longitudinal interneurons (CoBLs), are also found in larval zebrafish, which are also activated during both swimming and struggling (Liao and Fetcho, 2008).

Descending interneurons (dINs) provide excitatory input to the network by projecting directly to the motoneurons on the ipsilateral side and to other interneurons (Roberts and Clarke, 1982; Li et al., 2004; Li et al., 2006; Soffe et al., 2009). Despite the similarities in morphology, there are in fact two subtypes of descending interneurons: the dINs that fire once during each swimming cycle and are considered swimming CPG interneurons (Soffe et al., 2009; Berkowitz et al., 2010; Roberts et al., 2010) but barely fire during struggling (Li et al., 2007); and the repetitive-firing dINs (dINrs) that fire repetitively during each struggling cycle but not during swimming (Li et al., 2007).

The spinal cord of larval zebrafish also contains swim-specialized interneurons, the multipolar commissural descending interneurons (MCoDs) (Ritter et al., 2001; McLean et al., 2007; McLean and Fetcho, 2008), and struggle-specialized interneurons, the commissural longitudinal ascending interneurons (CoLAs) (Liao and Fetcho, 2008). The CoLAs are inhibitory interneurons and thought to reverse the body-bending propagation direction for struggling (Liao and Fetcho, 2008).

The multifunctional and specialized interneurons found in these two simple systems suggest a shared component providing the reciprocal inhibition between the two sides of the body (cINs and CoBLs) and specialized components for each behavior (dINs, dINrs, MCoDs and CoLAs) (Berkowitz et al., 2010). However, this hypothesis is hard to test by cell morphology and cell recording alone. The transparency of larval zebrafish and the powerful transgenic tools that can be used in zebrafish (McLean and Fetcho, 2011)

may enable researchers to selectively activate or inactivate each group of interneurons to test the hypotheses.

Both multifunctional and specialized interneurons are found in more complicated systems as well. The brainstem of cats and rodents is sufficient to generate rhythmic respiratory motor patterns, including eupnea, sighs, and gasps (Smith et al., 1991; Orem and Trotter, 1993; Ramirez et al., 1997; Lieske et al., 2000), and many other non-respiratory but stereotyped fictive behaviors, including coughing, swallowing and vomiting, which also involve respiratory muscles (Bolser, 1991; Milano et al., 1992; Bianchi and Gestreau, 2009). Intracellular recordings from mouse brainstem slices revealed multifunctional neurons that were activated during all three forms of respiratory motor patterns (Lieske et al., 2000). Many of the respiratory neurons were also activated during coughing (Gestreau et al., 1996; Shiba et al., 1999; Gestreau et al., 2000; Baekey et al., 2001) and swallowing (Oku et al., 1994; Gestreau et al., 1996; Gestreau et al., 2005). These multifunctional interneurons suggest shared CPG components in generating multiple rhythmic behaviors. However, some respiratory neurons were not activated during swallowing (Oku et al., 1994) or vomiting (Shiba et al., 2007), suggesting specialized CPG components as well.

Rhythmic limb movements require synergies among many joints and are multiphasic. They are more complicated than the diphasic swimming and respiratory motor patterns. Spinal turtles are able to generate rhythmic hindlimb behaviors and motor patterns, including scratching and swimming (Stein, 2005). Both extracellular and intracellular

recordings revealed that most interneurons that were rhythmically activated during forward swimming were also activated during scratching (Berkowitz, 2002; Berkowitz, 2008; Berkowitz et al., 2010), including a group of morphologically identified interneurons with dendrites projecting mainly in the transverse plane, the transverse neurons (T neurons) (Berkowitz et al., 2006). There are also scratch-specialized interneurons that were rhythmically activated during scratching, but were inhibited during forward swimming (Berkowitz, 2002; Berkowitz, 2008; Berkowitz et al., 2010) and interneurons that were rhythmically activated during scratching, but fired tonically during swimming (Berkowitz, 2002; Berkowitz et al., 2010).

Neural circuits with largely multifunctional neurons and some specialized neurons are consistent with the hypothesis that different motor patterns are generated by a largely but not completely shared network. However, again, without knowing each neuronal type's contribution to the network, the hypothesis is hard to test. Other approaches to investigate CPGs more systematically may provide additional information to answer this question.

Another approach: lesion studies

One approach is to determine the distribution of necessary CPG neurons by removing or inactivating parts of the central nervous system.

Motor patterns are relatively simple in limbless vertebrates such as lamprey, tadpole and larval fishes. Still, they can produce multiple rhythmic behaviors including forward

swimming and struggling (Fetcho et al., 2008; Roberts et al., 2010; Hsu et al., 2014). An isolated lamprey spinal cord can still generate forward swimming motor patterns featuring left-right alternation that is propagated from rostral to caudal segments (Cohen and Wallen, 1980; Buchanan, 1996). Spinal cord transections demonstrated that any four spinal cord segments are sufficient to generate a swimming motor pattern with rostral to caudal propagation. Similar results were found in dogfish (Grillner, 1974) and zebrafish (Wiggin et al., 2012). The lack of critical segments in axial swimming CPGs suggested a segmentally distributed rhythm generator. Unfortunately, to my knowledge, the struggling CPG was not studied in a similar manner in any of these systems to provide direct comparisons between the swimming and struggling CPG distributions.

Similarly, lesion studies of early-stage limbed animals did not provide enough information to compare the CPG distributions for two motor patterns. Both the rostral and caudal portion of the lumbosacral cord were sufficient to generate a locomotion-like rhythm in an *in vitro* chick embryonic spinal cord, with the rostral segments contributing more than the caudal segments (Ho and O'Donovan, 1993). In neonatal rat, despite some controversial results, it has usually been found that the rostral portion, but not the caudal portion, of the isolated lumbosacral spinal cord was sufficient to generate a locomotion-like rhythm (Cazalets et al., 1995; Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997). In spinal turtles, similarly, the caudal portion of the hindlimb enlargement is not necessary to generate a scratching motor pattern (Mortin and Stein, 1989) and cats (Deliagina et al., 1983; Arshavsky et al., 1984). Direct comparisons of

the distributions of different CPGs within a single species are needed to answer the question.

Another approach: interactions of two motor patterns

Another approach to understand the organization of the CPGs is to investigate the effects of the activation of one motor pattern on another ongoing motor pattern.

At one extreme, if two motor patterns are generated by two completely separate CPGs, simultaneous delivery of the stimulation for the two motor patterns would activate both of the CPGs without them affecting each other. If the two motor patterns involve the same motoneurons, an intracellular recording from the motoneurons would show a superimposition of two inputs. In fact, this prediction was confirmed in a study of locust flight and walking (Ramirez and Pearson, 1988) as well as cricket flight and stridulation (Hennig, 1990).

However, such cases have been rare. In more cases, there were some interactions between the two motor patterns. The question is, how strong are these interactions and to what extent do the two motor patterns share their CPGs?

Traditionally, if the rhythm of one motor pattern can be reset by another input, i.e., the rhythm is stopped and restarted at a new timing, it is believed that the input had accessed the CPG of the reset motor pattern. Fish can perform a dramatic C-start escape behavior when stimulated by various external cues. The escape behavior is triggered by

a pair of descending reticulospinal neurons, Mauthner cells, and would always override swimming (Wilson, 1959). One single action potential in the Mauthner cell during swimming could evoke the escape behavior and reset the swimming rhythm (Svoboda and Fetcho, 1996). In spinal turtles, an ongoing scratching rhythm could be reset by a quick hindlimb withdrawal triggered by a brief foot tap (Currie and Stein, 1989). In rodents, respiration could be inhibited and reset by swallowing in a moving animal (McFarland and Lund, 1993) and in an *in vitro* preparation (Yamanishi et al., 2010). The interruption of an ongoing rhythm by a fixed action pattern was normally explained by a general inhibition from the neurons involved in the fixed action pattern (Faber et al., 1989; Yamanishi et al., 2010; Moore et al., 2014).

An ongoing rhythmic motor pattern can also sometimes be reset by activation of the CPG of another rhythmic motor pattern. In mollusk *Pleurobranchaea californica*, escape swimming, which features rhythmic alternation between dorsal and ventral flexion, is generated by 7 neurons in the cerebropleural ganglion (Davis and Mpitsos, 1971; Jing and Gillette, 1999). Once this high-threshold behavior or motor pattern was triggered, it would override the rhythmic feeding motor pattern (Gillette et al., 1982), which features rhythmic alternation of protraction and retraction of the radula (Kupfermann, 1974; Cropper et al., 2004) by inhibiting the feeding command neuron I1 (Jing and Gillette, 1995; Gillette et al., 2000; Jing and Gillette, 2000; Gillette and Jing, 2001).

In more complicated systems such as spinal turtles, a brief swim stimulation could reset the ongoing scratching rhythm and vice versa (Juranek and Currie, 2000). In rodent brainstem preparations, respiration could reset the rhythm of whisking (Moore et al., 2013) and licking (Katakura et al., 1995; Nakamura et al., 1999).

The reset of an ongoing rhythm clearly demonstrates strong interactions between the CPGs for two motor patterns. However, in the mollusk swimming/feeding example, the separation of the key components in generating these two motor patterns suggests that the reset of an ongoing rhythm may not be a good indicator of the extent to which the CPGs for two motor patterns are shared.

Instead, the merging of two motor patterns may provide more information on the overlap of the CPGs. For example, when the saline level was intermediate, a semi-intact leech produced a behavior that was a hybrid between crawling, which was slow and usually evoked when the saline level was low, and swimming, which was fast and usually evoked when the saline level was high (Esch et al., 2002). The observation that swimming only happened during the elongation phase of crawling suggested coordination of the two motor patterns in both rhythm generation, which sets the pace of the motor pattern, and pattern generation, which sets timing and amplitudes of multiple muscles within a cycle. The majority of the interneurons were activated during both swimming and crawling motor patterns, consistent with shared components of their CPGs (Briggman et al., 2005). Similarly, a rabbit could merge scratching into hopping (Brown, 1911b), and a cat could merge scratching into walking (Carter and Smith,

1986), supporting shared CPG components in vertebrates. When stimulated in the transition zone, spinal turtles could produce a switch behavior in which some cycles were one form of scratching while other cycles were another form of scratching (Mortin et al., 1985). During simultaneous swim and rostral scratch stimulation, a spinal turtle could produce a hybrid behavior in which scratching and swimming were coordinated and each cycle fulfilled the goals of both behaviors (Earhart and Stein, 2000a). These examples were done with moving animals. Movement-related sensory feedback may help coordinate the behaviors.

In vitro preparations or immobilized animals can also merge two motor patterns. The crab stomatogastric ganglion is able to generate several rhythmic motor patterns, including gastric mill and pyloric rhythms. The two rhythms could be coordinated with each other under the control of modulatory commissural neuron 1 (Bartos and Nusbaum, 1997; Bartos et al., 1999). In spinal and immobilized turtles, a single stimulation in the transition zone or simultaneous stimulation of two locations could evoke switches or hybrids between two forms of scratching motor patterns (Robertson et al., 1985); simultaneous rostral scratch and swim stimulation could evoke a hybrid motor pattern (Juranek and Currie, 2000).

These interactions between two behaviors or two motor patterns are consistent with shared components or strong interactions between two CPGs. Further investigations of the interactions between CPGs in adult limbed vertebrates may help us understand to what extent the CPGs for different motor patterns are shared at the systemic level.

Turtles are an excellent vertebrate model system

The spinal cord of the red-eared turtle *(Trachemys scripta elegans)* is a great model system to address the extent to which the two motor patterns share their CPGs. The spinal cord contains 9 cervical segments, 10 dorsal segments, which are equivalent to the thoracic and lumbar segments in mammals, 2 sacral segments and several caudal segments (Ruigrok and Crowe, 1984). The five-segment hindlimb enlargement (D8-D10, S1- S2) that contains all the motoneurons innervating the hindlimb muscles (Ruigrok and Crowe, 1984) is equivalent to the lumbar 4 (L4)-L7 and S1 in cats (Romanes, 1951) and L1-L5 in rodents (McHanwell and Biscoe, 1981; Nicolopoulos-Stournaras and Iles, 1983).

As a diving animal, turtles have high tolerance to hypoxia (Hounsgaard and Nicholson, 1990; Lutz and Milton, 2004). Turtle tissue can stay healthy much longer than mammalian tissue when blood and thus oxygen are limited during many experimental conditions. This extended time is particularly valuable to allow for within-animal comparisons.

As a limbed animal, the adult turtle has a diverse behavioral repertoire, including locomotion (stepping, forward swimming, and backward swimming) (Lennard and Stein, 1977; Earhart and Stein, 2000a, b; Juranek and Currie, 2000) and scratching (rostral, pocket and caudal) (Stein and Grossman, 1980; Mortin et al., 1985; Robertson et al., 1985). The turtle spinal cord contains the CPGs for swimming and scratching without the need for brain input or movement-related sensory feedback (Stein, 2005).

These rhythmic motor patterns all involve the same hindlimb muscles and feature rhythmic alternation between the hip flexor and the hip extensor, but these motor patterns are also distinguishable from each other by the relative amplitudes and synergies of the hip flexor, hip extensor, and knee extensors.

Further, the onset and cessation of the swimming and scratching motor patterns can be controlled by the onset and offset of the stimulation. This facilitates within-animal comparisons, as well as a detailed investigation of motor pattern interactions.

Research on interactions between motor patterns and research on the cells involved in each motor pattern demonstrated strong interactions or shared components of forward swimming and scratching CPGs. However, the existence of scratch-specialized interneurons suggested specialized scratching CPG components. These results suggested that the swimming and scratching CPGs are neither completely shared, nor completely separate. To what extent are the swimming and scratching CPGs shared?

Research described in this dissertation attempts to answer three aspects of this question: 1) Does each spinal cord segment contribute equally to the generation of swimming and scratching motor patterns? (Chapter 2)

2) What are the effects of activating the swimming CPG on the generation of the scratching motor pattern and vice versa? (Chapter 3)

3) If there are effects of simultaneous stimulation, do these effects on the motor patterns happen at the motoneuron level or at the interneuron level? (Chapter 4)

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Chapter 2: Rostral spinal cord segments are sufficient to generate a rhythm for both locomotion and scratching, but affect their hip-extensor phases differently

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SUMMARY

Rostral segments of the spinal cord hindlimb enlargement are more important than caudal segments for generating locomotion and scratching rhythms in limbed vertebrates. But the adequacy of rostral segments has not been directly compared between locomotion and scratching. I separated caudal segments from immobilized, low-spinal turtles by sequential spinal cord transections. After the separation of the caudal four segments of the five-segment hindlimb enlargement, the remaining enlargement segment and five pre-enlargement segments still produced rhythms for forward swimming and both rostral and pocket scratching. The swimming rhythm frequency was usually maintained. Some animals continued to generate swimming and scratching rhythms even with no enlargement segments remaining, using only pre-enlargement segments. The pre-enlargement segments and rostral-most enlargement segment were also sufficient to maintain hip flexor (HF) motoneuron quiescence between HF bursts [which normally occurs during each hip extensor (HE) phase] during swimming. In contrast, the HF-quiescent phase was increasingly absent (i.e., HE-phase deletions) during rostral and pocket scratching. Moreover, respiratory motoneurons that

normally burst during HE bursts continued to burst during the HF quiescence of swimming even with the caudal segments separated. Thus, the same segments are sufficient to generate the basic rhythms for both locomotion and scratching. These segments are also sufficient to produce a reliable HE phase during locomotion but not during rostral or pocket scratching. I hypothesize that the rostral HE-phase interneurons that rhythmically inhibit HF motoneurons and interneurons are sufficient to generate HF quiescence during HE-biased swimming, but not during the more HF-biased rostral and pocket scratching.

INTRODUCTION

Animals perform distinct behaviors using the same motoneurons and muscles, even in the absence of both brain inputs and movement-related sensory feedback (Brown, 1911a; Jankowska, 2008; Goulding, 2009; Kiehn, 2011). They may achieve this through the reconfiguration of a single network of multifunctional interneurons (Morton and Chiel, 1994; Dickinson, 1995; Marder and Calabrese, 1996; Marder and Bucher, 2001; Briggman and Kristan, 2008; Kupfermann and Weiss, 2001). However, behaviorally specialized interneurons can also play a role in both invertebrates (Heitler, 1985; Ramirez and Pearson, 1988; Hennig, 1990; Jing and Weiss, 2001) and vertebrates (Ritter et al., 2001; Berkowitz, 2002; Berkowitz, 2007; Li et al., 2007; Liao and Fetcho, 2008; McLean and Fetcho, 2009; Soffe et al., 2009).

The turtle spinal cord can generate several kinds of rhythmic hindlimb behaviors, including locomotion [e.g., forward swimming (Lennard and Stein, 1977; Juranek and

Currie, 2000)] and several forms of scratching [e.g., rostral and pocket scratching (Mortin et al., 1985; Robertson et al., 1985)], each of which features rhythmic alternation between hip flexors (HF) and hip extensors (HE), but is otherwise distinct. Single-cell recording during forward swimming (henceforth, just "swimming") and scratching motor patterns revealed the existence of both multifunctional and scratching-specialized interneurons (Berkowitz, 2010). Simultaneous stimulation of swimming and scratching can evoke hybrid motor patterns (Earhart and Stein, 2000a), reset an ongoing rhythm (Juranek and Currie, 2000), increase the rhythm frequency, decrease the swim stimulation threshold, or interrupt the rhythm (Hao et al., 2011). These results suggest that swimming and scratching are generated by either a shared network or highly connected networks.

A spinal cord preparation without the caudal segments of the hindlimb enlargement is still able to generate rostral and pocket scratching motor patterns in turtles (Mortin and Stein, 1989), scratching in cats (Deliagina et al., 1983), and locomotor-like rhythms in chicks (Ho and O'Donovan, 1993) and neonatal rodents (Cazalets et al., 1995; Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997). However, the likelihood of HE-phase deletions, defined by the omission of an HE burst and the corresponding HF quiescence, increases during turtle rostral scratching in such preparations (Stein and Grossman, 1980; Mortin and Stein, 1989; Currie and Gonsalves, 1999; Stein and Daniels-McQueen, 2004).

It is unknown whether the caudal segments of the hindlimb enlargement are necessary for turtle swimming motor patterns or whether each spinal cord segment contributes equally to locomotion and scratching. Here, I sequentially separated caudal segments of the turtle hindlimb enlargement and evoked swimming and scratching motor patterns in the remaining preparation. I found that the caudal segments of the hindlimb enlargement were not necessary for swimming rhythm generation, similar to rostral and pocket scratching, cat scratching, and chick and neonatal rodent locomotor patterns. However, I also found that HE-phase deletions increased significantly for scratching but not for swimming in these reduced preparations, suggesting that the deleted segments contribute unequally to locomotion and scratching. Some findings have previously been described in an abstract (Hao et al., 2012).

METHODS

Animal preparations

Adult red-eared sliders, *Trachemys scripta elegans*, of both sexes (n = 20), weighing 400-1500 g**,** were submerged in crushed ice for at least 2 h to induce hypothermia before and during surgery (Lennard and Stein, 1977). The spinal cord was exposed and transected between the dorsal 2 (D2) and D3 roots. The spinal cord hindlimb enlargement [D8-D10 and sacral (S) 1-2] and two pre-enlargement segments [D6-D7] were also exposed. Several muscle nerves on one side of the turtles were dissected free for nerve recordings: the hip flexor (HF), ventral puboischiofemoralis internus, pars anteroventralis; the hip extensor (HE), flexor cruris, pars flexor tibialis internus; and the knee extensors (KEs), triceps femoralis, pars iliotibialis (IT), pars ambiens (AM), and/or

pars femorotibialis (FT) (Robertson et al., 1985). Only the FT-KE nerve recording is shown in each figure. In 5 animals, I also dissected branches of the D7 peripheral nerve innervating the respiratory muscles transverse abdominus (TA) and/or oblique abdominus (OA) (Currie and Gonsalves, 1997). After surgery, turtles were allow to warm to room temperature, then immobilized with gallamine triethiodide (8 mg/kg i.m.; Sigma-Aldrich, St. Louis, MO), and artificially ventilated throughout the experiment. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma.

Stimulation and spinal cord transections

Forward-swimming motor patterns were evoked by electrical stimulation in the D3 contralateral lateral funiculus (0.1-ms, 10–900 μA, bipolar pulses at 5–80 Hz) with a pair of 100-μm silver wires (California Fine Wire, Grover Beach, CA), insulated except at the tips, with one tip contacting the D3 face of the spinal cord and the other in the saline (Lennard and Stein, 1977; Juranek and Currie, 2000; Berkowitz, 2002; Berkowitz, 2008). Forward swimming features weak, brief and approximately in-phase bursts of the hip flexor (HF) and knee extensor (KE) alternating with strong and long hip extensor (HE) bursts (Lennard and Stein, 1977; Juranek and Currie, 2000).

Rostral and pocket scratching motor patterns were evoked by continual gentle rubbing of a single site in the receptive field of each scratch form at about 0.3 N, 3–4 Hz using a glass probe with a fire-polished tip attached to a force transducer (Grass Technologies/Astro-Med, West Warwick, RI) (Mortin et al., 1985). Rostral scratching

was evoked by stimulating at SP1 or SP2, in the D5-D6 dermatomes; pocket scratching was evoked by stimulating in the ventral pocket region in the D6-D8 dermatomes (Mortin and Stein, 1990). Rostral scratching features strong and long HF bursts, with a KE burst in approximately the second half of each HF burst alternating with shorter and weaker HE bursts. Pocket scratching features alternating HF and HE bursts of similar amplitude and duration, with KE bursts that largely overlap with HE bursts (Mortin et al., 1985; Robertson et al., 1985).

Three forms of motor patterns were evoked in pseudorandom order with an interval of at least 2 minutes between stimulations. After each kind of stimulation was applied three times, the spinal cord was transected completely midway between the dorsal roots of two adjacent segments by Moria Pascheff-Wolff spring scissors (Fine Science Tools, Foster City, CA) with all the spinal cord segments left in place. The spinal cord caudal to the cut was briefly lifted and viewed in cross section to verify complete transection. After the transection, the animal was allowed to rest for at least 30 min (except in the first four experiments) until spontaneous activity stopped and rostral scratching could be elicited.

Nerve recordings

Dissected nerves were submerged in mineral oil, surrounded by a wax well molded onto the turtle carapace. Recordings from each nerve were obtained extracellularly using a pair of 100-μm silver wires. Filtered and amplified (band-pass 0.1–1.0 kHz; x 1,000; A-

M Systems, Carlsborg, WA) nerve activities were recorded on a digital audio tape recorder (TEAC America, Montebello, CA).

Data analysis

Two preliminary animals were completely excluded from the data set due to the weak HF signal or failure to deliver swim stimulation. The remaining 18 animals were tested for rhythm generation. In some animals, a particular transection was omitted. If an animal was not tested in one preparation (e.g., D3-S2), but still generated a particular type of rhythm after the following transection (e.g., D3-S1), it was assumed for the graph in Figure 3 that this animal also would have generated the same rhythm in the untested (longer) preparation. If an animal could not generate a particular type of rhythm after one transection (e.g., D3-D9) but was not tested after the following transection (e.g., D3-D8), it was assumed for this graph that the animal still would not have generated this rhythm in the untested (shorter) preparation.

Five animals with OA and/or TA recordings were used for another specific purpose and not included in the quantitative analysis of motor pattern frequency, burst amplitude, and HE-phase deletions. One animal was only included in the rhythm generation analysis (Fig. 3) because swimming stopped after the separation of the S2 segment. In the remaining 12 animals, recordings were redigitized and quantitatively analyzed using Datapac software (Run Technologies, Laguna Hills, CA). Recordings were rectified and then smoothed with a time constant of 50 ms. The onset and offset of each burst (i.e., a clear increase and then decrease in the nerve's rectified and smoothed amplitude) were

determined in Datapac by positive- and negative-slope crossings using custom-selected thresholds. Cycle period was defined as the interval between two successive HF burst onsets. Burst duration was the interval between the onset and offset of a burst. Mean burst amplitude was also determined in Datapac. Only cycles completely within the period of stimulation were analyzed. HE-phase deletion cycles, defined by the absence of the quiescence between successive HF bursts (Stein and Grossman, 1980; Stein, 2008), were not included in cycle frequency or burst amplitude analyses.

For each form of motor pattern and type of spinal cord preparation, the cycle frequency, i.e., the reciprocal of the cycle period, was determined by averaging all cycles within each stimulation episode. If no motor pattern was evoked by a stimulation, the cycle frequency value was zero. In most cases, I evoked each form of motor pattern three times in each type of spinal cord preparation in each animal, obtaining three mean values. Statistical comparisons were made between types of spinal cord preparation within each animal (and within each form of motor pattern) using the nonparametric repeated-measures test (Friedman's test), followed by selected-pair comparisons (Dunn's test) if Friedman's test yielded significance (Instat 3, GraphPad Software, San Diego, CA). To meet the criteria of Friedman's Test, any missing data (e.g., when there were not three usable episodes, such as when all cycles in the episode had HE-phase deletions) were imputed, using the mean of all measured cycle frequency values within the animal (Quinn and Keough, 2002). In addition, I compared cycle frequencies between types of spinal cord preparation (for each form of motor pattern) across all 12 animals together by normalizing each cycle frequency value (with the D3-end

preparation value being 100% for each form of motor pattern in each animal). In two early animals, the D3-end data were imputed using the D3-Caudal 1 values because the D3-end values had not been obtained.

The frequency of HE-phase deletions was analyzed within each form of motor pattern and each animal in the 12 animals that produced regular motor patterns after removing the S2 and more caudal segments. For all motor patterns, an equal number of cycles were analyzed from the beginning of each episode; episodes with too few cycles were omitted from this analysis. The Chi-square test was used to evaluate whether any change in HE-phase deletion probability occurred as a result of the whole set of transections (Instat 3, GraphPad Software, San Diego, CA).

RESULTS

To determine which are the key spinal cord segments to produce forward swimming motor patterns (henceforth, just "swimming") and to determine whether the same or different segments are required for rostral and pocket scratching motor patterns (henceforth, just "scratching" when referring to both of these forms of scratching), I first eliminated spinal cord segments caudal to the hindlimb enlargement from the preparation under study by a transection between the sacral 2 (S2) and caudal 1 (Ca1) roots in immobilized spinal turtles (Fig. 1). Next, I sequentially eliminated segments from the caudal end of the preparation to the most rostral segment of the five-segment hindlimb enlargement, dorsal 8 (D8).

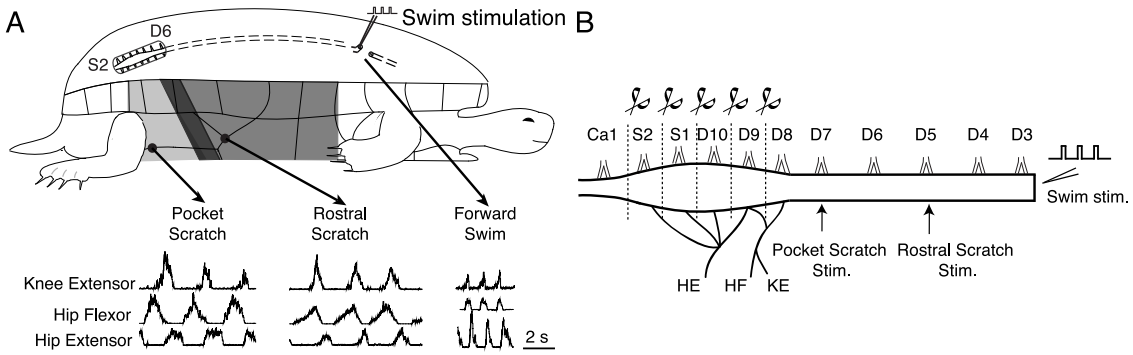

Figure 1. Diagram of the preparation.

A, immobilized, spinal turtle with the D2/D3 transection site, additional spinal cord segments exposed, swim stimulation, and receptive fields for rostral (medium shading) and pocket (light shading) scratching and the rostral-pocket transition zone (dark shading). Three traces below are examples of the motor patterns produced. B, diagram of the locations of the spinal cord transection sites in the hindlimb enlargement (scissors icons and dashed lines). Arrows below the spinal cord indicate the segments receiving the sensory input to evoke rostral and pocket scratching. KE, knee extensor; HF, hip flexor; HE; hip extensor; D, dorsal; S, sacral; Ca, caudal.

Rhythm generation

Figure 2 shows swimming and scratching from one animal following each transection. After the separation of four of the five segments of the hindlimb enlargement (i.e., in D3-D8 preparations), rhythmic HF bursts were still observed during both swim and scratch stimulation (Fig. 2E2-4). This was seen in 10 of the 18 animals [including 13 animals without transverse abdominus (TA) and oblique abdominus (OA) recordings and 5 animals with OA/TA recordings] (Fig. 3). Thus, at least 10/18 animals were able to generate both swimming and scratching rhythms with only the D3-D8 segments. For swimming, rostral scratching, and pocket scratching, the 4 caudal segments of the hindlimb enlargement were not necessary for rhythm generation.

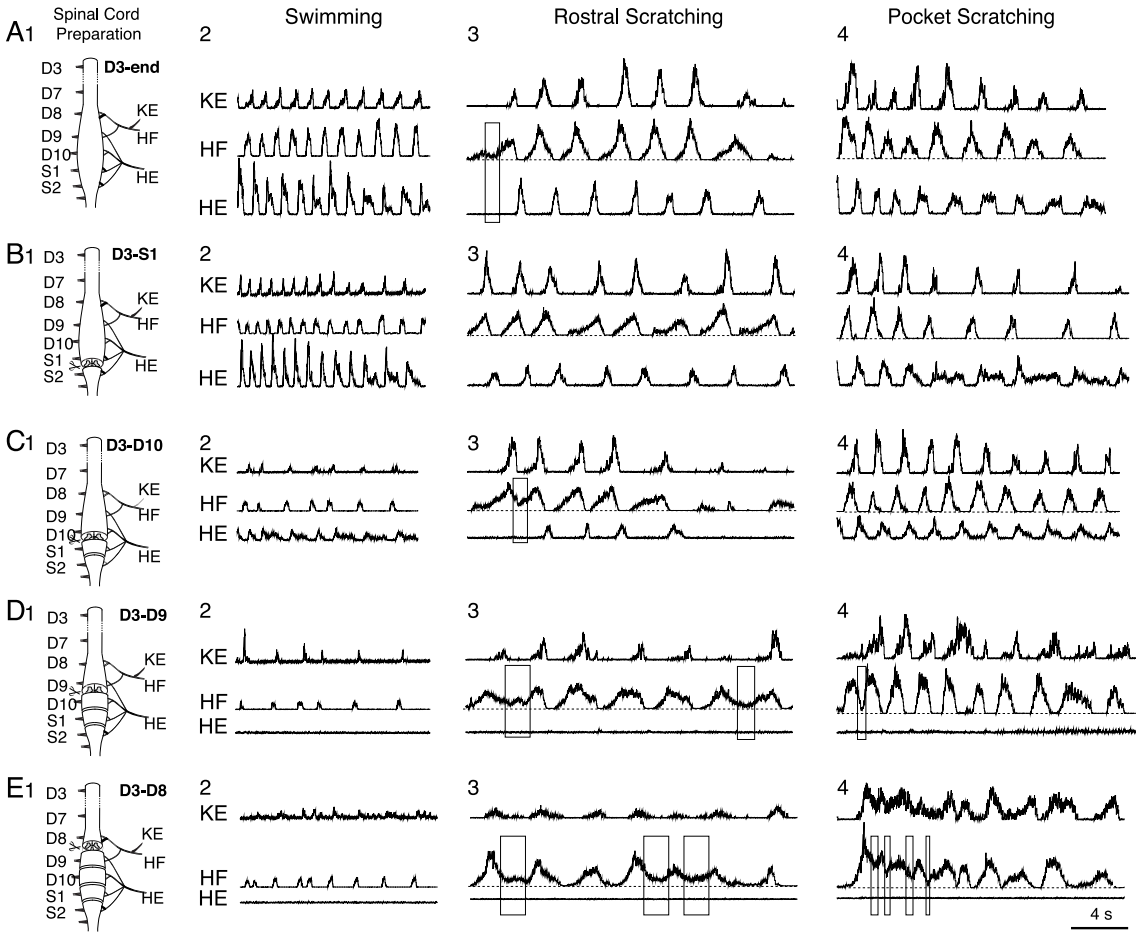

Figure 2. The effects of sequential elimination of the caudal segments of the spinal cord hindlimb enlargement on motor patterns during swim and scratch stimulation in one animal.

A1-E1, diagrams of the spinal cord preparation and the motor nerves recorded. Black boxes, the absence of HF quiescence, i.e., HE-phase deletions. Dashed lines, baseline of HF recordings.

The transections reduced the cycle frequencies for some but not all animals. In the animal shown in Fig. 2, the transections significantly reduced the mean cycle frequency for swimming (from 1.0 Hz in the D3-end preparation to 0.9 Hz in the D3-D8 preparation; $p = 0.03$) and pocket scratching (from 0.53 Hz to 0.34 Hz; $p = 0.008$), but not for rostral scratching (from 0.33 Hz to 0.31Hz; $p = 0.17$). One other animal showed a significant decrease for pocket scratching and one other animal showed a significant increase for swimming. For the remaining 9 animals (see Methods for animals used for

each type of analysis), the cycle frequencies in the D3-D9 or D3-D8 preparations were not significantly different from the values in the D3-end preparations. Taking all 12 animals together, the mean cycle frequency, normalized to its value in the D3-end preparation in each animal, was reduced to 72.9% in the D3-D8 preparation for swimming ($p = 0.19$), to 68.2% for rostral scratching ($p = 0.51$), and to 59.8% for pocket scratching ($p = 0.003$).

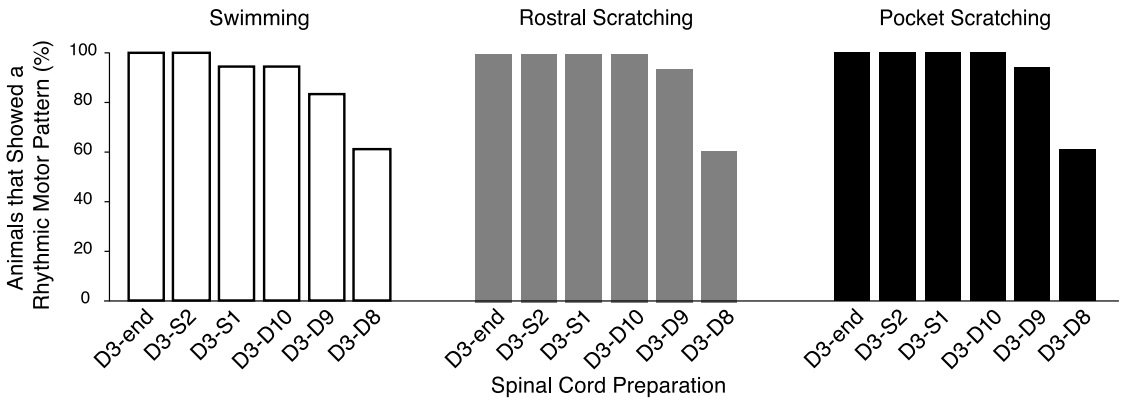

Figure 3. Percentage of animals that generated each type of rhythm after each of the caudal four segments of the hindlimb enlargement was eliminated from the preparation.

18 animals were tested, but not all tests were conducted in each animal (see Methods).

Loss of motoneuron pools

Following certain transections, the knee extensor (KE), HF, and HE burst amplitudes decreased, presumably because the motor pools were partially removed from the preparation being stimulated (Ruigrok and Crowe, 1984). Among the 12 animals I analyzed quantitatively (see Methods), the mean HF burst amplitude decreased significantly after the separation of the D10 segment in 3/12 animals for swimming, 0/12 animals for rostral scratching, and 0/12 animals for pocket scratching. The HF burst amplitude decreased after the separation of the D9 segment in 5/12 animals for swimming, 1/12 animals for rostral scratching, and 0/12 animals for pocket scratching.

Across all 12 animals, the normalized HF burst amplitude decreased significantly after the separation of the D9 segment for swimming, rostral scratching and pocket scratching (all by Friedman's test and then Dunn's test for individual comparisons**;** data not shown). The HE burst amplitude also gradually decreased following the transections. The HE nerve completely stopped responding to stimulation for swimming and scratching after the D10 (1/12 animals) or D9 (11/12 animals) segment was separated (data not shown).

HE-phase deletions

In D3-end preparations, swimming and scratching featured rhythmically alternating HF and HE bursts, i.e., HF nerve bursts occurred while the HE nerve was quiescent and vice versa. Occasionally, during scratching, two sequential HF bursts occurred without an HE burst and the corresponding HF quiescent period in between (HE-phase deletions, Fig. 2, black rectangles). The occurrence of HE-phase deletions during scratching increased following the elimination of caudal segments of the hindlimb enlargement (Fig. 2, A3-E3, A4-E4 and Fig. 4, B and C; data acquired from the same animal as in Fig. 2). HE-phase deletions were not observed during swimming in the animal shown in Fig. 2 (Fig. 4A). In fact, HE-phase deletions during swimming were seen in only one animal even after the transections (Fig. 4D).

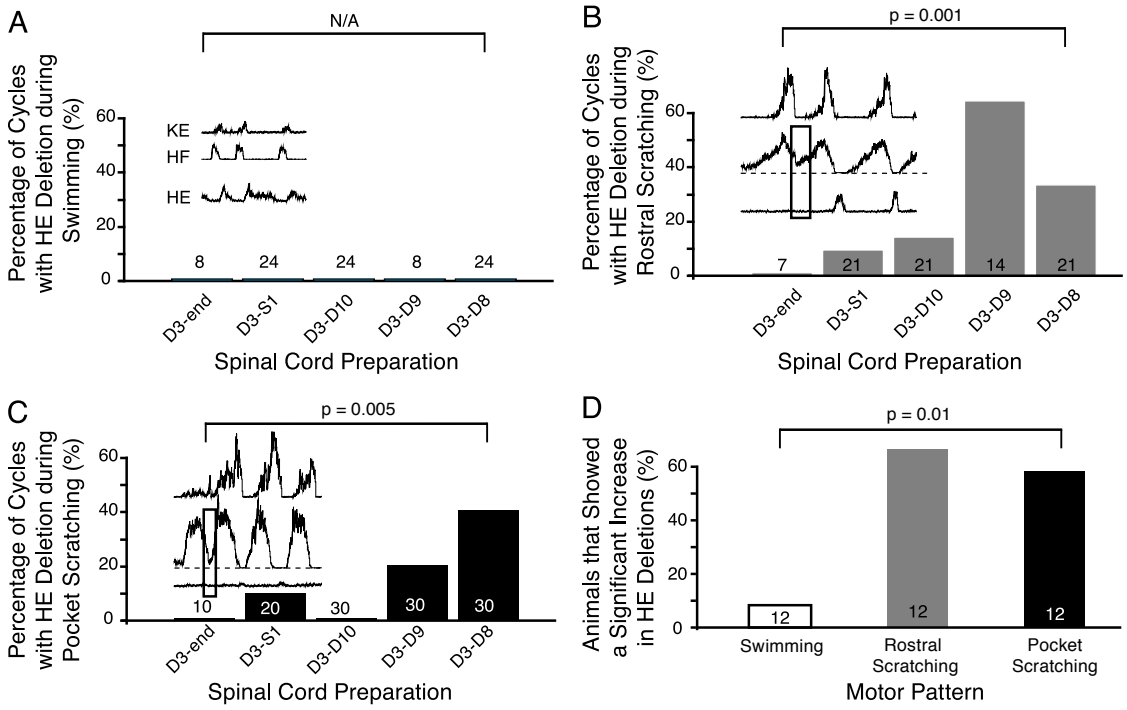

Figure 4. In contrast to scratching, HE-phase deletions were rarely observed during swimming even after the elimination of the caudal portion of the hindlimb enlargement.

A-C, the percentage of cycles that showed HE-phase deletion from the animal shown in Fig. 2 for swimming (A), rostral scratching (B), and pocket scratching (C). Insets in A-C are examples of motor patterns showing no HE-phase deletions during swimming (A) but one HE-phase deletion each during rostral scratching and pocket scratching (B-C, black boxes). D, percentage of animals that showed a significant change in occurrence of HE-phase deletions following segment eliminations. Numbers inside or above the bars in A-C, total number of cycles tested. Numbers inside the bars in D, total number of animals tested. p, significance by the Chi-square test.

Due to the loss of the HE motor pool in a D3-D8 preparation, HE-phase deletions could not be assessed by the absence of an HE burst; instead, HE-phase deletions were defined by two successive HF bursts without an HF-quiescent period in between in most animals (n = 13; see Methods). To further assess the presence or absence of an HE phase in preparations with the HE motor pool separated, I recorded from branches of the D7 nerve, OA and/or TA, which innervate respiratory muscles, in addition to HF and HE in some animals (n = 5). During a non-deletion scratching cycle in the D3-end preparation, OA fires in phase with HE while TA fires in phase with HF (Currie and

Gonsalves, 1997). I found that OA also fired in bursts in phase with HE during swimming in D3-end preparations (Fig. 5, A2-A4). In D3-D9 preparations, OA bursts still alternated with HF bursts during swimming and non-deletion scratching cycles, thus providing a positive indication of HE phases (Fig. 5 B2-B4). During scratching HE-phase deletion cycles, the absence of OA bursts coincided with the absence of quiescent periods between HF bursts (Fig. 5, B3 and B4), which directly supports the absence of an HE phase during scratching HE-phase deletions in the D3-D9 preparation.

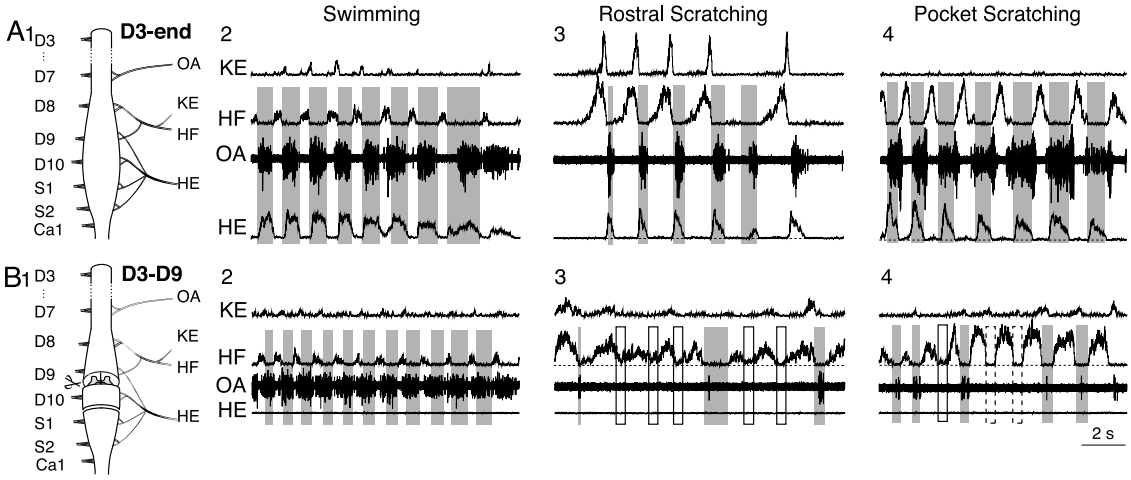

Figure 5. The oblique abdominus respiratory muscle nerve (OA) was activated during most normal HE phases but was silent during HE-phase deletions, even after the HE motor pool was separated, providing a positive indication of the HE phase in reduced preparations.

The OA traces shown are raw recordings without integration to show clearly the few spikes in B3 and B4. Gray shading indicates the quiescent period between two successive HF bursts, which was in phase with the HE burst (when present) and OA burst. Solid-line boxes, HE-phase deletions, as defined by lack of HF quiescence. Dashed-line boxes, occurrences of HF quiescence without corresponding OA spikes.

Interestingly, I observed the absence of OA activity during some non-deletion cycles (Fig. 5B4), which suggests that an OA deletion may be a more sensitive indicator of a weakened HE phase than is HF activity between HF bursts. Also, I observed in D3-D9 preparations that the OA bursts were stronger during swimming than during scratching (Fig. 5B2-4). This suggests that HE-phase excitatory inputs (to OA motoneurons and

probably also to HE motoneurons) are stronger during forward swimming than during rostral or pocket scratching in the preparation with caudal segments separated.

Rhythm generation in the D3-D7 preparation

Following the D7/D8 transection, no motor pattern could be observed in animals in which I only recorded from KE, HF, and HE due to the separation of all three motor pools. In two animals in which I recorded from OA and/or TA and stimulated D3-D7 preparation animals, however, I was able to assess the sufficiency of pre-enlargement segments for rhythmic activity for both scratching (previously assessed by Currie and Gonsalves, 1997) and swimming. In one of these animals, both swimming and scratching rhythms persisted in the D7 nerve branches in the D3-D7 preparation (Fig. 6). (In the other animal, pocket scratching rhythms persisted but not rostral scratching or swimming.) OA fired in rhythmic bursts during swimming while TA fired in rhythmic bursts during scratching. The rhythms produced by the D3-D7 preparation (Fig. 6C) were much less robust than those produced by the D3-end preparation (Fig. 6A), but similar to those produced by the D3-D8 preparation (Fig. 6B).

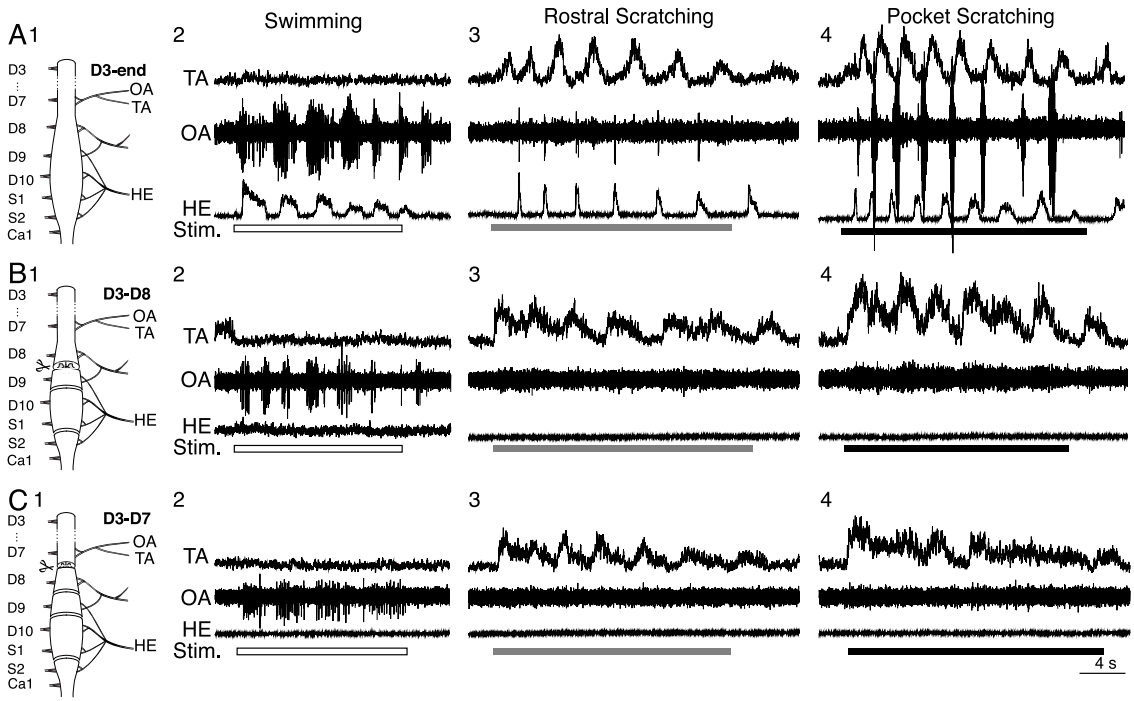

Figure 6. Pre-hindlimb enlargement segments (D3-D7) were sufficient to generate a rhythm during swim stimulation, as well as during rostral and pocket scratch stimulation.

Bars underneath the recordings, the duration of the swim (open bars), rostral scratch (gray bar), or pocket scratch (black bars) stimulation. TA, the transverse abdominus respiratory muscle nerve.

DISCUSSION

The D9-S2 segments are not necessary for swim rhythm generation

After separating the caudal four segments of the five-segment hindlimb enlargement in immobilized low-spinal turtles, descending swim stimulation was still able to evoke a swimming rhythm in remaining hindlimb and/or respiratory motor nerves. The turtle five-segment enlargement (D8-D10, S1- S2 (Ruigrok and Crowe, 1984)) is equivalent to the lumbar 4 (L4)-L7 and S1 in cats (Romanes, 1951) and L1-L5 in rodents (McHanwell and Biscoe, 1981; Nicolopoulos-Stournaras and Iles, 1983). Similarly, the caudal portion of the limb enlargement is not necessary to produce rhythms for mudpuppy locomotion (Wheatley et al., 1994), turtle rostral and pocket scratching

(Mortin and Stein, 1989; Currie and Gonsalves, 1997; Currie and Gonsalves, 1999), chicken embryo rhythmic activity (Ho and O'Donovan, 1993), rodent locomotor-like activity (Cazalets et al., 1995; Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997), and cat scratching (Berkinblit et al., 1978; Deliagina et al., 1983). Selective activation of a subgroup of glutamatergic interneurons in the rostral hindlimb enlargement can evoke locomotor-like activity in neonatal mice (Hagglund et al., 2010; Hagglund et al., 2013).

Furthermore, a turtle swimming rhythm could be generated by pre-enlargement segments alone (Fig. 6). Similar results have been reported for turtle rostral and pocket scratching (Mortin and Stein, 1989; Currie and Gonsalves, 1997) and chicken embryo rhythmic activity (Ho and O'Donovan, 1993). These additional results suggest that the entire hindlimb enlargement may be unnecessary for locomotion or scratching rhythm generation in limbed vertebrates.

The D9-S2 segments are not necessary to maintain swim rhythm frequency

There was no consistent change in cycle frequency for either swimming or rostral scratching following separation of caudal enlargement segments. This provides additional support for the conclusion that key circuits in swimming and scratching rhythm generation are located in the rostral portion of and rostral to the hindlimb enlargement.

This result is similar to findings for cat scratching (Deliagina et al., 1983) but different from previous findings for turtle scratching (Mortin and Stein, 1989), chicken embryo rhythmic activity (Ho and O'Donovan, 1993), and mudpuppy locomotion (Wheatley et al., 1994), which reported or showed a figure with decreased rhythm frequency in the reduced preparation**.** However, I did observe a significant decrease in rhythm frequency in one animal and in pocket scratching overall. Previous rhythm frequency decreases were shown for just one animal for turtle scratching and chick rhythmic activity. Thus, the difference between my results and previous findings may be explained by inter-animal variability. This interpretation is supported by the substantially different distributions of swimming- or scratching-activated neurons among turtles (Mui et al., 2012) and also by inter-animal variation in cellular and synaptic properties of well-defined invertebrate networks (Marder, 2011)**.**

The D9-S2 segments are not necessary to maintain the HF-HE alternation during swimming

During rhythmic motor patterns, occasional deletions of an extensor or flexor burst with or without the quiescence of its antagonist have been reported for turtle scratching motor patterns (Stein and Grossman, 1980; Robertson and Stein, 1988; Stein and Daniels-McQueen, 2002; Stein and Daniels-McQueen, 2003; Stein and Daniels-McQueen, 2004; Stein, 2008) and cat scratching and locomotion (Lafreniere-Roula and McCrea, 2005; Rybak et al., 2006). In this study, I focused on HE-phase deletions, which were defined by the absence of HF quiescence between successive HF bursts (Stein, 2008). This indirect definition of HE-phase deletions was used because the loss

of HE motoneurons (Ruigrok and Crowe, 1984) and thus the diminished HE activity in the D3-D9 preparation made it impossible to rely on the presence or absence of HE bursts themselves. The reliability of continuing HF activity as the indicator of HE-phase deletions was confirmed by the recording of the OA nerve, which generated bursts in phase with HE during non-deletion swimming and scratching. I observed that the likelihood of HE-phase deletions increased with caudal spinal cord transections during scratching, as previously found (Mortin and Stein, 1989; Currie and Gonsalves, 1999)**.** During swimming, however, I rarely observed HE-phase deletions even in the D3-D8 preparation.

A shared network or separate networks for the HE phase?

One explanation for the difference in HE-phase deletions between swimming and scratching is that the swimming and scratching networks that generate the HE phase are separate. In support of partly separate networks, sensory input can reset or modify the locomotor and scratching rhythms differently in decerebrate cats (Frigon and Gossard, 2010). In turtles, a higher percentage of active neurons were found in rostral segments during swimming rhythms than during scratching rhythms (Mui et al., 2012). In addition, both extracellular and intracellular recordings have found scratching-specialized interneurons (Berkowitz, 2002; Berkowitz, 2008). Elimination of HE-phase scratching-specialized interneurons by separation of caudal segments could be a reason for the increasing occurrence of HE-phase deletions during scratching but not during swimming.

However, additional evidence suggests that completely separate networks for swimming and scratching are not plausible. In moving turtles, simultaneous activation of swimming and rostral scratching can evoke a hybrid behavior (Earhart and Stein, 2000a). In immobilized turtles, swim stimulation can reset a rostral scratching rhythm and vice versa (Juranek and Currie, 2000). Applying swim and scratch stimulation simultaneously can cause modifications of the motor patterns, including increased rhythm frequency, blends of swimming and scratching motor patterns, interruptions of the rhythm, and recruiting a swimming rhythm using otherwise subthreshold swim stimulation (Hao et al., 2011). In single-neuron studies, both extracellular and intracellular recording have shown that the majority of interneurons were activated during both swimming and scratching (Berkowitz, 2002; Berkowitz, 2005, 2008). Interneurons active during locomotion and scratching have also been found in cats (Geertsen et al., 2011).

A second explanation for the difference in HE-phase deletions following separation of caudal segments is that the remaining interneurons inhibiting HF motoneurons are sufficient to maintain the HE phase during swimming but not during scratching. Currie and Gonsalves found a greater likelihood of HE-phase deletions during rostral scratching than during pocket scratching following separation of caudal segments (Currie and Gonsalves, 1999). They proposed that HE-phase inhibitory neurons were more strongly activated during pocket scratching than rostral scratching. An expanded version of this hypothesis could account for the current findings. Swim stimulation might activate HE-phase inhibitory interneurons more strongly than either rostral

scratching or pocket scratching does, generating quiescence between HF bursts during swimming but not always during scratching.

A third explanation of the difference in HE-phase deletions in swimming vs. rostral and pocket scratching would be that the excitation of HF motoneurons is greater during rostral and pocket scratching than during swimming and cannot always be overcome by the remaining HE-phase inhibition (even if this inhibition is the same for swimming and scratching). During rostral scratching, HF typically has longer and stronger bursts than HE (Robertson et al., 1985; Stein and Daniels-McQueen, 2004; Stein, 2008). During pocket scratching, HF and HE bursts are similar in duration and amplitude (Robertson et al., 1985). During forward swimming, by contrast, HE typically has longer and stronger bursts than HF (Lennard and Stein, 1977; Juranek and Currie, 2000). Therefore, I suggest that: 1) during swimming, the HF motoneurons receive weak excitatory inputs from the excitatory HF premotor interneurons (Fig. 7A, solid green waveform); 2) during swimming, the HE motoneurons receive strong excitatory inputs from HE premotor interneurons (Fig. 7C, solid green waveform); and 3) during rostral scratching (Fig. 7B and D), the scenario is reversed. For simplicity in this diagram, I assume that inhibitory inputs are of the same amplitude during swimming and scratching (Fig. 7A and C, solid red waveform).

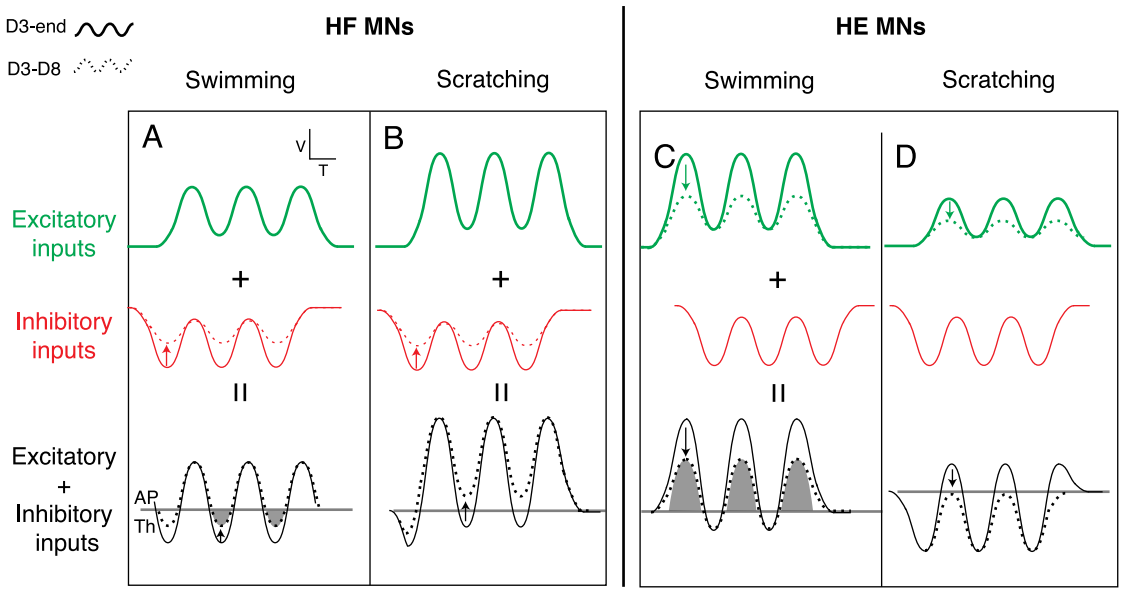

Figure 7. Schematic diagram showing one possible explanation of the difference in occurrence of HE-phase deletions between swimming and scratching after segment eliminations.

Black waveforms, the membrane potential oscillations achieved by a simple summation of excitatory (green) and inhibitory (red) rhythmic inputs. Gray horizontal lines, action potential threshold (AP Th). Dashed waveforms, membrane potential oscillations after the separation of the caudal portion of the hindlimb enlargement. Gray shading, remaining HE phase (in swimming but not scratching) after the separation of the caudal portion of the hindlimb enlargement. Arrows indicate the direction of change after the separation of the caudal portion of the hindlimb enlargement. Excitatory input to HF in the D3-end preparation is hypothesized to be weaker during swimming (green waveform in A) than during scratching (green waveform in B). Inhibitory input is shown as the same during swimming (red solid waveform in A) and scratching (red solid waveform in B) to simplify the scheme. In the D3-D8 preparation, the inhibitory inputs are hypothesized to be reduced to the same level for swimming (red dashed waveform in A) and scratching (red dashed waveform in B), which is still sufficient to periodically hyperpolarize below threshold the weak HF depolarizations during swimming but is not sufficient to hyperpolarize below threshold the strong HF depolarizations during rostral and pocket scratching. For HE motoneurons, the amplitudes of the excitatory (green waveforms in C and D) and inhibitory inputs (red waveforms in C and D) are reversed compared to the HF motoneurons. This results in reliable HE bursts during swimming but not scratching in the D3-D8 preparation.

Under this hypothesis, during non-deletion cycles, the rhythmic excitatory and inhibitory inputs cause the HF and HE motoneurons to fire rhythmically and alternately. After the separation of the caudal portion of the hindlimb enlargement, more of the excitatory HE premotor interneurons are likely separated compared with the excitatory HF premotor interneurons. This is based on the fact that the HE motor pool is more caudal than the HF motor pool (Ruigrok and Crowe, 1984) and the assumption that

excitatory premotor interneurons show a similar rostrocaudal distribution. The remaining inhibitory premotor inputs to HF (Fig. 7A and B, red dashed waveforms) are then sufficient to inhibit the HF motoneurons and sustain the HE phase during swimming (gray shadings), which features weak HF bursts. But the remaining inhibitory inputs are not sufficient to silence HF motoneurons during rostral scratching, which features stronger HF bursts. Similarly, the reduced excitatory HE premotor inputs (Fig. 7 C and D, green dashed waveforms) are sufficient to activate the HE motoneurons and sustain the HE phase during swimming (Fig. 7C, gray shading), which features strong HE activity. But these reduced excitatory inputs are not sufficient to excite the HE motoneurons during rostral scratching (Fig. 7D) because rostral scratching features weak HE activity.

My second and third explanations are not mutually exclusive and it is likely that separation of both inhibitory and excitatory inputs to motoneurons from caudal segments contribute to the relative increase in HE-phase deletions for rostral and pocket scratching but not forward swimming.

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Chapter 3: Strong interactions between spinal cord networks for locomotion and scratching

[The majority of this chapter has been published as: Hao Z-Z, Spardy LE, Nguyen EBL, Rubin JE, Berkowitz A (2011) Strong interactions between spinal cord networks for locomotion and scratching. J Neurophysiol 106:1766-1781. The published paper also contains computer simulations of three CPG models by Spardy, LE and Rubin, JE that are not included in this chapter.]

SUMMARY

Distinct rhythmic behaviors involving a common set of motoneurons and muscles can be generated by separate CNS networks, a single network, or partly overlapping networks in invertebrates. Less is known for vertebrates. Simultaneous activation of two networks can reveal overlap or interactions between them. The turtle spinal cord contains networks that generate locomotion as well as three forms of scratching (rostral, pocket, and caudal) with three different knee-hip synergies. Here, I report that in immobilized, spinal turtles, simultaneous stimulation for forward swimming and each form of scratching could 1) increase the rhythm frequency; 2) evoke switches, hybrids, and intermediate motor patterns; 3) reconstruct a swim motor pattern when the swim stimulation was subthreshold, and 4) disrupt rhythm generation entirely. The strength of swim stimulation could influence which effect was obtained. Thus, even pocket scratching and caudal scratching, which do not share a knee-hip synergy with forward swimming, can interact with swim stimulation to alter both rhythm and pattern generation. Collectively, these findings suggest that the spinal cord networks that

generate locomotion and scratching have important shared components or strong interactions between them.

INTRODUCTION

Animals perform a wide variety of behaviors with a limited number of neurons and muscles. Are different behaviors involving the same motoneurons and muscles generated by the same central nervous system (CNS) network or different networks? This question can conveniently be addressed for rhythmic behaviors, which are relatively simple and often generated by CNS networks.

Individual neuron recordings have shown that CNS neurons can be rhythmically activated during multiple rhythmic behaviors involving the same motoneurons (Morton and Chiel, 1994; Dickinson, 1995; Marder and Calabrese, 1996; Marder and Bucher, 2001; Briggman and Kristan, 2008; Kupfermann and Weiss, 2001). Vertebrate examples include tadpole and larval zebrafish swimming and struggling (Soffe, 1993; Li et al., 2007; Liao and Fetcho, 2008), turtle swimming and three forms of scratching (Berkowitz, 2010) and multiple mammalian respiratory rhythms (see references in (Berkowitz et al., 2010)). However, there are also behaviorally specialized neurons (Heitler, 1985; Ramirez and Pearson, 1988; Hennig, 1990; Jing and Weiss, 2001; Berkowitz, 2002; Li et al., 2007; Liao and Fetcho, 2008; Soffe et al., 2009). In some smaller nervous systems, recordings of each neuron involved can determine whether CNS networks generating different rhythmic behaviors are identical (Marder and

Bucher, 2001), completely separate (Heitler, 1985; Ramirez and Pearson, 1988; Hennig, 1990) or partly overlapping (Briggman and Kristan, 2008).

In larger nervous systems, however, individual interneuron recordings may be insufficient to demonstrate shared rhythm-generating networks. Another approach is to deliver stimuli for different motor patterns simultaneously and investigate interactions (Carter and Smith, 1986; Stein et al., 1986; Earhart and Stein, 2000a). I have taken this approach using the turtle spinal cord, which can generate locomotion (e.g., forward swimming) and three forms of scratching, each of which features rhythmic hip extensor and hip flexor alternation but is otherwise distinct. Many spinal cord interneurons are rhythmically activated during all the corresponding fictive motor patterns, but scratch-specific interneurons also exist (Berkowitz, 2010). Simultaneous stimuli for two forms of scratching can evoke hybrid motor patterns (Mortin et al., 1985; Stein et al., 1986), suggesting that the networks for the three forms of scratching have shared components.

The situation for scratching and swimming, however, is not clear. Simultaneous activation of rostral scratching and forward swimming, which share a knee-hip synergy, can evoke hybrids in moving animals (Earhart and Stein, 2000a). In immobilized animals, a brief rostral scratch stimulation can reset the rhythm of forward swimming and vice versa (Juranek and Currie, 2000), but prolonged simultaneous stimulation was not reported. Simultaneous stimulation for forward swimming and pocket or caudal scratching, each of which has a distinct knee-hip synergy, has also not been reported. Here, I investigated rhythmogenic network interactions by activating the forward

swimming network along with each scratch form's network in immobilized animals. Preliminary results from some of this work have been reported in abstracts (Nguyen and Berkowitz, 2007; Hao and Berkowitz, 2009; Hao et al., 2010).

METHODS

Animal preparations

Adult red-eared sliders (n = 19), *Trachemys scripta elegans,* of both sexes, weighing 300-900 g were placed in crushed ice for at least 2 h before surgery (Lennard and Stein, 1977). Animals were spinally transected between the dorsal 2 (D2) and D3 dorsal roots. Several muscle nerves on one side were dissected free for electroneurographic (ENG) recording: the hip flexor, ventral puboischiofemoralis internus, pars anteroventralis (HF), the hip extensor, flexor cruris, pars flexor tibialis internus (HE). and knee extensors (KE), triceps femoralis, pars iliotibialis (IT), pars ambiens (AM), and/or pars femorotibialis (FT) (Robertson et al., 1985). Only one of the knee extensors is shown in the figures unless other KEs provide extra information. After surgery, turtles were removed from the crushed ice and immobilized with gallamine triethiodide (8 mg/kg i.m.; Sigma-Aldrich, St. Louis, MO) and artificially respirated throughout the experiment. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma.

Stimulation procedures

Mechanical stimulation for fictive scratching was delivered to the receptive field of each scratch form using a glass probe with a fire-polished tip attached to a force

transducer (Grass Technologies/Astro-Med, West Warwick, R.I.). Fictive forward swim stimulation was delivered by electrical stimulation in the contralateral D3 lateral funiculus (0.1 ms, 10-1000 μA, bipolar pulses at 5-100 Hz) with a pair of 100-μm silver wires (California Fine Wire Company, Grover Beach, CA) insulated except at the tips, with one tip contacting the surface of the spinal cord and the other in the saline over the spinal cord. "Scratch/swim stimulation" refers to the combination of scratch and forward swim stimulation delivered at overlapping times.

ENG recordings

Exposed nerves were submerged in mineral oil surrounded by dental wax molded onto the turtle carapace. ENG recordings from each nerve were obtained using a pair of 100-μm silver wires. Amplified (1000 x) and filtered (band-pass 0.1-1.0 kHz, A-M Systems, Inc., Calsborg, WA) recordings were stored with a digital audio tape recorder (TEAC America, Montebello, CA).

Data analysis

ENG recordings were redigitized and analyzed off-line using Datapac software (Run Technologies, Laguna Hills, CA). Redigitized ENG recordings were then smoothed with a time constant of 50 ms. The onset and offset of each burst were determined in Datapac by positive- and negative-slope crossings using custom-selected thresholds. Cycle period was defined as the interval between two successive HF onsets. Burst duration was the interval between the onset and offset of a burst. Mean burst amplitude was also determined in Datapac. Other values were then calculated in Excel. Duty cycle

was the burst duration divided by the cycle period. Normalized burst amplitude was the mean amplitude of bursts of each nerve normalized to that nerve's mean for all scratching cycles of that form. Only cycles completely within the period of stimulation were analyzed. Cycles during a single-stimulation part of scratch/swim stimulations were not included in any quantitative analyses. Instead, cycles during scratch/swim stimulation were compared to single-stimulus scratch and swim control episodes.

Mean cycle frequency was the average of individual cycle frequencies, each of which was the reciprocal of the cycle period. For each form of stimulation, the same number of cycles from the beginning of stimulation was used for quantitative comparisons. All the episodes evoked using the same stimulation paradigm in that animal were included unless there were too few cycles in an episode.

The dual-referent phase values of KE and HE within the HF cycle were calculated as previously described (Berkowitz and Stein, 1994). Each HF cycle was divided into HF-on and HF-off phases. The onset of HF was assigned the phase value of 0.0 and 1.0 and the offset HF phase was assigned a phase value of 0.5. Other phase values were proportional within either the HF-on or the HF-off phase.

The non-parametric one-way ANOVA test (Kruskal–Wallis) and Dunn's test (Instat 3, GraphPad Software, Inc.) for selected-pair comparisons were performed to determine statistical significance.

RESULTS

Scratch and forward swim (henceforth, just "swim") stimulations were delivered at overlapping time periods (scratch/swim stimulation) in 19 animals. I observed a variety of motor patterns that differed from either pure-form scratching or pure-form swimming.

Mechanical stimulation in the receptive field of each scratch form (rostral, pocket, and caudal) alone evoked a pure-form scratching motor pattern. Electrical stimulation in the contralateral lateral funiculus (cLF) in the D3 segment elicited a pure-form swimming motor pattern (see Methods). All of these motor patterns were characterized by rhythmic alternation between hip flexor (HF) and hip extensor (HE) bursts. However, each motor pattern had a distinct set of nerve burst amplitudes and phases. During pure-form rostral scratching, HF bursts were stronger and longer than HE bursts (HF > HE) and knee extensor (KE) activity occurred during the latter portion of each HF burst (Fig. 8A).

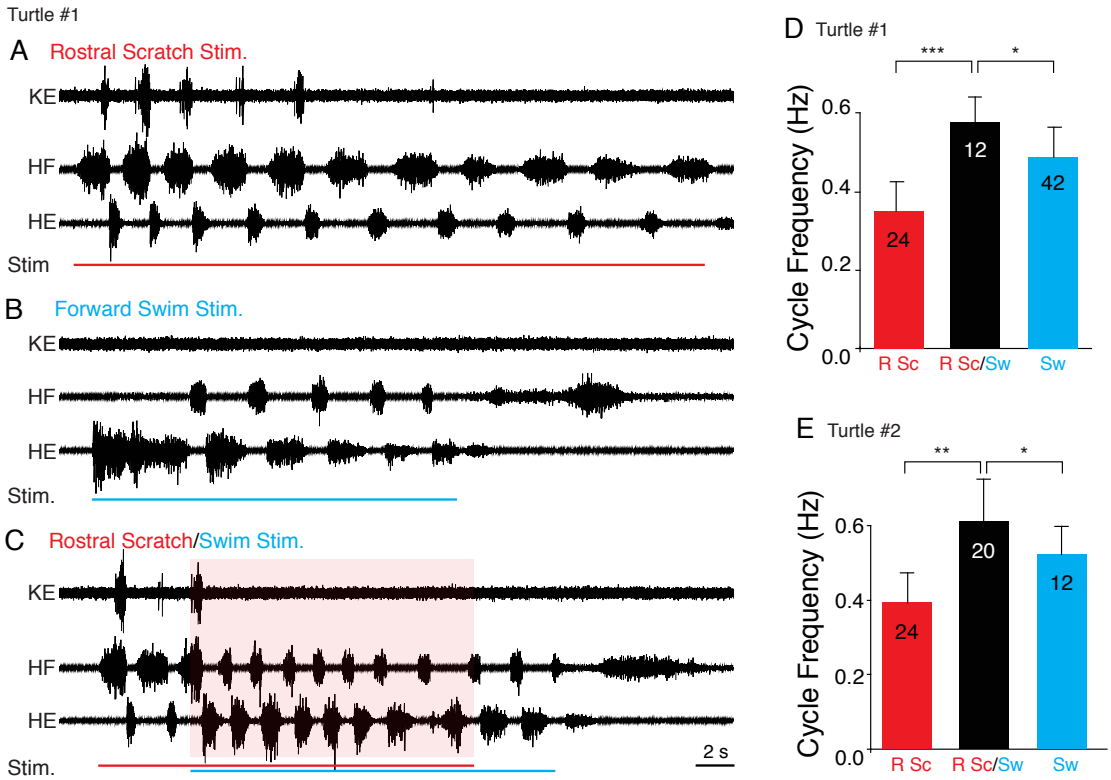

Figure 8. Rostral scratch/swim dual stimulation increased cycle frequency.
A-C, fictive motor patterns recorded from three hindlimb nerves (KE, knee extensor; HF, hip flexor; HE, hip extensor). A, Rostral scratching evoked by mechanical stimulation of ipsilateral shell bridge (SP1 site; stimulation period indicated by red bar). B, fictive swimming evoked by current pulses (300 μA, 40 Hz, blue bar) in dorsal (D) 3 contralateral lateral funiculus (cLF). C, motor pattern during rostral scratch/swim stimulation (red and blue bars). During dual stimulation (red shaded area), cycle frequency was higher than during either rostral scratch stimulation alone or swim stimulation alone. D, quantitative summary of all the episodes from this animal with the same stimulation parameters. E, summary of a second animal that showed a significant frequency increase during rostral scratch/swim dual stimulation. Error bars, standard deviation; number in each bar, number of cycles; *, $p<0.05$; **, $p<0.01$; *** $p<0.001$, using Dunn's test; R Sc, rostral scratch; R Sc/Sw, rostral scratch/swim dual stimulation; Sw, forward swim.

During pure-form pocket scratching, the amplitude and duration of HF and HE were similar and KE activity lasted from late in the HF burst to early in the HE burst (Fig. 9A).

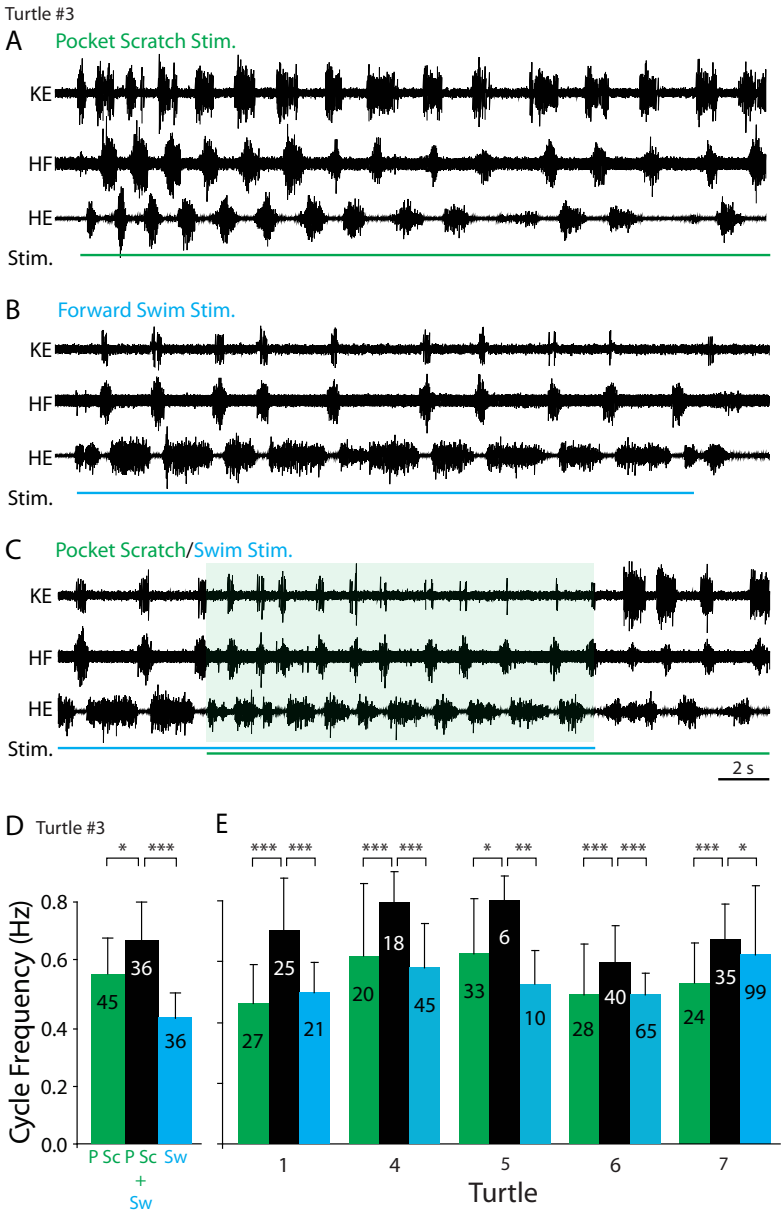

Figure 9. Pocket scratch/swim dual stimulation increased cycle frequency.
A, fictive pocket scratching evoked by mechanical stimulation of ipsilateral hindlimb femoral plate (stimulation period indicated by green bar). B, fictive swimming evoked by current pulses (400 μA, 40 Hz, blue bar) at cLF. C, motor pattern during pocket scratch/swim stimulation (green and blue bars). During dual stimulation (green shaded area), cycle frequency was higher than during either pocket scratch stimulation or swim stimulation alone. D, quantitative summary from all the episodes of this animal with the same stimulation parameters. E, summary of all the other animals that showed significant frequency increases during dual stimulation. P Sc, pocket scratch; P Sc + Sw, pocket scratch/swim dual stimulation; other symbols and abbreviations as in Figure 8.

During pure-form caudal scratching, HF bursts were weaker and shorter than HE bursts (HF < HE) and KE activity was present from the end of HE bursts until the beginning of HF bursts (Fig. 10A). During pure-form swimming (Figs. 8B, 9B and 10B), HF < HE (which differentiated swimming from rostral scratching) and KE bursts occurred at the end of HF bursts (which differentiated swimming from pocket and caudal scratching (Lennard and Stein, 1977; Robertson et al., 1985; Juranek and Currie, 2000; Stein, 2005).

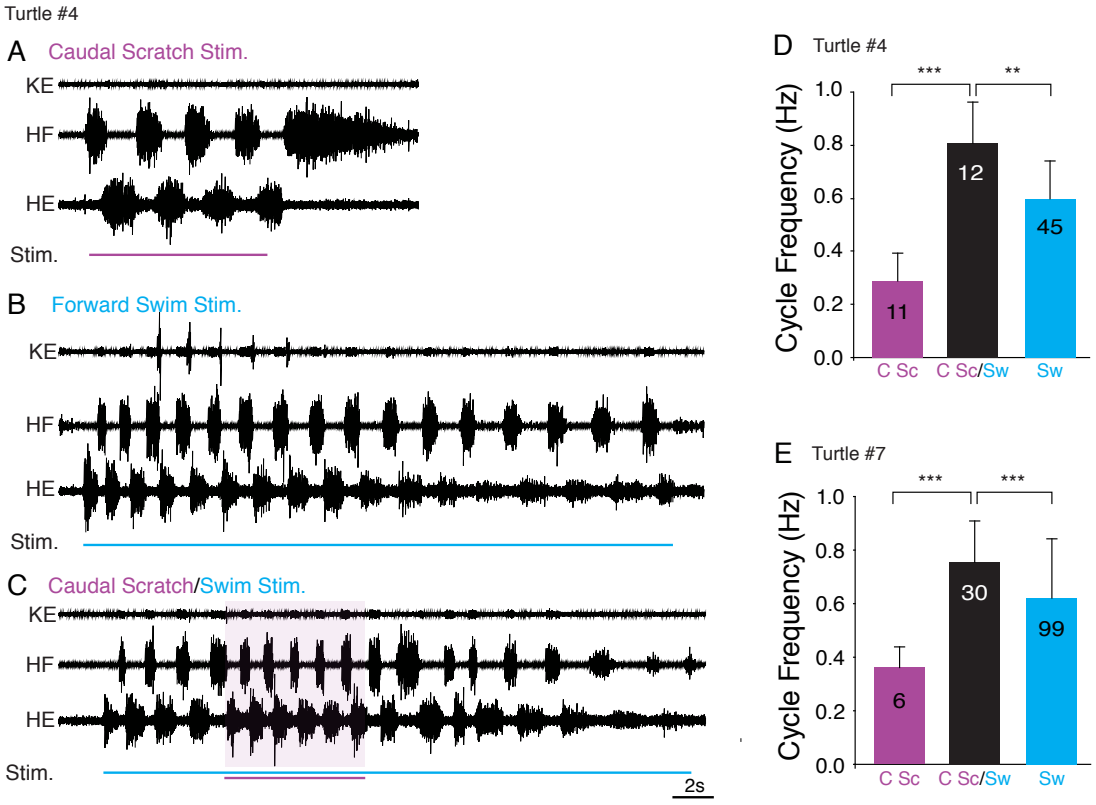

Figure 10. Caudal scratch/swim dual stimulation increased cycle frequency.
A, fictive caudal scratching evoked by mechanical stimulation of ipsilateral anal plate (stimulation period indicated by purple bar). B, fictive swimming evoked by current pulses (250 µA, 40 Hz, blue bar) at cLF. C, motor pattern during caudal scratch/swim stimulation (purple and blue bars). D, quantitative summary of this animal. E, summary of another animal that showed a significant frequency increase during caudal scratch/swim dual stimulation. C Sc, caudal scratch; C Sc/Sw, caudal scratch/swim dual stimulation; other symbols and abbreviations as in Figure 8.

Faster rhythms

Scratch/swim stimulation could significantly increase the frequency (i.e., decrease the cycle period) of resulting motor patterns. Cycle frequency during dual stimulation was higher than during scratch stimulation alone and swim stimulation alone for rostral scratch/swim (n = 3 animals), pocket scratch/swim (n = 6), and caudal scratch/swim stimulation (n = 2). Figure 8 shows an example of rostral scratch/swim stimulation that increased the cycle frequency. Figures 9 and 10, respectively, show increased cycle frequency evoked by pocket scratch/swim and caudal scratch/swim stimulation. In most cases, when faster rhythms were elicited, the motor patterns were swim-like (rostral: 2/3 animals, pocket: 5/6, caudal: 2/2). But I did observe intermediate motor patterns (during rostral scratch/swim stimulation, see below, Fig. 16) and scratch-like motor patterns (during pocket scratch/swim stimulation, data not shown) with increased cycle frequency.

Reduced cycle periods had briefer HE and HF bursts

To determine which components of the motor pattern caused the decreased cycle period during dual stimulation, I compared HF and HE burst durations during dual stimulation with those during scratch stimulation alone and swim stimulation alone. Neither HF nor HE burst durations were significantly longer during dual stimulation in any animal. For 5 out of 7 animals (except turtle # 5, HF, pocket scratch/swim and turtle #7, HF, caudal scratch/swim), HF and HE burst durations were each significantly briefer during dual stimulation than during at least one kind of single stimulation (scratch or swim). In several cases, burst duration was significantly briefer than during both scratch

stimulation alone and swim stimulation alone (HF: pocket, n = 4/6 animals; caudal, n = 1/2 animals; HE: pocket, n = 1/6 animals, caudal, n = 1/2 animals).

Cycle period was affected more by HE than HF duration within each stimulation paradigm

Although the reduced cycle periods often evoked by dual stimulation could be caused by significant shortening of HF or HE or both, within each stimulation paradigm (i.e., scratch, swim, or scratch/swim stimulation), HE burst duration usually changed with cycle period more than HF burst duration (Fig. 11, individual animals; Fig. 12, trends for all animals showing faster rhythm with dual stimulation). Also, the best-fit lines during dual stimulation (black lines) and swimming (blue lines) were clustered together and were different from those during scratching. This is consistent with the observation that faster rhythms during dual stimulation were mostly swim-like.

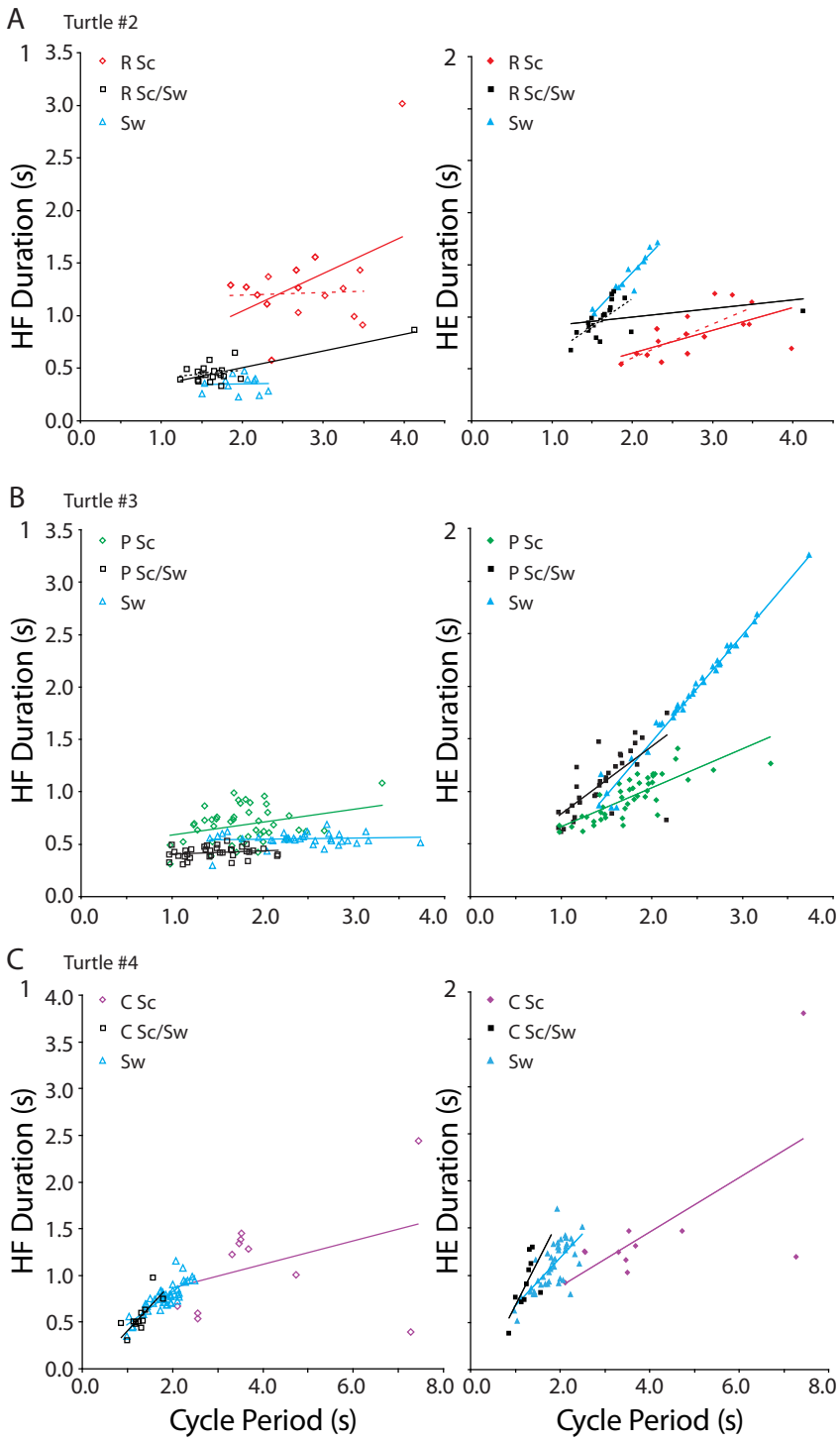

Figure 11. HF burst duration does not change with cycle period as much as HE burst duration for swim or scratch/swim stimulation.

A1-C1, HF burst duration as a function of cycle period. A2-C2, HE burst duration as a function of cycle period for the same episodes as in A1-C1, respectively. A, rostral scratch and swim; B, pocket scratch and swim; C, caudal scratch and swim. Color-coding and abbreviations are the same as in previous figures. Solid lines in A-C, best fit lines to all cycles. Dashed lines in A1 and A2, best-fit lines after deleting 2 outliers (1 for rostral scratch; 1 for dual stimulation) on the far right of A1.

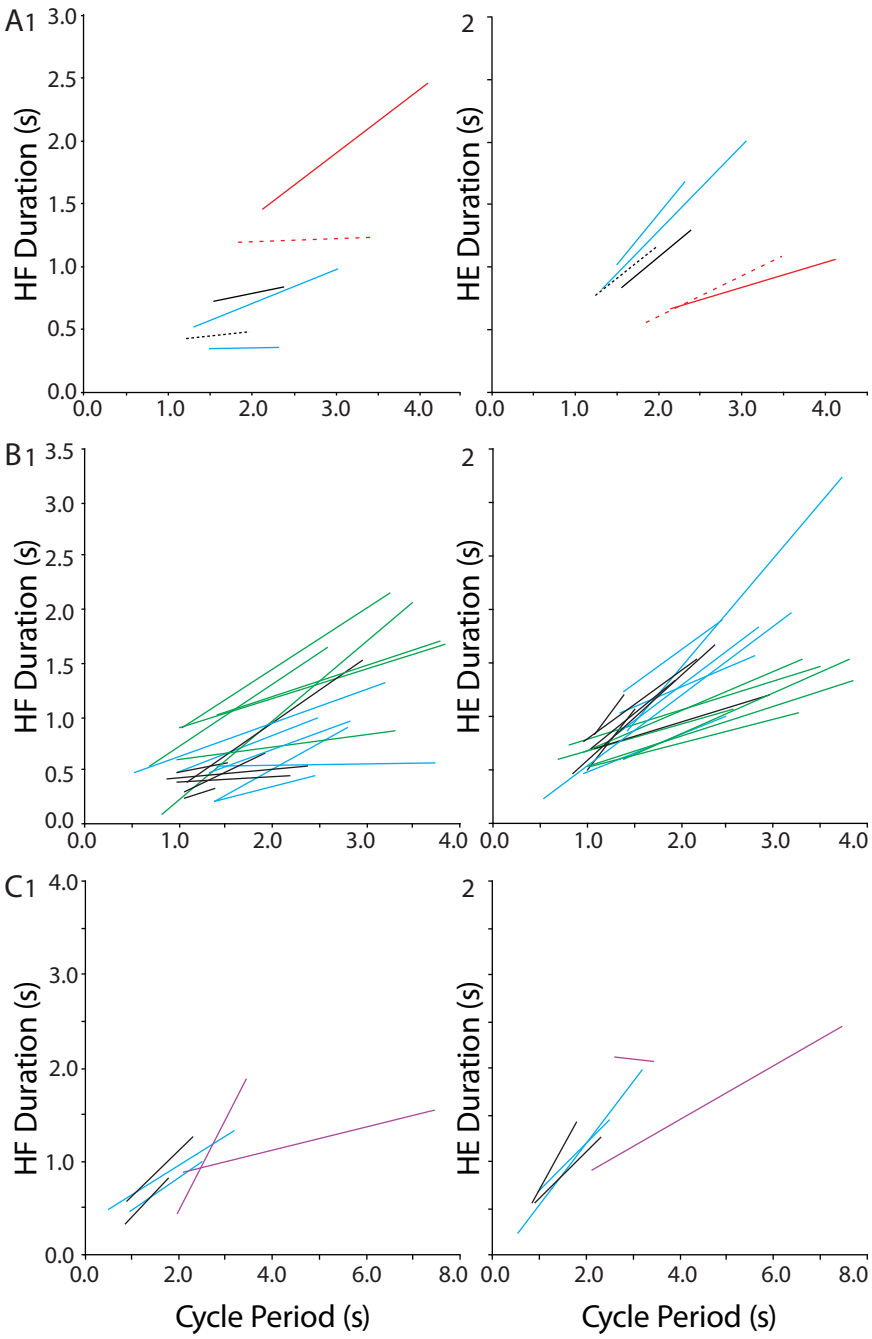

Figure 12. HF burst duration does not change with cycle period as much as HE burst duration for all animals showing increased cycle frequency with dual stimulation.

Best-fit lines only are shown. A1-A2, rostral scratch + swim stimulation (n = 2 animals). B1-B2, pocket scratch + swim stimulation (n = 6). C1-C2, caudal scratch and swim stimulation (n = 2).

Motor pattern blends

Scratch/swim stimulation could evoke motor patterns in which one or more cycles differed from pure-form scratching and pure-form swimming. Simultaneous stimulation in two scratch receptive fields or stimulation in a scratch transition zone can evoke blends of two scratch motor patterns, which can be either switches (when the scratch form changes on a cycle-by-cycle basis) or hybrids (when each cycle includes characteristics of both scratch forms (Mortin et al., 1985; Robertson et al., 1985; Stein et al., 1986). Simultaneous forward swim stimulation and rostral scratch stimulation in moving animals can also elicit hybrid motor patterns (Earhart and Stein, 2000a).

Switches and hybrids between fictive swimming and each of the three forms of fictive scratching were observed in my experiments. Figure 13 is an example of a rostral scratch/swim switch. The first two cycles during the dual stimulation were rostral scratch-like (HF > HE). Then the motor pattern switched to a swim-like motor pattern (HF < HE).

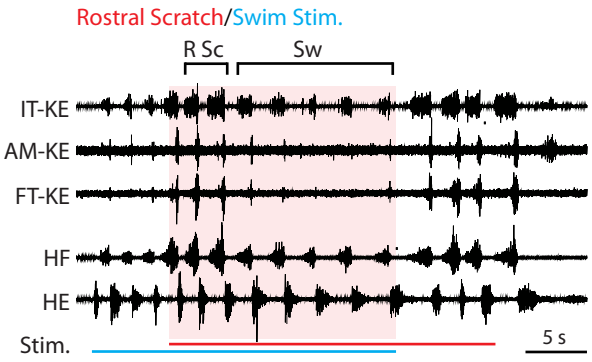

Figure 13. Example of a sudden motor pattern switch from rostral scratch-like (bracket marked R Sc) to swim-like (bracket marked Sw) during rostral scratch/swim dual stimulation (from turtle #6).

Figure 14 shows a pocket scratch/swim switch. In this case, the motor pattern gradually changed over several cycles from scratch-like to swim-like; once the swim stimulation ended, the motor pattern immediately changed back to scratch. Switch patterns were observed during rostral scratch/swim (n = 6 animals), pocket scratch/swim (n = 6), and caudal scratch/swim (n = 1, data not shown) stimulation.

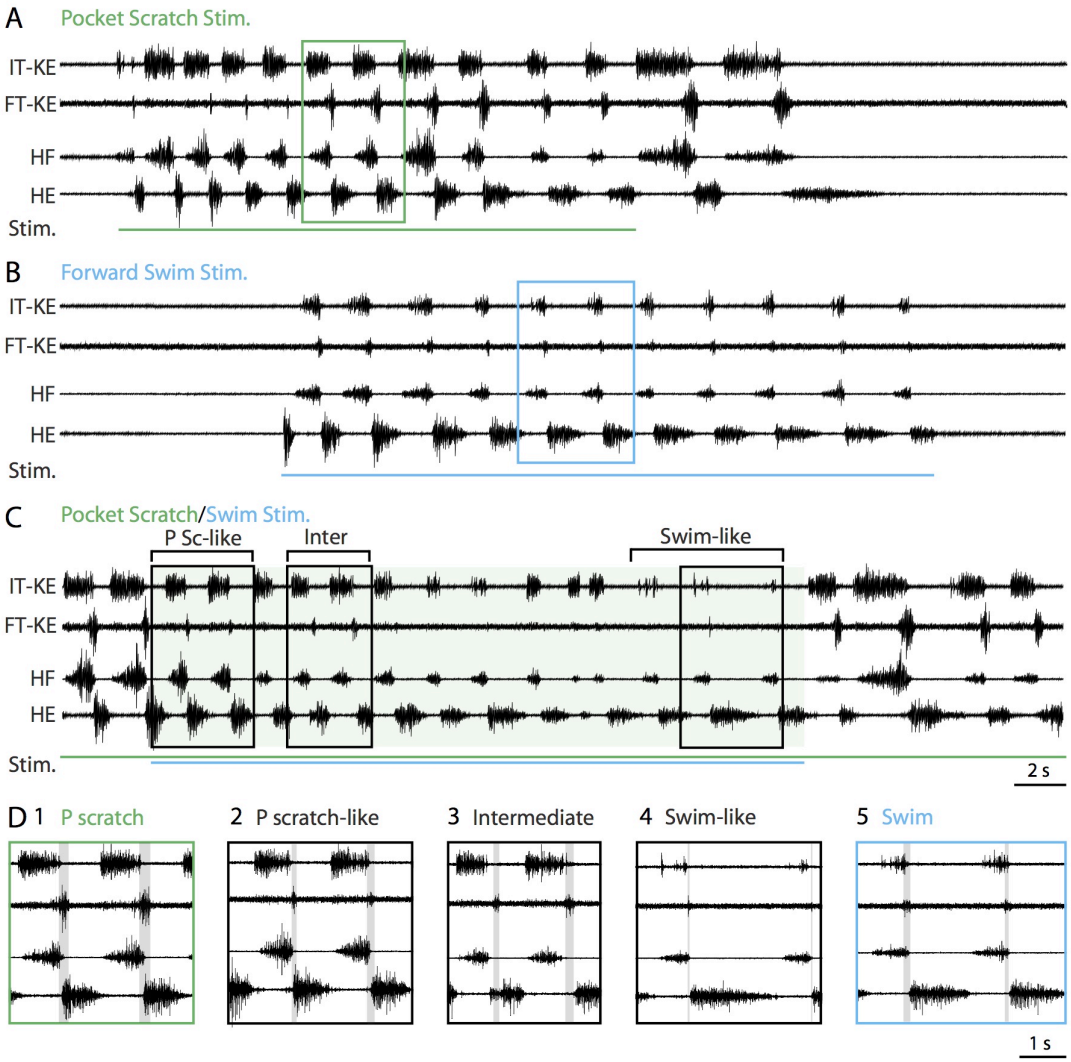

Figure 14. Example of a gradual motor pattern switch from pocket scratch-like to swim-like (from turtle #6).

A, pocket scratch stimulation alone. B, swim stimulation alone. C, pocket scratch/swim stimulation. At the beginning of the dual stimulation, the motor pattern was pocket scratch-like (bracket marked with P Sc-like). In the middle, the motor pattern was intermediate (Inter) between pocket scratching and swimming. At the end of the dual stimulation, the motor pattern was swim-like (Swim-like). D, time expansion of motor patterns indicated by boxes in A (1), C (2-4), and B (5). Gray shading indicates the duration of FT-KE bursts.

Hybrids of scratching and swimming were also observed during pocket scratch/swim stimulation (n = 1) and caudal scratch/swim stimulation (n = 3). Figure 15 shows a hybrid motor pattern between caudal scratching and swimming. During caudal scratch/swim dual stimulation, KE bursts included the normal KE phases of both caudal scratching and swimming, i.e., the onset of KE bursts was at the beginning of the HF burst (as during caudal scratching) and the offset was at the end of the HF burst (as during swimming). I also observed two KE bursts within each HF cycle (one caudal scratch-like and one swim-like) in another animal (data not shown).

Intermediate HF and HE amplitudes and durations were also observed during rostral scratch/swim stimulation (Fig. 16). The episode began with forward swim stimulation alone, continued with dual stimulation, and ended with rostral scratch stimulation alone. During the dual stimulation, the duty cycle (i.e., the percentage of each cycle during which the nerve was active) for HF and HE was intermediate between pure-form rostral scratching (HF > HE), and swimming (HF < HE). Also, the HF and KE burst mean amplitudes were significantly less than for rostral scratching (but not significantly different from swimming). The phase of the KE burst within the HF cycle did not differ significantly among swim stimulation alone, rostral scratch stimulation alone, and dual stimulation, except the offset phase of HF. I also observed intermediate motor patterns evoked by rostral scratch/swim stimulation in five other animals (data not shown).

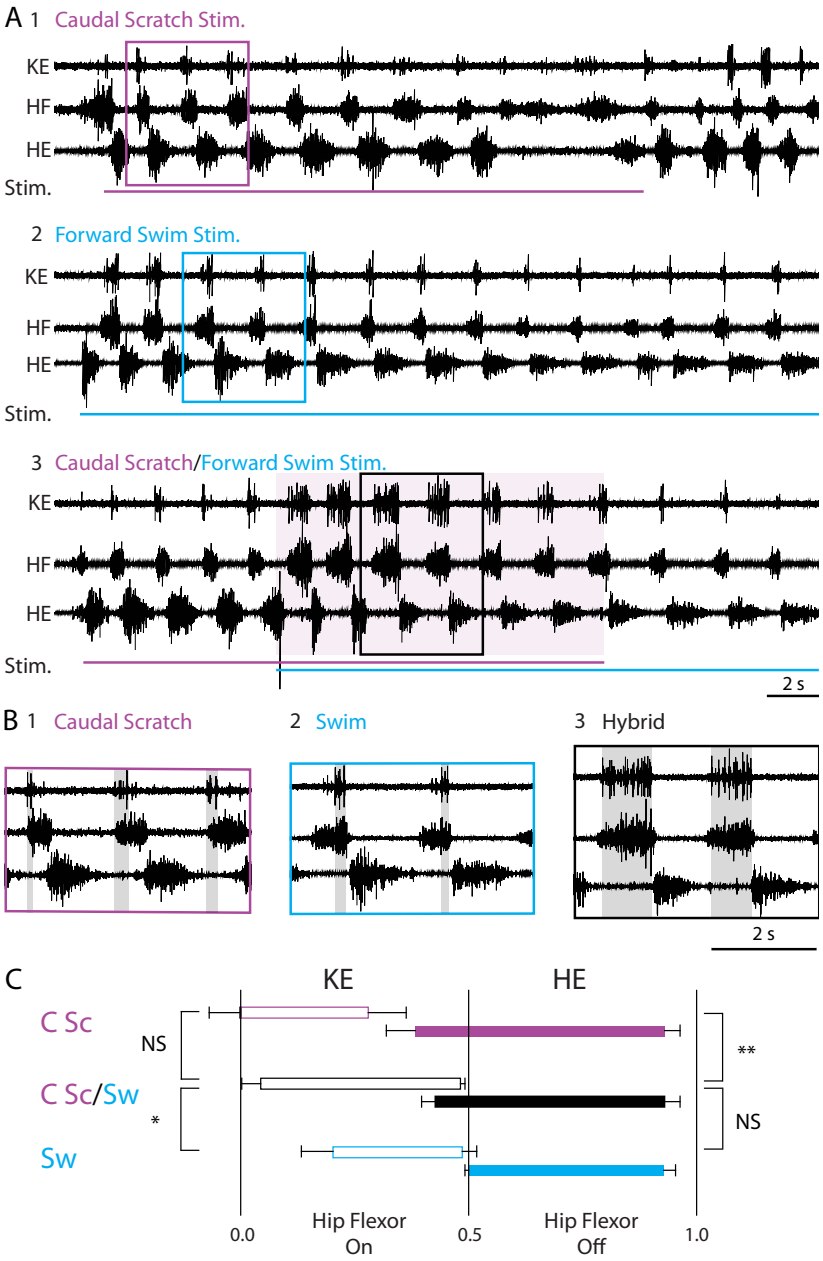

Figure 15. Example of a hybrid motor pattern between caudal scratching and swimming (from turtle #6).

A1-3, motor patterns during caudal scratch, swim (150 μA, 40 Hz), and caudal scratch/swim stimulation, respectively. B1-3, time expansion of the motor patterns indicated by color-coded boxes in A1-3. Gray shading indicates the KE burst durations. C, mean (± SD) onsets and offsets of KE (open bars) and HE (filled bars) bursts with respect to the onset and offset of HF bursts during single and dual stimulation. Statistical results for KE onset and offset changes are indicated (*, $p<0.05$; **, $p<0.01$, NS, not significant; using Dunn's test). Neither the HE onset phase nor the HE offset phase changed significantly, except the HE onset between caudal scratch/swim and swim ($p<0.01$).

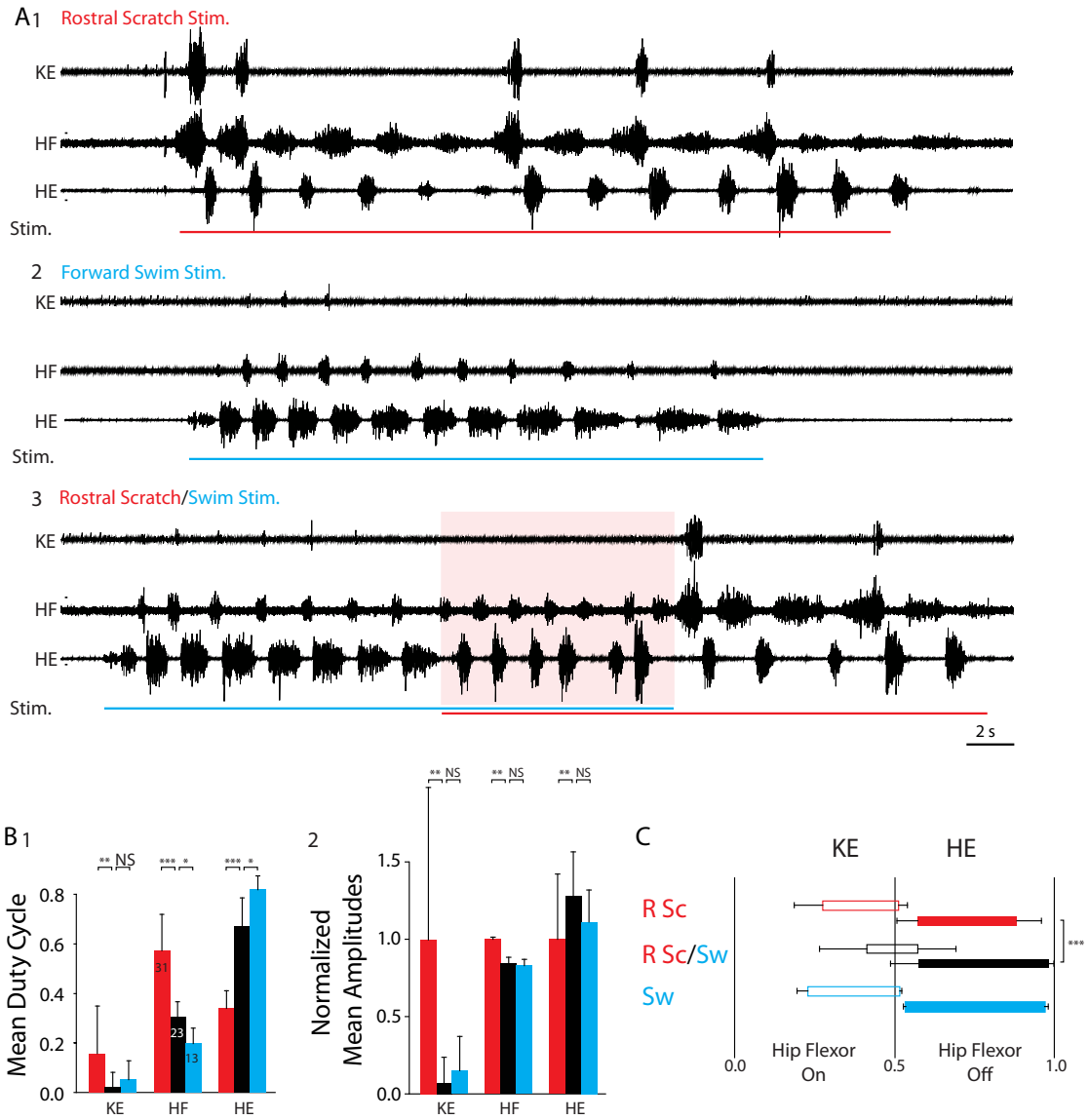

Figure 16. Example of an intermediate motor pattern between rostral scratching and swimming (from turtle #2).

A1-3, motor patterns during rostral scratch, swim (190 μA, 40 Hz), and rostral scratch/swim stimulation, respectively. B, quantitative summary of duty cycles (B1) and amplitudes (B2) of KE, HF and HE for all the episodes from this animal with the same stimulation parameters. C, mean (± SD) onsets and offsets of KE (open bars) and HE (filled bars) bursts with respect to the onset and offset of HF bursts during single and dual stimulation (not significant except as indicated). Red bars and R Sc, rostral scratch; black bars and R Sc/Sw, rostral scratch/swim stimulation; blue bars and Sw, swim.

Scratch stimulation could reconstruct the swim motor pattern when added to inadequate swim stimulation

In some cases, subthreshold swim stimulation (which evoked no response or tonic HE activity, n = 3 animals) or overly strong swim stimulation (which evoked tonic HE activity, n = 3) combined with scratch stimulation evoked a normal swim-like HF-HE alternation and knee-hip synergy. I call this a "reconstructed" swim motor pattern, by analogy to reconstructed scratch motor patterns evoked using dual stimulation in lesioned animals (Stein et al., 1998). Figure 17 shows an example of rostral scratch/subthreshold swim stimulation that evoked a swim-like motor pattern.

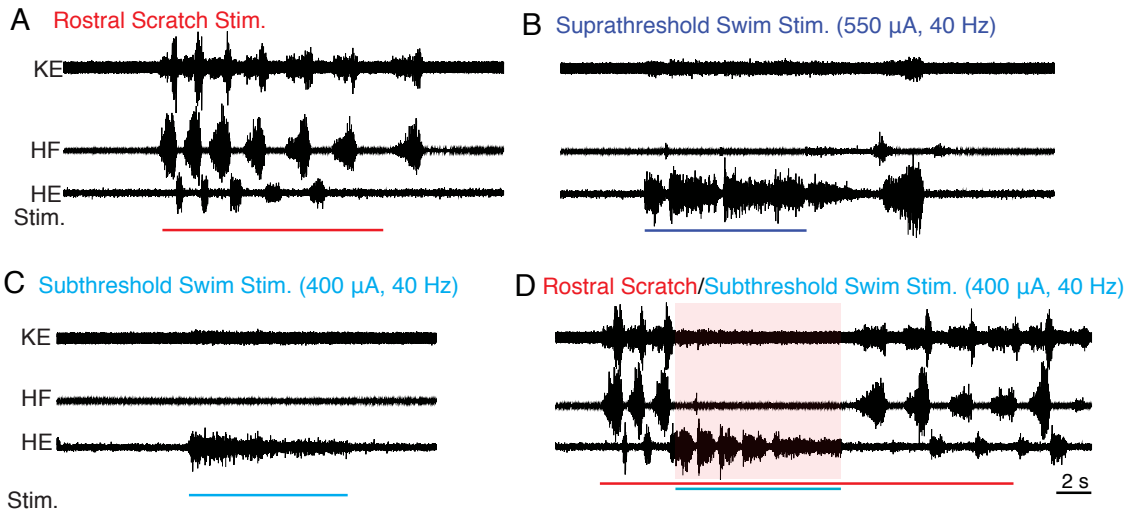

Figure 17. Example of rostral scratch stimulation reconstructing the swim motor pattern when added to subthreshold swim stimulation (from turtle #5).
A, rostral scratching (HF/HE alternation, HF > HE) evoked by rostral scratch stimulation. B, normal swimming (HF/HE alternation, HF < HE) evoked by suprathreshold swim stimulation (550 µA, 40 Hz, dark blue bar). C, tonic HE activity evoked by subthreshold swim stimulation (400 µA, 40 Hz, light blue bar). D, motor pattern during rostral scratch/subthreshold swim stimulation; during dual stimulation (red shading), the motor pattern was swim-like (HF/HE alternation, HF < HE).

The subthreshold swim stimulation alone evoked tonic HE activity instead of HF-HE alternation (Fig. 17C). The same swim stimulation combined with rostral scratch stimulation, however, led to HF-HE alternation with HF < HE (Fig. 17D), similar to

pure-form swimming in the same animal evoked by suprathreshold swim stimulation (Fig. 17B), but different from rostral scratching (Fig. 17A). When each form of scratch stimulation was added to overly strong swim stimulation (Fig. 18), a swim-like motor pattern could also be reconstructed with HF < HE and each KE burst at the end of an HF burst.

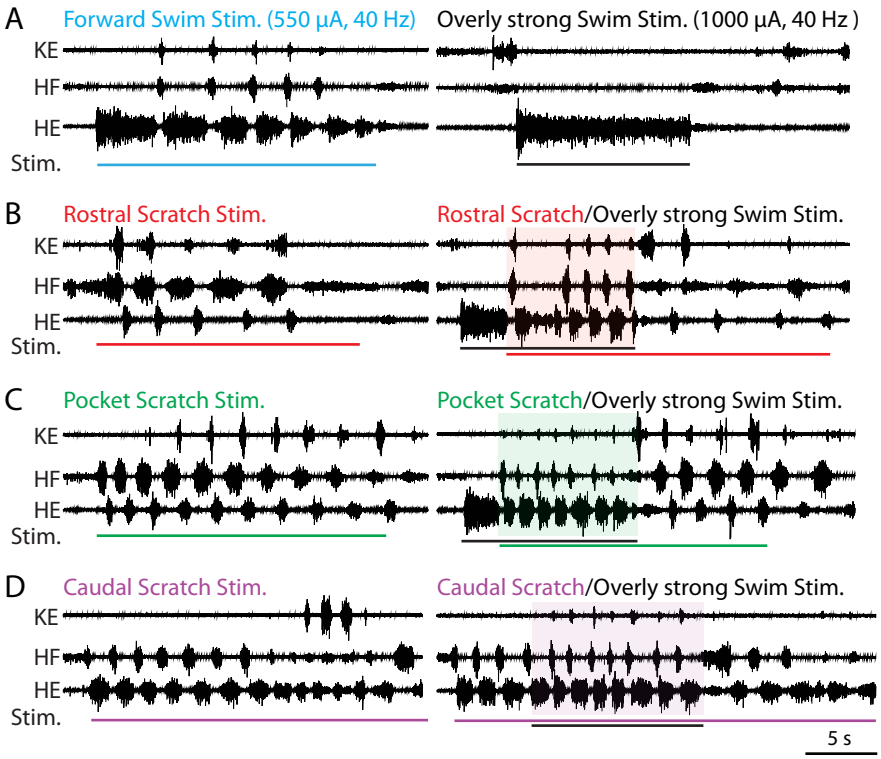

Figure 18. Example of scratch stimulation reconstructing the swim motor pattern when added to overly strong swim stimulation (from turtle #1).

A-D, left panel, normal swimming (550 µA, 40 Hz, light blue bar) and three forms of scratching. A, right panel, tonic HE activity during overly strong swim stimulation (1000 µA, 40 Hz, black bar). B-D, right panel, motor patterns during scratch/overly strong swim stimulation. During the dual stimulation (shaded areas), the motor patterns are swim-like (HF/HE alternation, HF<HE, KE at the end of HF).

Disruptions

In some cases, I observed that dual stimulation disrupted an ongoing rhythm, although the same scratch or swim stimulation alone elicited rhythmic pure-form scratching or swimming, respectively (rostral scratch/swim, n = 1; pocket scratch/swim, n = 4). Figure 19 shows several examples of disrupted rhythms during pocket scratch/swim

stimulation. When the swim stimulation was delivered during an ongoing pocket scratch rhythm, the HF bursts ceased while HE bursts continued rhythmically (i.e., there were HF deletions; pink asterisks in Fig. 19, C-D). After several HE cycles, the HF-HE alternation was restored to a swim-like rhythm. When the swim stimulation was stopped while pocket scratch stimulation was maintained, the motor pattern became pocket scratch-like. In another trial with the same dual stimulation paradigm, both HF and HE bursts ceased after two HF-HE cycles (i.e., rhythm cessation, pink bars in Fig. 19, D-E). A pocket scratch-like motor pattern reappeared immediately after swim stimulation was stopped. Both HF deletions and complete rhythm cessation could occur in the same trial (Fig. 19E). During the period of dual stimulation, HF-HE alternation persisted for one cycle, then there were two HF deletion cycles, then there was a complete shutdown of the rhythm. The rhythm was restored immediately after the swim stimulation was stopped. In most cases (n = 4 animals), rhythm cessation was observed only when the swim stimulation was delivered during ongoing scratch stimulation, not the reverse. In one animal, rhythm cessation was observed with either order of stimulation (i.e., pocket scratch stimulation during ongoing swim stimulation, or vice versa).

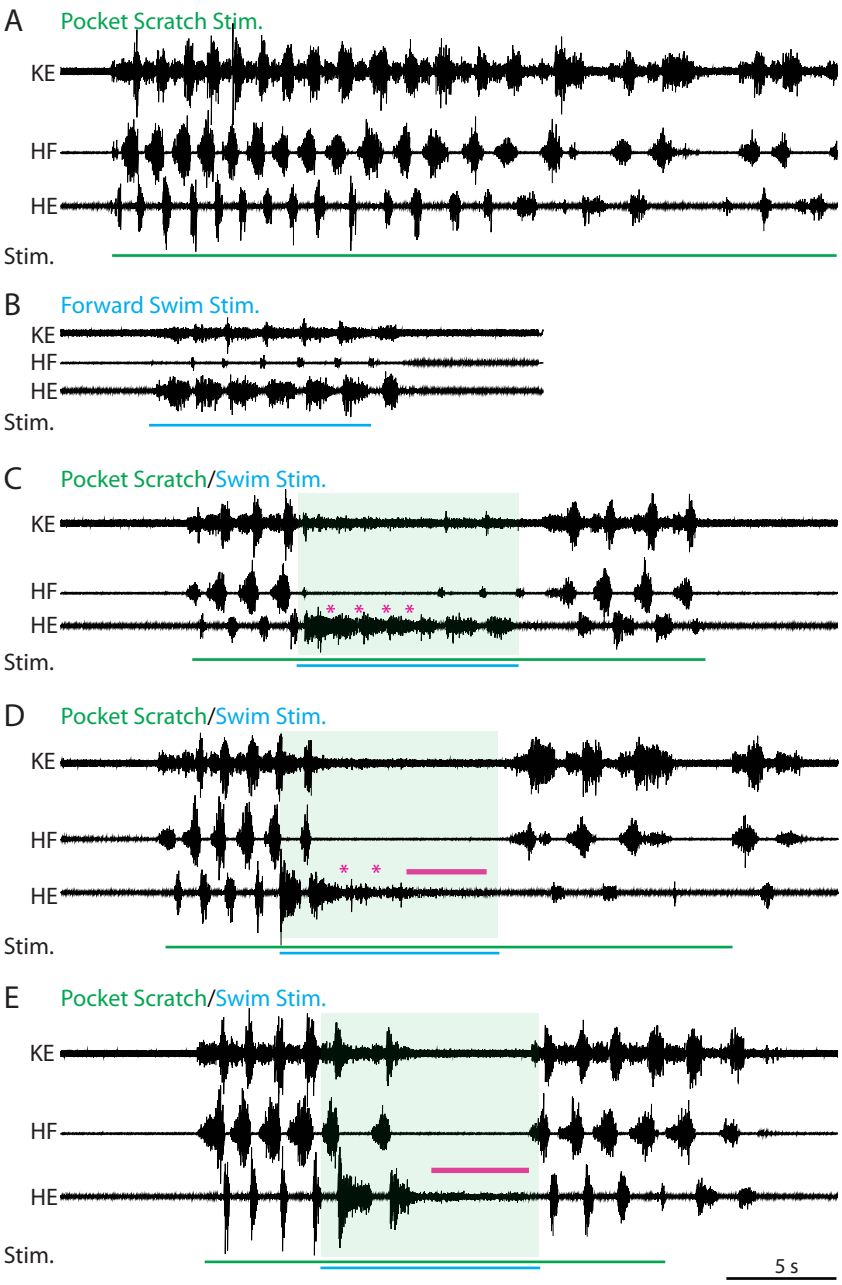

Figure 19. Example of pocket scratch/swim dual stimulation disrupting an ongoing rhythm (from turtle #5).

A, Motor pattern during pocket scratch stimulation. B, Motor pattern during swim stimulation. C-E, Motor pattern during pocket scratch/swim stimulation. During the dual stimulation (green shading), HF bursts could cease while the HE rhythm continued (i.e., HF deletions, pink asterisks, C-D). The rhythm could also cease completely (pink bars,D-E). In all cases, the rhythm was restored immediately after the swim stimulation was stopped.

Swim stimulation parameters could influence the effect of dual stimulation

To explore possible reasons for the varying effects of dual stimulation, I systematically adjusted the swim stimulation frequency and repeated the experiment in three animals. Different swim stimulation pulse frequencies combined with scratch stimulation could evoke different results of the types described above. In one animal, altering swim stimulation could produce frequency increases or swim reconstruction (Fig. 20). When the swim stimulation was very weak (400 μA, 5 Hz, no response; Fig. 20A, left), rostral scratch/swim stimulation evoked a rostral scratch-like motor pattern (Fig. 20A, right). With somewhat stronger subthreshold swim stimulation (300-400 μA, 10 Hz, no response; Fig. 20B, left), adding rostral scratch stimulation evoked HF-HE alternation with HF < HE, i.e., a reconstructed swim-like motor pattern (Fig. 20B, right). When this swim stimulation was stopped while the rostral scratch stimulation was maintained, the motor patterns were rostral scratch-like with HF > HE. Adding rostral scratch stimulation to suprathreshold swim stimulation (400 μA, 30 Hz) evoked a swim-like pattern with a cycle frequency that was higher than with swim or scratch stimulation alone (Fig. 20C). When overly strong swim stimulation (400 μA, 60 Hz) was delivered alone, I observed tonic HE activity and when this was combined with rostral scratch stimulation, I failed to restore the rhythm. I thus reduced the swim stimulation amplitude, while I kept stimulation frequency the same. This adjusted swim stimulation (300 μA, 60 Hz, which evoked tonic HE activity; Fig. 20D, left) combined with rostral scratch stimulation produced a swim-like motor pattern with HF < HE (Fig. 20D, right).

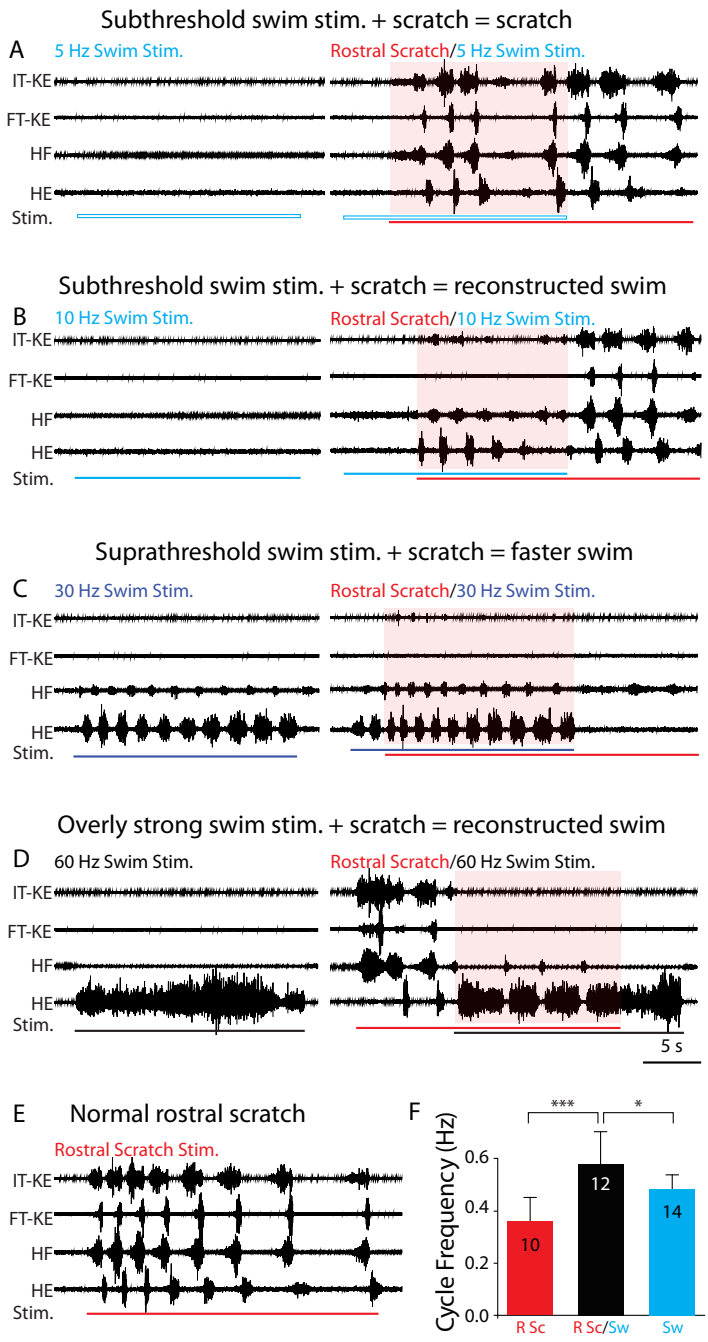

Figure 20. Swim stimulation parameters could influence the effect of rostral scratch/swim stimulation (from turtle #8).

A, Rostral scratch stimulation added to a subthreshold swim stimulation (400 µA, 5 Hz, open blue bar, left panel) evoked a rostral scratch-like motor pattern (right panel, HF > HE). B, Rostral scratch stimulation added to a stronger, but still subthreshold swim stimulation (400 µA, 10 Hz, light blue bar, left panel) evoked a swim-like motor pattern (right panel, HF < HE). C, Rostral scratch stimulation added to a suprathreshold swim stimulation (400 µA, 30 Hz, dark blue bar, left panel) evoked a swim-like pattern with increased cycle frequency (right panel, HF < HE; statistical analysis shown in F). D, Rostral scratch stimulation added to overly strong swim stimulation (300 µA, 60 Hz, black bar, left panel) reconstructed the swim rhythm (HF < HE). E, Motor pattern during rostral scratch stimulation alone (HF > HE). F, Quantitative summary of all the episodes from this animal with the same stimulation parameters as in C.

In another animal (Fig. 21), very weak swim stimulation (300 μA, 5 Hz, no response) combined with pocket scratch stimulation evoked a scratch-like motor pattern (Fig. 21A). Somewhat stronger swim stimulation (300 μA, 10-20 Hz) combined with pocket scratch stimulation could evoke a variety of results, including swim-like motor patterns, pocket scratch-like motor patterns, switches, and rhythm disruptions (Fig. 21B-C). Suprathreshold swim stimulation (300 μA, 40 Hz) combined with pocket scratch stimulation evoked a swim-like motor pattern that was significantly faster than with swim stimulation alone (Fig. 21D). Overly strong swim stimulation (300 μA, 50 Hz, which evoked tonic HE activity), combined with pocket scratch stimulation evoked a normal swim-like motor pattern (Fig. 21E). When the swim stimulation was still stronger (300 μA, 60 Hz), HE tonic activity persisted even when pocket scratch stimulation was delivered (data not shown).

In summary, scratch stimulation combined with weak swim stimulation evoked scratch-like motor patterns; scratch stimulation combined with subthreshold swim stimulation sometimes evoked swim-like motor patterns; scratch stimulation combined with near-threshold swim stimulation evoked modified motor patterns, such as switches, hybrids and intermediate motor patterns; scratch stimulation combined with suprathreshold swim stimulation usually produced faster swim-like motor patterns.

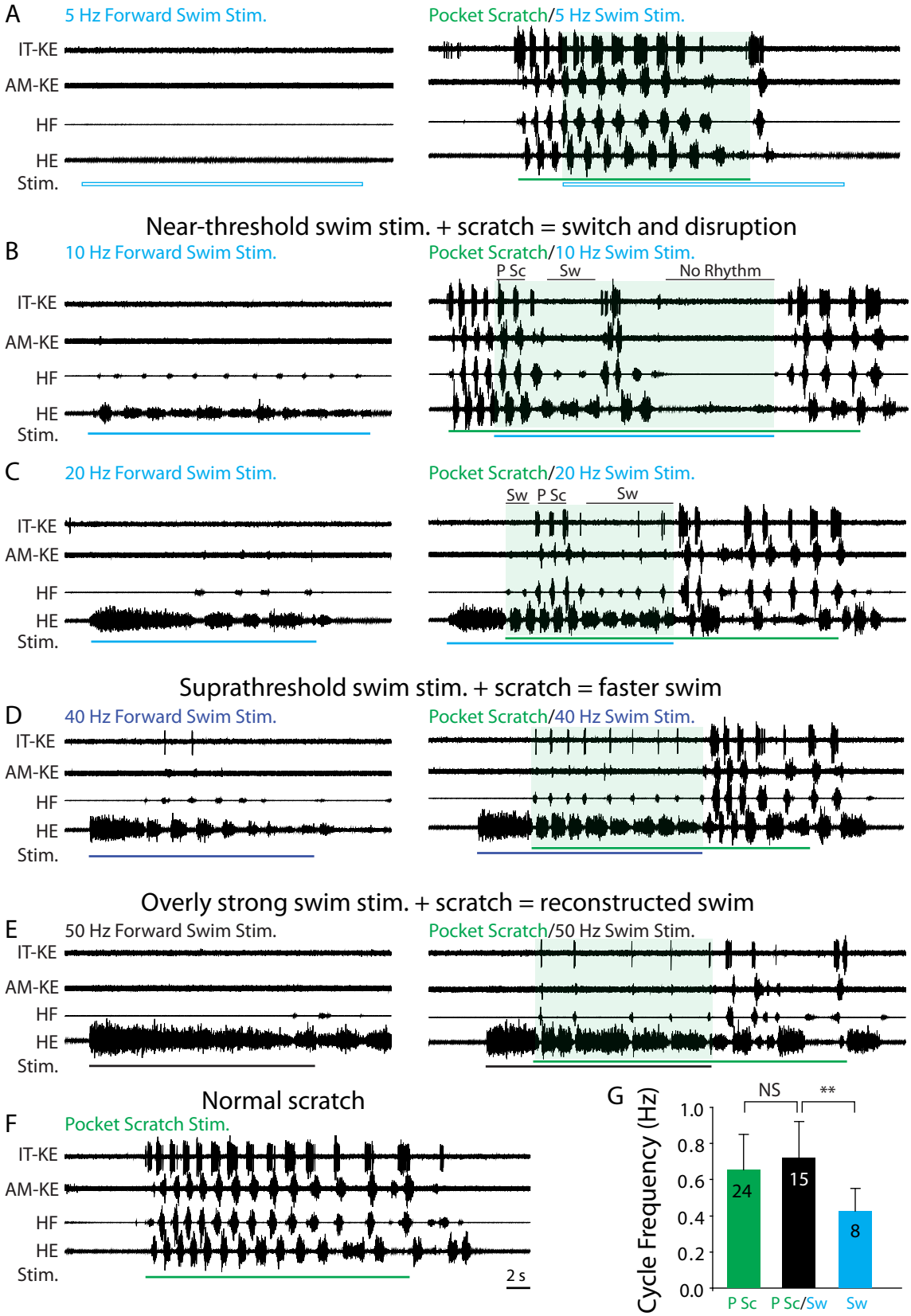
A
5 Hz Forward Swim Stim.
Pocket Scratch/5 Hz Swim Stim.
IT-KE
AM-KE
HF
HE
Stim.
Near-threshold swim stim. + scratch = switch and disruption
B
10 Hz Forward Swim Stim.
Pocket Scratch/10 Hz Swim Stim.
P Sc
Sw
No Rhythm
C
20 Hz Forward Swim Stim.
Pocket Scratch/20 Hz Swim Stim.
Sw
P Sc
Sw
Suprathreshold swim stim. + scratch = faster swim
D
40 Hz Forward Swim Stim.
Pocket Scratch/40 Hz Swim Stim.
Overly strong swim stim. + scratch = reconstructed swim
E
50 Hz Forward Swim Stim.
Pocket Scratch/50 Hz Swim Stim.
Normal scratch
F
Pocket Scratch Stim.
2 s
G
Cycle Frequency (Hz)
NS
**
1.0
0.8
0.6
0.4
0.2
0.0
24
15
8
P Sc
P Sc/Sw
Sw

Figure 21. Swim stimulation parameters could influence the effect of pocket scratch/swim stimulation (from turtle #9).

A-E, left panel, Motor patterns during swim stimulation with different pulse frequencies. A, right panel, Pocket scratch/subthreshold swim (300 μA, 5 Hz) stimulation evoked a pocket scratch-like motor pattern (strong KE that outlasts HF). B, right panel, Pocket scratch/near-threshold swim (300 μA, 10 Hz) stimulation evoked a switch between scratch-like (P Sc) and swim-like patterns (Sw; HF < HE, brief KE at the end of HF), followed by rhythm disruption (No rhythm). C, right panel, A second example of a switch motor pattern evoked by pocket scratch/near-threshold swim (300 μA, 20 Hz) stimulation. D, right panel, Faster swimming evoked by pocket scratch/suprathreshold swim (300 μA, 40 Hz) stimulation (statistical analysis shown in G). E, right panel, Pocket scratch stimulation added to overly strong swim (300 μA, 50 Hz) stimulation reconstructed the swim rhythm. F, Motor pattern during pocket scratch stimulation alone. G, Quantitative summary of all the episodes from this animal with the same stimulation parameters as in D.

DISCUSSION

I found that simultaneous activation of swimming and scratching central networks could produce motor patterns with altered cycle periods, phases, and amplitudes. The swim rhythm could also be reconstructed or disrupted by scratch stimulation.

Faster rhythms indicate scratch-swim interactions in rhythm generation

Scratch/swim dual stimulation could produce motor patterns that were faster than with either individual stimulation. These faster motor patterns were stable from cycle to cycle, which suggests that scratch and swim inputs were integrated at or prior to rhythm generation. Such integration might occur via strong interactions between swimming and scratching rhythm generators or via a single rhythm-generating module. The latter possibility is supported by the finding that two simultaneous inputs to one central pattern generator (CPG) could increase rhythm frequency (McCrohan and Kyriakides, 1992). Preliminary evidence for a faster rhythm during scratch/swim dual stimulation had been found previously [Fig 7B in (Juranek and Currie, 2000); Fig 6d in (Berkowitz, 2002)], but not explored systematically.

Faster motor patterns during scratch/swim dual stimulation were usually swim-like

Dual stimulation often evoked motor patterns having shorter cycle periods, with amplitudes and relative phases similar to forward swimming. This statement is supported by the linear regressions between burst duration and cycle period, which were similar for dual stimulation and swimming. Also, HE burst duration usually changed with cycle period more than HF burst duration during both dual stimulation and swimming, but not scratching. Earlier research on vertebrate stepping also showed that stance phase duration changed more with cycle period than swing phase duration (Jacobson and Hollyday, 1982; Nilsson et al., 1985; Bekoff et al., 1989; Wisleder et al., 1990; Reilly and Elias, 1998; Earhart and Stein, 2000b). These findings differ, however, from findings during turtle forward swimming in moving animals (Earhart and Stein, 2000a). The similarity of motor patterns between dual stimulation and swimming suggests that the faster motor patterns during dual stimulation share mechanisms of rhythm and pattern generation with swimming.

Modified motor patterns indicate interactions in pattern generation

In some cases, the motor patterns during dual stimulation switched between scratch-like and swim-like. Switches could be sudden (Fig. 13), when a scratch-like cycle was followed immediately by a swim-like cycle or vice versa. Sudden switching has been reported in separate (Heitler, 1985) and partly shared (Mortin et al., 1985; Stein et al., 1986; Jing et al., 2001) networks. My switches could also occur gradually over several cycles (Fig. 14), which is hard to explain by mutual inhibition between two separate

networks and suggests instead that scratch and swim inputs interact at or prior to pattern generation. Gradual switches were also reported for tadpole swimming and struggling (Green and Soffe, 1996), which are generated by partly shared networks (Soffe, 1993; Li et al., 2007), and for turtle rostral and pocket scratching (Robertson et al., 1985).

In other cases, each of several cycles during dual stimulation could have characteristics of both scratching and swimming (i.e., hybrids, Fig. 15), or be intermediate between them (i.e., intermediate motor patterns, Fig 16). In such merged motor patterns (Bellman and Krasne, 1983) in some other systems, the cycle periods of the two rhythms differed substantially, and either the faster rhythm slowed down to match the slower rhythm (observed in isolated nervous systems (Dickinson et al., 1990; Meyrand et al., 1991)), or the faster rhythm repeated several cycles during just one phase of the slower rhythm's cycle (observed in moving animals (Brown, 1911b; Carter and Smith, 1986; Marder and Weimann, 1992; Esch et al., 2002) and isolated nervous systems (Bartos and Nusbaum, 1997; Bartos et al., 1999). In moving animals, movement-related sensory feedback might account for this kind of coordination. In isolated nervous systems, mutual inhibition between components of the two central networks could account for this coordination instead. The cycle periods of turtle scratching and swimming motor patterns, however, were usually within the same range and movement-related sensory feedback did not exist in my immobilized animals. The hybrid motor patterns I report here and those between two forms of scratching in moving animals (Mortin et al., 1985) and immobilized animals (Robertson et al., 1985; Stein et al.,

1986) involve modifications of each cycle to include characteristics of both motor patterns, which supports a strong interaction between CPGs or a unitary CPG.

Interactions can happen at the input level

In some cases, subthreshold swim stimulation, which by itself evoked tonic HE activity, when combined with scratch stimulation could produce a swimming motor pattern with normal HF-HE alternation (Figs. 17, 18). These results suggest that scratch inputs converge with swim inputs or have access to the swim rhythm generator. The normal swim motor pattern obtained during such dual stimulation is analogous to reconstructed rostral scratch motor patterns obtained in spinally hemisected animals when contralateral and ipsilateral rostral scratch stimulations were combined (Stein et al., 1998). Residual excitation following scratching has also been shown to summate with scratch (Currie and Stein, 1988) and swim stimulation (Earhart and Stein, 2000b). Thus, I speculate that the swim rhythm in my experiments was reconstructed by addition of scratch excitatory input to the swim CPG. In some other cases, however, suprathreshold swim stimulation, which by itself evoked normal HF-HE alternation, when combined with scratch stimulation abolished HF and sometimes also HE rhythmic activity (Fig. 18). Both swim rhythm reconstruction and rhythm cessation could be observed within the same animal, so the different effects cannot simply be due to inter-animal variability. Rather, these results suggest that scratch and swim inputs can access common rhythm-generating circuitry to cause either summation or interference.

Factors that may underlie the variety of effects

By varying the swim stimulation amplitude and frequency, I found that the type of dual-stimulation effect depended on the strength of the swim stimulation (Figs. 20, 21). Thus, the level of excitation in the swim network may influence whether and how an intermediate motor pattern is produced. This may be analogous to the crayfish choice among feeding, escaping, or some intermediate behavior, which depends on food size (Bellman and Krasne, 1983). Also, in the crustacean stomatogastric nervous system, increasing the robustness of one motor pattern can cause some neurons to switch gradually from one firing pattern to another through hybrid firing patterns (Marder and Weimann, 1992); a similar mechanism might underlie hybrid and intermediate motor patterns in turtles.

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Chapter 4: Turtle swim and scratch stimuli are integrated to generate one rhythm prior to motoneurons

SUMMARY

Does the spinal cord use a single network to generate locomotor and scratching rhythms or two separate networks? Simultaneous swim and scratch stimulation ("dual stimulation") in immobilized, spinal turtles evokes a single rhythm in hindlimb motor nerves with a frequency often greater than during swim stimulation alone or scratch stimulation alone (Hao et al., 2011). This suggests that either a single network generates both rhythms or the two rhythms are integrated into one. If the latter, integration could occur in motoneurons themselves or earlier, in spinal interneurons. Here, I recorded intracellularly from hindlimb motoneurons during dual stimulation. Motoneuron membrane potentials displayed a regular oscillation at a higher frequency during dual stimulation than during swim or scratch stimulation alone. In contrast, arithmetic addition of the oscillations during swimming alone and scratching alone with various delays always generated irregular oscillations. Also, the standard deviation of the dual-referent phase-normalized membrane potential was similar during dual stimulation and swimming or scratching alone. In contrast, the standard deviation was greater when pooling cycles of swimming alone and scratching alone. This shows that dual stimulation generates a single rhythm prior to motoneurons. Thus, either swimming and scratching share a rhythm generator or the two rhythms are integrated into one by interneurons.

INTRODUCTION

The central nervous system (CNS) can generate many rhythmic behaviors that are appropriate for an animal's circumstances even without brain inputs and movement-related sensory feedback. How is the CNS organized to generate these different behaviors? In particular, are different rhythmic behaviors that involve the same motoneurons and muscles generated by the same set of neurons or different sets?

One approach to answer this question is to map each circuit in detail. In some invertebrate circuits, it is possible to identify and record from each neuron and investigate its contribution to a behavior by selectively activating or silencing it (Marder et al., 2005). In some cases, two different rhythmic behaviors may be generated by two completely separate neural networks (Heitler, 1985; Ramirez and Pearson, 1988; Hennig, 1990). In other cases, two rhythmic behaviors are generated by one shared network (Jing and Weiss, 2001; Marder and Bucher, 2001; Kupfermann and Weiss, 2001) or by partly shared networks (Briggman and Kristan, 2008).

Using single-neuron recording in vertebrate circuits, researchers have found both shared interneurons, such as for cat locomotion and scratching (Geertsen et al., 2011), turtle swimming and scratching (Berkowitz, 2002; Berkowitz, 2005, 2008; Berkowitz, 2010), and tadpole and larval zebrafish swimming and struggling (Soffe, 1993; Li et al., 2007; Liao and Fetcho, 2008), and behaviorally specialized interneurons, such as tadpole struggle-specialized neurons (Li et al., 2007), zebrafish struggle-, or escape-specialized neurons (McLean et al., 2007; Liao and Fetcho, 2008; McLean et al., 2008; Satou et al.,

2009), and turtle scratch-specialized neurons (Berkowitz, 2002). However, with the huge number of neurons in vertebrate circuits, an individual cell's effect on the whole network is generally so small that it is hard to demonstrate by selectively activating or silencing the neuron. Recent studies with transgenic mice provide a very powerful tool to investigate a subtype of neurons as a group. However, most research on mouse rhythmic behaviors so far has focused on the locomotor rhythm (Bonnot et al., 2002; Kiehn, 2006; Ziskind-Conhaim et al., 2010) and the networks generating other rhythmic behaviors involving the same motoneurons and muscles are still unclear (Moore et al., 2014). A direct comparison between networks that generate different rhythmic behaviors in limbed vertebrates is still very challenging.

Another approach to understand the organization of vertebrate rhythm-generating networks is to activate simultaneously the networks that generate two different motor patterns and assess network interactions via motor output (Carter and Smith, 1986; Stein et al., 1986; Currie and Stein, 1988; Ramirez and Pearson, 1988; Hennig, 1990; Svoboda and Fetcho, 1996; Earhart and Stein, 2000a; Juranek and Currie, 2000; Berkowitz and Hao, 2011; Hao et al., 2011). The turtle spinal cord contains sufficient circuitry to generate several rhythmic hindlimb motor patterns without brain inputs or movement-related sensory feedback. These motor patterns include forward swimming and three forms of scratching (rostral, pocket and caudal) (Stein, 2005). These motor patterns can be reliably evoked by electrical or mechanical stimulation, respectively. Simultaneous swim and scratch stimulation can alter the motor patterns in multiple ways. For instance, when combined with a scratch stimulation, 1) a suprathreshold

swim stimulation could evoke a motor pattern with a cycle frequency that was higher than either swimming or scratching alone, 2) a suprathreshold swim stimulation could evoke a motor pattern that switched between swimming and scratching, and 3) an overly high-frequency swim stimulation could evoke a normal swimming motor pattern even though the swim stimulation by itself could only evoke tonic hip-extensor activity (Hao et al., 2011).

These changes in cycle frequency and motor patterns demonstrate that scratch-evoking stimulation can influence both the rhythm and the pattern of swimming and vice versa. But these results do not indicate whether the interactions happen at the level of interneurons or motoneurons. Here, I intracellularly recorded from motoneurons that were involved in the two motor patterns *in vivo* while delivering swim and scratch stimulation simultaneously to trigger the effects mentioned above (Hao et al., 2011). I predicted that if the swim and scratch networks largely overlap or converge prior to motoneurons, the motoneuron membrane potential would oscillate with one rhythm; if the swim and scratch networks only converge in motoneurons, however, the motoneuron membrane potential would display summation of two oscillations. I observed clear and regular oscillations of motoneuron membrane potentials at one rhythm during simultaneous swim and scratch stimulation. These results support the hypothesis that the swim- and scratch-evoking inputs converge and generate a single rhythm prior to motoneurons, which suggests shared components for swimming and scratching rhythm generation. To my knowledge, this is the first demonstration of such

strong interactions at the interneuronal level between pathways for different rhythmic limb movements in adult vertebrates.

METHODS

Animal preparations

Adult red-eared turtles *Trachemys scripta elegans*, of both sexes (n = 14), weighing 270-570 g, were prepared for recording as described previously (Robertson et al., 1985; Berkowitz, 2001). Briefly, animals were anesthetized by hypothermic analgesia 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 extracellular recordings and stimulation: the hip flexor (HF), ventral puboischiofemoralis internus, pars anteroventralis; the hip extensor (HE), flexor cruris, pars flexor tibialis internus; and the knee extensors (KEs), triceps femoralis, pars iliotibialis (IT-KE), pars ambiens (AM-KE), and/or pars femorotibialis (FT-KE) (Robertson et al., 1985). After the surgery, turtles were warmed to room temperature for 0.5 h, then immobilized with gallamine triethiodide (8 mg/kg i.m.; Sigma-Aldrich, St. Louis, MO) and artificially ventilated throughout the experiment. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma.

Stimulation procedures

Forward-swimming motor patterns were evoked by electrical stimulation in the D3 contralateral lateral funiculus (0.1-ms, 100–300 μA, bipolar pulses at 10–60 Hz) with a

pair of 100-μm silver wires (California Fine Wire, Grover Beach, CA), insulated except at the tips, with one tip contacting the D3 face of the spinal cord and the other in the saline (Lennard and Stein, 1977; Juranek and Currie, 2000; Berkowitz, 2002). The swim stimulation amplitude and frequency were usually adjusted to evoke a swimming motor pattern with a cycle frequency that differed from the scratching cycle frequencies. Rostral, pocket and caudal scratching motor patterns were evoked by continual gental rubbing of a single site in the receptive field of each scratch form at ~3 N, ~3-4 Hz using a glass probe with a fire-polished tip (Mortin et al., 1985; Hao et al., 2014). "Swim/scratch dual stimulation" refers to the combination of swim and scratch stimulation delivered at overlapping times.

Electrophysiology

Dissected nerves were submerged in mineral oil, surrounded by a wax well molded onto the turtle carapace. Recordings from each nerve were obtained extracellularly using a pair of 100-μm silver wires and amplified and filtered (x 1000; band-pass 0.1–1.0 kHz; A-M Systems, Carlsborg, WA); these nerve recording electrodes were also used to stimulate motoneurons antidromically.

Intracellular recordings (n = 21 cells) were obtained from the ipsilateral hindlimb enlargement using sharp electrodes, pulled by a P-97 puller (Sutter Instrument Company, Novato, CA) and filled with 3 M potassium chloride (Fisher Scientific) or 4 M potassium acetate (Mallinckrodt Baker, Inc., Paris, TY) with resistances of 40–120 MΩ (Robertson and Stein, 1988; Berkowitz, 2005). The meninges were torn at the site

of each electrode penetration. Both nerve and single-cell recordings were stored on a digital audio tape recorder (TEAC America, Montebello, CA).

Antidromic stimulation

One of the dissected nerves was stimulated (0.5-10 V, 0.1-ms, 1-100 Hz or a single pulse) to evoke antidromic action potentials in the intracellularly recorded motoneuron (Fig. 22). A motoneuron was confirmed when 1) the delay between the stimulation and the onset of the action potential was less than 1 ms with a threshold lower than 2 V (Robertson and Stein, 1988); or 2) the onsets of the action potentials evoked by a train of antidromic stimulation were consistent (Brock et al., 1953).

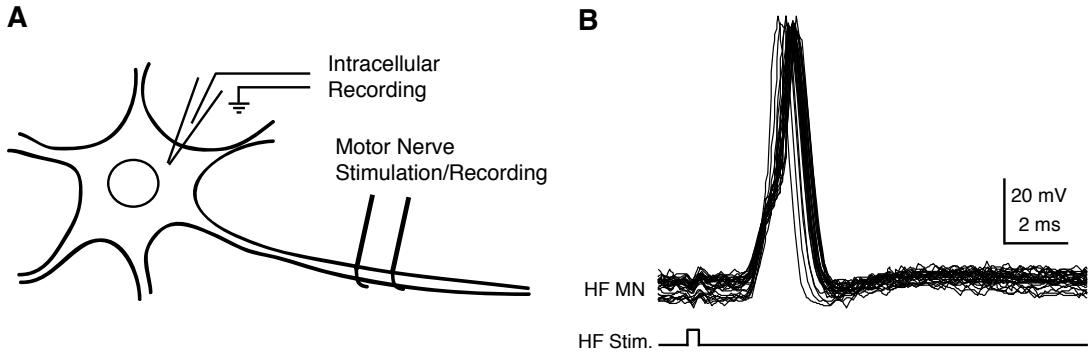

Figure 22. Motoneurons were confirmed by antidromic stimulation of motor nerves.

A, experimental design. B, the superimposition of the intracellular recording (HF MN, upper traces) during a train of 40-Hz electrical pulses (bottom trace) to the hip flexor (HF) nerve. HF, hip flexor; MN, motoneuron; Stim. , stimulation.

Data analysis

Six cells were not used for quantitative analysis because they were lost before delivery of swim and/or scratch stimulation alone; they behaved qualitatively like the other cells. Recordings of the remaining 15 cells were redigitized and analyzed using Datapac software (Run Technologies, Laguna Hills, CA). Action potentials were deleted and

remaining membrane potentials linearly smoothed using a sliding 50-ms window. The superimpositions of swimming and scratching membrane potentials were created using Matlab (Mathworks, Inc, Natick, MA) by adding the two membrane potentials linearly (Sirois et al., 2013). Dual-referent phase-normalized membrane potential averages and their standard deviations (SDs) were calculated with respect to the HF burst and HF-quiescent period of each cycle (Berkowitz and Stein, 1994). Only cycles completely within the period of stimulation were analyzed. HE-phase deletion cycles, defined by the absence of the quiescence between successive HF bursts (Stein and Grossman, 1980; Stein, 2008), at the beginning of an episode were not included in the quantitative analysis.

Statistics

Statistical comparisons of SDs were made within each form of motor pattern across animals using the repeated-measure nonparametric ANOVA test (Friedman's test) followed by selected pair comparisons (Dunn's test, Instat 3, GraphPad Software, San Diego, CA).

RESULTS

Electrical stimulation of the contralateral D3 lateral funiculus evoked pure-form forward swimming motor patterns (henceforth, just "swimming"). Mechanical stimulation of the ipsilateral body surface evoked pure-form rostral, pocket or caudal scratching motor patterns (henceforth, just "scratching"), depending on the stimulation location. Swim and each form of scratch stimulation delivered at an overlapping time

period (swim/scratch dual stimulation) could increase the rhythm frequency and/or alter the motor pattern itself (Hao et al., 2011). Here, I intracellularly recorded from 15 HF motoneurons, 2 HE motoneurons, and 4 KE motoneurons, which were confirmed by antidromic stimulation of the respective motor nerves (Fig. 22; see Methods), and analyzed their responses to the swim/scratch dual stimulation.

Motoneuron membrane potentials during swim/rostral scratch dual stimulation

During swimming, the hip flexor (HF) and hip extensor (HE) nerves were activated rhythmically and alternately, with HF bursts briefer and weaker than HE bursts (Lennard and Stein, 1977; Juranek and Currie, 2000) (Fig. 23A). Rostral scratching also featured rhythmic alternation between HF and HE bursts, while the HF bursts were longer and stronger than HE bursts (Robertson et al., 1985) (Fig. 23B). During swim stimulation, HF motoneurons displayed brief depolarizations in phase with HF nerve bursts and fired only a few action potentials (Fig. 23A). During scratch stimulation, each HF motoneuron's membrane potential displayed large and regular oscillations that were in phase with the HF nerve bursts and it fired action potentials at a higher rate than during swimming (Robertson and Stein, 1988) (Fig. 23B). When rostral scratch stimulation was added to an ongoing swim stimulation, the motor pattern cycle frequency could increase (Fig. 23C). The HF motoneuron membrane potential oscillated regularly and in phase with the HF nerve bursts. After the end of the swim stimulation, the cycle frequency slowed down and the motor pattern became rostral scratch-like. In this case, the motor pattern during the dual stimulation switched from swim-like (HF < HE) to scratch-like (HF > HE), back to swim-like. Matching the motor pattern, the

membrane potential gradually switched from swim-like (brief depolarization with few action potentials) to rostral scratch-like (strong depolarization with many action potentials) and then switched back to swim-like on the last cycle.

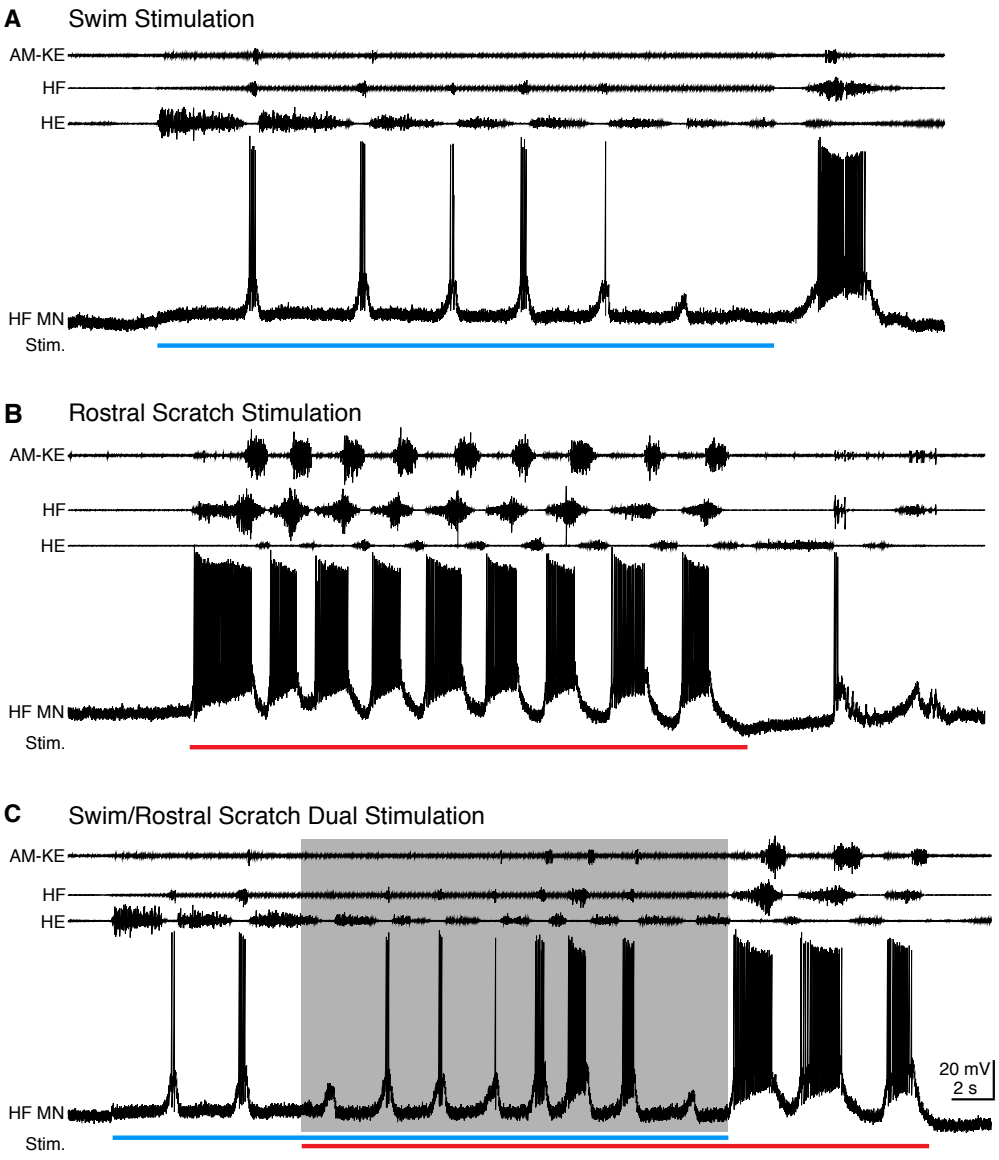

Figure 23. When the cycle frequency was increased by the swim/rostral scratch dual stimulation, this HF motoneuron membrane potential still oscillated regularly.

A-C, extracellular recordings from limb nerves (top three traces; AM-KE, ambiens knee extensor; HF, hip flexor; HE, hip extensor) and the intracellular recording of the motoneuron shown in Figure 22 (HF MN, fourth trace). A, recordings during swim stimulation [current pulses at 25 Hz; blue bar indicates the stimulation period (Stim.)]. B, recordings during rostral scratch stimulation (red bar). C, when swim/rostral scratch dual stimulation (blue and red bars) evoked a motor pattern with a higher cycle frequency (shaded area), the membrane potential of the HF motoneuron oscillated regularly at the same rate as the motor pattern.

In many other cases, the motor pattern could be swim-like during the whole swim/rostral scratch dual stimulation and the membrane potential then oscillated regularly and matched the motor patterns (data not shown). Regardless of the motor pattern, the membrane potential oscillated regularly and in phase with the nerve.

I predicted that if the swim and rostral scratch networks are separate and only converge onto motoneurons, the membrane potential oscillations of the motoneurons would be approximately a superimposition of those during swim stimulation alone and scratch stimulation alone. Alternatively, if the swim and scratch inputs are integrated prior to motoneurons, I would only observe one rhythmic input to the motoneurons.

To test between these two hypotheses, I first deleted the action potentials and smoothed the remaining membrane potentials to better visualize the waveform oscillations (see Methods). The membrane potential still oscillated regularly during swim/rostral scratch dual stimulation (Fig. 24A). The cycle frequency was higher during dual stimulation than during swim stimulation alone (Fig. 24B-H, blue traces) and rostral scratch stimulation alone (Fig. 24B-H, red traces). Then I linearly summed the membrane potential during swim stimulation alone and scratch stimulation alone with various delays between them. The summation yielded waveforms that were irregular with additional peaks, troughs or prolonged cycle durations (Fig. 24B-H, black traces), which were never observed during swim/rostral scratch dual stimulation. Also, I never observed a summation that generated a consistently increased cycle frequency.

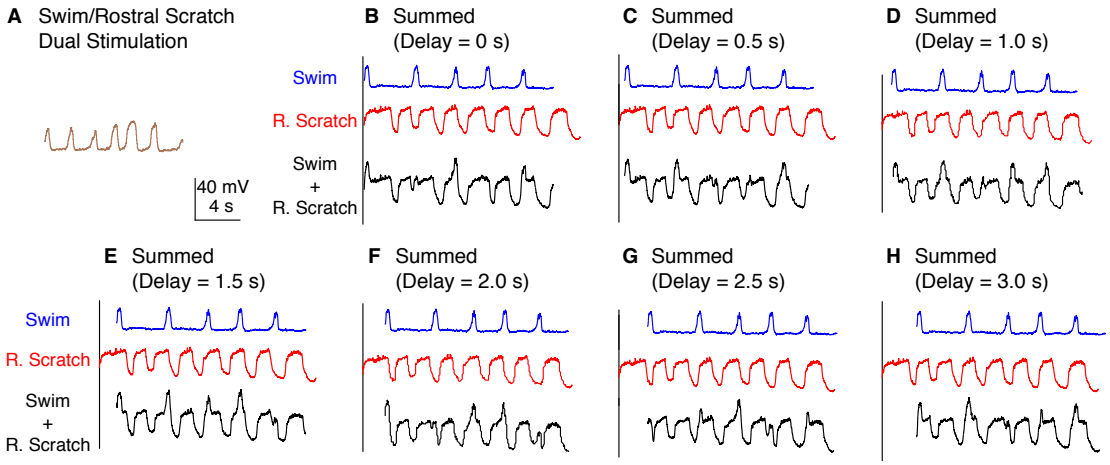

Figure 24. Linear summations of the membrane potential oscillations during swim stimulation alone and rostral scratch stimulation alone did not produce regular oscillations.

A, the membrane potential oscillations during swim/rostral scratch dual stimulation shown in Figure 23C. B-H, linear summations (Swim + R. Scratch, black traces) of the membrane potential oscillations during swim stimulation alone (Swim, blue traces) and rostral scratch stimulation alone (R. Scratch, red trace) at various delays.

To quantitatively analyze the consistency of the membrane potential oscillations during swim/rostral scratch dual stimulation, I calculated dual-referent phase-normalized membrane potential averages (solid curves) and SDs (dots) with respect to the HF-on and HF-off phases of each cycle (see Methods) (Fig. 25A-C). In the cell illustrated, the SDs during the swim/rostral scratch dual stimulation (Fig. 25C, E, F) was greater than during swim stimulation alone (Fig. 25A, E, F), but was smaller than during rostral scratch stimulation alone (Fig. 25B, E, F). The SD during dual stimulation was also smaller than the SD when I pooled all cycles during swim stimulation alone and rostral scratch stimulation alone (Fig 25D, E, F).

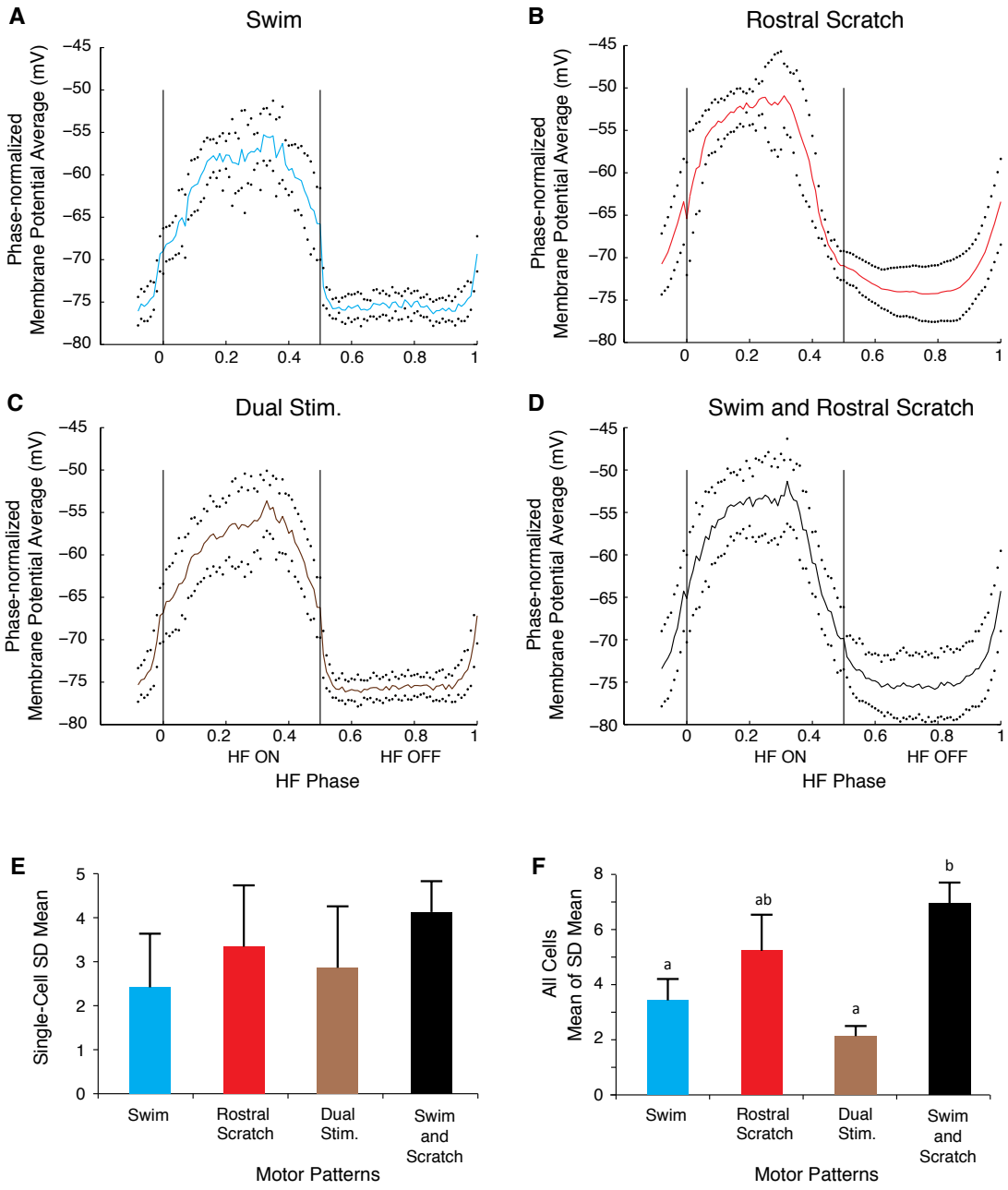

Figure 25. The motoneuron membrane potential oscillations during swim/rostral scratch dual stimulation were as regular as those during swim stimulation alone or rostral scratch stimulation alone.

A-C, dual-referent phase-normalized membrane potential average and the SD (black dots) across all cycles for the cell in Figure 23 with the same stimulation parameters during swim stimulation (A, blue curve), rostral scratch stimulation (B, red curve) and swim/rostral scratching dual stimulation (C, brown curve). D, dual-referent phase-normalized membrane potential average of all cycles during swim stimulation alone and rostral scratch stimulation alone within the cell. E, the mean of the SD within each stimulation paradigm for this cell. Error bars, SD. F, the mean of the mean SD in E across all 10 cells with the same stimulation paradigm. Error bars, SE. a, b, indicate significant differences by Friedman's test ($p = 0.003$) followed by Dunn's test.

These findings were consistent across the 10 motoneurons for which I collected enough data during swim, rostral scratch, and swim/rostral scratch dual stimulation. The SDs during swim/rostral scratch dual stimulation was not significantly different from SDs during either swim or rostral scratch stimulation alone, but was significantly smaller than the SD of the pooled swim stimulation alone and scratch stimulation alone cycles (Fig. 25F; Friedman's test, $p = 0.003$, followed by Dunn's test: swim stimulation vs. pooled, $p < 0.05$; dual stimulation vs. pooled, $p < 0.01$; other groups were not significantly different).

Motoneuron membrane potentials during swim/pocket scratch dual stimulation

Pocket scratching featured rhythmic alternation between HF and HE nerve bursts with the AM-KE and FT-KE nerve bursts continuing during part of the HE nerve bursts (Robertson et al., 1985) (Fig. 26B). During swim/pocket scratch dual stimulation, the motor pattern was swim-like, with AM-KE nerve bursting at the end of each HF nerve burst. The depolarization phase of the motoneuron membrane potential was swim-like while the hyperpolarization phase was less consistent, like that during pocket scratch stimulation (Fig. 26C). The membrane potential oscillation during the swim/pocket scratch dual stimulation was regular (Fig. 27A) whereas the linear summation of the membrane oscillation during swim stimulation alone (Fig. 27B-H, blue traces) and pocket scratch stimulation (Fig. 27B-H, green traces) at various delays created double bursts or prolonged excitations (Fig. 27B-H, black traces).

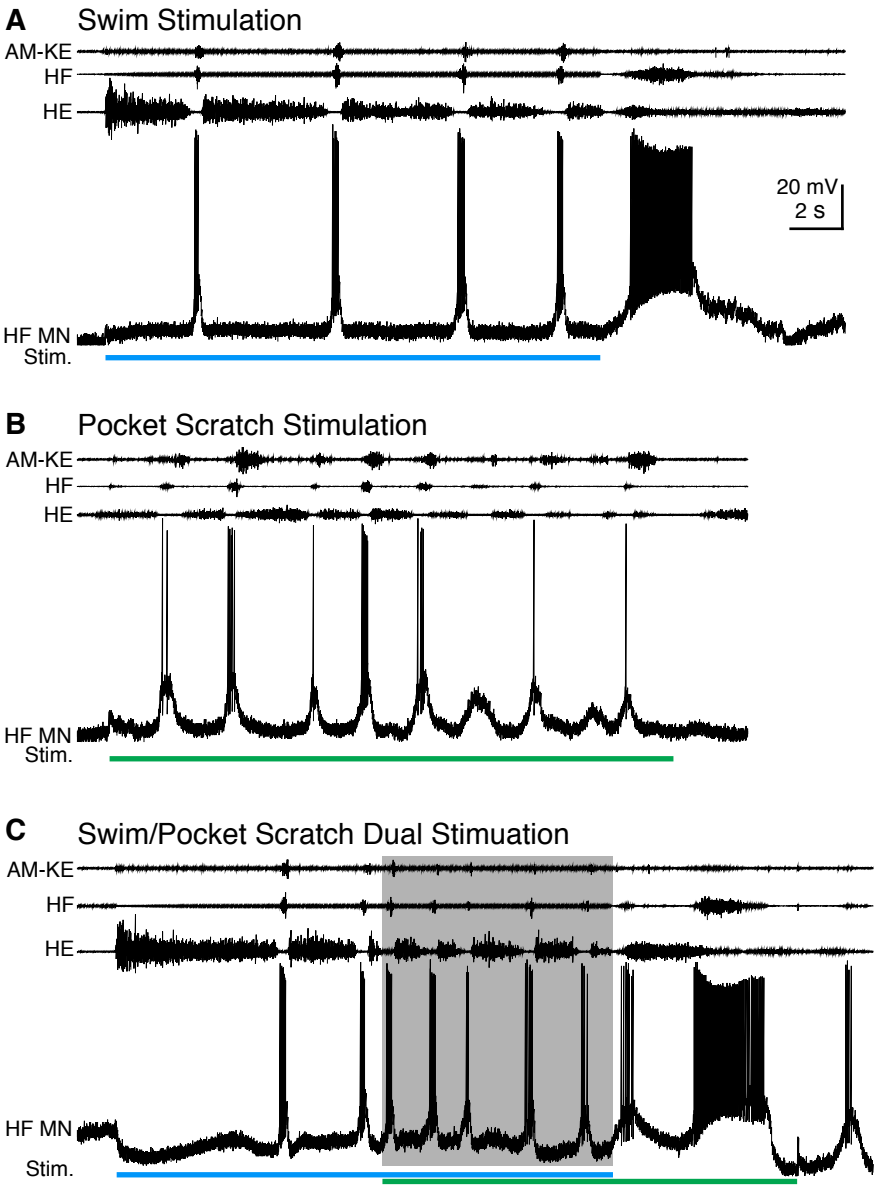

Figure 26. When the cycle frequency was increased by swim/pocket scratch dual stimulation, this HF motoneuron membrane potential oscillated regularly.

A-C, extracellular recordings from limb nerves and the intracellular recording of the same HF motoneuron shown in Figures 22 and 23. A, recordings during swim stimulation alone (current pulses at 40 Hz; blue bar). B, recordings during pocket scratch stimulation alone (green bar). C, when swim/pocket scratch stimulation (blue and green bars) evoked a motor pattern with a higher cycle frequency (shaded area), the membrane potential of the HF motoneuron oscillated regularly at the same rate as the motor pattern.

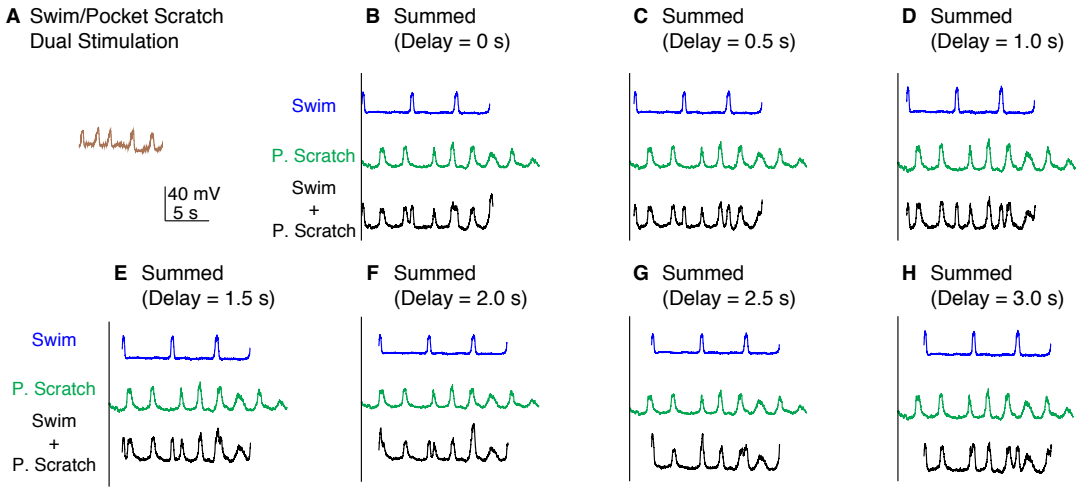

Figure 27. Linear summations of the membrane potential oscillations during swim stimulation alone and pocket scratch stimulation alone did not produce regular oscillations.

A, the membrane potential oscillations during swim/pocket scratch dual stimulation shown in Figure 26C. B-H, linear summations (Swim + P. Scratch, black traces) of the membrane potential oscillations during swim stimulation alone (Swim, blue traces) and pocket scratch stimulation alone (P. Scratch, green trace) at various delays.

In three cases, the cycle frequencies during swim stimulation alone and pocket scratch stimulation alone were similar. In these cases, I observed regular membrane potential oscillations when I added the membrane potentials from swim stimulation alone and pocket scratch stimulation alone. However, when I added the membrane potentials in phase, the peak-to-trough amplitudes were larger than either during swim stimulation alone or scratch stimulation alone. During dual stimulation, however, for two of the three cells that had similar swimming and scratching cycle frequencies, their mean peak-to-trough oscillation amplitude (mean ± SD, 16.1 ± 1.9 mV and 14.4 ± 3.7 mV,) was smaller than during swim stimulation alone (24.1 ± 2.7 mV, and 13 ± 5.4 mV) or pocket scratch stimulation alone (20.9 ± 3.4 mV and 17.8 ± 3.1mV, respectively). For the third cell, though, the mean oscillation amplitude during the dual stimulation (21.8 ±

1.6 mV) was higher than that during swim stimulation alone (10.6 ± 5.8 mV) and pocket scratch stimulation alone (12.7 ± 2.6 mV).

The SD during the swim/pocket scratch dual stimulation for this cell (Fig. 28C, E, brown bar) and across all 13 cells for which I collected enough data during swim, pocket scratch, and swim/pocket scratch dual stimulation (Fig. 28F) was smaller than or equal to those during swim stimulation alone (Fig. 28A, E, F), pocket scratch stimulation alone (Fig. 28B, E, F), and all cycles pooled from both swim stimulation alone and pocket scratch stimulation alone (Fig. 28D, E, F) (Friedman's test, $p = 0.017$, followed by Dunn's test : swim stimulation vs. pooled, $p < 0.05$; dual stimulation vs. pooled, $p < 0.05$; other groups were not significantly different, Fig. 28F).

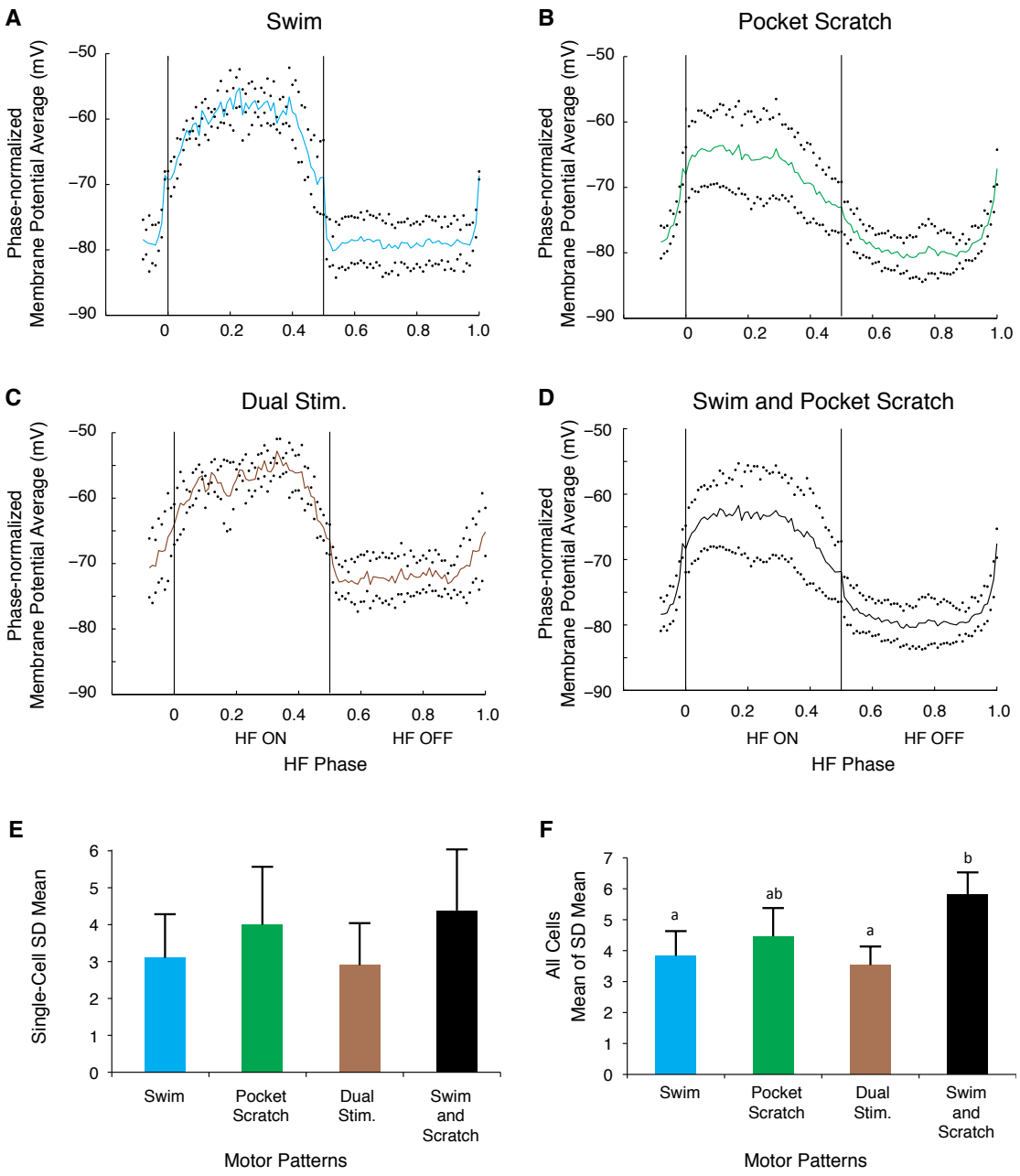

Figure 28. The motoneuron membrane potential oscillations during swim/pocket scratch dual stimulation were as regular as those during swim stimulation alone or pocket scratch stimulation alone.

A-C, dual-referent phase-normalized membrane potential average and the SD (black dots) across all cycles of the cell in figure 26 with the same stimulation parameters during swim stimulation (A, blue curve), pocket scratch stimulation (B, green curve) and swim/pocket scratch dual stimulation (C, brown curve). D, dual-referent phase-normalized membrane potential average of all cycles during swim stimulation alone and pocket scratch stimulation alone within the cell. E, the mean of the SD within each stimulation paradigm. Error bars, SD. F, the mean of the mean SD in E across all 13 cells with the same stimulation paradigm. Error bars, SE. a, b, c, indicate significant differences by Friedman's test ($p = 0.017$) followed by Dunn's test.

Increased cycle frequency during swim/caudal scratch stimulation

Caudal scratching featured rhythmic alternation between HF and HE bursts with the HF bursts weaker and briefer than the HE bursts (Robertson et al., 1985) (Fig. 29A). FT-KE bursts occurred at approximately opposite phases for swimming (end of each HF burst; Fig. 29A) and scratching (start of each HF burst; Fig. 29B). Fig. 29 shows an example of an AM-KE motoneuron recording.

Compared to the brief and regular depolarization of this motoneuron during swim stimulation, the depolarization of this AM-KE motoneuron during caudal scratch stimulation was prolonged. During swim/caudal scratch dual stimulation, the motor pattern was swim-like with the AM-KE nerve burst at the end of each HF burst. The depolarizing phases of the membrane potential were sharp and swim-like, but the membrane potential stayed depolarized longer and in this respect was caudal scratch-like (Fig. 29C).

The membrane potential oscillation during swim/caudal scratch dual stimulation was regular (Fig. 30A) whereas the linear summation of the membrane oscillations during swim stimulation alone (Fig. 30B-H, blue traces) and caudal scratch (Fig. 30B-H, purple traces) stimulation alone at various delays created irregular oscillations with an oscillation amplitude that varied from cycle to cycle.

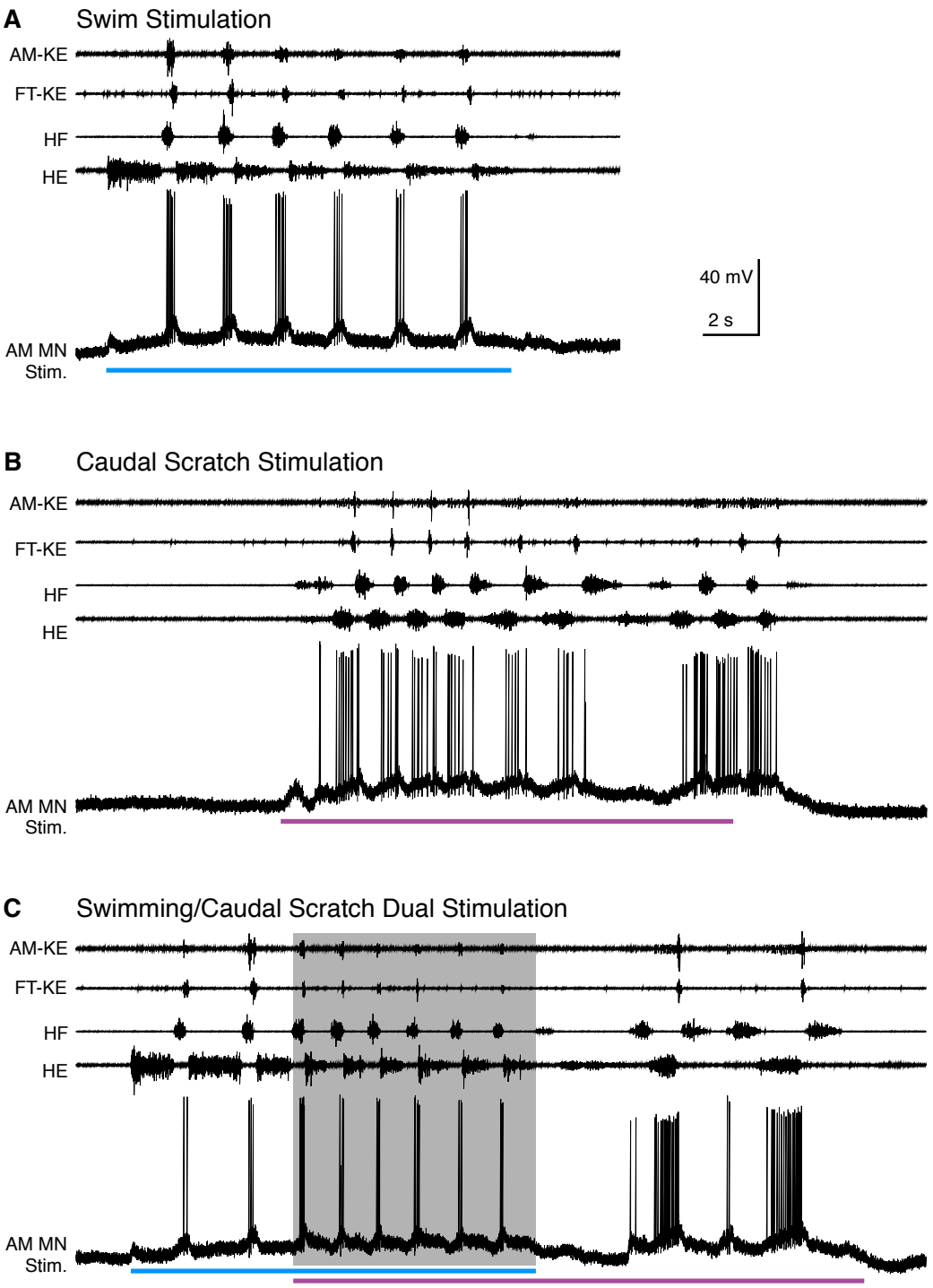

Figure 29. When the cycle frequency was increased by swim/caudal scratch dual stimulation, this AM-KE motoneuron membrane potential oscillated regularly.
A-C, extracellular recordings from limb nerves (top four traces; AM-KE, ambiens knee extensor; FT-KE, femorotibialis knee extensor; HF, hip flexor; HE, hip extensor) and the intracellular recording of an AM-KE motoneuron. A, recordings during swim stimulation alone (current pulses at 40 Hz, blue bar). B, recordings during caudal scratch stimulation alone (purple bar). C, when swim/caudal scratch stimulation (blue and purple bars) evoked a motor pattern with a higher cycle frequency (shaded area), the membrane potential of the AM-KE motoneuron oscillated regularly at the same rate as the motor pattern.

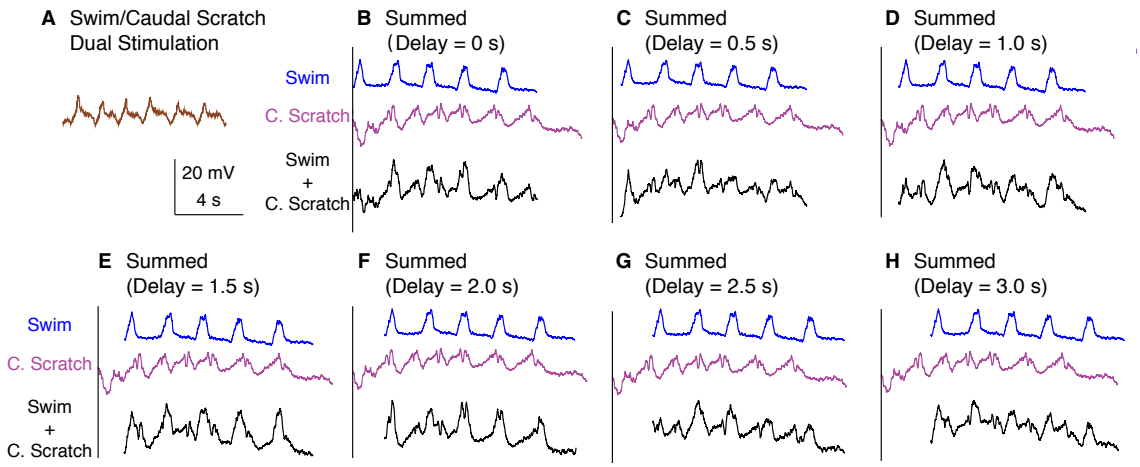

Figure 30. Linear summations of the membrane potential oscillations during swim stimulation alone and caudal stimulation alone did not produce regular oscillations.

A, the membrane potential oscillations during swim/caudal scratch dual stimulation shown in Figure 29C. B-H, linear summations (Swim + C. Scratch, black traces) of the membrane potential oscillations during swim (Swim, blue traces) and caudal scratch (C. Scratch, purple traces) stimulation alone at various delays.

The SD of the phase-averaged membrane potential during the swim/caudal scratch dual stimulation for this cell (Fig. 31C, E) and across all 8 cells for which I collected enough data during swim, caudal scratch, and swim/caudal scratch dual stimulation (Fig. 31F) was smaller than or equal to those during swim stimulation alone (Fig. 31A, E, F), caudal scratch stimulation alone (Fig. 31B, E, F), and cycles pooled from both swim and caudal scratch stimulation alone (Fig. 31D, E, F) (Friedman's test, $p = 0.013$ followed by Dunn's test, $p>0.05$ for each single comparison, Fig. 31F).

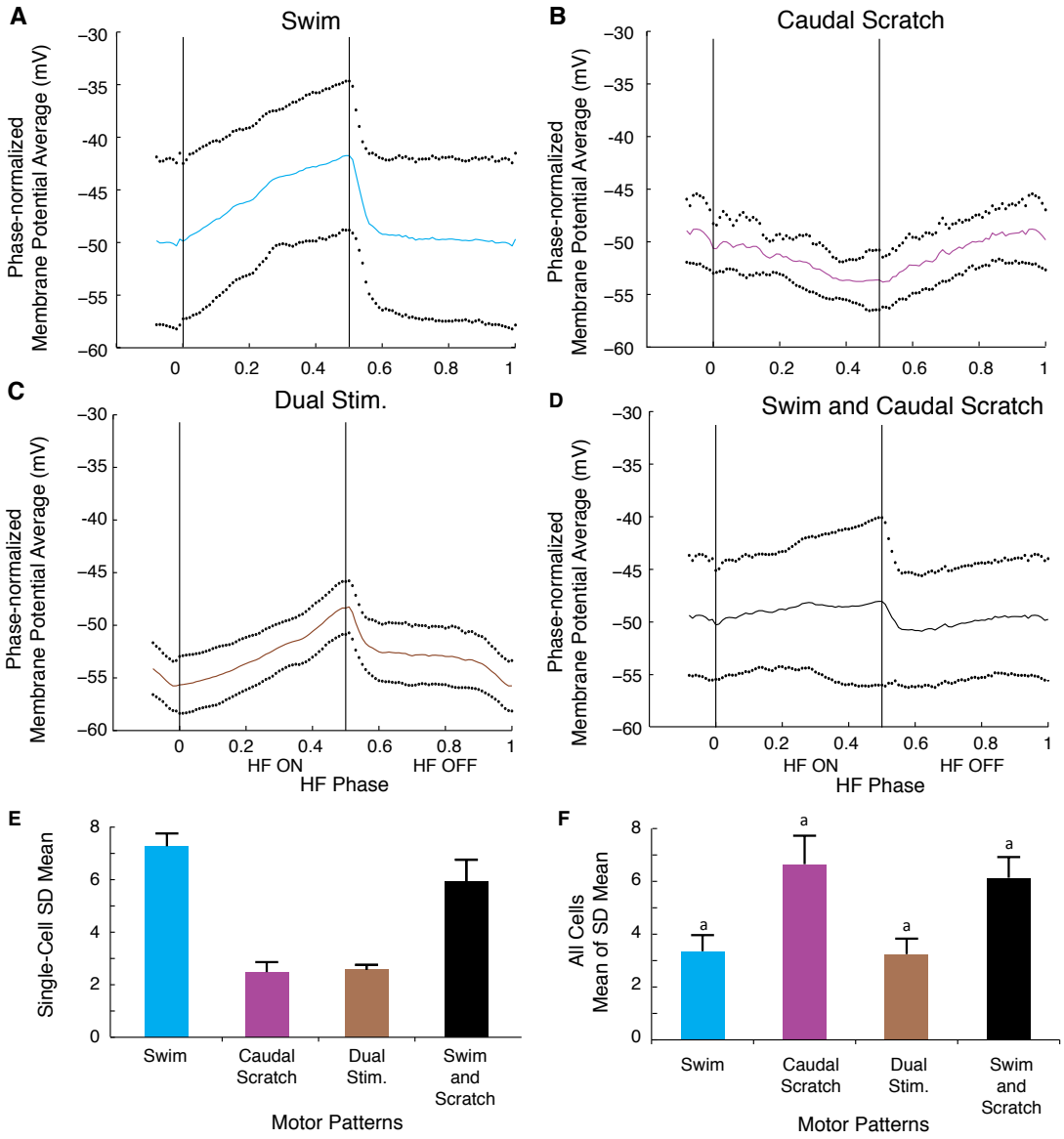

Figure 31. The motoneuron membrane potential oscillations during swim/caudal scratch dual stimulation were as regular as those during swim or caudal scratch stimulation alone.

A-C, dual-referent phase-normalized membrane potential average and the SD (black dots) across all cycles of the cell in figure 29 with the same stimulation parameters during swim stimulation (A, blue curve), caudal scratch stimulation (B, purple curve) and swim/caudal scratching dual stimulation (C, brown curve). D, dual-referent phase-normalized membrane potential average of all cycles during swim stimulation alone and caudal scratch stimulation alone within the cell. E, the mean of the SD within each stimulation paradigm for this cell. Error bars, SD. F, the mean of the mean SD in E across all 8 cells with same stimulation paradigm. Error bars, SE. a, no significant differences among tested group pairs by Friedman's test ($p = 0.013$) followed by Dunn's test (not significant).

Altered motor patterns

In addition to increasing the cycle frequency, swim/scratch dual stimulation could alter the motor pattern, depending on the stimulation parameters (Hao et al., 2011). One of the effects was that the motor pattern could switch between swim-like and scratch-like within one episode. I recorded from two HF motoneurons when such switches occurred. Figure 32 shows one clearer example from the cell shown in Figure 23. During rostral scratch stimulation alone, the AM-KE nerve bursts were strong, the HF nerve bursts were much stronger and longer than the HE bursts (Fig. 32B) and the depolarizations of the HF motoneuron were also stronger and triggered more action potentials. During typical swimming evoked by 25-Hz electrical pulses (Fig. 32A), AM-KE bursts, when they occurred, were very weak, the HF nerve bursts were much weaker and briefer than the HE bursts and the depolarizations of the HF motoneuron were also weak and triggered only a few action potentials. A 13-Hz swim stimulation evoked weaker swimming and the depolarization of the recorded HF motoneuron was subthreshold (Fig. 32C). When rostral scratch stimulation was added to this weak swim stimulation (Fig. 32D), the motor pattern switched between swim-like and scratch-like. When the swim stimulation ceased, the motor pattern stayed rostral scratch-like until the end of the rostral scratch stimulation. During this switching, the membrane potential of the HF motoneuron matched the motor output and showed no sign of summing two distinct inputs.

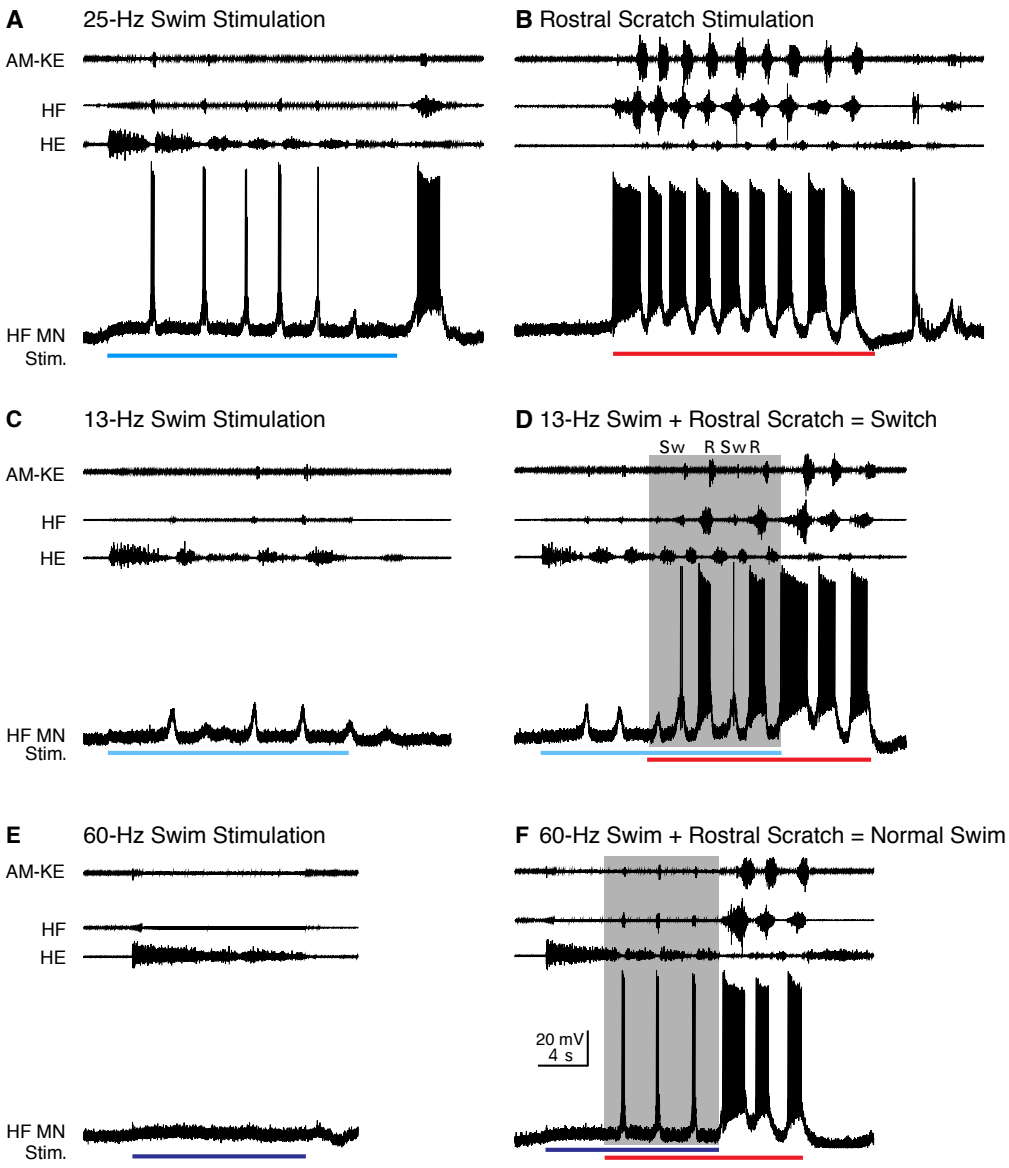

Figure 32. The motoneuron membrane potential oscillated regularly when scratch inputs altered the motor pattern evoked using different swim stimulation frequencies.

A, typical swimming evoked by 25-Hz swim stimulation (blue bar). B, typical rostral scratching evoked by rostral scratch stimulation (red bar). C, weaker swimming evoked by 13-Hz swim stimulation (light blue bar). Notice the motor pattern was still swim-like although this motor neuron remained subhreshold for action potentials. D, when rostral scratch stimulation was added to the 13-Hz swim stimulation, the motor pattern switched between swim-like (Sw) and rostral scratch-like (R). The shape of the membrane potential also switched between swim-like and rostral scratch-like with the motor pattern. After the end of the swim stimulation, the rostral scratch stimulation alone evoked a typical rostral scratch motor pattern and motoneuron membrane potential oscillation. E, an overly high-frequency swim stimulation (60 Hz, dark blue bar) failed to evoke any swim rhythm. Note that there are no membrane potential oscillations in the HF motoneuron either. F, adding rostral scratch stimulation to the overly high-frequency swim stimulation (shaded area) evoked a swim-like motor pattern similar to typical swimming shown in A. The membrane potentials were also swim-like during the dual stimulation. The motor pattern and membrane potential changed to rostral scratch-like when the swim stimulation ended and the rostral scratch stimulation continued.

In other cases (n = 2), an overly high-frequency swim stimulation could evoke tonic HE activity with no HF bursts (Fig. 32E). During the stimulation, the membrane potential of the HF motoneurons depolarized slightly without any noticeable oscillation (Fig. 32E). Adding the rostral scratch stimulation evoked the typical swim-like motor pattern with HE-biased HF-HE alternation (Fig. 32F). During this swim-like motor pattern, the HF motoneurons depolarized regularly and briefly as during normal swimming (Fig. 32F). When the overly strong swim stimulation stopped and rostral scratch stimulation continued, both the motor pattern and the HF motoneuron membrane potential oscillations immediately changed backed to rostral scratch-like. Similar results were seen during swim/pocket scratch stimulation in the same cell (data not shown).

DISCUSSION

In the previous chapter, I showed that swim/scratch dual stimulation could evoke a motor pattern that differed from swim stimulation alone and scratch stimulation alone in both the rhythm rate and the motor pattern. These results demonstrated strong interactions between swimming and scratching networks in both rhythm and pattern generation. However, this previous study recorded from motoneurons extracellularly and thus could not distinguish whether the interactions happened at the motoneuron level or instead at the interneuron level. Motoneurons might integrate separate swimming and scratching oscillatory inputs, resulting in irregular motoneuron membrane potential oscillations, yet generate action potentials rhythmically because only the peaks of the oscillations (which occur regularly) are suprathreshold. In this study, I recorded intracellularly from motoneurons to monitor the inputs they received

and to assess whether the convergence of swimming and scratching inputs occurs in motoneurons or prior to motoneurons.

Swimming and scratching inputs could affect each other's rhythm generation prior to motoneurons

During swim/scratch dual stimulation, I often observed a motor pattern that was faster than either swimming or scratching alone (Chapter 3, Figs. 23, 26 and 29). This demonstrates that swimming and scratching inputs interact when generating a rhythm. Here, I directly recorded from motoneurons during dual stimulation and observed regular motoneuron oscillations with a standard deviation similar to pure-form swimming and pure-form scratching (Figs. 24, 27 and 30). The regularity of the membrane potential oscillations during swim/scratch dual stimulation is consistent with the hypothesis that the interaction between swimming and scratching rhythm generation happens prior to motoneurons.

The regularity of motoneuron membrane potential oscillations suggests that motoneurons do not integrate two different rhythmic inputs

The intracellular recordings during dual stimulation displayed regular oscillations (Figs. 23C, 26C, 29C, 32D and 32F). In contrast, linear summations of oscillations from swim stimulation alone and scratch stimulation alone were irregular and had additional peaks, troughs or prolonged excitation (Figs. 24, 27 and 30). However, the membrane conductance of a neuron and the driving force for each ion would change with the membrane potential oscillation, which would make the summation of two rhythmic

inputs unlikely to be linear. Despite this, the predicted irregularity of the membrane potential oscillation, if there were two different rhythmic inputs, should still be evident, although the oscillation amplitudes might differ from a linear summation. Thus, the linear summation of two inputs should still provide an informative comparison qualitatively.

The irregularities I observed during the linear summations of oscillations were probably due to the differences between the swimming and scratching cycle frequencies. The linear summations were very similar to those generated by simultaneous activation of two separate networks (Ramirez and Pearson, 1988; Hennig, 1990). However, I rarely observed these kinds of irregularities during the swim/scratch dual stimulation, which is consistent with the hypothesis that the pathways for swimming and scratching converge prior to motoneurons.

It is true that when swimming and scratching have the same cycle frequency, the linear summation of the two oscillations could be regular if the two oscillations are in phase. However, I do not think this occurred in my experiments because: 1) for 12/15 cells, I was able to adjust the swim stimulation so that the cycle frequency was distinct during swim stimulation alone and scratch stimulation alone; 2) in 2 of the remaining 3 cells, the oscillation amplitude during the dual stimulation was not increased relative to that during swim stimulation alone or scratch stimulation alone, although linear summation of the two separate oscillations had a higher peak-to-trough amplitude; 3) in the remaining 1 cell, the oscillation amplitude during the dual stimulation did increase

compared to those during swim stimulation alone and scratch stimulation alone. However, during the dual stimulation, the oscillation had a higher cycle frequency than during either swim stimulation alone or scratch stimulation alone, whereas the linear summations did not alter the cycle frequency.

Swimming and scratching inputs could affect motor pattern selection prior to motoneurons

During switches between swimming and scratching, the motoneuron membrane potentials also oscillated regularly and switched quickly between swim-like and scratch-like (Fig. 32D). In switches, the membrane potential during each cycle was either swim-like or scratch-like, matching the motor output. Similar switches have been reported in other systems with partly shared networks (Mortin et al., 1985; Stein et al., 1986; Jing and Weiss, 2001). Switches between two motor patterns have also been reported for motor patterns generated by separate networks. But these switches tended to have longer delays (Heitler, 1985; Hennig, 1990) or irregular motoneuron membrane potentials (Heitler, 1985).

In other cases, I observed that overly high-frequency swim stimulation, which evoked tonic HE activity by itself, when combined with scratch stimulation could evoke normal swimming (Hao et al., 2011) (Fig. 32 E-F). During the overly high-frequency swim stimulation alone, the motoneuron membrane potential remained relatively flat and showed no sign of oscillations. During the dual stimulation, the membrane potential was swim-like and matched the motor output. This result argues against the possibility that

during the high-frequency swim stimulation, motoneurons received subthreshold swim-like input, and scratch input merely provided tonic excitation of the motoneurons to make the existing swim oscillations suprathreshold. Rather, this result suggests that the scratch input converged with swim input prior to the motoneuron and affected swim generation at the interneuronal level.

Persistent inward currents are unlikely to account for these findings

Various ion channels may shape membrane potential oscillations. For example, L-type Ca^{2+} channels may induce bistable plateau phases that outlast the depolarizing current in turtle spinal cord slices (Hounsgaard and Kiehn, 1989; Alaburda et al., 2002). Both swim and scratch stimulation might enhance such plateau phases (Delgado-Lezama et al., 1997; Alaburda and Hounsgaard, 2003), which could play a role in integrating swimming and scratching synaptic inputs during swim/scratch dual stimulation. However, these plateau potentials are overwhelmed by the high synaptic conductance during scratching *in vitro* (Alaburda et al., 2005). Similarly, in my experiment, the activation of the scratching network during swim/scratch dual stimulation would increase the synaptic conductance and it is unlikely that motoneuron plateau properties by themselves substantially smoothed irregular inputs during swim/scratch dual stimulation.

Partly shared networks

Collectively, my results are consistent with strong interactions or shared components between swimming and scratching networks at the interneuronal level. These findings

do not contradict earlier results suggesting some differences between spinal networks for two rhythmic behaviors (Ritter et al., 2001; Berkowitz, 2002; Li et al., 2007; McLean et al., 2007; Berkowitz, 2008; Liao and Fetcho, 2008; McLean and Fetcho, 2008; Satou et al., 2009; Frigon and Gossard, 2010; Mui et al., 2012; Hao et al., 2014). Rather, these results make it unlikely that the swimming and scratching pathways are completely separate prior to motoneurons. Thus, to the extent that there are swim and/or scratch-specialized spinal interneurons involved, they appear to have their effects predominately or exclusively on interneurons, not motoneurons.

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Chapter 5: Conclusions

The aim of this dissertation is to answer the question: to what extent are the swimming and scratching CPGs shared in adult limbed animals? In red-eared turtles, I have found that:

The D3-D8 segments of the spinal cord contribute to swimming and scratching differently because these segments were sufficient to maintain the HE-phase during swimming but not so during scratching (Chapter 2).

The swimming and scratching CPGs are not completely separate because swim stimulation could alter the scratching motor pattern and vice versa. The shared or interacting CPG components include the networks that: 1) activate the swimming and scratching CPGs, 2) determine the motor pattern cycle frequencies, and 3) set the synergies among muscles for the motor patterns (Chapter 3).

The shared components or the interactions between the two CPGs occur at the interneuronal level, rather than the motoneuron level, because the motor rhythm and pattern during simultaneous swim and scratch stimulation were determined prior to motoneurons (Chapter 4).

These findings demonstrate that there are shared components or strong interactions between swimming and scratching CPGs at the interneuronal level. However, the detailed mechanisms of how the two CPGs affect each other are still unknown.

Intracellular recordings from interneurons during simultaneous swim and scratch stimulation, followed by immunocytochemistry analysis on the morphology and neurotransmitter of recorded neurons, may provide further information to reveal these mechanisms.