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ELECTROPHYSIOLOGICAL DIFFERENCES BETWEEN SPECIALIZED AND MULTIFUNCTIONAL TURTLE SPINAL CORD NEURONS INVOLVED IN SCRATCHING AND SWIMMING MOTOR PATTERNS

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ELECTROPHYSIOLOGICAL DIFFERENCES BETWEEN SPECIALIZED AND MULTIFUNCTIONAL TURTLE SPINAL CORD NEURONS INVOLVED IN SCRATCHING AND SWIMMING MOTOR PATTERNS

A THESIS APPROVED FOR THE SCHOOL OF BIOLOGICAL SCIENCES

BY THE COMMITTEE CONSISTING OF

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Abstract

I examined the electrophysiology of both multifunctional and specialized neurons located in the turtle spinal cord, which are activated during swimming, scratching, and/or flexion reflex. My research analyzes these neurons to determine the electrophysiological differences between neurons that are activated during multiple motor patterns (multifunctional) and neurons that are activated only during a specific movement (scratch-specialized, flexion reflex-selective, and swim-specialized). The differences between multifunctional and specialized neurons could elucidate more about the organization of the central pattern generators (CPGs) located within the spinal cord.

During analysis of intracellular recordings of multifunctional and scratch-specialized neurons, it was determined that most neurons preferred to fire during one phase of activity for both scratching and swimming. Furthermore, the multifunctional neurons could be divided into two groups (hip flexor-on phase preference and hip flexor-off phase preference) based on phase preference.

Analysis of spiking activity in multifunctional and scratch-specialized neurons showed that multifunctional neurons tend to spike more rhythmically than scratch-specialized neurons. When analyzing the peak and trough phases of both multifunctional and specialized neurons it was determined that the multifunctional neurons are probably rhythmically modulated by inhibition more than excitation because the trough phases of activity for both scratching and swimming were significantly correlated for multifunctional neurons, while the peak phases were not. I hypothesize that alternation between the hip extensor and flexor, which are vital for successful scratching and swimming, are occurring due to these multifunctional CPG neurons. Furthermore, since the scratch-specialized neurons and flexion reflex-selective neurons were not as strongly rhythmically modulated, it is likely they are not part of the CPG and instead are responsible for starting scratching activity and flexion reflex, respectively.

Also, this study showed the first swim-specialized neuron, which was tonically excited during swimming, but inactive during scratching. Similar to scratch-specialized neurons, this neuron was not strongly rhythmically modulated, so it is not likely to be part of the CPG. It was activated by each swim stimulation pulse at a relatively short latency. I hypothesize that similar to scratch-specialized neurons, the swim-specialized neuron is responsible for starting the motor pattern.

Taken together, this study provides more insight into the organization of the spinal cord CPG for turtles.

Chapter I- Introduction

Scratching, walking, running, swimming, and flying are all rhythmic behaviors produced by the motor output of both vertebrates and invertebrates alike (Clarac, 2008; Gabriel et al., 2008; Stein, 1989; Wilson, 1961). These behaviors would not be possible without complex networks of neurons, called Central Pattern Generators (CPGs), that are generating these motor outputs without the need for sensory feedback (Guertin, 2013; Steuer & Guertin, 2019). There are three main types of CPG organization within studied organisms: dedicated, reorganizing, and multifunctional (Briggman & Kristan, 2008; Grillner et al., 2008; Morton & Chiel, 1994).

A dedicated CPG is composed of groups of neurons that are specialized for only one type of movement and are either inactive or actively inhibited during all other motor patterns. The cricket singing and flying CPGs are a great example of dedicated organization, as both motor patterns have a separate set of interneurons active only during a specific motor pattern (Hennig, 1990). During flying, the neurons that are specialized for flying are activated, whereas the neurons specialized for singing are inhibited. This type of dedicated CPG organization means that the neurons for one activity will only fire during a specific behavior (Briggman & Kristan, 2008). Furthermore, dedicated organization is seen in other species such as locust walking and flight, as well as wing retraction and swimming in the mollusk *Clione* (Briggman & Kristan, 2008; Morton & Chiel, 1994; Ramirez & Pearson, 1988; Satterlie & Spencer, 1985).

Reorganizing CPGs have been studied extensively in the stomatogastric ganglion (STG) of crustaceans (Hooper & DiCaprio, 2004; Marder et al., 2005; Marder & Bucher, 2007). In this type of CPG the network organization is constantly changing, and that can include removal/addition of certain types of neurons, changes in neurons' synaptic connections, and even changes in the intrinsic properties of the neurons (Morton & Chiel, 1994). Also, the CPGs can fuse and form a distinct superordinate CPG, which shows the constant reorganization of the STG. The neurons within this type of organization often reorganize, and some will contribute more to one CPG than another (Briggman & Kristan, 2008; Morton & Chiel, 1994).

Multifunctional CPGs have been studied in many organisms, such as hatchling tadpoles, larval zebrafish, and leeches. Unlike both the dedicated and reorganizing circuitry, the multifunctional circuitry allows for both specialized and multifunctional neurons. The dedicated circuit requires separate pools of neurons that are specialized for one motor pattern, whereas in a reorganizing CPG the various groups of neurons are frequently being added/deleted, changing intrinsically, and restructuring to produce various motor outputs; these two types of organization seem to be at opposite ends of the organizational spectrum, with one being very strict in the organization of the circuitry, and the other allowing for more variation among the neurons producing various motor patterns.

However, the multifunctional organization is intermediate, because some of the circuits are shared, as seen with multifunctional neurons, and other parts of the circuitry contain specialized neurons that only contribute to one type of movement (Briggman & Kristan, 2008; Hooper & DiCaprio, 2004; Morton & Chiel, 1994). For example, there are both multifunctional neurons and specialized neurons found in the hatchling tadpole spinal cord, and both play a role in the production of hatchling tadpole swimming and struggling. The commissural interneurons (cINs) are multifunctional neurons that contribute to both swimming and struggling, whereas the repetitive-firing descending interneurons (dINrs) are specialized, and only help produce the

struggling motor pattern within the hatchling tadpole (Berkowitz et al., 2010; Briggman & Kristan, 2008).

As previously stated, the hatchling tadpole is an example of an organism with multifunctional organization of CPGs within the spinal cord. However, it is one of the least complex vertebrates to be studied for this type of organization, as it has no limbs and is typically 2 days post-fertilization; these attributes allowed for the neurons present to be heavily studied and mapped at this stage of development (Berkowitz et al., 2010). The hatchling tadpole has been studied in depth via intracellular recordings, and the connectivity for each CPG has been heavily researched. Each form of motor activity in hatchling tadpoles is initiated via distinctive sensory inputs, which in turn creates a unique output. For example, the swimming CPG can be activated via a single electrical or mechanical stimulus and produces left/right alternation of the trunk of the body, from head to tail at 10-25 Hz. Alternatively, while the same left/right trunk alternation is utilized during the struggling CPG, it requires repeated/prolonged stimulation of the skin, and produces a higher amplitude body waves at 2-10 Hz in the opposite tail-head direction (Kahn et al., 1982; Kahn & Roberts, 1982; Roberts et al., 2010; Soffe, 1991). These activities, while activated by the same sensory neurons, produce distinct motor outputs.

The individual neurons involved in hatchling tadpole CPGs have been classified based on morphology, neurotransmitter phenotype, and physiology, which are useful for comparisons to more complex species (Berkowitz et al., 2010). The CPGs for hatchling tadpole swimming, short-latency flexion, and struggling have been mapped extensively, and both specialized and multifunctional neurons are present (Berkowitz et al., 2010; Roberts et al., 2010). The unique connectivity of the interneurons for these CPGs is essential for producing different motor patterns, as all of the motor patterns produced by the hatchling tadpole share muscles, Rohon-Beard sensory neurons, and motor neurons (Clarke et al., 1984; Roberts et al., 1999). The 2-days post-fertilization hatchling tadpole only has ten different types of neurons. This includes not only interneurons, but sensory and motor neurons as well.

Seven types of interneurons are found in the hatchling tadpole spinal cord, and they consist of both multifunctional and specialized cells. Two of the types of neurons are specialized for swimming and are named after their morphology and physiology. The descending interneurons (dINs) are excitatory, specialized, and fire only once per swimming cycle. These neurons are essential to the production of swimming in the hatchling tadpole, as they are the main rhythmic excitatory drive behind swimming activity (Berkowitz et al., 2010; Soffe et al., 2009). However, there are also the dINrs, which fire during struggling rather than swimming. These neurons are morphologically similar to the dINs but differ by repetitively firing during struggling. These neurons are not active during swimming because they do not receive enough stimulation to fire, and require the repeated input that produced via prolonged stimulation of the skin for struggling (Berkowitz et al., 2010; Li et al., 2007).

Excitatory commissural interneurons (eCINs) are stimulated by Rohon-Beard sensory neurons and are considered sensory pathway neurons. These neurons and the dorsolateral ascending interneurons, and dorsolateral commissural interneurons (eCINs, dla's, and dlc's, respectively) get excitatory input from Rohon-Beard cells, are glutamatergic, and excite other interneurons and motoneurons. The eCINs are active only during struggling and are classified as specialized neurons, whereas the dlc's/dla's are active for struggling and help with activation of the swimming episode (Berkowitz et al., 2010). However, it is crucial to note that all the neurons

that are classified as specialized that are present within the hatchling tadpole are excitatory rather than inhibitory. Furthermore, the presence of specialized neurons solely being excitatory within the hatchling tadpole could provide a template for what to expect in more complex species' neurons.

Multifunctional neurons in the hatchling tadpole spinal cord are active for more than one type of motor output. The aINs and the commissural interneurons (cINs) are both multifunctional, inhibitory, glycinergic, premotor neurons that are active during struggling and swimming in the hatchling tadpole. The aINs have axons that ascend on the same side of the spinal cord, whereas the cINs are commissural and cross to the other side of the spinal cord and have T-branches of the axon that both ascend and descend (Dale & Roberts, 1985; Roberts & Clarke, 1982; Soffe et al., 1984). Additionally, both the aINs and cINs fire repetitively during each struggling cycle, but typically only fire once, if that, during swimming cycles (Berkowitz et al., 2010; Li et al., 2007). Conversely, the aINs and cINs can be differentiated by firing time during swimming activity, as the aINs typically fire later in the swimming cycle than the cINs (Berkowitz et al., 2010; Li et al., 2001, 2002; Li, 2004; Li et al., 2007). However, even within the multifunctional neurons, preference for one motor pattern can be seen, as both the aINs and cINs fire strongly during struggling, but only fire sporadically during swimming (Berkowitz et al., 2010; Li et al., 2007).

In addition to the specialized neurons of the hatchling tadpole all being excitatory, the multifunctional neurons within the hatchling tadpole are all inhibitory. This could suggest a pattern for more complex organisms and provide insights into the overall organization of other organisms' CPGs. Much time has been spent categorizing neurons within the hatchling spinal cord, and much is known about their morphology, physiology, connections, and neurotransmitter phenotype. The hatchling tadpole being so well studied, provides an example for the possibilities that can arise in more complex organisms, and provides predictions of the neurons involved in various other motor patterns.

The larval zebrafish also displays multifunctional organization and is more complex than the hatchling tadpole. Most studies are typically conducted 3-10 days post-fertilization in the larval zebrafish, which is further along developmentally than the previously discussed hatchling tadpole. This organism, while more complex than the hatchling tadpole, is not as intricate as a limbed animal. However, unlike the hatchling tadpole, the larval zebrafish's CPG organization is not as well understood. In 3-to-10-day post-fertilization larval zebrafish, the movements of the body are similar to the hatchling tadpole and consist of struggling, swimming, and escape. These motor patterns require body bending like the hatchling tadpole and consist of alternating left and right axial body movements (Berkowitz et al., 2010).

Both the swimming and struggling CPGs of the larval zebrafish share similarities to that of the hatchling tadpole. In the larval zebrafish, swimming propagates from the head to tail at 100 Hz and requires body bends akin to the hatchling tadpole swimming CPG. Furthermore, larval zebrafish struggling resembles that of the hatchling tadpole, in that the movement occurs from tail to head at 20 Hz, which is a slower rate than the swimming CPG. Additionally, similar to what is seen in the hatchling tadpoles, the swimming CPG requires a single electrical pulse much weaker than what would initiate an escape, whereas struggling requires repeated stimulation (Berkowitz et al., 2010; Budick & O'Malley, 2000; Liao & Fetcho, 2008; Müller & van Leeuwen, 2004; Ritter et al., 2001). However, dissimilar to the hatchling tadpole, larval

zebrafish swimming eventually reaches a point where swimming can be split into two types as the larvae develop: “slow swimming” and “fast swimming”. The slow swimming seen in larval zebrafish primarily uses the tail, whereas “fast swimming” involves the movement of the entire body and typically follows the escape response (Berkowitz et al., 2010; Budick & O’Malley, 2000). Furthermore, the “faster” swimming has larger body bends before eventually slowing down (Berkowitz et al., 2010).

Similar to what is seen in the hatchling tadpole (dINs), specialized neurons are present in larval zebrafish, and these neurons can be excitatory. The multipolar commissural descending interneurons (MCoDs) are specialized swimming neurons, which are active for both “slow” and “fast” swimming, whereas the circumferential ipsilateral descending interneurons (CiDs) are excitatory neurons that are highly active for escape. The sensory pathway neurons, the commissural primary ascending interneurons (CoPAs) and dorsal longitudinal ascending interneurons (DoLAs) have been compared to the hatchling tadpole dlc’s and dla’s, respectively. The CoPAs are directly excited by Rohon-Beard sensory neurons, are glutamatergic/excitatory, and have similar morphology to hatchling tadpole dlc’s. These neurons are known for initiation of non-escape swimming, similar to how the dlc’s help initiate swimming in hatchling tadpoles (Berkowitz et al., 2010). The similarity between the sensory pathway interneurons of the hatchling tadpole and larval zebrafish could indicate that more complex species could have types of neurons with physiology similar to the aforementioned organisms.

However, unlike the hatchling tadpole, which only has excitatory specialized interneurons, the larval zebrafish contains neurons that are inhibitory and specialized. The commissural longitudinal ascending interneurons (CoLAs) are inhibitory glycinergic neurons that are only active during struggling (Berkowitz et al., 2010; Liao & Fetcho, 2008). The commissural bifurcating longitudinal interneurons (CoBLs) are glycinergic, inhibitory, and are active during both swimming and struggling, while sometimes being active during escape (Berkowitz et al., 2010; McLean et al., 2007). The presence of inhibitory specialized neurons in the CPGs of a more complex organism could indicate that as the complexity of the species increases, so does the complexity of the connectivity of the CPGs themselves.

Multifunctional neurons contribute to swimming, struggling, and escape CPGs found within the larval zebrafish spinal cord. The CoBLs, CiDs, and MCoDs are all multifunctional neurons that are active during more than one type of motor output. Multifunctional neurons such as the cINs/aINs in the hatchling tadpole provide evidence that neurons can be active for more than one type of motor output in various vertebrate CPGs. The CoBLs are glycinergic, inhibitory, and are active during both swimming and struggling, while sometimes being active during escape. These neurons have been said to be homologous to hatchling tadpole cINs, as both have T-branches of the commissural axon that goes both rostrally and caudally (Berkowitz et al., 2010; McLean et al., 2007). Another multifunctional neuron in the larval zebrafish is the CiAs, which are stated to be homologous to hatchling tadpole aINs, since they are both inhibitory, and active for swimming and struggling (Berkowitz et al., 2010; McLean et al., 2007). These homologues are helpful because they indicate that there are similarities of neuron types between species, which can be useful for hypothesizing neuron types and morphological/physiological properties in other species’ CPGs. Furthermore, all multifunctional neurons seen in the larval zebrafish are inhibitory, which is similar to the findings in the

hatchling tadpole. This indicates that it's possible species more complex than the larval zebrafish could have all inhibitory multifunctional neurons as well.

The adult turtle is a good organism to study the CPGs of the spinal cord because as a limbed vertebrate it has various joints that contain flexors and extensors, such as the hip flexor and hip extensor (HF and HE), as well as the knee extensors (KEs). Furthermore, not only are there various joints on a single limb, but the left and right side of the body can be taken into account as well for different motor patterns (Stein, 2005). Furthermore, adult organisms are much more complex than larval or hatchling stages, which means their CPGs would most likely be more complex. Also, since the turtle frequently dives underwater for as long as an hour, it is far more resistant to hypoxia than mammals (Berkowitz, 2010; Lutz & Milton, 2004). This makes studying the CPGs of the spinal cord for scratching, swimming, and flexion reflex of the adult turtle an ideal choice for determining the organization of CPGs for multiple limb movements within an adult spinal cord.

The turtle spinal cord can be transected, therefore removing brain input, and still produce flexion reflex, three different forms of scratching, and swimming (Stein, 2005). Furthermore, these spinal circuits can be defined as CPGs due to not requiring sensory feedback, which can be eliminated via gallamine-induced paralysis that acts as a neuromuscular blocking agent (Robertson et al., 1985). The different forms of activity produced by the turtle have been studied in depth, but much remains to be uncovered about the specifics of CPG organization within this vertebrate's spinal cord.

The three forms of scratching (rostral, pocket, and caudal) can be elicited by mechanically stimulating different areas on the turtle shell and skin near the hindlimb, and can be differentiated by the timing of KE activity in relation to the HF and HE activity cycle (Mortin et al., 1985; Robertson et al., 1985). During a rostral scratching episode, the HF and HE are continuously alternating, while the KE burst occurs around the second half of the HF burst. In the pocket scratching episode, while the HF and HE are alternating, the KE burst typically occurs during a HE burst. Whereas during caudal scratch, while HF and HE are alternating, the KE burst occurs after the HE burst but before the next HF burst (Berkowitz et al., 2010). Furthermore, there are also transition zones, which are located between the rostral and pocket scratching zones, as well as between the pocket and caudal scratching zones. These zones can produce not only either type of the scratching they're located between, but blended motor patterns called hybrids or switches; hybrids contain a mixture of both types of scratching in each cycle, whereas a switch will shift between two motor patterns (Mortin et al., 1985; Robertson et al., 1985). The presence of hybrids indicates that the specific types of scratching share circuitry and reveals more about the organization of the CPGs within the spinal cord of the turtle.

Swimming, as seen in other vertebrates such as larval zebrafish and hatchling tadpoles, does not require sensory feedback in the turtle (Juranek & Currie, 2000). This provides evidence for the presence of a swimming CPG. This fictive swimming, which can occur without sensory feedback or input from the brain, is produced by electrical stimulation of the contralateral dorsolateral funiculus (cDLF) at the third post-cervical segment (D3) of the spinal cord (Juranek & Currie, 2000). Similar to rostral scratching, the swimming motor pattern shows HF and HE alternation, with the KE burst occurring at a similar time to what is observed in rostral scratching. However, rostral scratching and swimming can be differentiated by the amplitude/duration of the HF, HE, and KE bursts. The HE burst within a swimming cycle is both

larger in amplitude and duration than the HF, whereas the HE burst is smaller in amplitude/duration in rostral scratching. Also, the shape of HE is more triangular during fictive swimming than what is seen during rostral scratching with the amplitude starting out large and gradually decreasing. Furthermore, the KE bursts during rostral scratching are larger in amplitude and duration than what is observed in swimming (Berkowitz et al., 2010).

Flexion reflex differs from both swimming and scratching because the motor output is nonrhythmic. This response is produced in turtles from a tap to the dorsal foot and generates an initial short-latency/large-amplitude single burst of the HF before a brief pause that leads to a smaller amplitude/longer lasting second burst of HF. This motor pattern seems similar to what is observed in the hatchling tadpole, with the activity of the flexion reflex response (Berkowitz et al., 2010). This similarity in motor patterns between species could provide a framework for organization within a more complicated organism, such as an adult limbed vertebrate like the turtle.

These three types of scratching, flexion reflex, and swimming have been studied in depth, but much remains to be elucidated about the organization of the CPGs within the turtle spinal cord. However, previous evidence from less complex species can help predict what occurs in a more developmentally advanced adult limbed vertebrate (Berkowitz, 2007, 2008, 2010; Berkowitz et al., 2010). Furthermore, evidence is present within the turtle that indicates there is shared componentry within CPGs for scratching and swimming. For example, with the presence of transition zones for scratching motor patterns, there are sometimes hybrids of two forms of scratching. These hybrids indicate that the CPGs for the different types of scratching are not completely separate (Mortin et al., 1985; Robertson et al., 1985).

Further evidence of shared elements can be seen by dual stimulation of rostral scratching and swimming (Hao et al., 2011; Juranek & Currie, 2000). The dual stimulation can trigger a cycle or more of scratching in the middle of a swimming motor pattern and resets the bursting rhythm of swimming activity. Additionally, the opposite can occur if you start fictive swimming in the middle of a rostral scratching episode. The ability for both motor patterns to reset the rhythm of the other indicates that there are strong interactions between their components, and they possibly share some circuitry. This finding provides more evidence for the presence of shared circuitry between scratching and swimming CPGs.

However, one of the largest pieces of evidence that the CPGs are not entirely separate is the extracellular and intracellular recordings from individual neurons inside the turtle spinal cord (Berkowitz, 2002, 2007, 2008, 2010; Berkowitz et al., 2010). These studies showed the presence and activity of not only multifunctional neurons but also specialized neurons. One study was even able to classify a type of multifunctional neuron by morphology as well as activity. The T-neuron is a type of multifunctional neuron that is active during all forms of scratching, flexion reflex, and swimming (Berkowitz, 2002, 2008, 2010; Berkowitz et al., 2010). The presence of multifunctional neurons, similar to what is seen in hatchling tadpoles and larval zebrafish, indicates that there are some shared aspects of the CPGs for scratching, swimming, and flexion reflex.

As previously stated, the hatchling tadpole and larval zebrafish show multifunctional organization, and contain multifunctional neurons that are active for both swimming and struggling (Berkowitz et al., 2010). These organisms can be used as a roadmap for possible CPG

organization within the turtle spinal cord. In both the hatchling tadpole (aINs/cINs) and larval zebrafish (COBLs/CiAs/CoSAs), the multifunctional neurons were all inhibitory, and contribute to alternation of the left and right side during motor patterns (Berkowitz et al., 2010).

There is also the presence of scratch-specialized neurons within the turtle spinal cord. These neurons are only activated during one or more types of scratching and not during swimming. There are also flexion reflex-selective neurons, which are only activated by a brief tap to the dorsal hindlimb (Berkowitz, 2007, 2008; Berkowitz et al., 2010). The presence of specialized neurons within the turtle spinal cord could provide evidence that the CPGs for scratching and swimming are not totally shared, so they might be partially shared/separate instead (Berkowitz, 2002, 2008, 2010; Berkowitz et al., 2010). Thus, the answer to separate or shared CPGs is much more complicated than a yes or no answer, and the CPGs most likely are partially shared, similar to hatchling tadpole and larval zebrafish.

The inhibitory struggle-specialized cells in the larval zebrafish (CoLAs) suggest there might be inhibitory scratch-specialized neurons in turtles. These neurons could inhibit the HE during scratching, which could explain the decreased amplitude/duration of HE during scratching when compared to swimming. This is important, because understanding how each type of neuron contributes to a specific CPG can help determine CPG organization.

As stated previously, studies of the organization of the CPGs at earlier stages of development have occurred in both larval zebrafish and hatchling tadpole, and they've shown, via intracellular recordings, the contributions of individual neurons to multiple motor patterns (Berkowitz et al., 2010). However, much is still unknown about the overall organization of the turtle spinal cord CPGs. Unlike both the hatchling tadpole and larval zebrafish, the adult turtle is limbed which makes it more complex, and it is also further along developmentally than either of the aforementioned species. Furthermore, the data for interneurons found within the spinal cord of the adult limbed turtle are more limited than for both the hatchling tadpole and larval zebrafish, which indicates a gap in knowledge for more complex species that should be studied. The information from a higher-level organism such as the adult limbed turtle when compared to that of a larval zebrafish or hatchling tadpole, could provide hypotheses about CPG organization that could be tested in future experiments on higher order species, furthering knowledge about the process of decision making of complex movements.

Chapter II- Electrophysiological differences between specialized and multifunctional turtle spinal cord neurons involved in scratching and swimming motor patterns

Introduction

How do organisms' nervous systems determine which motor pattern is produced, when the movements in question share the same muscles and motor neurons? The key to producing the correct motor pattern, in many invertebrate and vertebrate nervous systems, is central pattern generators (CPGs) (Guertin, 2013; Guertin & Steuer, 2009; Steuer & Guertin, 2019), but how are these CPGs organized? There are three types of organization described in both invertebrates and vertebrates: dedicated, reorganizing, and multifunctional (Briggman & Kristan, 2008; Grillner et al., 2008; Morton & Chiel, 1994).

In invertebrates, dedicated organization can be seen within CPGs for cricket singing and flying, and locust walking and flight (Hennig, 1990; Ramirez & Pearson, 1988). This type of organization is composed entirely of specialized CPG neurons, which are only active during a particular motor pattern (Briggman & Kristan, 2008). However, invertebrates have also been shown to have reorganizing CPGs that contain changing groups of neurons, where synaptic connections and intrinsic neuronal properties are constantly rearranging (Briggman & Kristan, 2008; Morton & Chiel, 1994). This is seen especially in the stomatogastric ganglion (STG) of crustaceans (Marder & Bucher, 2001, 2007).

Multifunctional organization, composed of both specialized neurons and multifunctional neurons, has been observed in vertebrates, such as the hatchling tadpole and larval zebrafish (Berkowitz et al., 2010; Roberts et al., 2010). In these species, both multifunctional and specialized interneurons are present; the ascending interneurons (aINs) in tadpoles and commissural bifurcating longitudinal interneurons (CoBLs) in zebrafish, each contribute to both swimming and struggling motor patterns, whereas the repetitive-firing descending interneurons (dINrs) in tadpoles and excitatory commissural interneurons in tadpoles (eCINs) are both specialized for struggling (Berkowitz et al., 2010). However, while much is known about the organization of the CPGs of these organisms, there is still much to be uncovered for adult limbed vertebrates.

The turtle is good organism for studying CPG organization in an adult limbed vertebrate, as it produces motor patterns for three forms of scratching, swimming, and flexion reflex without sensory feedback or brain input (Juranek & Currie, 2000; Lennard & Stein, 1977; Mortin et al., 1985; Robertson et al., 1985; Stein et al., 1982). Furthermore, because turtles will often dive underwater for long periods of time, they have evolved greater resistance to hypoxia compared to mammals, which makes them ideal for physiological studies (Lutz & Milton, 2004).

Both extracellular and intracellular recordings of the neurons within the turtle spinal cord have shown the presence of multifunctional interneurons that are activated for scratching, swimming, and flexion reflex, as well as neurons that are specialized for scratching, and other neurons that are specialized for flexion reflex (Berkowitz, 2002, 2007, 2008, 2010). Hybrid motor patterns, which are a mixture of two distinct motor patterns (Berkowitz & Hao, 2011), have been seen in both dual stimulation of different scratch motor patterns, as well as dual stimulation of scratching and swimming (Earhart & Stein, 2000; Hao et al., 2011; Hao & Berkowitz, 2017; Stein et al., 1986). This evidence, along with the presence of multifunctional neurons, indicates the presence of some shared circuitry for distinct motor patterns. There are previous studies analyzing intracellular recordings of both specialized and multifunctional neurons in the turtle spinal cord, but since these studies combined electrophysiology and morphology (Bannatyne et al., 2020; Berkowitz, 2008), those cells where dye injection was unsuccessful were not analyzed. Here, we analyze electrophysiological data from intracellular recordings without accompanying morphology to elucidate electrophysiological properties of multifunctional and specialized neurons, suggesting how they may contribute to swimming, scratching, and flexion reflex.

Methods

Data Acquisition

The data analyzed in this study were from turtle spinal cord intracellular recording experiments from 2011-2017 (Bannatyne et al., 2020). These cells were originally recorded with the purpose of morphology and electrophysiology dual analysis. However, the cells analyzed in this study did not have successful morphology data, so were left unanalyzed for the aforementioned study. This current study is analyzing these neurons for the first time.

Animal Preparation

Adult red-eared sliders were used in this study (*Trachemys scripta elegans*, 360-850 g, both sexes, n= 44). The turtle was first placed in ice to induce hypothermic analgesia and underwent three main procedures while continuously covered in ice: First, the spinal cord was transected between dorsal spinal segments 2 and 3 (D2-D3). Second, dorsal segments 6 through 10 (D6-D10) and sacral segments 1 & 2 (S1-S2) were exposed. Third, several right hindlimb motor nerves were prepared for electroneurographic recordings (ENGs). These nerves included: the hip flexor, ventral puboischiofemoralis internus, pars anteroventralis (HF), the hip extensor, flexor cruris, pars flexor tibialis internus (HE), and one or more of the knee extensors: triceps femoralis, pars iliotibialis (IT), pars ambiens (AM), and/or pars femorotibialis (FT). The immobilization agent, gallamine triethiodide (8 mg/kg; Sigma-Aldrich, St. Louis, MO), was administered intramuscularly after these procedures were completed, and the turtle was artificially respirated for the duration of the experiment. These procedures have been described previously (Berkowitz, 2001). The Institutional Animal Care and Use Committee (IACUC) of the University of Oklahoma approved all procedures.

Motor Pattern Stimulation

ENGs were obtained by pairs of 100-μM silver wires wrapped around the nerves, suspended in a pool of mineral oil. Fictive scratching was evoked by mechanical stimulation with a glass probe in the proper zone (Fig.1). Fictive forward swimming was evoked by electrical stimulation of the contralateral D3 lateral funiculus (0.1-ms, 200- to 400-μA pulses at 40 Hz). Fictive flexion reflex was elicited by a tap to the dorsum of the foot, with the same glass probe used for fictive scratching, or by electrical stimulation to the skin of the dorsal foot (Fig. 1) (Currie and Stein, 1989).

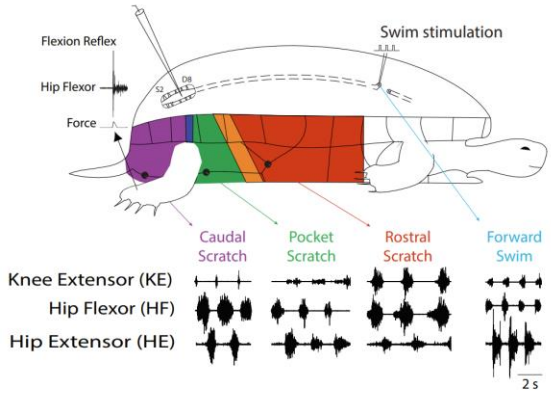


Fig. 1. Diagram of experimental turtle preparation. Intracellular recordings of neurons located in the spinal cord between spinal segments D8 and S2. In vivo fictive scratching and limb withdrawal were induced by either tickling in the respective scratching zones, or electrical stimulation of the dorsum of the foot, respectively. Fictive swimming was induced via electrical stimulation of the D3 dorsolateral funiculus. Motor output was monitored via nerve recordings of the knee extensor (KE), hip flexor (HF), and hip extensor (HE).

Intracellular recordings

All neurons were located on the right side in one of the five segments of the hindlimb enlargement (D8, D9, D10, S1, and S2).

Cell Criteria

All neurons (n=71) had to be < 1000 μM from the dorsal surface, to largely exclude motor neurons. The cells had to have clear postsynaptic potentials to ensure the recordings were intracellular rather than extracellular, and at least 30-mV action potential amplitudes. If the signal for HF nerve activity was too low, HE was used for analysis instead. Cells were required to have been recorded during at least two full cycles of swimming, as well as at least two full cycles of one type of scratching (rostral, pocket, or caudal). Cells not meeting these requirements were not used in this study.

Electrophysiological Analysis

Dual-referent phase histograms (Berkowitz and Stein, 1994) (Fig. 2) of intracellularly recorded neurons were calculated with respect to the onset and offset of bursts of a nerve, usually HF but in some cases HE, during scratching and/or swimming motor patterns, provided that at least two cycles of the rhythm occurred, using a custom script written in Spike2 (Cambridge Electronic Design). If the signal for HF nerve activity was too low, HE was used for analysis instead. The phase histogram data were then used to calculate the mean vector (Batschelet 1981; Berkowitz and Stein 1994; Drew and Doucet 1991; Mardia 1972). The mean vector length (MVL) indicates the degree of rhythmic modulation; an MVL of 0 would indicate no phase preference with respect to the hip flexor cycle, whereas an MVL of 1 would indicate that all action potentials occurred in the same phase (one tenth) of each cycle. The mean vector angle (MVA) indicates the neuron's spiking phase preference within the hip flexor cycle; an MVA of 0–180° (or 0–0.5) would indicate a phase preference during hip-flexor activity and an MVA of 180–360° (or 0.5–1.0) would indicate a phase preference within the hip-flexor interburst intervals. The null hypothesis that the interneuron's firing occurred at random with respect to the hip flexor cycle was evaluated using the Rayleigh test (Batschelet 1981; Berkowitz and Stein 1994; Drew and Doucet 1991; Mardia 1972). MVAs were used in analyses only if the phase histogram passed the Rayleigh test with $p < 0.05$. The scratch MVL used for comparison to the swim MVL was the average of the MVLs for all available forms of scratching. The scratch MVA used for comparison to the swim MVA was for the form of scratching that passed the Rayleigh test with the lowest p value, unless phase histograms for multiple forms of scratching had $p < 0.001$, in which case the one among these with the highest MVL was used (Berkowitz 2001, 2002,2008; Berkowitz and Stein 1994). Cycles of scratching motor patterns that showed HE deletions (Robertson et al., 1985) were excluded from oscillation amplitude analyses, peak/trough data, and MVL/MVA analyses, so as not to skew the data. Furthermore, the cell had to fire at least ten action potentials during these motor patterns to be used for MVA and MVL analysis.

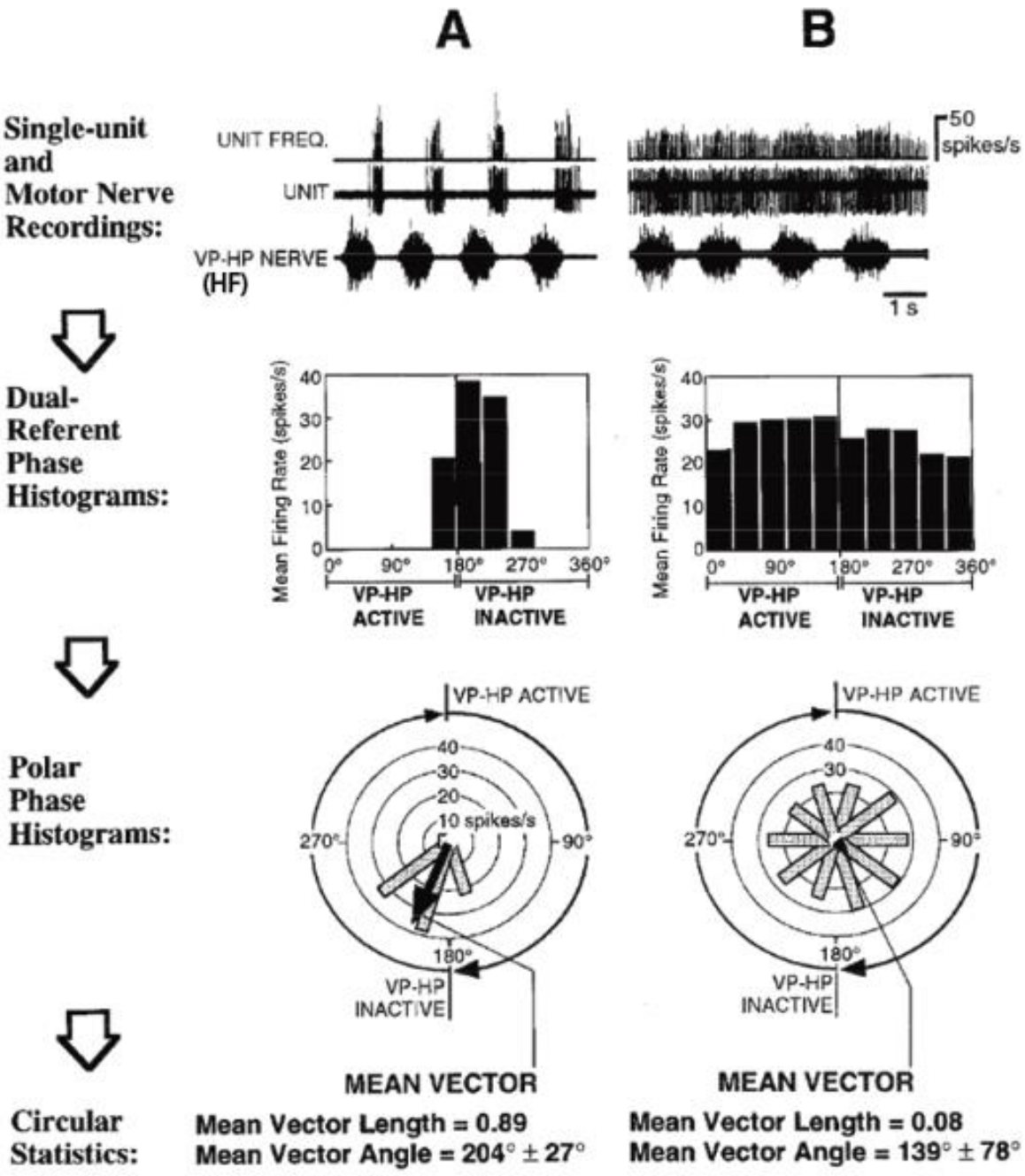

A
B
Single-unit and Motor Nerve Recordings:
UNIT FREQ.
UNIT
VP-HP NERVE (HF)
50 spikes/s
1 s
Dual-Referent Phase Histograms:
Mean Firing Rate (spikes/s)
40
30
20
10
0
0°
90°
180°
270°
360°
VP-HP ACTIVE
VP-HP INACTIVE
Polar Phase Histograms:
VP-HP ACTIVE
40
30
20
10 spikes/s
270°
90°
180°
VP-HP INACTIVE
MEAN VECTOR
Circular Statistics:
Mean Vector Length = 0.89
Mean Vector Angle = 204° ± 27°
Mean Vector Length = 0.08
Mean Vector Angle = 139° ± 78°

Fig.2 Method for phase analysis of neuron activity within HF nerve activity cycle during fictive scratching and fictive swimming. The transformation of concurrent interneuron spiking and motor-nerve recordings (top row) into dual-referent phase histograms (second row) and descriptive circular statistics (bottom two rows) is illustrated for a very strongly rhythmic neuron (A) and a very weakly rhythmic neuron (B). Dual Referent Phase Histogram: Each VP-HP (HF) nerve activity cycle was defined to begin at the onset of a burst of nerve activity and to end at the onset of the next burst of nerve activity. Each nerve burst (or nerve-active period) was divided into five equal time bins; each nerve-inactive period was also divided into five equal time bins. The interneuron mean firing rate was calculated for each of the 10 bins of each nerve activity cycle, by dividing the number of interneuron spikes in the bin by the bin duration. The interneuron mean firing rates for the first bin of each of several nerve activity cycles were themselves averaged, to obtain a mean value of the mean firing rate for the first bin. The same calculation was performed for each of the other nine bins, yielding the interneuron mean firing rates during 10 phases of the HF activity cycle. Mean Vector: the nerve activity cycle was treated as a circle, with 0 defined as the onset of HF nerve activity and 180 defined as the offset of HF nerve activity. Each value of interneuron mean firing rate per bin was treated as a vector. Each vector's length was defined as the mean firing rate for a particular bin and each vector's angle was defined as the angle of the center of the bin within the cycle. Thus, the distribution of interneuron mean firing rates within the HF nerve activity cycle was represented by 10 vectors, one for each of the 10 phases of the cycle. The vector sum of these 10 vectors, normalized to the sum of the mean firing rates, is called the mean vector (Batschelet, 1981). Figure from Berkowitz and Stein.

Membrane Potential Oscillations

Action potentials were deleted, and voltage values interpolated for these brief intervals, in Spike2; multiple cycles of membrane potentials were averaged to create a dual-referent phase-averaged membrane potential, from which the peak phase, trough phase, and oscillation amplitude were measured (Berkowitz 2005, 2008). If the peak phase was calculated with respect to the onset and offset of HE, then 0.5 was added to or subtracted from the peak phase to assess all peak phases with respect to the HF activity cycle (Berkowitz, 2008). If more than one form of scratching (rostral, pocket, caudal) was evoked, then the motor pattern with the highest oscillation amplitude was used for comparison of peak phase, trough phase, and oscillation amplitude for scratching. In some cases, 1.0 (= 360˚) was added or subtracted to the peak or trough phase for display purposes (Fig. 8); it is important to note that adding or subtracting 1.0 does not change the phase preference.

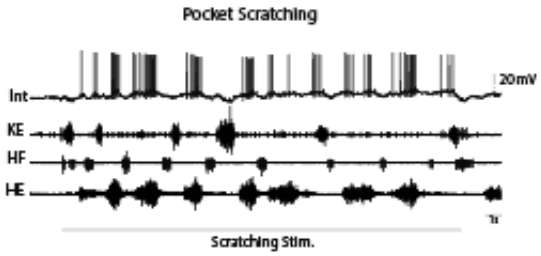
Statistics

As in previous studies (Berkowitz, 2002, 2005, 2008), for linear variables such as oscillation amplitude and mean vector length (MVL), the Kruskal-Wallis and Dunn's nonparametric tests were used to determine if the p-value was less than 0.05. Furthermore, the Pearson's correlation coefficient was used to determine correlation within linear scatterplots, such as oscillation amplitude and MVL. To determine if correlation was present between circular parameters, such as the MVA, and trough/peak phase comparisons, the circular-circular correlation coefficient (r_C) was used to assess significance (Batschelet, 1981; Berkowitz, 2008).

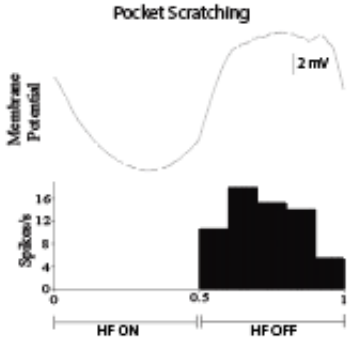
Results

Seventy-one neurons activated during fictive scratching, fictive forward swimming, and/or fictive flexion reflex were analyzed. The neurons included multifunctional (n=47), scratch-specialized (n=19), flexion reflex-selective (n=4), and swim-specialized (n=1). Each multifunctional neuron (e.g., Fig. 3A-D) is activated during both scratching (Fig. 3A-B) and swimming (Fig. 3C-D), whereas each scratch-specialized neuron (e.g., Fig. 3E-H) increases its spike rate during scratching (Fig. 3E-F), but not during swimming (Fig. 3G-H).

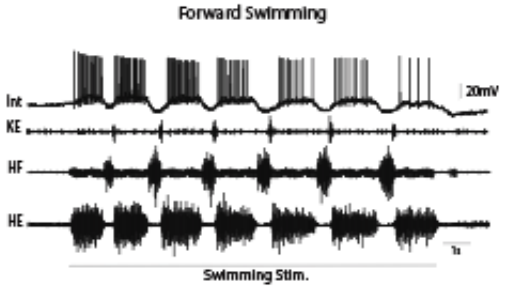
A


Pocket Scratching
Int
KE
HF
HE
20mV
1s
Scratching Stim.

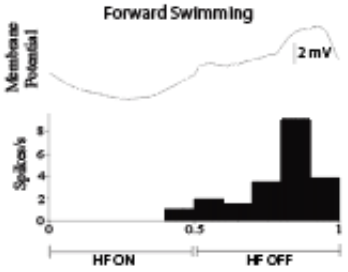
B


Pocket Scratching
Membrane Potential
2 mV
Spikes/s
16
12
8
4
0
0
0.5
1
HF ON
HF OFF

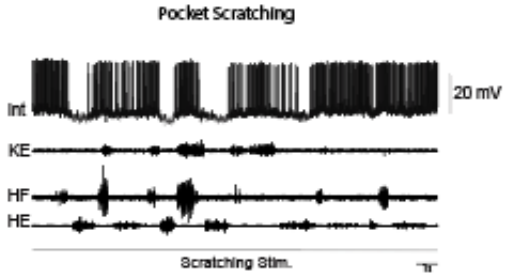
C


Forward Swimming
Int
KE
HF
HE
20mV
1s
Swimming Stim.

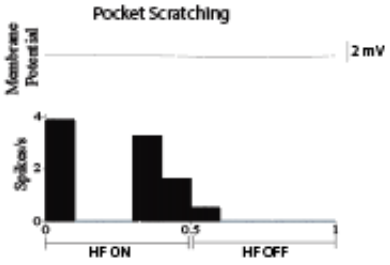
D


Forward Swimming
Membrane Potential
2 mV
Spikes/s
0
0.5
1
HF ON
HF OFF

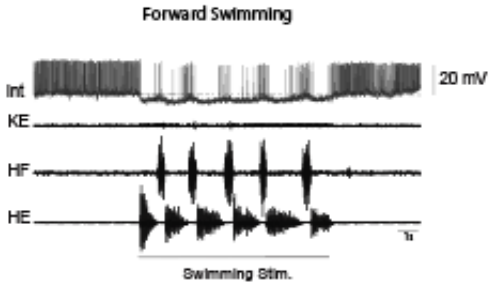
E


Pocket Scratching
Int
KE
HF
HE
20 mV
Scratching Stim.
1s

F


Pocket Scratching
Membrane Potential
2 mV
Spikes/s
4
0
0
0.5
1
HF ON
HF OFF

G


Forward Swimming
Int
KE
HF
HE
20 mV
1s
Swimming Stim.

H

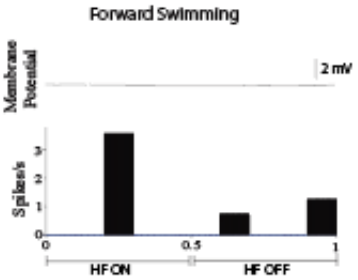

Forward Swimming
Membrane Potential
2 mV
Spikes/s
3
2
1
0
0
0.5
1
HF ON
HF OFF

Fig. 3. Comparisons of multifunctional and scratch-specialized intracellular recordings during scratching and swimming. A-D: a multifunctional neuron that is rhythmically excited for pocket scratching and forward swimming A&C: ENG and intracellular recordings during pocket scratching and swimming. B&D: phase histogram of membrane potential and spike rate during pocket scratching and forward swimming. E-H: a scratch-specialized cell that is rhythmically activated during pocket scratching. F: phase histogram of membrane potential and spike rate during pocket scratching. G: the same scratch-specialized neuron was rhythmically inhibited during swimming. H: phase histogram of membrane potential and spike rate during forward swimming.

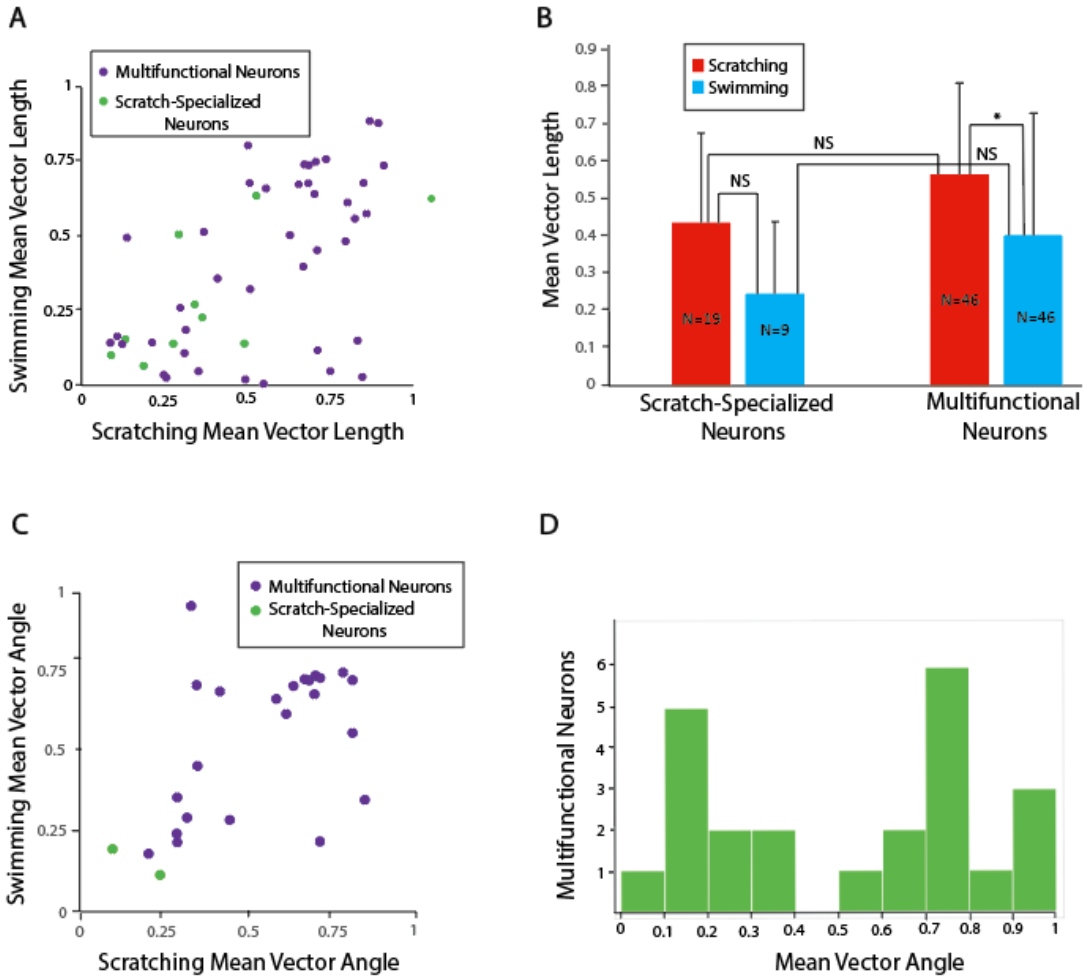


Fig. 4. Comparisons of rhythmicity and phase preference in multifunctional and scratch-specialized cells. A: mean vector length for scratching and swimming in multifunctional (n=40) and scratch-specialized neurons (n=9). B: bar graphs comparing the mean vector length for scratching and swimming in scratch-specialized and multifunctional neurons C: mean vector angle during scratching and swimming of multifunctional (n=23) and scratch-specialized (n=2) neurons. D: histogram indicating two distinct groups of multifunctional neurons based on firing phase preference. *, $p<0.05$; NS, not significantly different.

The mean vector is composed of both an MVL and MVA (see Methods). The information provided by the mean vector helps with analyzing firing activity of an individual neuron during a motor pattern: MVL (Fig. 4A-B) and MVA (Fig. 4C-D) are used to determine a neuron's rhythmicity and phase preference for firing action potentials within the hip-flexor activity cycle, respectively. If the MVL is significant (Rayleigh test $p<0.05$), then the MVA reliably shows the neuron's firing phase preference during the HF activity cycle.

Analysis of the MVLs shows that a neuron that is highly rhythmic for one motor pattern tends to be highly rhythmic for both motor patterns (Fig. 4A; $r=0.57$, $p<0.01$). Also, it seems that multifunctional neurons tend to be more rhythmic than any other neuron type. Neurons that are not highly rhythmic for scratching seem to also have low rhythmicity for swimming as well. When comparing the average MVL for scratching and swimming between scratch-specialized neurons and multifunctional neurons (Fig. 4B), there was a statistically significant difference between scratching and swimming in multifunctional neurons. Although, no statistically significant differences were observed between multifunctional and scratch-specialized neurons as a whole, a statistically significant difference was observed between the MVLs of scrath-specialized neurons and multifunctional neurons when only analyzing neurons that spiked sufficiently for both motor patterns; the scratch-specialized neurons had a significantly lower MVL than multifunctional neurons during scratching, which would indicate that the multifunctional neurons were significantly more rhythmically modulated during scratching than scratch-specialized neurons were (Fig. 4B; Kruskal-Wallis, followed by Dunn's test, $p<0.05$ between scratching and swimming MVLs of multifunctional neurons).

Analysis of the MVAs for both scratching and swimming indicated similar phase preferences for the different motor patterns. Twenty-three multifunctional neurons (49%) out of forty-seven possible multifunctional neurons and two (5%) of the nineteen scratch-specialized neurons were significantly rhythmic (Rayleigh test $p < 0.05$). For MVA comparisons (n=25), a significant correlation ($r_C<0.01$) between the scratching MVA and swimming MVA was determined via a circular-circular correlation test; this indicates that neurons have correlated phase preferences between scratching and swimming motor patterns (Fig. 4A). Although scratch-specialized neurons do not increase their firing rate during swimming, they can still fire action potentials during swimming, which allows for MVA to be calculated. The trend for neurons to have correlated phase preferences can be observed via the scatterplot for both multifunctional

neurons (green circles; n=23) and scratch-specialized neurons (purple circles; n=2) across many neurons (Fig. 4C); most neurons tend to have similar phase preferences for scratching and swimming. The mean angular difference (MVA) between the scratching and swimming MVAs was 0.02, indicating that phase preferences were nearly the same during scratching and swimming.

Multifunctional neurons appear to be bimodal in their firing phase preferences, with two peaks, at 0.1-0.2 and 0.7-0.8 (Fig. 4D). This could indicate that multifunctional neurons comprise two groups, one for HF-on phase and another for HF-off phase.

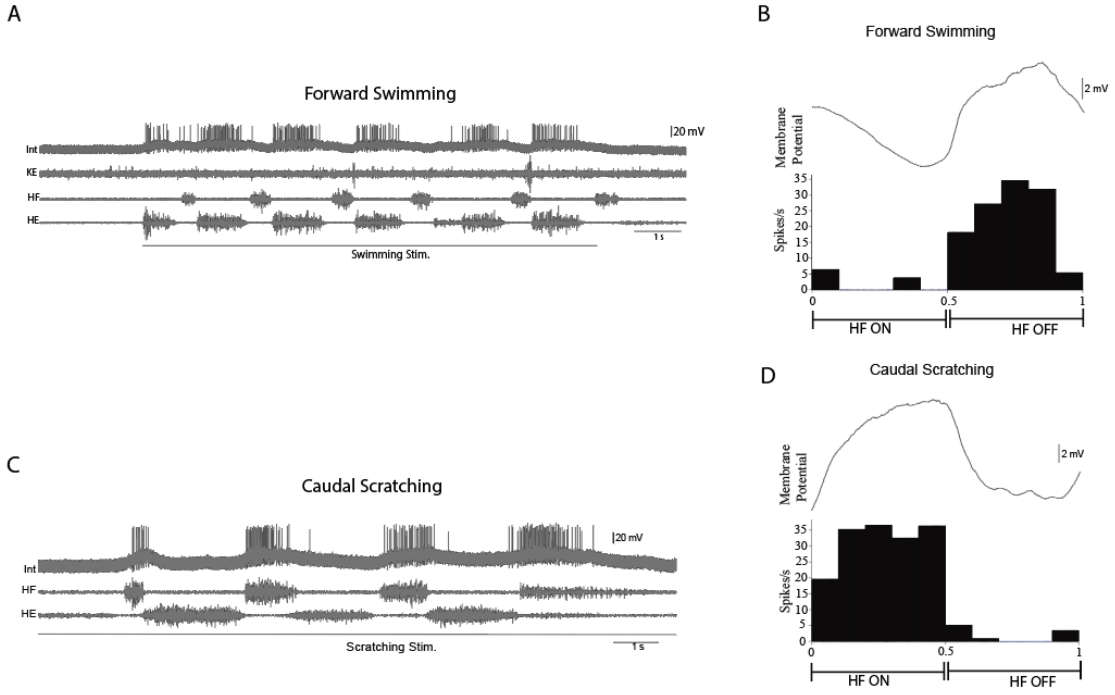


Fig. 5 A multifunctional neuron with opposite phase preferences during caudal scratching and swimming. A&C: intracellular recording of forward swimming and caudal scratching B&D: phase histogram of membrane potential and spike rate during swimming and caudal scratching.

Although neurons overall tended to have the same phase preferences within the hip activity cycle for scratching and swimming, they could have opposite phase preferences. Unlike most multifunctional neurons, the neuron shown in Figure 5 had a phase preference for HF-off during forward swimming (Fig. 5B), but HF-on during caudal scratching (Fig. 5D).

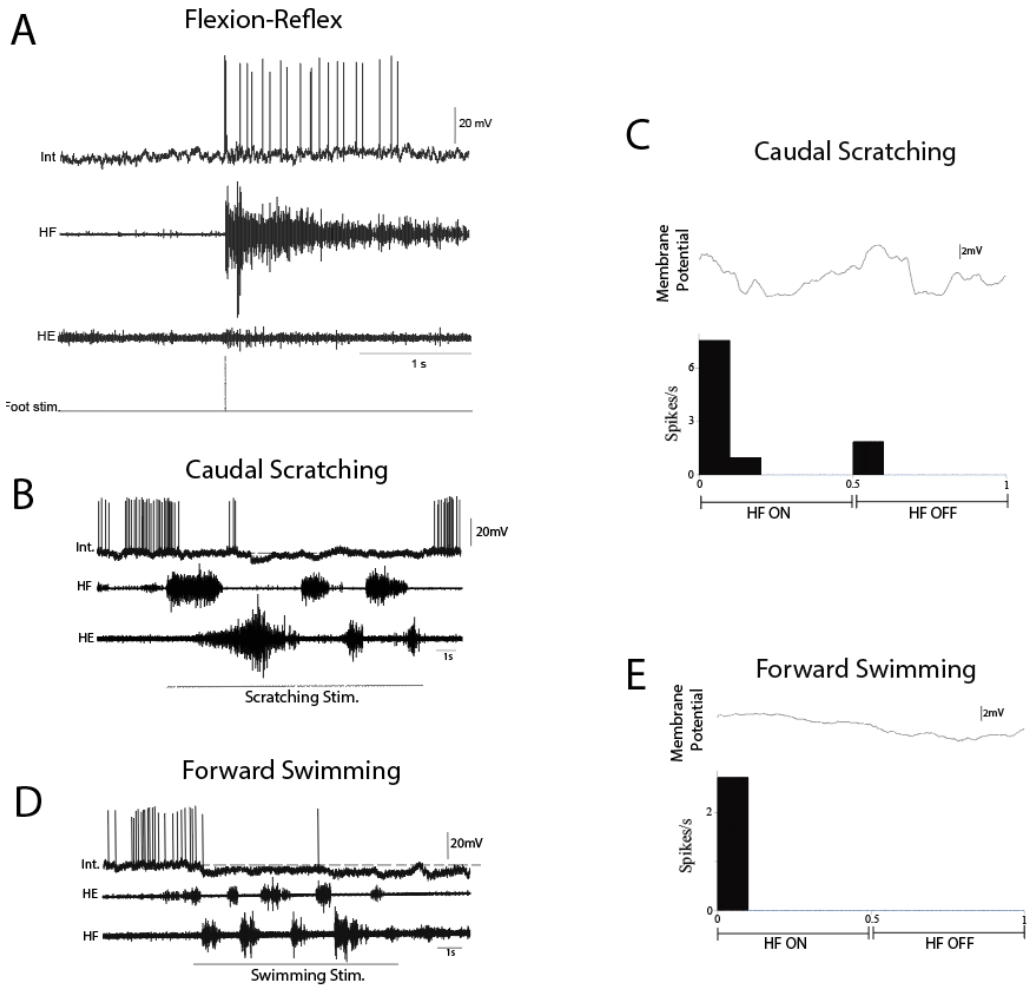


Fig. 6 Flexion reflex-selective neuron. A: intracellular recording during flexion reflex stimulation. B: Same neuron during caudal scratching. C: phase histogram of membrane potential and spike rate during caudal scratching. D: intracellular recording from same flexion reflex-selective neuron during forward swimming. E: phase histogram of membrane potential and spike rate during swimming. The dashed line represents the membrane potential prior to the motor pattern.

Flexion reflex-selective neurons are activated during flexion reflex, but not during scratching or swimming (e.g., Fig. 6). Furthermore, in addition to being inhibited during scratching and swimming (Fig. 6B&D), evidenced by the membrane potential dropping below the baseline (dashed line in Fig. 6B&D) during scratching and swimming, they also did not show strong rhythmic oscillation; the oscillation amplitude was only about 2 mV for both scratching and swimming for this neuron. Although statistical tests could not be performed because there were too few cells, the average oscillation amplitude of flexion reflex-selective neurons during

scratching and swimming was 3.7 ± 1.7 mV and 1.6 ± 0.5 mV, respectively, which is much lower than both multifunctional (9.6 ± 9 mV, 6.3 ± 5.4 mV) and scratch-specialized (7.3 ± 4.7 mV, 4.2 ± 2.4 mV) neurons. Overall, the flexion reflex-selective neurons were slightly rhythmically modulated during scratching and swimming, but not as much as multifunctional or scratch-specialized neurons.

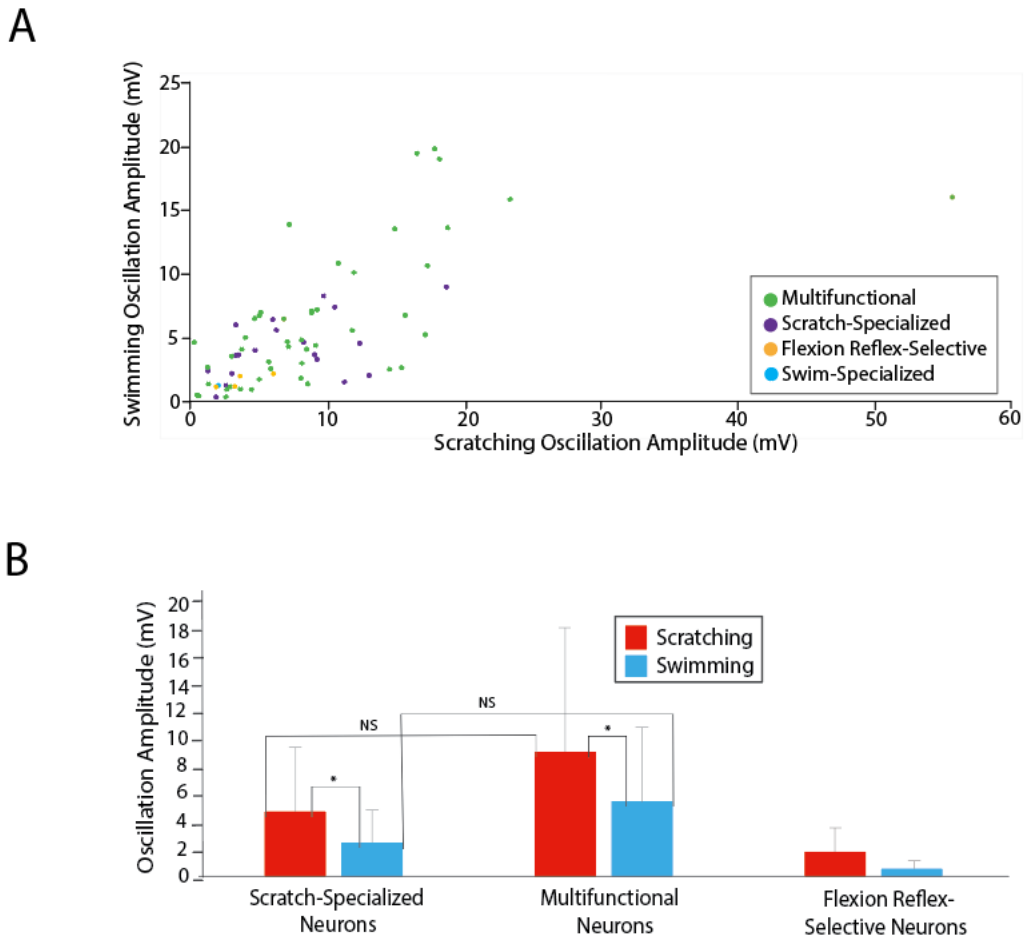


Fig. 7 Oscillation amplitudes of scratch-specialized neurons, flexion reflex-selective neurons, multifunctional neurons, and a swim-specialized neuron during scratching and swimming. A: scatterplot of scratching oscillation amplitudes compared to swimming oscillation amplitudes ($p<0.01$, $r=.66$). B: bar graph comparing the mean oscillation amplitudes for scratching and swimming between scratch-specialized, multifunctional, and flexion reflex-selective neurons. *, $p<0.05$; NS, not significantly different.

Membrane potential oscillation amplitudes allow for analysis of rhythmicity for multifunctional, scratch-specialized, flexion reflex-selective, and swim-specialized neurons during scratching and swimming, whether or not they fired action potentials (Fig. 7A). The scratching oscillation amplitude and swimming oscillation amplitude were strongly correlated for

neurons overall ($p<0.01$, $r=0.66$). The rhythmicity seen in this larger group of neurons mimics MVL results; neurons that were strongly rhythmically modulated, were modulated for both scratching and swimming. More generally, similarly to what was seen in the MVL analysis, multifunctional neurons tended to have larger rhythmic oscillations than any type of specialized neuron (Fig. 7A-B).

Both scratch-specialized neurons and multifunctional neurons had significantly larger average scratching oscillation amplitude than swimming oscillation amplitude (Kruskal-Wallis followed by Dunn's test; $p < 0.05$). Neither the swim-specialized neuron nor the flexion reflex-selective neurons had enough data to perform the statistical test.

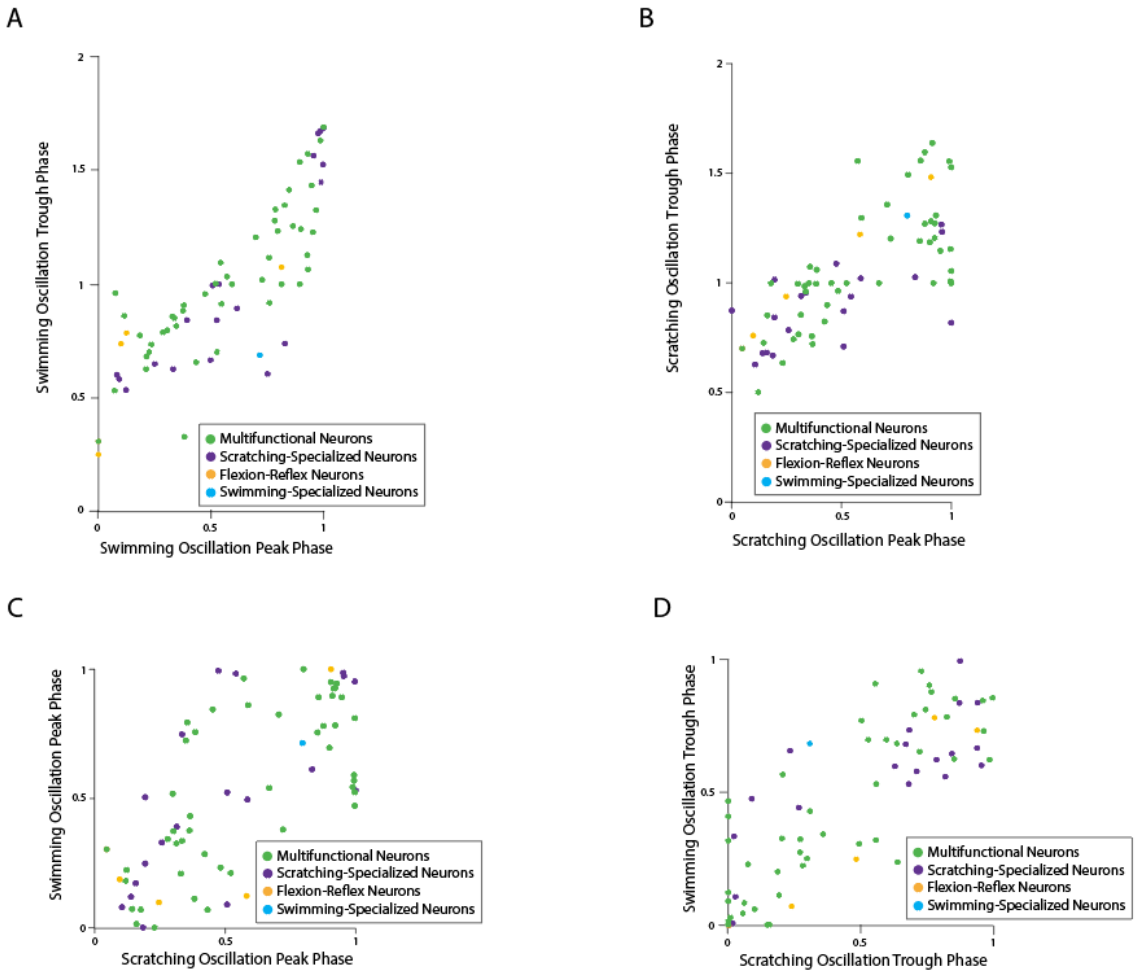


Fig. 8 Comparisons of the oscillation peak and trough phases of all neuron types (multifunctional, scratch-specialized, flexion reflex-selective, and swim-specialized neurons). Circular-circular correlation tests were performed for peak to trough comparisons, but only for scratch-specialized and multifunctional neurons, as the other neuron types did not have enough neurons to test ($n<5$). A, B, D: Swim oscillation peak vs. swim oscillation trough, scratch oscillation peak vs. scratch oscillation trough, and scratch oscillation trough vs. swim oscillation trough showed correlation ($p<0.01$) for multifunctional neurons only. C: Scratching oscillation peak vs. swimming oscillation peak showed no significant correlation between individual neuron types ($p >0.01$).

Analyzing the peak and trough phases of phase-averaged membrane potentials during scratching and swimming could help determine if specific cell types are being rhythmically modulated via excitation or inhibition. The circular-circular correlation test was used to assess significant correlation between pairs of phases for trough-trough, peak-peak, and peak-trough comparisons during scratching and swimming; when there was a significant correlation, the mean angular difference (MVA) between the pair was calculated. There was correlation between the trough and peak phases for multifunctional neurons, during both swimming (Fig. 8A: circular-circular correlation=0.57, $p<0.01$, MVA=0.45) and scratching (Fig. 8B: circular-circular correlation=0.41, $p<0.01$, MVA=0.48). Trough-trough phase comparisons (Fig. 8D: circular-circular correlation=0.5, $p<0.01$, MVA=0.004) and peak-peak phase comparisons (Fig. 8C: circular-circular correlation=0.24, $p>0.01$) showed that neurons have similar phase preferences for scratching and swimming, whereas the trough and peak phase differed from each other by about half of a cycle of activity.

The peak phases of swimming and scratching appeared correlated in multifunctional and scratch-specialized neurons (Fig. 8C) but were not significantly correlated ($p>0.05$). In contrast, the trough phases of swimming and scratching were significantly correlated ($p<0.01$) in multifunctional neurons (Fig. 8D). This suggests that inhibition largely creates the rhythmic modulation observed in both scratching and swimming motor patterns in multifunctional neurons.

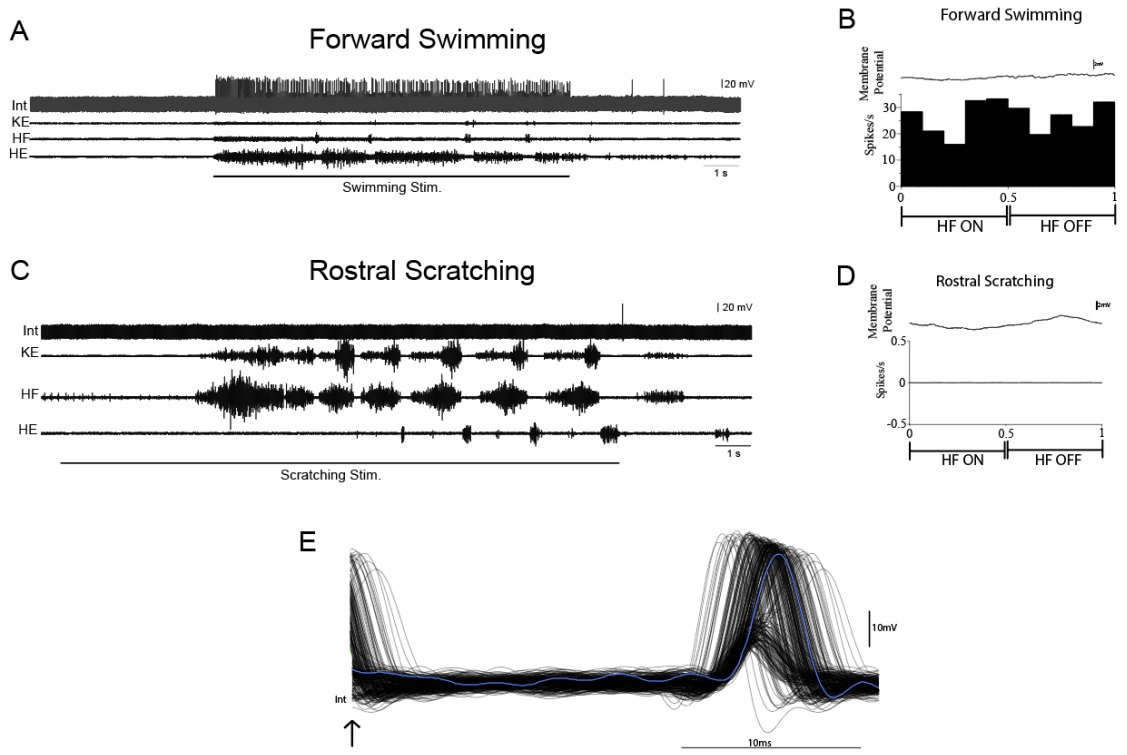


Fig. 9 A swim-specialized neuron. A: Intracellular recording of a swim-specialized neuron during swimming stimulation. C: Intracellular recording of the same neuron during rostral scratch stimulation. B&D: phase histogram of membrane potential and spike rate during forward swimming and rostral scratching. E: Superimposition of this neuron's response to each swim stimulation pulse (arrow).

Previous studies have described scratch-specialized neurons and flexion reflex-selective neurons, but a swim-specialized neuron has not been described previously (Fig. 9). This neuron was tonically activated during swimming and did not appear to have rhythmic oscillations during swimming (Fig. 9A-B). This neuron was not active during rostral scratching stimulation but had one spike after stimulation was complete (Fig. 9C). However, it did have a small membrane oscillation, with a defined peak and trough, during rostral scratching (Fig. 9D). When superimposing the neuron's response to each swim stimulation pulse the neuron had an excitatory post-synaptic potential and usually a spike, at a variable latency of about 20 ms (Fig. 9E); this suggests that the neuron was excited oligosynaptically by one or more of the cDLF axons being stimulated.

Discussion

This study suggests that multifunctional neurons are more strongly rhythmically modulated than behaviorally specialized neurons during scratching and swimming. Forty-nine

percent of multifunctional neurons had a significant mean vector length, indicating rhythmic spiking, as compared to only 5% of scratch-specialized neurons. Multifunctional neurons tended to have a higher mean vector length on average than scratch-specialized neurons. This tendency did not reach statistical significance when comparing to scratch-specialized neurons as a whole, but was statistically significant when comparing to the set of scratch-specialized neurons that had enough spikes during both scratching and swimming to calculate their mean vector lengths. Multifunctional neurons also were statistically significantly more rhythmically modulated during scratching than swimming. Previous studies looking at both intracellular and extracellular recordings of multifunctional neurons in turtles have also found similar results, but this study included a larger number of neurons than previous intracellular studies (Bannatyne et al., 2020; Berkowitz, 2002, 2008). Evidence from this study as well as previous intracellular studies (Berkowitz, 2007, 2008) suggest that oscillation amplitudes are larger on average in multifunctional neurons than in scratch-specialized, flexion reflex-selective, or swim-specialized neurons. The stronger rhythmic modulation in multifunctional neurons suggests that multifunctional neurons, or at least a subset of them, are part of the CPG for both scratching and swimming.

Significant correlation was found in multifunctional neurons between the trough phases, but not the peak phases, of membrane potential oscillations during both scratching and swimming; this suggests that rhythmic inhibition plays a greater role than rhythmic excitation in creating the rhythmic oscillations of multifunctional neurons, as also suggested by previous turtle intracellular studies involving fewer neurons (Bannatyne et al., 2020; Berkowitz, 2008). One recent study also indicated that multifunctional neurons mainly release inhibitory neurotransmitters (Bannatyne et al., 2020), so it is possible that while these CPG neurons are being inhibited, they are also inhibitory. Multifunctional neurons also tend to be rhythmic and inhibitory in tadpole and larval zebrafish swimming and struggling, involving left-right alternation, which is similar in concept to HF and HE alternation seen in the turtle hindlimb (Berkowitz et al., 2010). I hypothesize these inhibitory multifunctional neurons create the alternation between hip flexors and hip extensors during both scratching and swimming motor patterns.

In this study we also found that multifunctional neurons have similar phase preferences in scratching and swimming motor patterns in turtles; these preferences were assessed by both spike rate and membrane potential oscillation peak phases. This has been suggested in previous studies, but for membrane potential oscillations, the current study (n=71) has almost twice the number of neurons as the previous intracellular studies (n=37 and n=30, respectively) (Bannatyne et al., 2020; Berkowitz, 2008). The multifunctional neurons typically fire during the same phase of the hip activity cycle during different motor patterns, as previously suggested (Berkowitz, 2002, 2005, 2008; Berkowitz & Stein, 1994). Multifunctional neurons tended to depolarize in the middle of the HF-on phase or the middle of the HF-off phase. However, I also found an unusual neuron that fired during opposite phases of the hip activity cycle during scratching vs. swimming, which might indicate this neuron is involved in exciting or inhibiting a different motor pool, such as knee extensors.

Scratch-specialized neurons in this study also were mostly not significantly rhythmically modulated during scratching and swimming, consistent with previous studies (Berkowitz, 2002, 2008). This suggests that scratch-specialized neurons are likely not part of the CPG for either scratching or swimming. The flexion reflex-selective neurons we studied had even smaller rhythmic oscillations during scratching and swimming than scratch-specialized neurons. I hypothesize that instead, scratch-specialized and flexion reflex-selective neurons are involved in triggering the motor patterns for scratching or flexion reflex, respectively.

This is the first study to identify a turtle swim-specialized neuron, to the best of my knowledge. This neuron was tonically excited with no apparent rhythmic modulation of its membrane potential during swimming and was silent during scratching. It is possible, similar to what was suggested for other behaviorally specialized turtle neurons, that the swim-specialized neurons are solely used to select swimming over scratching, and not to create the swim rhythm. Furthermore, the swim-specialized neuron had a ~20-ms latency between stimulating the axons involved in swimming and an EPSP, indicating that not only is the neuron oligosynaptically connected but that it's a possible intermediary between the axons being stimulated and the swim CPG. Swim-specialized neurons have been observed in species such as the hatchling tadpole, larval zebrafish, and leech (Berkowitz et al., 2010; Kristan et al., 2005). Although many neurons were analyzed here, only one was swim-specialized. It has been previously suggested in the leech that specialized neurons that are not as prevalent for a motor pattern were possibly more recent in the evolutionary process, and the more recent motor patterns evolved by sharing componentry with the more primitive motor patterns (Briggman & Kristan, 2006); this is my suggestion for turtle swim-specialized neurons.

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