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An *in vivo* Assay for Simultaneous Monitoring of Neuronal Activity and Behavioral Output in the Stomatogastric Nervous System of Decapod Crustaceans

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SHORT ABSTRACT

We present an experimental method to simultaneously monitor behavioral output and neuronal activity of a central pattern generator in the stomatogastric nervous system of decapod crustaceans.

ABSTRACT

Central pattern generators (CPGs) generate rhythmic output patterns and drive vital behaviors such as breathing, swallowing, locomotion and chewing [1-10]. While most insights into the rhythm generating mechanisms of CPGs have been derived from isolated nervous system preparations, the relationship between neural activity and corresponding behavioral expression is often unclear. The stomatogastric system of decapod crustaceans is one of the best characterized neural system for motor pattern generation [9-12] and many mechanisms of motor pattern generation and selection have been discovered in this system. Since most studies are limited to the isolated nervous system, little is known about the actual behavioral output of this system. For example, it is unknown whether the observed flexibility in the motor patterns is present *in vivo* and whether distinct motor activities drive corresponding behavioral patterns. We present a method which allows electrophysiological recordings of CPG neurons and the simultaneous monitoring of the behavioral output of the stomatogastric nervous system. For this, we use extracellular hook electrodes either for recording or stimulation of neurons in the gastric mill CPG that drive the chewing movements of three teeth in the foregut of the animal. Electrodes are applied in tethered, but otherwise fully intact crabs (*Cancer pagurus*) and an endoscope is used to monitor tooth movements. Nerve and video recordings of the endoscopic camera are synchronized and motion tracking techniques are used to analyze gastric mill movements. This approach thus allows testing the behavioral relevance of the neural activity patterns produced by central pattern generators.

KEYWORDS. Electrophysiology, sensorimotor, crab, gastric mill, central pattern generator, behavior, video motion tracking

INTRODUCTION

Central pattern generators are assemblies of neurons that produce rhythmic activity without requiring phasic input signals, and often drive rhythmic muscle movements. They underlie vital behaviors such as breathing, swallowing, and chewing, as well as locomotion and saccadic eye movements, and often continue to function even in isolated nervous system (i.e. *in vitro*) preparations [1-10]. Due to their extraordinary accessibility for neuronal manipulations *in vitro*, CPGs have contributed much to our understanding of the mechanisms of neuronal plasticity and modulation of nervous system output. Yet, most insights into the rhythm generating mechanisms have been derived from isolated ganglion preparations. However, functional behavior also requires the activation of muscles and sensory feedback, and the interaction of many central pattern generators may be necessary for the animal to successfully evade dangers, find food and mates [13]. These complex interactions, and in particular the contribution of flexibility in the CPG motor patterns to the generated behavior are far from understood.

While CPGs are present in all vertebrates species, invertebrates often serve as valuable model organisms for a detailed investigation of the mechanisms underlying pattern generation [14,15]. The knowledge aggregated from studies on various invertebrate species encompasses the subcellular level [16], the network level [11] and the behavioral level [17-21]. However, in those systems in which a detailed characterization of the nervous system is feasible, access to the behavioral output is often limited. One invertebrate model system in which the neuronal basis of motor pattern generation [11,22], as well as the anatomy of the structure of effectors [23-25] has been characterized exceptionally well is the stomatogastric nervous system of decapod crustaceans. Neurons in this system form several distinct CPGs that innervate a complex digestive system consisting of the oesophagus, the foregut, the mid- and hindgut (Figure 2) [26,27]. After ingestion, food is chewed in the gastric mill which consists of two lateral teeth and one medial tooth [28]. The chewing movements are controlled by the gastric mill CPG which is composed of several interconnected motor neurons in the stomatogastric ganglion [29,30]. The stomatogastric ganglion contains a second CPG that controls the pyloric region of the foregut which further filters the food and passes it on to the midgut for digestion [31,32]. All neurons which are

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part of these two CPGs as well as their synaptic connections and intrinsic properties are identified, and several distinct gastric mill motor patterns that are elicited by sensory and modulatory pathways have been characterized *in vitro* [12]. For example, stimulation of the ventral cardiac neurons (VCNs), mechanoreceptors in the foregut, elicits a gastric mill rhythm [33] that is distinct from that elicited by the inferior ventricular neurons (IVN) which relay chemosensory information from the antennae to the gastric mill CPG. In particular the phase relationship of the gastric mill neurons and the structure of the motor neuron bursts are distinct. For example, while in the IVN version of the rhythm the bursts of the gastric mill protractor neuron LG (lateral gastric neuron) consists of continuous firing, the LG burst is separated into several burstlets due to interactions with the pyloric rhythm in the VCN version of the gastric mill rhythm [33]. Whether such differences are retained at the behavioral level is unclear, since the relationship between motor patterns and behavioral output (chewing) has not been investigated thoroughly, and little is known about the interaction of neuronal activity patterns and musculoskeletal system. In fact, it is not even clear whether those distinct motor patterns are present *in vivo*.

The methods presented here allow researchers to record motor neurons in the stomatogastric nervous system to measure CPG activity *in vivo* and the concurrent behavioral output by monitoring the chewing movement of the gastric mill teeth. Gastric mill motor neurons can also be stimulated with activity patterns that have been observed *in vitro* in order to characterize their influence on tooth movement (and hence their behavioral relevance). We use extracellular hook electrodes for recording or stimulation of nerves that contain the axons of pro- and retractor neurons of the gastric mill CPG. Electrodes are applied in the tethered, but otherwise fully intact preparation and an endoscope is used to monitor tooth movements. Nerve recordings and video recordings of the endoscopic camera are digitally recorded and motion tracking techniques are used to analyze gastric mill movements.

This approach thus allows the experimenter to do two things: (1) record the central pattern generator activity in the fully intact animal and thus learn about the behavioral relevance of these patterns *in vivo*. (2) The extracellular electrodes can also be used for stimulation, which allows the selective activation of identified motor neurons as well. This enables studying the dynamics of the musculoskeletal system and its response to varying motor neuron activity patterns.

PROTOCOL

1. Construction of extracellular electrodes (Figure 1)

The extracellular electrodes will be custom made using standard laboratory material. Each electrode consists of two steel wires bent to hooks on which the recorded nerve rests. Both wires must not touch and are isolated from the general bath to increase the signal-to-noise ratio. In principle, a single hook plus a general ground positioned elsewhere in the body cavity would be sufficient to record motor neuron activities, but better signal-to-noise ratios are obtained using the two-hook electrodes.

1.1) Use two 5 cm pieces of insulated silver wire (0.1 mm or 0.075 mm, insulation 0.01 mm (PFTE), Good Fellow, Cambridge, England) to form 2 hooks.

1.2) Remove the insulation from each wire end and bend the bare ends into hooks. The hooks will hold the nerve during the experiment. Bend hooks such that a silicone tube (see step 5) can be slid over them with ease: a diameter of 0.5 - 1.5 mm for each hook is sufficiently small. Note that the hook diameter should be adjusted to the diameter of the nerve(s) to be recorded from.

1.3) Place both wires next to each other on a flat surface 0.5 − 1 mm apart, then place a syringe needle (16 gauge) alongside the two wires. Use Parafilm® (Pechiney Plastic Packaging, Chicago, IL, USA) to fix both wires to the syringe needle such that the hooked ends of the wire stick out 0.8 - 1 cm from the tip of the needle. Carefully warm up the Parafilm® with a gas lighter to secure the bond between wires and needle.

1.4) Slide a piece of silicone tubing (1.5 cm length, I.D. 1.8 mm, O.D. 1.9 mm) over parafilm and wires all the way to the plastic screw-end of the needle.

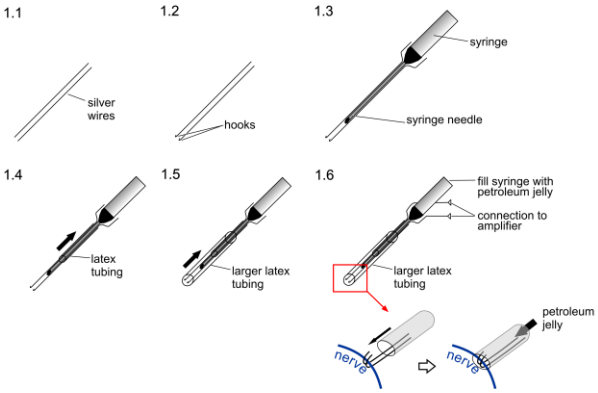

Figure 1. Schematic of electrode construction. For stability, the hooks are fixed to the syringe tip by a piece of small diameter latex tubing. The nerve makes electrical contact with both hooks and the larger diameter silicon tubing insulates hooks and nerves from the general ground when slid down. The syringe tube is filled with petroleum jelly which is ejected when the syringe plunger is pressed. The petroleum jelly then encloses hooks and nerve and increases signal-to-noise ratio. Detected action potentials are recorded with a differential amplifier. For details, see protocol.

1.5) Slide a second piece of silicone tubing (1 cm length, I.D. 1.9 mm, O.D. 2 mm) over the first silicone tube. During the experiment, this second silicone tube will be moved forward over the hooks in order to seal them from the general ground.

1.6) Fill a 5 ml syringe with petroleum jelly. 2.5ml is sufficient for 5 experiments. The advantage of not filling the syringe fully is that the plunger is at a position more suitable and less obstructive for experimentation.

1.7) Connect the syringe to the syringe tip and the distant end of the recording wires to shielded wires that link to the differential amplifier (Model 1700, A-M Systems, Carlsborg, USA). Use micro-pin connectors to connect silver wires and shielded wires and fix them to the syringe with duct tape.

1.8) Attach the body of the finished electrode to a ball joint mount.

2. *In vivo* preparation (Figure 2)

2.1) Anesthetize the crab (purchased at Feinfisch GmbH, Neu-Ulm, Germany) by packing it in ice for 20 - 40 min.

2.2) Tether the animal and place it in the plexiglass holder (design by H.G. Heinzel, University of Bonn, Germany).

2.3) Open the dorsal carapace using a precision drill (Proxxon GmbH, Germany), creating a rectangular window. In rostrocaudal direction, the window ranges from the frontal to the metagastric part of the carapace; medio- laterally it ranges from the orbital to the branchial lobe [34]. Aim for an approximate size of 4 x 4 cm.

2.4) After opening the carapace, remove the hypodermis to reveal the dorsal side of the stomach. Take care to keep the ophthalmic artery, which proceeds rostrocaudally along the midline of the carapace, intact in order to maintain the integrity of the blood stream and therefore the health of the animal.

2.5) Removing connective tissue from the motor nerves with fine spring scissors. In the representative experiments shown, the dorsal ventricular nerve (*dvn*) was used. The *dvn* contains the axons of several CPG neurons, including the lateral gastric motor neuron (LG). The LG neuron is part of the gastric mill CPG and controls several muscles which elicit the protraction of the bilaterally symmetric lateral teeth.

2.6) Connect a saline flask to a swivel joint that is attached to the plate of the crab holder with neodym magnets. Start superfusing the wound with cooled (5-10°C) saline (NaCl: 440 mMol/l, KCl: 11 mMol/l, $CaCl^{2+}*2H_2O$: 13 mMol/l, $MgCl^{2+}*6H_2O$: 26 mMol/l, trisma base: 11 mMol/, maleic acid: 5 mMol/l, pH 7.4 - 7.6).

3. Application of extracellular electrodes

Position the extracellular hook electrodes using dovetail rails fixed to the base plate of the crab-holder.

3.1) Attach the swivel joint of each extracellular electrode to the dovetail rails at the base plate of the crab-holder. Several electrodes can be attached, which allows the simultaneous recording or stimulation of multiple motor neurons.

3.2) Position each electrode roughly above the desired nerve, then use a stereomicroscope (Stemi 2000, Zeiss, Oberkochen, Germany) to fine position the hooks and load the nerve onto it (Figure 1.6, bottom). Slide the hook ends under the nerve, then carefully lift the nerve into the crook of the wire with a minuten pin or a hand-held hook.

3.3) Once the nerve is in place, move the insulation tubing forward so that it covers nerve and hooks. Use fine forceps (#5, Fine Science Tools GmbH, Heidelberg, Germany) to do this. Carefully push the plunger of the syringe to apply petroleum jelly. The petroleum jelly will seal the nerve and hooks from the general ground.

3.4) Switch on the extracellular amplifier and record the electrode signal using a data acquisition board (CED Power 1401, Cambridge Electronic Design, Cambridge, UK) and software (Spike 2, ver 7.10; Cambridge Electronic Design, Cambridge, UK).

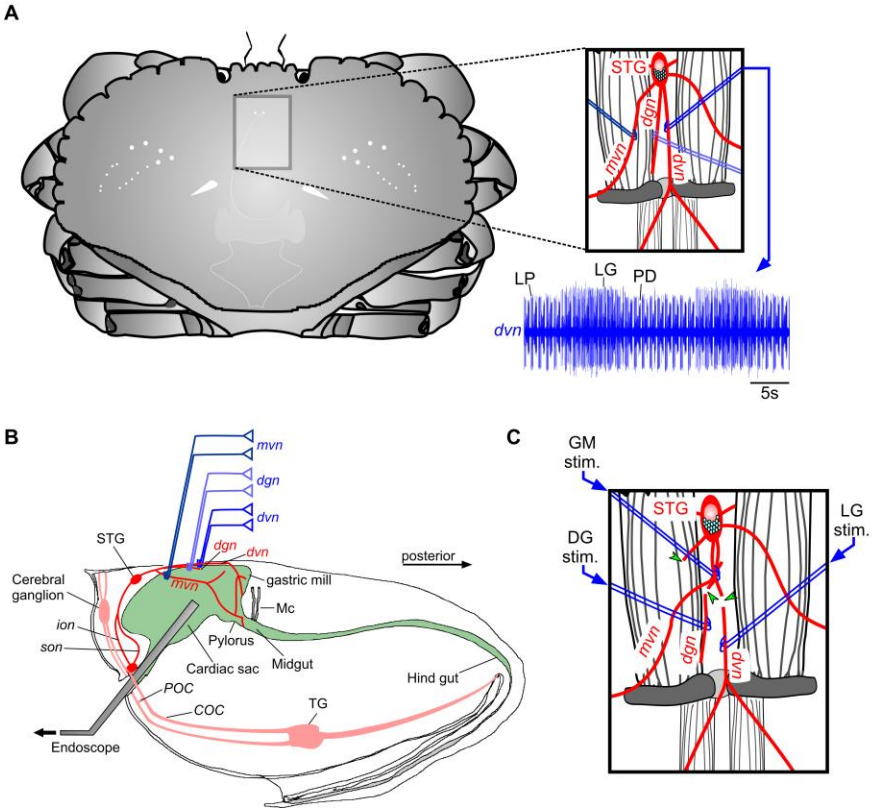

Figure 2. Electrode application *in vivo*. A. Left: dorsal view of *C. pagurus*. The opening in the carapace is indicated. Right: magnification showing STG and motor nerves, and the location of the electrodes, as well as a recording of the dorsal ventricular nerve (*dvn*). The application of several simultaneous recording electrodes is possible (3 are shown). LP, lateral pyloric neuron; LG, lateral gastric neuron; PD, pyloric dilator neuron; *mvn*, medial ventricular nerve; *dgn*, dorsal gastric nerve. B. Sagittal view. The digestive tract is shown in green. The endoscope is shown in its recording position inside the cardiac sac. *COC*, circum oesophageal commissure; *ion*, inferior esophageal nerve; Mc, midgut caecum; *POC*: post oesophageal commissure; TG, thoracic ganglion. C. Arrangement of electrodes and nerves during stimulation of motor neurons. Green arrows mark transection sites. DG, dorsal gastric neuron; GM, gastric mill neuron.

4. Endoscopy

The behavioral output of the gastric mill is the movement of the three gastric mill teeth. To monitor the opening and closing of the teeth, use an endoscope of 6 mm diameter and *300 mm* length (*30°* view angle) will be used (Panoview, Richard Wolf GmbH, Knittlingen, Germany). The endoscope contains a LED unit to illuminate the inside of the stomach and is connected to a video camera (TECAM-3, Richard Wolf GmbH, Knittlingen, Germany, 640x480 pixels, framerate: 25 Hz) that is synchronized with the electrophysiological data via the plugin software "spike2video" provided with the Spike2 software package.

The video data is translated into two-dimensional data, which can then be analyzed. For this, software written for Matlab (Mathworks, Natick, USA) for auto tracking points in

video files can be used [35]. This software tracks any change in contrast within a pre-definable window of 9-100 pixels edge length. The change in contrast is determined via an extended Kalman filter [36,37]. As tracking points the tips of the lateral teeth and the tip of the medial tooth can be used, in addition to small markings or other segments of stark contrast. The software saves coordinates and the number of the corresponding frame to an ASCII file.

Application of the endoscope:
4.1) The endoscope consists of a rigid tube allowing stable recording conditions and an attached video camera. The camera is fixed to the eyepiece of the endoscope. Clamp the endoscope to a tripod to ensure a stable positioning of endoscope and camera.
4.2) Pry open the mandibles with forceps and insert the endoscope through the esophagus into the foregut. Tilt the animal inside the crab holder relative to the endoscope until the gastric mill is visible.
4.3) Turn on the camera and start the recording computer software.

5. Motor neuron stimulation
The hook electrodes can also be used for stimulation of the motor neurons. This can be done by connecting the hook wires to a stimulator unit (Master8, AMPI, Jerusalem, Israel, or comparable) instead of the extracellular amplifier to generate the voltage pulses. Use a duration of 1 ms for each pulse to elicit a single action potential per pulse. To ensure the recruitment of only the relevant motor neurons, use the threshold voltage that first elicits tooth movement.
Before stimulation, transect the motor nerves that connect STG and muscles. This prevents synaptic influences of the stimulated neurons onto other STG neurons. The activity of the gastric mill teeth is elicited by three motor neuron classes which project through specific nerves. The LG neuron projects via the dorsal ventricular nerve (*dvn*) and it is the largest unit on this nerve (resulting in the lowest threshold during stimulation). After *dvn* transection, stimulate the nerve posterior to the transection site to specifically activate LG [38]. LG stimulation will activate the protractor muscles of the lateral teeth [30]. The medial tooth retractor neuron DG (dorsal gastric neuron) can be activated in a similar fashion. Here, transect the dorsal gastric nerve *(dgn)* and stimulate posterior to the transection site to activate the retractor muscles. In contrast to retraction, medial tooth protraction is mediated by the activity of four motor neurons: the four gastric mill motor neurons (GMs) project to multiple protractor muscles via several nerves. GM axons are found in the *dvn*, the *dgn* and the bilateral symmetric medial ventricular nerves (*mvn*). To activate all GM neurons, transect all three nerves and place them on a single hook electrode. Since the GMs leave the *mvn* about 5mm lateral to the STG, cut the mvn such that the severed nerve can still be stimulated. Stimulate laterally of the transection site to elicit orthodromic action potentials in the GMs. For the *dgn* and *dvn*, stimulate anterior to the transection site (posterior would activate the medial tooth retractor and lateral tooth protractor muscles, respectively; see above). Anterior stimulation will elicit antidromic action potentials in the GM neurons that will invade the STG and from there travel to the protractor muscles. By stimulating all three nerves, all GM neurons will be activated and contractions of the protractor muscles will be elicited.
Many stimulus patterns can be used to activate the motor neurons. To apply different patterns, trigger the stimulator with timing signals generated in the computer. Connect the digital outputs of the data acquisition board (the Power 1401) to the trigger inputs of the stimulator. The recording software (Spike2) allows to replay recorded data and also to create stimuli based on averaged data. This enables the application of various stimulus patterns to several motor neurons simultaneously. Each previously recorded action potential will then cause a single action potential in the axons of the stimulated motor neurons (LG, DG, GMs). The rhythmic movements of gastric mill teeth can then be recorded via the endoscope in the foregut. This setup also enables the researcher to construct stimuli with varying stimulus properties (such as tonic or phasic neuron activation) and test the effects of such stimuli on the behavior in the *in vivo* preparation.

REPRESENTATIVE RESULTS
Figure 3 shows a representative experiment in *Cancer pagurus*. Here, the *dvn* and the *dgn* were recorded extracellularly along with the movement of one of the lateral teeth (Fig. 3A). The *dvn* trace shows the activities of the pyloric dilator (PD) and lateral pyloric (LP) neurons (see also Figure 2A). These neurons are main players in the pyloric rhythm which drives the filtering of food in the pylorus of the foregut (Figure 2B; [39]). In addition to PD and LP, LG can clearly be identified as the largest unit. LG innervates an ensemble of gastric mill muscles which elicit the closing of the lateral gastric mill teeth (protraction). The gastric mill rhythm cycles at frequencies that are approximately 10 times lower than the pyloric rhythm [12]. Correspondingly, the bursts of the LG neuron span several bursts of the pyloric neurons. The *dgn* shows the activity patterns of the single gastric mill retractor neuron DG and the four protractor neurons GM. DG is the retractor motor neuron for the medial tooth and typically active in alternation to LG (lateral tooth protraction) and GM (medial tooth protraction) during gastric mill rhythms [12]. In the example in Fig. 3, the gastric mill rhythm was spontaneously active and all three classes of gastric mill motor neurons were bursting. DG and LG showed pyloric-timed interruptions in their burst activity (shown with outlined arrows for DG). These burst patterns resemble previously described burst activities from *in vitro* preparations after stimulation of the mechanosensitive VCN [40], indicating that this particular motor pattern exists in the intact animal as well. The corresponding movement of the lateral tooth is shown above the extracellular recording traces. To quantify the tooth movement across preparations, a relative measure is used: the horizontal width of the medial tooth cusp was determined in each video recorded via the endoscope camera and each movement trajectory was then divided by the pixel width of the medial tooth cusp, correcting for errors due to the position of the endoscope. All movement trajectories shown here are thus in MU (Medial tooth Units, i.e. normalized to the width of the medial tooth, [29]). The movement trace shows that

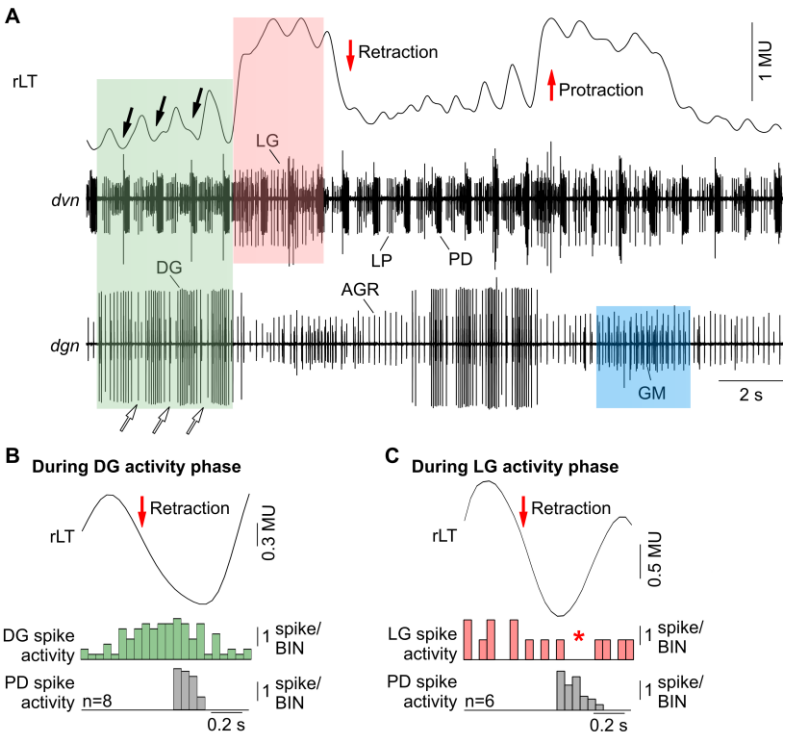

Figure 3. Representative data from an *in vivo* experiment. A. Extracellular recording of the *dgn* and *dvn* and simultaneous recording of the position of the right lateral tooth (rLT) using the endoscope. The *dvn* shows the firing of the pyloric neurons LP and PD, as well as of the gastric mill protractor neuron LG. The pyloric rhythm is constitutive and continuously active. The gastric mill rhythm was spontaneously active (spontaneous chewing) in this example. The movement of the lateral tooth shows components of both rhythms: fast small movements that were timed with the pyloric rhythm (small retractions, black arrows) while slower, more pronounced protractions were timed with the gastric mill rhythm (in particular, the LG protractor neuron activity). The tooth was retracted during the LG interburst, which coincided with the activity of the retractor neuron DG (on the *dgn*). Outlined arrows indicate pyloric-timed interruptions in the DG burst. Activity phases are color coded (red: LG; green: DG; blue: GMs). B. Average of the lateral tooth movement during the DG activity phase, triggered on the beginning of the PD neuron burst. The two bottom traces show histograms of DG and PD spike activities (BINwidth 50 ms), respectively. DG activity and tooth movement were timed by the pyloric rhythm. C. Similar to B., but during the LG activity phase and showing a histogram of LG spike activity. *: pyloric-timed gap in the LG burst.

both activity patterns (the pyloric and gastric mill rhythms) are reflected in the behavioral output, i.e. the tooth movement. Long-lasting pro and retractions followed the phasing of the gastric mill neurons, i.e when LG was active, the lateral tooth was protracted and when DG was active, the tooth was retracted. However, the pyloric timed interruptions in the firing activities of LG and DG also influenced the tooth movement: small tooth retractions (black arrows in Fig. 3) occurred during the activity phase of the pyloric PD neuron. When DG was active, these retractions roughly occurred in phase with the pyloric-timed burstlets of the DG neuron. This is consistent with the hypothesis that the DG retractor neuron contributes to the retraction movement of the lateral teeth as well. Figure 3B shows an average of the lateral tooth movement during the DG activity phase, plus a histogram of PD and DG activities, triggered on the beginning of the PD neuron burst. Retraction began with the start of the DG burstlet and the trough of the movement trajectory (the maximum retraction) coincided with the end of the DG burstlet, immediately after the PD burst ended. During the LG activity phase, small retractions were also timed by the pyloric rhythm. The average shown in Fig. 3C demonstrates that the trough of the retraction occurred at the beginning of the PD burst and when LG spike activity was minimal (* in Fig. 3C). In summary, the fast pyloric rhythm and the slow gastric mill rhythm were represented in the movement trajectory of the lateral tooth. Hence, the behavioral output of these motor patterns can be studied. Figure 4 shows the results of a stimulation experiment. Here, the LG, GM and DG motor neurons were stimulated extracellularly *via* the hook electrodes. For stimulation, previously recorded activity patterns of these neurons during different types of gastric mill rhythms were used. The averaged activity patterns of the gastric mill motor neurons after stimulation of the post-esophageal commissure neurons (POC) [41], the VCN [40] and the IVN [42,43], respectively, were used to construct the stimulus patterns. When compared, these activity patterns are different from one another [33,43], indicating that they *may* represent different versions of the gastric mill motor pattern. For example, the phasing of the motor neurons varies between POC, VCN and IVN-elicited patterns. In particular the phasing of the medial tooth pro- (GM) and retractor (DG) neurons varies strongly.

Stimulation with these three patterns resulted in clearly discernible movements of the gastric mill teeth: Figure 4 shows the movements of the medial tooth and one lateral tooth along with the stimulus patterns used. During all stimulation patterns, medial and lateral teeth showed strong pro- and retraction movements. However, the phasing of these tooth movements differed between stimulation patterns: For example, the protraction of the medial tooth preceded that of the lateral tooth during the IVN gastric mill rhythm, while it was clearly delayed during the VCN gastric mill rhythm. Similarly, the timing of medial tooth retraction depended on the stimulus pattern. Consequently, the structure of the neuronal patterns was faithfully reproduced by the tooth movement, which supports the idea that IVN, POC and VCN indeed elicit functionally distinct chewing behaviors.

DISCUSSION

In this paper we demonstrate a method that allows recording and distinguishing different versions of a rhythmic motor pattern in the associated behavior. There is an extensive literature establishing that individual identified CPGs generate distinct motor patterns in the isolated nervous system [1,5,6,9,10,12,44,45].

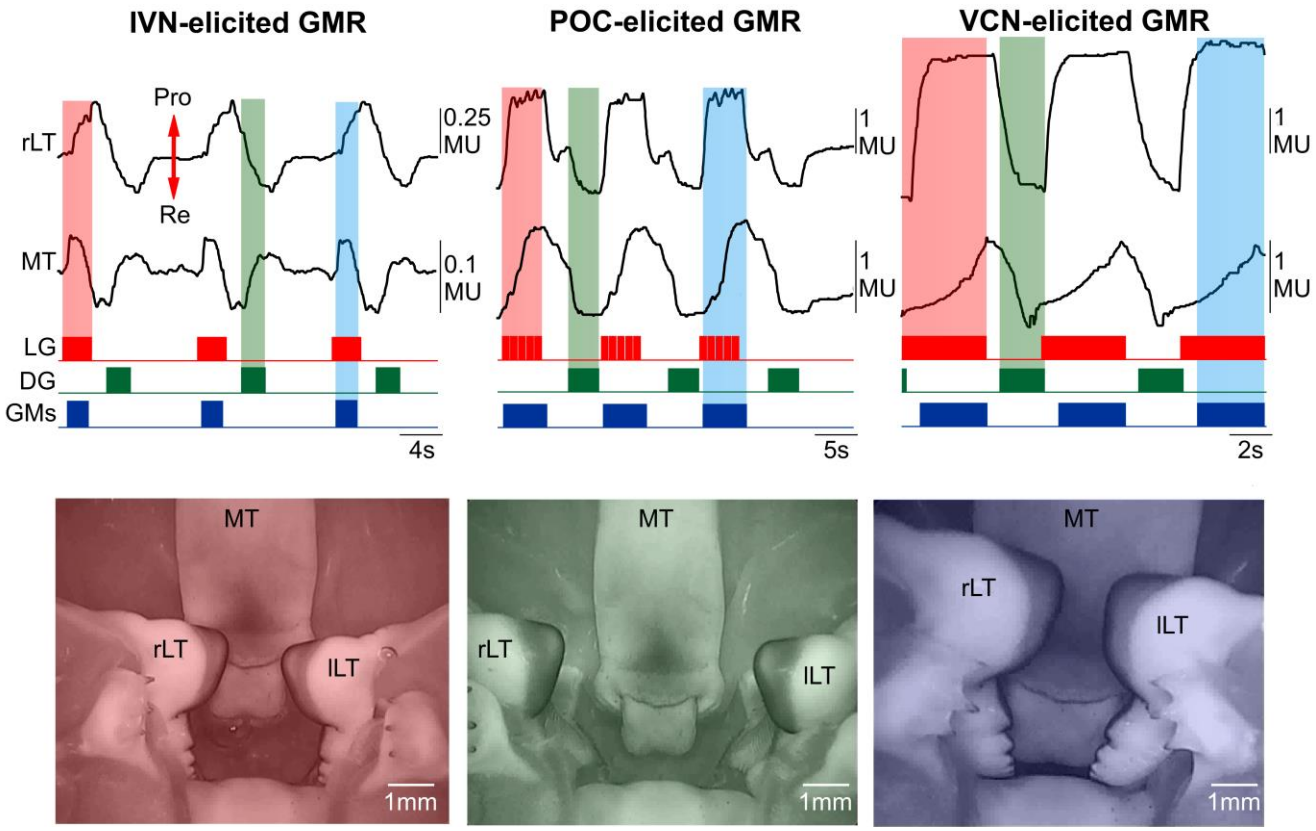

Figure 4. Representative data from a stimulation experiment. The hook electrodes in this configuration were used to selectively stimulate specific motor neurons (LG, DG, GMs) and the elicited tooth movements were recorded via the endoscope. Three different versions of the gastric mill rhythm (IVN, POC, VCN) were used for stimulation. The movement traces of the right lateral tooth (top) and medial tooth (2nd from top) are shown, as well as the stimulation patterns that elicited the movements (bottom three traces). Stimulation phases are color coded (red: LG, green: DG, blue: GMs). The bottom photos show the positions of the teeth in those phases. lLT, left lateral tooth; rLT, right lateral tooth; MT, medial tooth; MU, medial tooth unit.

Only few studies, however, have established whether these different CPG motor patterns also drive distinct movement patterns *in vivo* and hence, whether they are behaviorally relevant. We show an easy-to-produce design for electrodes which can be used to record CPG output activity from the crustacean stomatogastric nervous system in the tethered, but otherwise intact animal (*in vivo*). We also present a detailed description of the operation techniques necessary to apply these electrodes *in vivo*. The behavioral output, i.e. the movement of the three gastric mill teeth, was recorded simultaneously with an endoscopic camera. The endoscope was inserted into the oesophagus and monitored the movement of the medial and two lateral gastric mill teeth. Hence, the relation of motor neuron activity and movement output can be determined using this experimental setup.

The gastric mill CPG can elicit various activity patterns *in vitro* [41], [40], [42,43] that differ in burst patterns, neuronal phase relationship and firing rates. While these activity patterns are distinct from one another, it is unclear whether they indeed exist *in vivo*, and whether they elicit distinct movement outputs of the gastric mill teeth. By monitoring gastric mill motor neuron activity and gastric mill tooth movements, the approach presented here can thus be used to demonstrate the presence of particular motor patterns, and test their behavioral relevance. In our example, we recorded a spontaneously active gastric mill rhythm that roughly resembled the VCN motor pattern. However, sensory or neuromodulatory pathways could be stimulated to activate gastric mill activity, and to investigate the impact of motor neuron patterns on the movement output. For example, it would be possible to mechanically stimulate the VCN in the cardiac gutter to elicit a VCN type gastric mill rhythm and the corresponding tooth movements. Alternatively, the effects of neuromodulatory substances (such as hormones) on the motor patterns and the behavior could be tested.

A more straightforward way of testing the movement output of distinct motor patterns is to use the electrodes for stimulation with the corresponding motor neuron activity. In this case, the motor patterns are pre-defined and taken from previous experiments. In our case we used those of the IVN, POC and VCN gastric mill rhythms [40,41,43]. The movement output of the gastric mill teeth was clearly distinct for these three patterns, indicating that they indeed elicit distinct behavioral output. Future experiments could include manipulations of the stimulation patterns (such as the interburst firing frequency) to determine the response dynamics of the musculoskeletal system. In addition, the effects of neuromodulators on the musculoskeletal system can be tested.

The combination of endoscopy and electrophysiological recordings thus enables researchers to monitor the activity of a rhythmic motor system along with the behavioral output. It will broaden our understanding of interactions between nervous system, sensory organs and musculoskeletal system and help to determine the functional relevance of motor patterns.

DISCLOSURES

The authors have nothing to disclose.

REFERENCES

1. Marder, E. & Calabrese, R. L. Principles of rhythmic motor pattern generation. *Physiol. Rev.* **76** (3), 687–717, (1996).
2. Kiehn, O. Locomotor circuits in the mammal spinal cord. *Annu. Rev. Neurosci.* **29** (1), 279–306, doi:10.1146/annurev.neuro.29.051605.112910 (2006).
3. Katz, P. S. & Hooper, S. L. Invertebrate central pattern generators. *CSH Monogr.* **49** (0), 251–279, doi:10.1101/087969819.49.251 (2007).
4. Chevallier, S., Nagy, F. & Cabelguen, J.-M. Muscarinic control of the excitability of hindlimb motoneurons in chronic spinal-transected salamanders. *Europ. J. Neurosci.* **28** (11), 2243–2253, doi:10.1111/j.1460-9568.2008.06506.x (2008).
5. Doi, A. & Ramirez, J.-M. Neuromodulation and the orchestration of the respiratory rhythm. *Resp. Physiol. & Neurobiol.* **164** (1-2), 96–104, doi:10.1016/j.resp.2008.06.007 (2008).
6. Berkowitz, A. Multifunctional and specialized spinal interneurons for turtle limb movements. *Ann. N Y Acad. Sci.* **1198**, 119–132, doi:10.1111/j.1749-6632.2009.05428.x (2010).
7. Manira, El, A. & Kyriakatos, A. The role of endocannabinoid signaling in motor control. *Physiol.* **25** (4), 230–238, doi:10.1152/physiol.00007.2010 (2010).
8. Büschges, A., Scholz, H. & Manira, El, A. New moves in motor control. *Curr. Biol.* **21** (13), R513–24, doi:10.1016/j.cub.2011.05.029 (2011).
9. Harris-Warrick, R. M. Neuromodulation and flexibility in central pattern generator networks. *Curr. Opin. Neurobiol.* **21** (5), 685–692, doi:10.1016/j.conb.2011.05.011 (2011).
10. Marder, E. Neuromodulation of neuronal circuits: Back to the future. *Neuron* **76** (1), 1–11, doi:10.1016/j.neuron.2012.09.010 (2012).
11. Nusbaum, M. P. & Beenhakker, M. P. A small-systems approach to motor pattern generation. *Nature* **417** (6886), 343–350, doi:10.1038/417343a (2002).
12. Stein, Wolfgang Modulation of stomatogastric rhythms. *J. Comp. Physiol. A* **195** (11), 989–1009, doi:10.1007/s00359-009-0483-y (2009).
13. Holmes, P., Full, R. J., Koditschek, D. & Guckenheimer, J. The dynamics of legged locomotion: Models, analyses, and challenges. *SIAM Rev.* **48** (2), 207–304 (2006).
14. Marder, E. & Rehm, K. Development of central pattern generating circuits. *Curr. Opin. Neurobiol.* **15** (1), 86–93 (2005).
15. Sattelle, D. B. & Buckingham, S. D. Invertebrate studies and their ongoing contributions to neuroscience. *Invertebrate Neuroscience* **6** (1), 1–3, doi:10.1007/s10158-005-0014-7 (2006).
16. Hille, B. Ionic channels: molecular pores of excitable membranes. *Harvey Lectures* **82**, 47 (1986).
17. Tóth, T. I., Knops, S. & Daun-Gruhn, S. A neuromechanical model explaining forward and backward stepping in the stick insect. *J. Neurophysiol.* (2012).
18. Tang, S. & Juusola, M. Intrinsic activity in the fly brain gates visual information during behavioral choices. *PLoS ONE* **5** (12), e14455, doi:10.1371/journal.pone.0014455 (2010).
19. Giles, A. C. & Rankin, C. H. Behavioral Analysis of Learning and Memory in C. elegans. *Learn. Memory* **4**, 1–13 (2008).
20. Baxter, D. A. & Byrne, J. H. Feeding behavior of Aplysia: a model system for comparing cellular mechanisms of classical and operant conditioning. *Learn. Memory* **13** (6), 669–680, doi:10.1101/lm.339206 (2006).
21. Calabrese, R. L. Behavioral choices: how neuronal networks make decisions. *Curr. Biol.* **13** (4), R140–2 (2003).
22. Selverston, A. I. & Mulloney, B. Organization of the stomatogastric ganglion of the spiny lobster. *J. Comp. Physiol.* **91** (1), 33–51, doi:10.1007/BF00696155 (1974).
23. Pearson, J. *Cancer, the edible crab*. 1–254 (Price Six Shilling and Sixpence: London, 1908).
24. Maynard, D. & Dando, M. The structure of the stomatogastric neuromuscular system in *Callinectes sapidus, Homarus americanus* and *Panulirus argus*. *Phil. Trans. R. Soc. B* **268** (898), 161-220 (1974).
25. Govind, C., Atwood, H. & Maynard, D. Innervation and neuromuscular physiology of intrinsic foregut muscles in the blue crab and spiny lobster. *J. Comp. Physiol. A* **96** (3)**,** 185-204 (1975).
26. Icely, J. D. & Nott, J. A. *Microscopic Anatomy of Invertebrates.* (Wiley-Liss, 1992).
27. Yonge, C. M. Studies on the Comparative Physiology of Digestion. *J. Exp. Biol.* (1), 343–389 (1924).
28. Heinzel, H. G. Gastric mill activity in the lobster. I. Spontaneous modes of chewing. *J. Neurophysiol.* **59** (2), 528–550 (1988).
29. Heinzel, H. G., Weimann, J. M. & Marder, E. The behavioral repertoire of the gastric mill in the crab, *Cancer pagurus*: an in situ endoscopic and electrophysiological examination. *J. Neurosci.* **13** (4), 1793–1803 (1993).
30. Russell, D. F. Neural basis of teeth coordination during gastric mill rhythms in spiny lobsters, *Panulirus interruptus*. *J. Exp. Biol.* **114**, 99–119 (1985).
31. Bal, T., Nagy, F. D. R. & Moulins, M. The pyloric central pattern generator in Crustacea: a set of conditional neuronal oscillators. *J. Comp. Physiol. A* **163** (6), 715–727, doi:10.1007/BF00604049 (1988).
32. Marder, E. & Bucher, D. Central pattern generators and the control of rhythmic movements. *Curr. Biol.* **11** (23), R986–96 (2001).
33. Beenhakker, M. P., Blitz, D. M. & Nusbaum, M. P. Long-lasting activation of rhythmic neuronal activity by a novel mechanosensory system in the crustacean stomatogastric nervous system. *J. Neurophysiol.* (91), 78–91, doi:10.1152/jn.00741.2003 (2004).
34. Kennedy, V. S. & Cronin, L. E. *Anatomy of the postlarval blue crab*. In: *The Blue Crab: Callinectes Sapidus*. A Maryland sea grant book (Kennedy, V. S., Cronin, L. E, eds.). pp1–78. University of Maryland: Maryland Sea Grant College (2007).

35. Hedrick, T. L. Software techniques for two- and three-dimensional kinematic measurements of biological and biomimetic systems. *Bioinsp. Biomim.* **3** (3), 034001, doi:10.1088/1748-3182/3/3/034001 (2008).
36. Pillow, J. W., Ahmadian, Y. & Paninski, L. Model-based decoding, information estimation, and change-point detection techniques for multineuron spike trains. *Neur. Comp.* (23), 1–45 (2010).
37. Fahrmeir, L. Posterior mode estimation by extended Kalman filtering for multivariate dynamic generalized linear models. *J. Am. Stat. Assoc.* **87** (418), 501–509 (1992).
38. Stein, W., Smarandache, C. R., Nickmann, M. & Hedrich, U. B. S. Functional consequences of activity-dependent synaptic enhancement at a crustacean neuromuscular junction. *J. Exp. Biol.* **209** (7), 1285–1300, doi:10.1242/jeb.02133 (2006).
39. Hooper, S. L. & DiCaprio, R. A. Crustacean motor pattern generator networks. *Neurosign.* **13** (1-2), 50–69 (2004).
40. Beenhakker, M. P. & Nusbaum, M. P. Mechanosensory activation of a motor circuit by coactivation of two projection neurons. *J. Neurosci.* **24** (30), 6741–6750, doi:10.1523/JNEUROSCI.1682-04.2004 (2004).
41. Blitz, D. M., White, R. S., Saideman, S. R., Cook, A., Christie, A. E., Nadim, F. & Nusbaum, M. P. A newly identified extrinsic input triggers a distinct gastric mill rhythm via activation of modulatory projection neurons. *J. Exp. Biol.* **211** (6), 1000–1011, doi:10.1242/jeb.015222 (2008).
42. Hedrich, U. B. S. & Stein, W. Characterization of a descending pathway: activation and effects on motor patterns in the brachyuran crustacean stomatogastric nervous system. *J. Exp. Biol.* **211** (16), 2624–2637, doi:10.1242/jeb.019711 (2008).
43. Hedrich, U. B. S., Smarandache, C. R. & Stein, W. Differential activation of projection neurons by two sensory pathways contributes to motor pattern selection. *J. Neurophys.* **102** (5), 2866–2879, doi:10.1152/jn.00618.2009 (2009).
44. Dickinson, P. S. Neuromodulation of central pattern generators in invertebrates and vertebrates. *Curr. Opin. Neurobiol.* **16** (6), 604–614, doi:10.1016/j.conb.2006.10.007 (2006).
45. Blitz, D. M. & Nusbaum, M. P. Modulation of circuit feedback specifies motor circuit output. *J. Neurosci.* **32** (27), 9182–9193, doi:10.1523/jneurosci.1461-12.2012 (2012).