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Bistable Behavior Originating in the Axon of a Crustacean Motor Neuron

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Le, Thuc, Derek R. Verley, Jean-Marc Goillard, Daniel I. Messinger, Andrew E. Christie, and John T. Birmingham. Bistable behavior originating in the axon of a crustacean motor neuron. *J Neurophysiol* 95: 1356–1368, 2006. First published November 16, 2005; doi:10.1152/jn.00893.2005. Both vertebrate and invertebrate motor neurons can display bistable behavior in which self-sustained tonic firing results from a brief excitatory stimulus. Induction of the bistability is usually dependent on activation of intrinsic conductances located in the somatodendritic area and is commonly sensitive to action of neuromodulators. We have observed bistable behavior in a neuromuscular preparation from the foregut of the crab *Cancer borealis* that consists of the gastric mill 4 (gm4) muscle and the nerve that innervates it, the dorsal gastric nerve (*dgn*). Nerve-evoked contractions of enhanced amplitude and long duration (>30 s) were induced by extracellular stimulation when the stimulus voltage was above a certain threshold. Intracellular and extracellular recordings showed that the large contractions were accompanied by persistent firing of the dorsal gastric (DG) motor neuron that innervates gm4. The persistent firing could be induced only by stimulating a specific region of the axon and could not be triggered by depolarizing the soma, even at current amplitudes that induced high-frequency firing of the neuron. The bistable behavior was abolished in low-Ca²⁺ saline or when nifedipine or flufenamic acid, blockers of L-type Ca²⁺ and Ca²⁺-activated nonselective cation currents, respectively, was applied to the axonal stimulation region of the *dgn*. Negative immunostaining for synapsin and synaptotagmin argued against the presence of synaptic/modulatory neuropil in the *dgn*. Collectively, our results suggest that bistable behavior in a motor neuron can originate in the axon and may not require the action of a locally released neuromodulator.

INTRODUCTION

Spiking of a neuron in response to a brief excitatory stimulus usually persists for only as long as the stimulus is present. More rarely, however, a neuron will generate self-sustained tonic spiking that continues long after the stimulus has ended. When the prolonged firing is a characteristic of the neuron itself, rather than a network property, it results from the generation of a plateau potential (Weidmann 1951), and the neuron is said to be “bistable.” Plateau production is possible when a persistent inward current (PIC) causes the steady-state current–voltage (*I*–*V*) relationship to be “N”-shaped such that there are two quasi-stable membrane potentials corresponding to *I* = 0: a truly stable state near rest and a long-lived depolarized level above the threshold for spike production (Schwindt and Crill 1977, 1980a). The lifetime of the depolarized level varies from system to system, and the duration of the associated persistent firing can last fractions of a second

(Lee and Heckman 1996) or many minutes (Egorov et al. 2002). Bistable neurons have been identified in motor, sensory, and central nervous systems and in both vertebrates (Di Prisco et al. 1997; Egorov et al. 2002; Hounsgaard et al. 1984; Hsiao et al. 1998; Morisset and Nagy 1996; Reuveni et al. 1993; Russo and Hounsgaard 1994; Schwindt and Crill 1980a) and invertebrates (Dembrow et al. 2004; DiCaprio 1997; Dickinson and Nagy 1983; Katz and Harris-Warrick 1989; Lechner et al. 1996; Ramirez and Pearson 1991). Regardless of the system, one feature common to all documented bistable neurons is that the biophysical mechanisms responsible for the bistability are located in the somatodendritic rather than axonal region of the neuron (Bennett et al. 1998; Gutman 1991; Heckman et al. 2003; Hounsgaard and Kiehn 1993; Lee and Heckman 1996; Reuveni et al. 1993; Schiller et al. 2000; Schwindt and Crill 1980b; Svirskis et al. 2001).

Bistable motor neurons in both vertebrates and invertebrates share two additional common traits. First, all previously documented occurrences of bistability in motor neurons have involved the action of a neuromodulator (Kiehn 1991), typically a monoamine, that assists in the production of the plateau potential (Conway et al. 1988; Hounsgaard and Kiehn 1985, 1989; Hounsgaard et al. 1988; Hsiao et al. 1998; Kiehn and Harris-Warrick 1992b; Lee and Heckman 1998; Ramirez and Pearson 1991). Second, although recent studies have identified a role for a persistent Na⁺ current in some systems (Elson and Selverston 1997; Hsiao et al. 1998; Lee and Heckman 1999; Li and Bennett 2003; Powers and Binder 2003), production of plateau potentials in motor neurons often depends on the activation of an L-type Ca²⁺ current (Carlin et al. 2000; Hounsgaard and Kiehn 1989; Li and Bennett 2003; Russo and Hounsgaard 1994; Svirskis et al. 2001) and in some cases on a Ca²⁺-activated nonselective cation current *I*_{CAN} (Morisset and Nagy 1999; Reikling and Feldman 1997; Zhang et al. 1995).

One motor neuron in which the biophysical mechanisms underlying bistability have been studied in detail is the dorsal gastric (DG) neuron in the stomatogastric ganglion (STG) of the crab *Cancer borealis*. The stomatogastric nervous system (STNS), of which the STG is part, produces both the gastric mill motor pattern that is responsible for movement of teeth within the stomach and the pyloric motor pattern that controls the muscles of the pylorus, a filtering apparatus (Harris-Warrick et al. 1992). The DG neuron is part of the gastric mill circuit (Weimann et al. 1991) and innervates the gastric mill 4 (gm4) muscle [nomenclature from Maynard and Dando

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(1974)]. In a series of papers, Harris-Warrick and coworkers showed that serotonin released by a stretch-sensitive neuron (Katz and Harris-Warrick 1989) triggers DG plateau potentials by acting directly and indirectly on at least four currents including a Ca^{2+} conductance and a Ca^{2+} -activated nonselective cation current (Kiehn and Harris-Warrick 1992a,b; Zhang and Harris-Warrick 1995; Zhang et al. 1995).

Herein we also describe observations of bistability in DG, although our findings are distinct from those presented in the earlier studies. Rather than injecting current into the soma, we stimulated extracellularly the nerve containing the DG axon and observed persistent firing of the DG neuron and contractions of the gm4 muscle with enhanced amplitudes and durations. The magnitude of these effects depended on the amplitude of the stimulus. As in the previous inquiries, we found that calcium and calcium-dependent conductances play an essential role in producing the bistability. However, we conclude that the biophysical mechanisms responsible for bistability are located in the axon. Moreover, we saw no evidence that local neuromodulation plays a role in initiating the bistable behavior in this system. Some of these data previously appeared in abstract form (Verley et al. 2005).

METHODS

Animals and solutions

Adult male *Cancer borealis* were obtained from seafood suppliers in the Boston, MA area and from the Marine Biological Laboratory (Woods Hole, MA) and maintained in aerated aquaria at 10–12°C. In physiology experiments, we used saline with the following composition (in mM): 440 NaCl, 11 KCl, 13 CaCl_2 , 26 MgCl_2 , 5 maleic acid, and 11 Trizma base, pH 7.4–7.5. Low-calcium saline was identical to that just described except the Ca^{2+} concentration was reduced to 0.1 mM. The modulators proctolin (American Peptide Company, Sunnyvale, CA), TNRNFLRFamide (American Peptide Company), *C. borealis* tachykinin-related peptide Ia (CabTRP Ia, a gift from M. P. Nusbaum, Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, PA), serotonin (Sigma, St. Louis, MO), and γ -aminobutyric acid (GABA, Sigma) were dissolved in deionized water at 10^{-3} or 10^{-2} M and stored at -20°C before being diluted in saline and then bath-applied to the preparation. The peptide [Asn¹³]orcokinin (orcokinin, Bachem, San Carlos, CA) was dissolved in DMSO at 10^{-3} M and stored at -20°C before use. Nicardipine (Sigma) and flufenamic acid (FFA, Sigma) stock solutions were made in DMSO immediately before use and then diluted so that the final concentration of DMSO was 0.1%. We confirmed that DMSO at this concentration had no effect on muscle contraction or spike generation. Tetrodotoxin (TTX, Sigma) was prepared from a stock solution (10^{-4} M) and dissolved in a 750-mM sucrose solution to reach a final concentration of 10^{-6} M.

Contraction measurements

The gm4 muscle is innervated solely by the single DG motor neuron whose axon projects from the STG to the muscle by the dorsal gastric nerve (*dgn*) (Weimann et al. 1991). Neuromuscular preparations were dissected from the stomach and pinned flat in 5-ml Sylgard 182-lined (Dow Corning, Midland, MI) petri dishes. For most physiology experiments, the preparation consisted of the gm4 muscle and the *dgn*, with the nerve cut just below the STG. During recording sessions the bath volume was approximately 3 ml, and the preparation was continuously superfused (4–5 ml/min) with physiological saline cooled by an ice bath to a temperature regulated between 9 and 11°C. During contraction experiments in particular, care was taken to

regulate the temperature to within a few tenths of a degree because we observed the amplitude of muscle contractions to be temperature dependent (Birmingham, unpublished observations).

Contractions of the gm4 muscles were measured using a FT03 force transducer (Astro-Med, West Warwick, RI). One insertion of the gm4 muscle was pinned down to the dish, whereas the other was attached to the transducer with a short (about 3 cm) piece of size 6/0 silk suture thread (Fine Science Tools, Foster City, CA). The transducer was positioned so that the muscle was stretched just past its relaxed length. A stainless steel pin stimulation electrode was inserted into an insulating (petroleum jelly) well around the end of the nerve furthest from the muscle. The electrode was driven by a pulse stimulator (Model 2100, A-M Systems, Carlsborg, WA) to induce muscle shortening, and the force transducer measured the increased tension. The transducer signal was amplified (Brownlee Precision Model 440, San Jose, CA) by a factor of 10,000 and recorded using a Digidata 1322A acquisition system (Axon Instruments, Union City, CA).

Intracellular and extracellular recordings

Measurements of excitatory junctional potentials (EJPs) from the gm4 muscle were made using conventional 2 M KAc-filled microelectrodes with resistances of 7–10 M Ω . The *dgn* was stimulated extracellularly as during contraction measurements. EJPs were measured with an Axoclamp 2B intracellular amplifier (Axon Instruments), amplified tenfold using the Brownlee amplifier, and recorded using the Digidata acquisition system. For intracellular recordings from the DG neuron, the complete STNS consisting of the paired commissural ganglia (CoGs), the esophageal ganglion (OG), the STG, and a subset of the motor nerves was dissected out of the animal and pinned out in a Sylgard-coated dish. The STG was then desheathed and petroleum jelly wells were placed on the motor nerves. To prevent activation of the gastric mill rhythm by descending modulatory inputs, we built a petroleum jelly well filled with a sucrose/TTX solution (750 mM sucrose, 10^{-6} M TTX) on the stomatogastric nerve. Intracellular recordings from the DG soma were made using an Axoclamp 2A and 20- to 40-M Ω glass microelectrodes filled with 0.6 M K_2SO_4 and 20 mM KCl. DG was identified by correlating intracellular recordings with extracellular recordings of spikes in the *dgn*.

Extracellular measurements from the *dgn* were made using either pin or glass suction electrodes amplified by an A-M Systems 1700 differential amplifier and recorded using the Digidata acquisition system. In addition to the axon of DG, axons from the gastric mill (GM) motor neurons and the anterior gastric receptor (AGR) (Simmers and Moulins 1988), a muscle stretch receptor that does not innervate gm4, are known to project down the *dgn*. If present, electrical coupling between the DG, AGR, and/or GM neurons is too weak for any one of these neurons to activate the others (MP Nusbaum, unpublished observations). In extracellular recordings, the largest amplitude unit corresponded to DG spiking. Spontaneous AGR spiking (Simmers and Moulins 1988) was typically observed in extracellular recordings but was sometimes absent in preparations in which the recording was made very close to the gm4 muscle or from one portion of a branched *dgn*. The number of distinct spikes observed in a particular experiment varied from one to four.

Whole-mount immunohistochemistry

In anatomical studies one of two preparations was used: the STNS containing the *dgn* cut just anterior to its insertions onto the gm4 muscle or the *dgn* with the gm4 muscles attached. In either case, the preparation was dissected free of the foregut in chilled (about 10°C) saline and then pinned in a Sylgard 184-lined (Dow Corning) petri dish. The saline differed slightly from that used in physiology experiments in that it was buffered with HEPES acid (10 mM) instead of maleic acid/Trizma base, and its pH was adjusted to 7.4 with NaOH. Preparations were then fixed at 4°C for 12–24 h in a freshly prepared

solution of 4% paraformaldehyde (EM grade, catalog No. 15710; Electron Microscopy Sciences, Hatfield, PA) in 0.1 M sodium phosphate (P) buffer (pH 7.4). After fixation, tissues were rinsed five times over nearly 5 h in a solution of P buffer containing 0.3% Triton X-100 (P-Triton) and then incubated for about 72 h in primary antibody diluted to an appropriate working concentration (see *Antibodies*) in P-Triton containing 10% normal donkey serum (NDS, catalog No. 017-000-121; Jackson ImmunoResearch Laboratories, West Grove, PA). After incubation in primary antibody, tissues were again rinsed five times over nearly 5 h in P-Triton and then incubated in a 1:300 dilution of secondary antibody (see following text) for 12–24 h. As with the primary, incubation in secondary antibody was done in P-Triton containing 10% NDS. After incubation in secondary antibody, preparations were rinsed five times over about 5 h in P and mounted between a glass microscope slide and coverslip using Vectashield mounting medium (catalog No. H-1000; Vector Laboratories, Burlingame, CA). Incubations in primary and secondary antibody were done at 4°C with gentle agitation. All rinses were done at room temperature (18–24°C) without agitation. Secondary antibody incubation was conducted in the dark, as was all subsequent processing. Likewise, slides were stored in the dark at 4°C until examined.

Antibodies

Antibodies to the vesicle-associated proteins synapsin or synaptotagmin were used as general markers for extraganglionic neuropil. This strategy was previously used to identify areas of synaptic/modulatory neuropil in the STNS of a variety of crustacean species (Christie et al. 2004a; Goillard et al. 2004; Skiebe and Ganeshina 2000; Skiebe and Wollenschlager 2002). The synapsin antibody used was a mouse monoclonal antibody (SYNORF1; Klagges et al. 1996) generated against a glutathione S-transferase-fusion protein containing a portion of a *Drosophila* synapsin homologue. This antibody was used at a final concentration of 1:100. The synaptotagmin antibody used was a rabbit polyclonal antibody (DSYT-2; Littleton et al. 1993) generated against amino acid residues 134–474 of *Drosophila* synaptotagmin. This antibody was used at a final concentration of 1:750.

The secondary antibodies used in our experiments were donkey anti-mouse IgG conjugated to Alexa Fluor 488 (catalog No. A-21202; Molecular Probes, Eugene, OR) or Alexa Fluor 594 (catalog No. A-21203; Molecular Probes) and donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (catalog No. A-21206; Molecular Probes) or Alexa Fluor 594 (catalog No. A-21207; Molecular Probes).

Confocal and epifluorescence microscopy

Fluorescently labeled tissue was viewed and data collected using one of two Bio-Rad MRC 600 laser scanning confocal microscopes (Bio-Rad Microscience, Hemel Hempstead, UK), a Bio-Rad Radiance 2000 laser scanning confocal microscope, or a Nikon Eclipse E600 epifluorescence microscope (Tokyo, Japan). Specifications for each of the microscope systems used in this study were previously published, as were methods for figure production (Fu et al. 2005; Messinger et al. 2005).

Statistics

The SigmaStat program (version 3.1; Systat Software, Point Richmond, CA) was used for data analysis. In physiology experiments where the stimulation voltage was changed from 1 to 7 V or when a neuromodulatory substance or pharmacological agent was applied, a paired *t*-test was used to test for statistical significance. For experiments in which the effects of a neuroactive substance were tested for both stimulation voltages, a two-way ANOVA was used. Pairwise comparisons were then made using the Holm–Sidak method. Error bars on plots correspond to SEs.

RESULTS

High-voltage extracellular stimulation of the dgn induces contractions of the gm4 muscle that have enhanced amplitudes and durations

In the crustacean STNS, as well as in many other nervous systems, extracellular stimulation is commonly used to produce muscle contractions in neuromuscular preparations. A pin or suction electrode is used to apply voltage pulses across a nerve containing the motor axon, with each pulse eliciting an action potential when the stimulus voltage is above a threshold value. Beyond this threshold, no dependency on stimulation voltage is expected. Nerve-evoked contractions of the gm4 muscle in the crab *C. borealis* were previously recorded during investigations of neuromodulation of the stomatogastric musculature (Jorge-Rivera and Marder 1996, 1997; Jorge-Rivera et al. 1998). Using an experimental setup nearly identical to that in the studies mentioned earlier, we found the threshold voltage for gm4 contraction to be about 0.5–0.7 V for the 1-ms unipolar pulses used. When we applied a 12.5-Hz train of 1-V pulses, we observed a contraction (Fig. 1) that was qualitatively very similar to that described previously (Jorge-Rivera and Marder 1996, 1997; Jorge-Rivera et al. 1998). The stimulation frequency was chosen to be comparable to that of the DG neuron during a gastric mill rhythm (Beenhakker et al. 2004). The traces above the contraction show simultaneous measurements of extracellular activity in the *dgn* (recorded at a spot between the stimulation electrode and the muscle) and EJPs recorded intracellularly from a gm4 fiber. Also seen in the extracellular recording is tonic spiking of AGR.

As we increased the stimulation voltage above threshold, we observed that gm4 contraction depended on the amplitude of the stimulus in a highly nonlinear manner. Figure 2A shows contractions elicited in response to a 3-s duration, 12.5-Hz

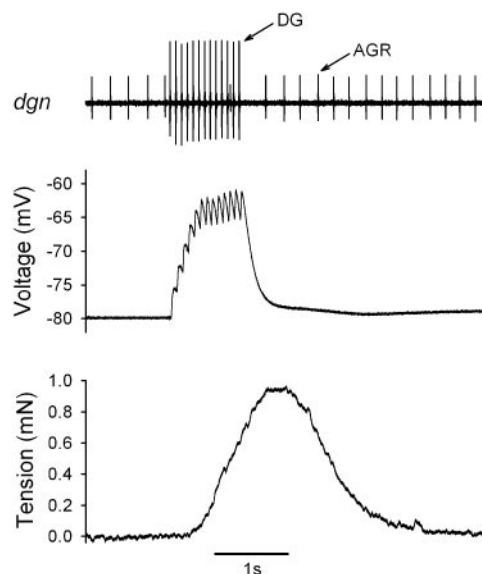


FIG. 1. Extracellular stimulation of the dorsal gastric nerve (*dgn*) results in contraction of the gastric mill 4 (gm4) muscle. Simultaneous recordings of extracellular activity in the *dgn*, membrane potential from a gm4 fiber, and tension exerted by gm4 in response to 1-s, 12.5-Hz, 1-V *dgn* stimulation. Large and small spikes in the extracellular recording correspond to the dorsal gastric (DG) motor neuron and anterior gastric receptor (AGR) neuron, respectively. Stimulation artifacts are smaller than the DG spike and are not observable in the extracellular recording.

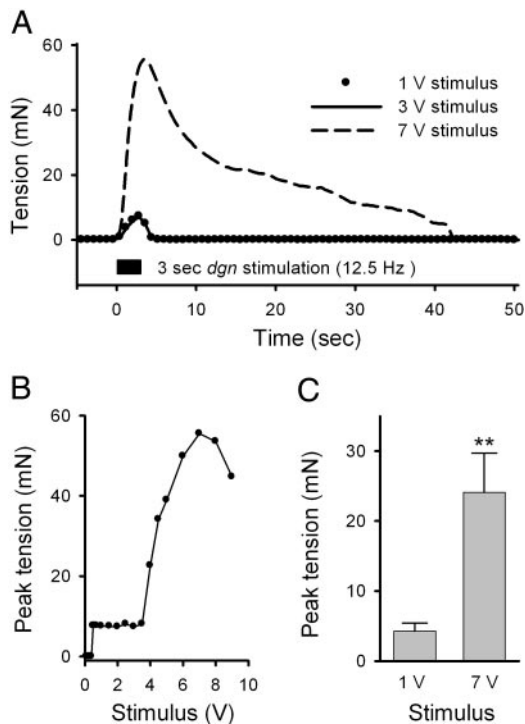


FIG. 2. Contraction of gm4 depends on stimulus voltage. *A*: muscle tension in response to 3-s, 12.5-Hz *dgn* stimulations with amplitudes 1, 3, and 7 V. *B*: peak tension as a function of stimulus voltage for preparation shown in *A*. *C*: average peak tension for 1- and 7-V stimuli over 10 experiments (paired *t*-test, $**P < 0.01$).

stimulation of the *dgn* for stimulation voltages of 1, 3, and 7 V in one experiment. In this experiment we used a realistic stimulus duration (Beenhakker et al. 2004), which had not been possible when making intracellular measurements from the muscle fiber. Contraction measurements were made 1 min apart and in order of increasing voltage at different amplitudes from 0.1 to 9 V (Fig. 2*B*). In this experiment the threshold voltage was found to be between 0.4 and 0.5 V. As expected, the 1- and 3-V contractions were indistinguishable, and in each case the muscle relaxed fully within 1 s after the stimulus ceased. The contraction elicited by the 7-V stimulus, on the other hand, was approximately seven times larger in amplitude, and muscle tension persisted for nearly 40 s after the end of the stimulus (Fig. 2*A*). A plot of the peak tension for each contraction as a function of stimulus voltage is shown in Fig. 2*B*. The peak tension remained constant for stimulation voltages between 0.5 and 3.5 V and began to increase with the 4-V stimulus. In this experiment, the peak tension actually decreased for stimulation voltages >7 V. This, however, is not representative of other experiments and most likely reflects the fact that the mechanism underlying the enhanced contractions sometimes decreased with repeated stimulation. In many cases, however, the ability to produce enhanced contractions in response to high-voltage stimulation remained largely undiminished after 2–3 h of intermittent stimulation and outlasted the experiment itself.

In general, we found that enhanced contractions were first observed at stimulation voltages between 4 and 5 V (and never <3 V), and that no further increase in contraction amplitude or duration resulted for voltages >7 or 8 V. Figure 2*C* summarizes the results from ten such experiments and shows that the

peak tension measured in response to 7-V stimuli was significantly greater than that for 1-V stimuli (paired *t*-test, $**P < 0.01$). In these experiments, the duration of the enhanced contraction varied from just a few seconds to >1 min (data not shown). Contractions with enhanced amplitudes and durations were also observed for high-voltage stimulation when we adjusted the other stimulation parameters, i.e., the stimulus train frequency (5 and 25 Hz), the train duration (0.5 and 1 s), and the pulse width (0.5 ms). In the last case, the threshold voltages for normal contraction and enhanced contraction were larger than those for 1-ms pulses (data not shown).

Persistent firing of the DG motor neuron contributes to the production of the enhanced contractions

To investigate whether the behavior shown in Fig. 2 originated in the muscle or the nerve, we made simultaneous measurements of contraction, *dgn* activity, and EJPs in response to high-voltage stimulation. Figure 3 shows recordings in response to a 1-s duration, 12.5-Hz train of 7-V pulses for the same preparation shown in Fig. 1. The simultaneous extracellular nerve recording and intracellular muscle recording demonstrate that the DG neuron fired action potentials for >20 s after the end of the stimulus, with a corresponding increase in the number of EJPs and the duration of the contraction. In this

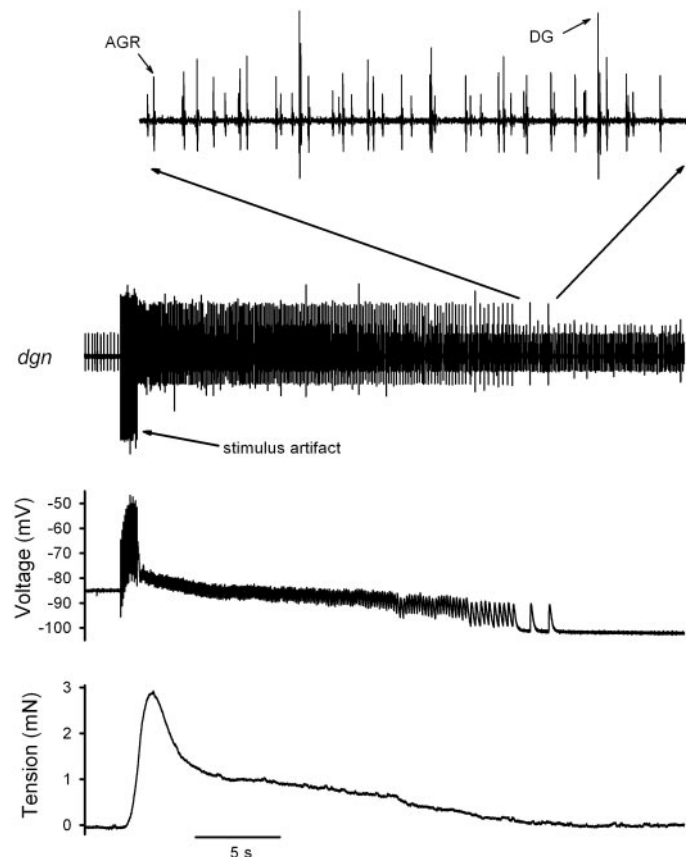


FIG. 3. Long-duration contractions generated by high-voltage *dgn* stimulation are correlated with persistent DG spiking. Simultaneous extracellular (*dgn*), intracellular (gm4), and tension recordings in response to 1-s, 12.5-Hz, 7-V *dgn* stimulation for same preparation as in Fig. 1. Expanded view of extracellular recording shows at least 4 distinct spikes in the *dgn*. Unidentified spikes are presumed to correspond to activity of 2 gastric mill (GM) motor neurons.

particular experiment the firing rate of AGR increased appreciably, and the expanded extracellular trace reveals two other spikes (one larger and one smaller than the AGR spike) that we assume to be GM spikes. In other experiments the firing rate of AGR remained unchanged after an extracellular stimulation that caused persistent DG spiking (data not shown).

Persistent DG firing cannot be elicited by current injection into the soma

The bistability in DG reported by Kiehn and Harris-Warrick (1992b) was induced by current injection into the neuron's soma in the presence of serotonin. In contrast, the behavior illustrated in Figs. 1–3 was observed in preparations in which the soma was not present. To investigate whether the two mechanisms were related, we did experiments in which the connection between the *dgn* and the STG

was preserved so that we could both record from and inject current into the DG cell body. In these experiments, the activity in the stomatogastric nerve was blocked with sucrose/TTX to prevent descending modulatory inputs from inducing the gastric rhythm. Figure 4, A–C shows simultaneous intracellular DG and extracellular *dgn* recordings in response to three different stimulus protocols. The response to a 5-s-duration injection of current into the soma is shown in Fig. 4A. Although the DG orthodromic instantaneous firing rate was ≥ 20 Hz during the duration of the stimulus, no persistent firing resulted, consistent with previous results in which the induction of plateau properties from the soma required the presence of a neuromodulator (Kiehn and Harris-Warrick 1992b; Weimann et al. 1993). Figure 4B shows that an extracellular 1-V, 50-Hz stimulus depolarized the DG soma but was unable to produce persistent firing. The *inset* shows that each pulse produced one antidromic

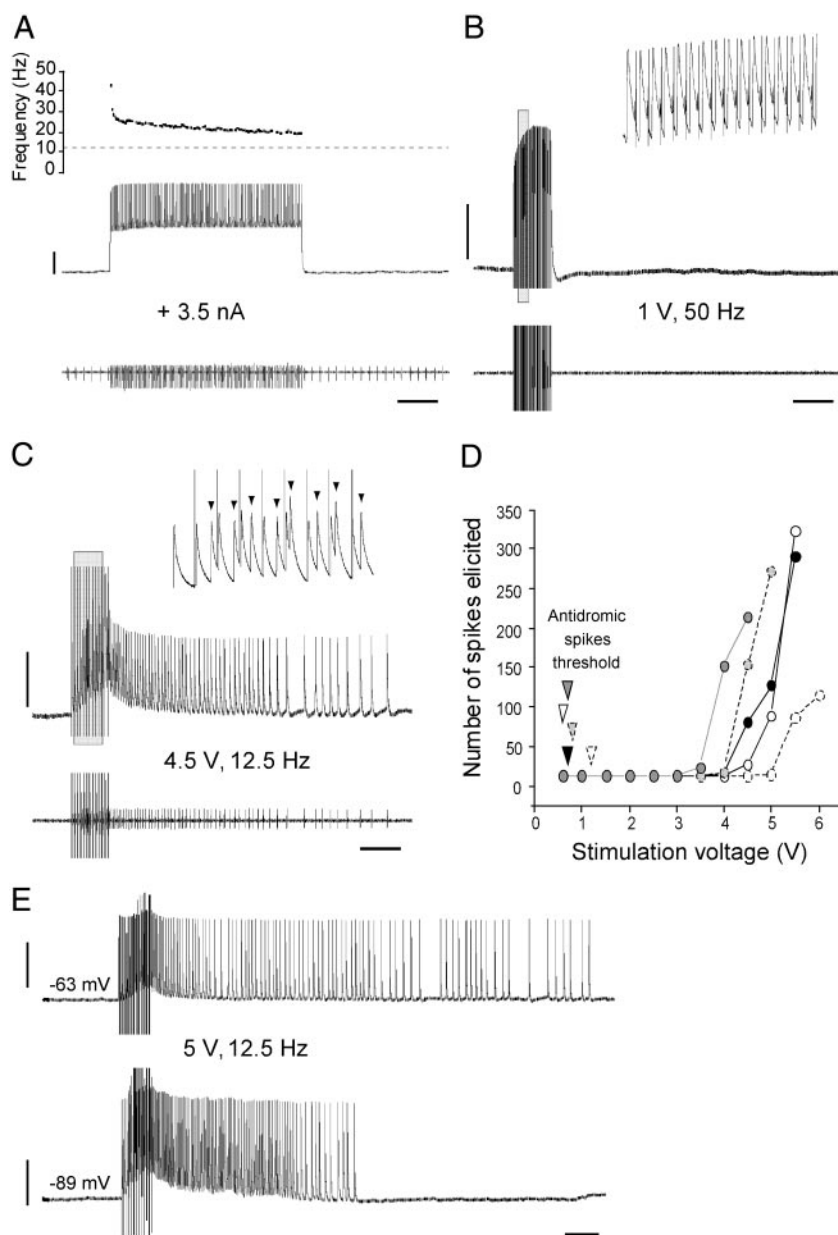


FIG. 4. Persistent firing can be induced only by high-voltage stimulation of the *dgn*. *A*: DG was depolarized by injecting current (+3.5 nA, 5 s) into the soma to elicit high-frequency orthodromic spiking. Instantaneous firing frequency was >20 Hz during the entire 5 s but persistent firing was not elicited. Dotted line shows 12.5 Hz. *B*: extracellular stimulation of the *dgn* at high frequency (50 Hz) and low voltage (1 V) for 1 s was unable to elicit persistent firing. *Inset*: each stimulus pulse induced a single antidromic spike recorded intracellularly from the DG soma. *C*: stimulation of the *dgn* in the same preparation at 12.5 Hz, 4.5 V for 1 s induced persistent firing that was recorded intracellularly in DG. *Inset*: extra spikes (black arrowheads) were produced during the train of stimulations. *D*: plot of total number of DG spikes produced in response to 1-s, 12.5-Hz extracellular stimulation as a function of stimulus voltage for 5 preparations. Arrowheads on the left of the graph indicate the thresholds for production of antidromic spikes. Between the threshold for spike production and the onset of persistent firing, exactly 13 spikes were produced in each preparation—one for each stimulus pulse. *E*: hyperpolarization of the DG soma from -63 to -89 mV reduced the persistent firing elicited by a 1-s, 12.5-Hz, 5-V extracellular stimulation of the *dgn*. Scale bars: time, 1 s; voltage, -60 to -50 mV in A–C, E (top trace), -90 to -80 mV in E (bottom trace). A and E recordings are from one experiment; B and C from another experiment.

spike. When the same preparation was stimulated at 4.5 V, 12.5 Hz, persistent DG firing was induced (Fig. 4C). The *inset* shows that extra spikes were also elicited between stimulus pulses such that the firing rate was >12.5 Hz during stimulation. Figure 4D summarizes the dependency on stimulation voltage of the DG spiking in five experiments when a 12.5-Hz extracellular stimulus was used. The minimum stimulus voltage for extra spiking varied from 3.5 to 5.5 V. From the threshold up to this voltage, 13 spikes were generated, one for each pulse. In none of these five preparations did stimulation of the *dgn* with high-frequency (≥ 40 Hz), 1-V pulses or depolarization of DG produce persistent firing. For each of the latter measurements, sufficient current was injected to produce spiking of ≥ 20 Hz. In two experiments we hyperpolarized the soma to between -75 and -90 mV while stimulating the *dgn* extracellularly to see whether persistent firing could be affected. The duration of the persistent firing was shortened by 20–80% but could not be suppressed by hyperpolarization of the cell body (Fig. 4E).

Persistent DG firing can be induced only at proximal sites on the axon

By varying the location of the extracellular stimulation electrode on the *dgn*, we determined that persistent firing could be induced from only a portion of the nerve. Figure 5A shows that when the electrode was relatively close (about 6 mm) to the STG, persistent firing was elicited by a 5.2-V, 12.5-Hz, 1-s stimulation. In contrast, persistent spiking could not be produced in the same preparation when the stimulation electrode was 15.5 mm away from the STG, even when the stimulus amplitude was increased to 9 V. We did a total of four experiments, and in each case we found persistent firing was never elicited beyond a distance of 10–15 mm from the STG, even by stimulation voltages more than twice as large as those used to generate the persistent firing by stimulating at a more proximal site.

Figure 5B shows intracellular and extracellular recordings of orthodromic spikes generated by current injection into the DG soma (*top*) and spikes from a persistent DG spike train induced by extracellular stimulation of the *dgn* (*bottom*). By comparing the arrival times of the spikes at the recording sites, we determined the spike initiation zone for persistent firing to be in the axon 4.9 mm distant from the STG. In each of four experiments, we located this peripheral spike initiation site in the axon. The average distance from the site to the STG was 5.5 ± 1.0 mm. Axonal spike initiation zones were nearby the stimulation sites, but in different experiments they were found to be either closer to or further from the STG, relative to the stimulation site.

Persistent DG firing and enhanced gm4 contractions are eliminated in low- Ca^{2+} saline

Previous studies referenced in the INTRODUCTION identified Ca^{2+} as having both direct (I_{Ca}) and indirect roles (I_{CAN} , neuromodulatory release) in the induction of bistability in many systems. If any of these mechanisms contributes to the generation of persistent DG axonal firing, we should expect the extra spikes to disappear when Ca^{2+} is removed from the saline. Persistent firing of DG (Fig. 6A) and enhanced contrac-

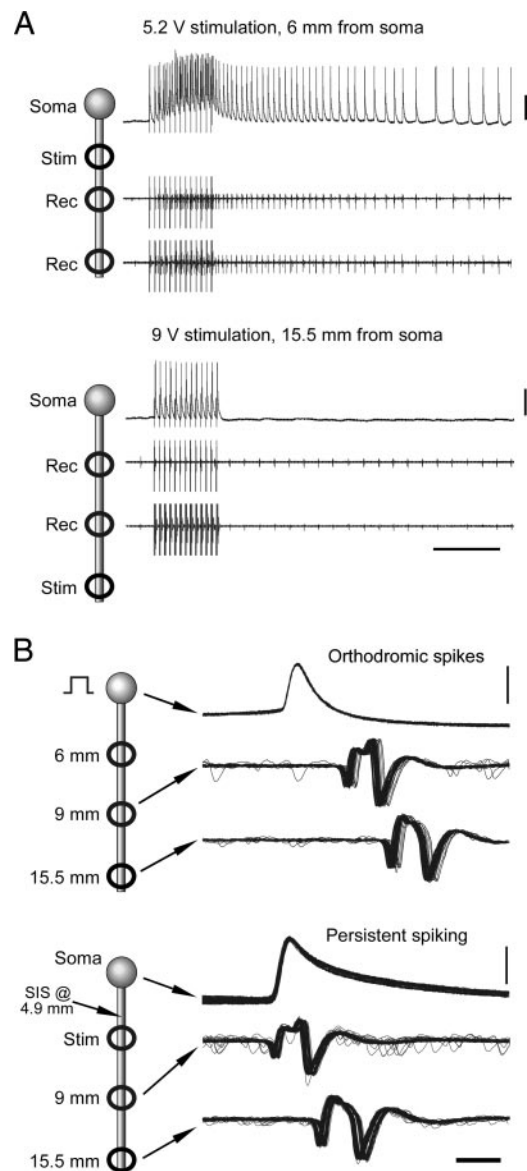


FIG. 5. Persistent firing can be induced only at proximal sites on the axon. Three petroleum jelly wells were built on the *dgn* at distances of 6, 9, and 15.5 mm from the stomatogastric ganglion (STG). One well was used for stimulation, whereas extracellular recordings were made in the other 2 wells, and an intracellular recording was made from the DG soma. *A*: when the *dgn* was stimulated in the 6-mm well for 1 s at 12.5 Hz, 5.2 V, persistent firing was elicited that was observed in all 3 recordings. When the *dgn* was stimulated in the 15.5-mm well, no persistent firing was recorded, even for a stimulation voltage of 9 V. Scale bars: time, 1 s, voltage, -60 to -50 mV. *B*: spiking was elicited in this same preparation by both current injection into the DG soma (*top*, represented by a square pulse) and extracellular stimulation of the *dgn* in the well 6 mm from the STG (*bottom*), with the latter producing persistent spiking. From the measured distances between recordings sites and the timing of the spikes, the spike initiation site (SIS) for persistent firing was deduced to be 4.9 mm from the STG. Scale bars: time, 5 ms, voltage, 10 mV.

tion of gm4 (Fig. 6B) were abolished when low- Ca^{2+} saline was placed in the stimulation well but reappeared when the calcium was returned. Sustained firing after the end of the stimulus was entirely suppressed in each of four experiments when low- Ca^{2+} saline was placed in the well. However, low- Ca^{2+} saline did not abolish the DG spikes elicited by each pulse (data not shown).

Enhanced contractions can be blocked using specific pharmacology against Ca^{2+} and calcium-activated currents

To investigate whether L-type Ca^{2+} channels and I_{CAN} were involved in DG axonal bistability, we tested the effects of the L-type calcium channel blocker nifedipine (a dihydropyridine) (Bean 1989; Hurley and Graubard 1998) and flufenamic acid (FFA), a nonspecific Ca^{2+} -activated current-blocking agent (Del Negro et al. 2005; Di Prisco et al. 2000; Gögelein et al. 1990; Guinamard et al. 2004; Lee et al. 1996; Mironov and Langohr 2005; Morisset and Nagy 1999), on the neuromuscular preparation. In all experiments the *dgn* was not desheathed and agents were applied specifically to the stimulation well, and not to the whole preparation, to prevent direct effects of the drugs on the musculature. When the stimulation electrode was bathed with nifedipine ($100 \mu\text{M}$), the enhanced contractions induced at 7 V disappeared, and the contractions induced at 1 and 7 V were identical (Fig. 7A). This effect of nifedipine was reversible. Figure 7B summarizes the results of five experiments and shows that the peak tension in control

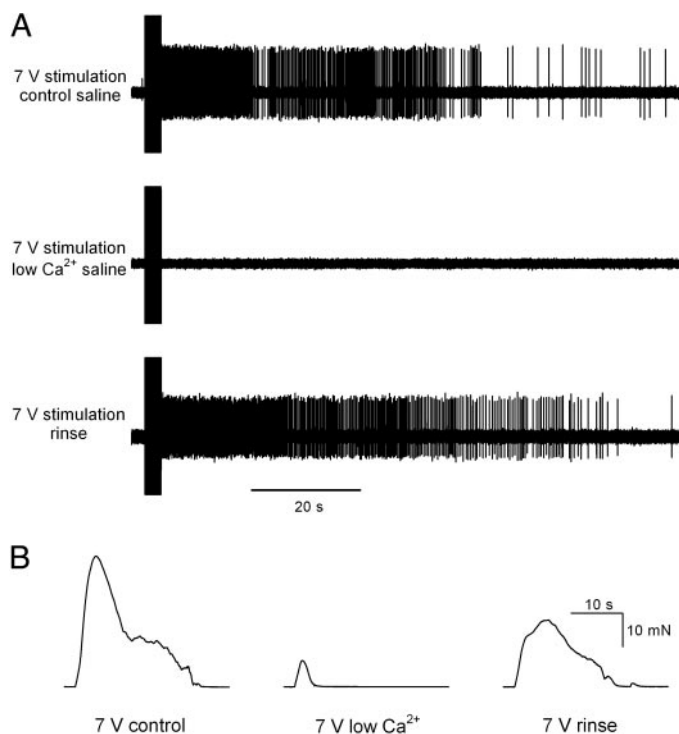


FIG. 6. Persistent firing and enhanced contractions disappear in low- Ca^{2+} saline. **A**: persistent firing produced in response to 3 s, 12.5 Hz, 7 V stimulation disappeared when low- Ca^{2+} saline was placed in the stimulation well but returned when control saline was reapplied. This last condition is labeled "rinse." Sustained firing after the end of the stimulus was entirely suppressed in 4 out of 4 experiments when low- Ca^{2+} saline was placed in the well. **B**: enhanced gm4 contraction amplitude and duration measured in a different preparation disappeared when low- Ca^{2+} saline was placed in the stimulation well but returned when control saline was reapplied. All of the low- Ca^{2+} experiments required that the stimulation well be removed temporarily and the nerve immersed in the low- Ca^{2+} saline for about 1.5 h to completely suppress the persistent firing or enhanced contraction. After the well was remade with low- Ca^{2+} saline inside, the saline outside the well could be exchanged, if desired. Persistent firing and enhanced contractions reappeared within 15 min of returning the Ca^{2+} to the saline in the well. In **A** the saline inside and outside the well was the same for each condition (control, low Ca^{2+} , rinse). However, we observed in other experiments that the absence or presence of calcium in the saline outside the well did not influence the DG spiking. For the contraction experiment, control saline was used outside the well in all cases.

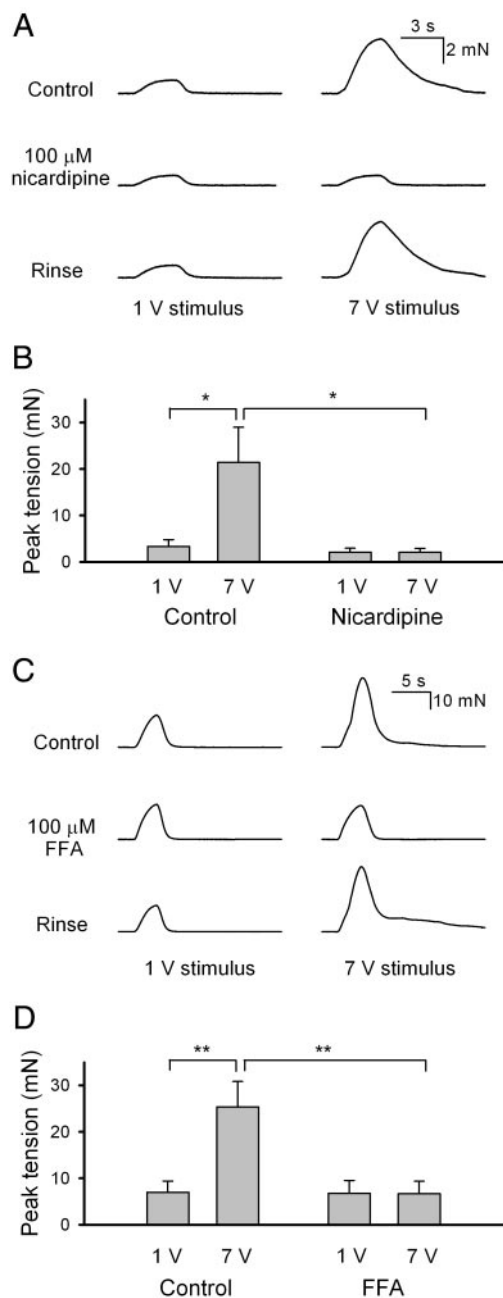


FIG. 7. Enhanced contractions are eliminated in the presence of nifedipine ($100 \mu\text{M}$) and flufenamic acid (FFA) ($100 \mu\text{M}$), blockers of L-type Ca^{2+} channels and I_{CAN} , respectively. **A**: contractions elicited in response to 3-s, 12.5-Hz stimulation at 1 and 7 V in control saline, nifedipine, and after rinsing. **B**: average peak tension for 5 experiments in which each of the 4 conditions (Control, 1 and 7 V; nifedipine, 1 and 7 V) was tested. Peak tension in control saline at 7 V was significantly different from the peak tension in control saline at 1 V and that measured in the drug at 7 V (Holm-Sidak method, $*P < 0.05$). **C**: contractions elicited in a different preparation in response to 3-s, 12.5-Hz stimulation at 1 and 7 V in control saline, FFA, and after rinsing. **D**: average peak tension for 6 experiments in which each of the 4 conditions (Control, 1 and 7 V; FFA, 1 and 7 V) was tested. Peak tension in control saline at 7 V was significantly different from the peak tension in control saline at 1 V and that measured in the drug at 7 V (Holm-Sidak method, $**P < 0.01$).

saline at 7 V was significantly different from both the peak tension in control saline at 1 V and that measured in the drug at 7 V (Holm-Sidak method, $*P < 0.05$). In each of these experiments, the effect of nifedipine took ≥ 20 but < 35 min

to develop. When FFA (100 μM) was applied to the stimulation well, enhanced gm4 contractions disappeared within 5–10 min and completely recovered on washout. A representative experiment is shown in Fig. 7C and a summary in Fig. 7D. Again, the control 7-V peak tension was significantly different from both the 1-V control value and the peak tension at 7 V in the presence of FFA (Holm–Sidak method, $n = 6$, $**P < 0.01$). Application of lower concentrations (10 μM) of either nicardipine or FFA reduced but did not completely block the enhancement of the contractions elicited at high stimulation voltages.

Lack of physiological and anatomical evidence of involvement of neuromodulator in generating bistability

The STNS in *C. borealis* is modulated by a host of neuroactive compounds that are released locally or circulate as hormones. Jorge-Rivera et al. showed that ten of these substances modulate nerve-evoked gm4 contractions. Two substances (histamine, allatostatin-3) decreased gm4 contraction amplitude, whereas eight [crustacean cardioactive peptide (CCAP), dopamine, octopamine, proctolin, red pigment concentrating hormone (RPCH), SDRNFLRFamide, serotonin, and TNRNFLRFamide] increased it (Jorge-Rivera and Marder 1996, 1997; Jorge-Rivera et al. 1998). Although several of the neuromodulators were shown to have subtle effects on the relaxation rate of the gm4 contraction (Jorge-Rivera et al. 1998), there is no evidence that any of them can produce persistent firing or prolonged gm4 contractions.

The three substances that induced the largest (>200%) increase in contraction amplitude in the previous studies were proctolin, serotonin, and TNRNFLRFamide (Jorge-Rivera et al. 1998). In our own experiments, we confirmed that in no case did application of one of these three modulators (10^{-7} M proctolin, $n = 6$; 10^{-5} M serotonin, $n = 4$; 10^{-7} M TNRNFLRFamide, $n = 5$) to the stimulation well and/or outside the well produce prolonged gm4 contractions in response to low (about 1 V)-voltage, 3-s, 12.5-Hz stimulation, although each modulator did increase the contraction amplitude. In the presence of each modulator, the dependency of the contraction on stimulation voltage was very similar to what had been observed under control conditions: contractions were prolonged and were further increased in amplitude at or above the voltages at which enhanced behavior had been observed under control conditions. We also assayed the effects on contraction of three modulators that had not previously been tested: CabTRP Ia (10^{-7} M, $n = 4$), GABA (10^{-5} M, $n = 7$), and orokinin (10^{-7} M, $n = 4$). None of these triggered bistable behavior. In all experiments in which a neuromodulator was applied to the *dgn*, the nerve was not desheathed and was cut below the STG.

In the crustacean STNS, a number of extraganglionic neuropil regions have been identified and modulation at these sites documented. Interestingly, despite the extensive mapping of neuromodulators in the STNS of *C. borealis*, immunohistochemical investigations of the *dgn* have produced negative results in every instance. Antibodies tested include those for proctolin (Marder et al. 1986), FLRFamide (Marder et al. 1987), tachykinin-related peptide (Goldberg et al. 1988), serotonin (Katz et al. 1989), pigment-dispersing hormone (Mortin and Marder 1991), buccalin and myomodulin (Christie et al.

1994), allatostatin (Skiebe and Schneider 1994); cholecystokinin (Christie et al. 1995), GABA (Swensen et al. 2000), orokinin (Li et al. 2002), histamine (Christie et al. 2004a), and mandibular organ-inhibiting hormone (MOIH) (Hsu et al. 2004).

Recent mass spectrometric measurements revealed the existence of many previously unknown peptides in neurosecretory structures in *C. borealis* (Li et al. 2003). Clearly, it is possible that an untested neuromodulator could be present in the *dgn* and released onto the DG axon in the proximal portion of the nerve by a calcium-dependent process. As a first step toward addressing this possibility, we immunolabeled preparations consisting of either the intact STNS containing the *dgn* cut just anterior to its insertions onto the gm4 muscle or the *dgn* with the gm4 muscles attached with antibodies directed against the vesicle-associated proteins synapsin or synaptotagmin. Because these proteins are usually found ubiquitously on clear synaptic or dense-core vesicles, respectively, they have been used extensively in the crustacean STNS as markers for synaptic/neurohemal structures (Christie et al. 2004b; Goillard et al. 2004; Skiebe and Ganeshina 2000; Skiebe and Wollenschlager 2002).

In all STNS preparations examined ($n > 6$), extensive synapsin labeling was seen throughout the nervous system, including extraganglionic neuropil patches in the stomatogastric and lateral ventricular nerves; however, no immunoreactivity was noted anywhere in the *dgn* proper in nerve–muscle preparations ($n = 4$) (Fig. 8, B–F). As expected, however, DG–gm4 neuromuscular junctions did show intense synapsin immunoreactivity (Fig. 8, G and H). Extensive synaptotagmin labeling was similarly observed in multiple locations throughout the nervous system in every STNS preparation examined ($n > 6$; data not shown); however, no labeling was noted anywhere in the *dgn* in nerve–muscle preparations labeled with the synaptotagmin antibody ($n = 3$; data not shown).

DISCUSSION

The data presented in this paper suggest that high-voltage extracellular stimulation of the *dgn* can induce bistable behavior in the DG axon. The increased DG firing observed during the stimulus train resulted in enhancement of the gm4 contraction amplitude. The persistent DG firing produced a dramatic increase in the duration of the contraction. DG spiking and gm4 contraction amplitude were graded functions of the stimulus voltage, suggesting that there are multiple levels or a continuum of sustained DG firing states.

Axonal bistability is dependent on familiar channels

One of the more impressive examples of bistability occurs in neurons of the rat entorhinal cortex, in which plateau potentials lasting ≥ 13 min (Egorov et al. 2002) can be induced. Plateau production was suppressed by nifedipine (a dihydropyridine) (50 μM) or FFA (10 μM), blockers of L-type Ca^{2+} currents and I_{CAN} , respectively. The bistability described in our study appears to depend on similar currents.

Calcium channels in the crustacean STNS are not easily placed into vertebrate categories (Bean 1989; Ertel et al. 2000; Triggle 1999). Currents that show largely L-type behavior have been identified in a number of crustacean species (Golowasch and Marder 1992; Hurley and Graubard

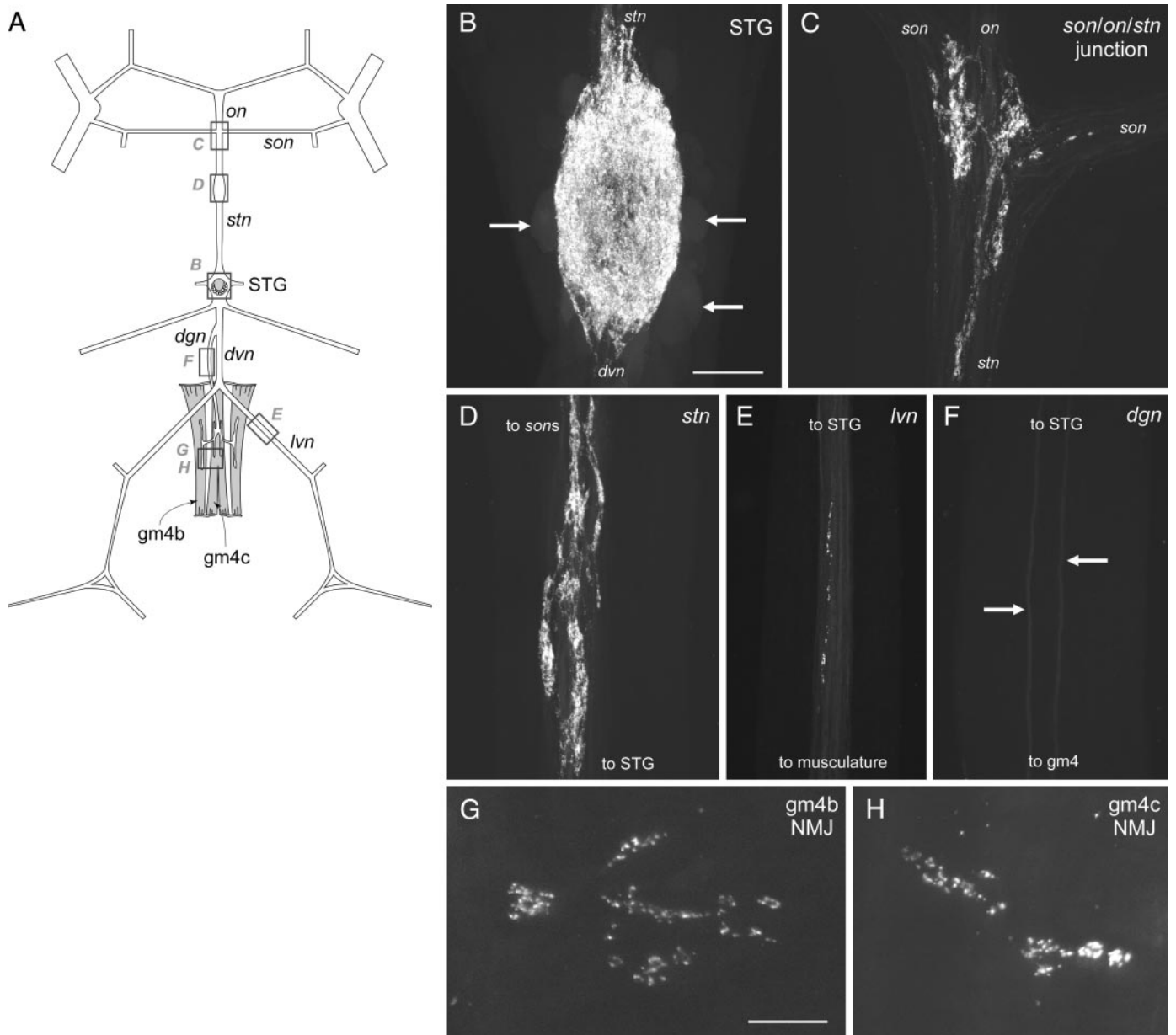


FIG. 8. Synapsin-like labeling is found throughout the STNS of *Cancer borealis* but not in the *dgn*. *A*: schematic representation of the *C. borealis* STNS, with the gm4 muscle included, showing the locations of the micrographs presented in *B–H*. The gm4 muscle is composed of the medial gm4c group and 2 lateral gm4b groups of muscle fibers (Maynard and Dando 1974). *B*: synapsin-like labeling was seen in the neuropil of the STG, a location known to contain extensive synaptic/modulatory interactions. *C–E*: extraganglionic synapsin labeling was also routinely seen in a number of the interconnecting regions and motor nerves including the junction of the *sons*, *on*, and *stn* (*C*); within the *stn* itself (*D*); and in the *lvn* (*E*). *F*: no discrete regions of synapsin-like labeling were seen anywhere within the *dgn*. This micrograph was collected approximately 7 mm posterior to the STG, within the region of the *dgn* in which DG axonal bistability was induced. *G* and *H*: although no synapsin-like labeling was seen in the *dgn* proper, staining was evident at NMJs on both (*G*) gm4b and (*H*) gm4c muscle fibers that are innervated by DG via the *dgn*. Intensity of background staining in micrographs *B–F* was increased to allow visualization of the cell bodies and/or axon tracts present in the ganglion/nerves shown. Arrows denote such labeling of STG somata in *B* and two axons in *F*. Scale bar equals 100 μm in *B* and 25 μm in *G*. Images *B–F* are shown at the same scale and are brightest pixel projections of serial optical sections (33, 6, 6, 2, and 5, respectively) collected at 1.95- μm steps. Images *G* and *H* are shown at the same scale and are brightest pixel projections of 31 and 81 serial optical sections, respectively, collected at 0.20- μm steps. All micrographs shown in this figure were collected from the same preparation. DG, dorsal gastric neuron; *dgn*, dorsal gastric nerve; *dvn*, dorsal ventricular nerve; gm4, gastric mill 4 muscle; *lvn*, lateral ventricular nerve; NMJ, neuromuscular junction; *on*, esophageal nerve; *son*, superior esophageal nerve; STG, stomatogastric ganglion; *stn*, stomatogastric nerve; STNS, stomatogastric nervous system.

1998; Johnson et al. 2003; Turrigiano et al. 1995; Zhang and Harris-Warrick 1995); however, their physiological properties and sensitivities to pharmacological or neuromodulatory agents show differences, even within the same species (Johnson et al. 2003). In the lateral pyloric (LP) neuron of *C. borealis*, I_{Ca} has N- or L-type kinetics but is insensitive

to nifedipine (Golowasch and Marder 1992). Sensitivity to the dihydropyridines was not tested in the DG cell in *C. borealis*, and although the current was blocked by Cd^{2+} , as in LP, activation occurred at somewhat lower voltages (Zhang and Harris-Warrick 1995). Our observation that bistability in the DG axon in *C. borealis* was completely

eliminated in the presence of nifedipine (100 μM) is consistent with the pharmacological profile of calcium channels in unidentified STG neurons in *Cancer productus*, in which nifedipine and nicardipine were effective blockers (Hurley and Graubard 1998). The IC_{50} for nicardipine found in that study was 17 μM and a complete block of the Ca^{2+} current was not obtained at the highest concentration (about 30 μM) applied. Although physiological characterization of Ca^{2+} currents in the STNS has thus far been limited to regions that are accessible from the somata of motor neurons, it is interesting to note that an antibody to L-type channels has been observed to stain discontinuous patches along peripheral motor axons in the spiny lobster *Panulirus interruptus* (French et al. 2002). We believe that L-type-like calcium channels are also located in motor axons in *C. borealis*.

The Ca^{2+} -activated nonselective cation current I_{CAN} has been described in only one stomatogastric neuron, specifically the DG neuron in *C. borealis* (Zhang et al. 1995). We found that bistability in the DG axon was completely eliminated by FFA (100 μM). Sensitivity to FFA was not measured in the DG cell body, nor, to the best of our knowledge, has it been tested in any invertebrate preparation. Thus although we can only speculate about the similarities between the current in the DG axon and the one recorded in the somatodendritic region, we conclude that the axonal current is sufficiently similar to those in vertebrate preparations to be blocked by the same agent. The high FFA concentration required in our experiments may indicate that FFA is a less-effective blocker of I_{CAN} in the DG axon than in other preparations and/or it could reflect the fact that the *dgn* was not desheathed during application.

The dependency on stimulation voltage of spiking and contractions in the DG/gm4 system is reminiscent of the graded response to repeated electrical stimulation in rat entorhinal cortical neurons (Egorov et al. 2002) and lamprey reticulospinal neurons (Di Prisco et al. 1997, 2000), two systems in which plateau potential production is dependent on I_{CAN} . In the latter case, the frequency and duration of the persistent firing were correlated with intracellular calcium (Di Prisco et al. 1997, 2000), and we suggest that calcium concentration may play a similar role in our system.

Bistability without action by characterized neuromodulators

The negative results in this and previous investigations do not rule out the possibility that an as yet uncharacterized neuroactive substance mediates bistability in the DG axon. However, if an unknown modulator encapsulated in vesicles were present in the *dgn*, we would have expected positive immunoreactivity to synapsin and/or synaptotagmin, as has been documented in other regions of extraganglionic neuropil in the STNS (Skiebe and Ganeshina 2000; Skiebe and Wollenschlager 2002). The lack of staining for either protein in the *dgn* argues against the presence of synaptic contacts or extraganglionic neuropil in the nerve. We conclude that, if local release of a neuromodulator is involved in generating the bistability, it occurs in an unconventional manner.

Two distinct sources of bistability in DG

Motor neurons in the STG have a standard monopolar invertebrate structure with a single primary neurite that exits

the ganglion as an axon or axons (King 1976a,b). Secondary processes branch off this neurite and into the neuropil region of the ganglion where synaptic connections are made. Spikes are generated in the primary neurite and, unlike in classic bipolar vertebrate neurons, the dendritic and axonal regions are not neatly separated by the cell body. In our experiments, persistent firing induced by extracellular stimulation originated in the axon itself at a spot approximately 0.5 cm away from the spike initiation zone for orthodromic firing.

Our results suggest that generation of the axonal plateau potential underlying the persistent firing involves a current or currents whose activation requires a sustained period of depolarization. This would explain why the bistability could or could not be induced by different stimulation protocols. When the amplitude of extracellular stimuli was large enough, the membrane potential would remain sufficiently depolarized between pulses to allow eventual activation of the current involved in the production of persistent firing. In contrast, orthodromic spiking, at least at the frequencies produced by depolarization of the soma, could never activate the current because the membrane would repolarize between the passages of each spike. The observation that hyperpolarization of the DG soma reduced persistent firing shows that the proximal portion of the axon is not electrotonically distant from the soma. This result is not surprising, given the results of a previous study in *C. borealis* in which hyperpolarizing current injected into the soma of the lateral gastric (LG) neuron controlled the activity of a peripheral spike initiation zone that was several cm from the STG (Meyrand et al. 1992). The likely explanation for this phenomenon in both neurons is a large axonal length constant, such that the voltage decreases very slowly with distance when propagated along the axon. The length constant (λ) of the pyloric dilator (PD) cell in the lobster *Homarus americanus* has been measured to be approximately 2 mm (D Bucher, unpublished observations). If the length constant of the DG axon were close to this value, negative current injection into the soma would produce hyperpolarization of the axon in the spike initiation region (about 0.5 cm or nearly 2–3 λ from the soma) that would be 5–15% of the somatic value. For the experiment shown in Fig. 4E, the hyperpolarization at the spike initiation site would be -2 to -3 mV. This could influence the duration of the persistent firing because, at the end of the spike train, the membrane potential in that portion of the axon is probably very close to the threshold for spiking. Our inability to generate bistability at distal sites of the axon points to heterogeneity in the DG axon. It appears that only in the section closer to the STG does the axon have the complement of channels necessary for generating bistability.

Induction of bistability in both the DG soma and axon depends on a Ca^{2+} current with L-type properties and on I_{CAN} ; however, neuromodulatory action appears to be required only in the former case. Without a detailed biophysical model of the neuron and/or direct measurements of the axonal currents, we can only speculate as to why this is true. Clearly, the properties of the channels involved in producing these two currents and/or their densities could vary throughout the neuron. In any case, we would expect both local excitability and cellular morphology to play crucial roles in

determining the ease of plateau potential production in each region of the DG neuron.

Possible physiological significance of DG axonal bistability

Crustacean axons can possess active conductances beyond those in the Hodgkin–Huxley model (Connor et al. 1977; French et al. 2002) and some axons, like vertebrate dendrites (Heckman et al. 2003; Major and Tank 2004), appear to have signal-processing capabilities. Several previous studies have demonstrated that, in the presence of a neuromodulator, axons in the STNS can modify the spike trains that are transmitted to synaptic targets (Bucher et al. 2003; Goillard et al. 2004; Meyrand et al. 1992). The bistable behavior we observed in the DG axon did not involve a neuromodulator, but it did require a very strong extracellular stimulation of the *dgn* that was 5–10 times greater than that needed to elicit spikes. Given the lack of either neuromodulatory release or electrical connections between DG and other axons within the *dgn*, how could bistable behavior be elicited *in vivo*? The simplest scenario involves the assistance of a neuromodulatory substance that circulates as a hormone and facilitates activation of the current(s) underlying the plateau. This might be achieved, for example, if the neurohormone shifted the current's activation voltage downward and/or decreased the leak conductance to increase the membrane time constant. A hormonal action of dopamine has similarly been proposed as being responsible for uncovering a peripheral spike initiation zone in a STG motor axon in *H. americanus* (Bucher et al. 2003). Under the scenario we put forward, it seems possible that bistable behavior in the DG axon and the resulting enhanced gm4 contraction could be elicited at times by the only physiologically relevant electrical stimulus we can identify: orthodromic DG spiking generated during a gastric mill rhythm.

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