Chapter 2

Long-Distance Modulation of Sensory Encoding via Axonal Neuromodulation

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Additional information is available at the end of the chapter

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Abstract

The neuromodulatory system plays a critical role in sensorimotor system function and animal behavior. Its influence on axons, however, remains enigmatic although axons possess receptors for a plethora of modulators, and pathologies of the neuromodulatory system impair neuronal communication. The most dramatic neuromodulatory effect on axons is ectopic spiking, a process common to many systems and neurons during which action potentials are elicited in the axon trunk and travel antidromically towards the site of sensory transduction. We argue that ectopic action potentials modify sensory encoding by invading the primary spike initiation zone in the periphery. This is a particularly intriguing concept, since it allows the modulatory system to alter sensory information processing. We demonstrate that aminergic modulation of a proprioceptive axon that elicits spontaneous ectopic action potentials changes spike frequency, which determines the burst behavior of the proprioceptor. Increasing ectopic spike frequency delayed the peripheral burst, caused reductions in spike number and burst duration, and changes in sensory firing frequency. Computational models show these effects depend on slow ionic conductances to modulate membrane excitability. Thus, axonal neuromodulation provides a means to rapidly influence sensory encoding without directly or locally affecting the sites of stimulus reception and spike initiation.

Keywords: proprioception, neuromodulation, sensory encoding, action potential, axon

1. Introduction

Flow of information in neurons of the sensory nervous system, as in the central nervous system, is usually thought of as unidirectional. The function of sensory neurons is to supply the central nervous system with information from the periphery, and this information is transmitted through action potentials (APs) propagating along the sensory axons. While this

concept was introduced early in the history of neuroscience in Cajal's neuron doctrine, it has been challenged many times. Such challenges include examples from retrograde transport from the synaptic terminals to the soma, which affects slow homeostatic processes [1], to APs that backpropagate from the axon initial segment into the dendritic regions where they modulate postsynaptic signaling and contribute to coincidence detection on fast time scales [2, 3]. Even axons, which are traditionally seen as faithful unidirectional conductors, can propagate APs backwards towards the axon origin [4]. Propagation direction depends on where APs are initiated, which is typically a spike initiation zone (SIZ) at the axon initial segment, near the axon hillock. Here, the excitability of the neuronal membrane is at its highest and integrated synaptic or sensory information has easy access.

The last decades have shown that membrane excitability, including that of the axon, is subject to changes depending on a diverse set of intrinsic and extrinsic conditions. The neuromodulatory system, for example, plays a critical role in sensory processing as a major contributor to the plasticity maintaining sensorimotor system function and animal behavior [5]. It typically targets local signal encoding, transmission, and AP initiation by modulating ion channel conductances though metabotropic (typically G-protein coupled) receptors. Modulator influences on long distance communication, however, remain enigmatic even though axons possess receptors for a plethora of modulators [4], and pathologies of the neuromodulatory system impair neuronal communication. Recent evidence suggests that neuromodulator-induced changes in axon membrane excitability facilitate AP propagation dynamics [6–8], and may serve to adapt sensory functions to different behavioral conditions.

The most dramatic change in axonal excitability is ectopic AP generation, a process common to many systems and neurons [4, 9–13]. In this case, axon trunk excitability increases to superthreshold levels, and APs are generated spatially distant from the primary SIZ. Since the axon membrane surrounding the AP initiation site is not refractory, APs propagate in both directions, orthodromically towards the axon terminal and antidromically towards the dendritic sites of signal integration. Excitability changes leading to ectopic spiking can be caused by various influences, including slow changes in local or global neuromodulators, external conditions such as temperature, or in different hormonal or pathological states. On faster time scales, antidromic APs can be elicited by axo-axonic synapses, such as those present in hippocampus [14], cortex [15], and most sensory neurons [16–18]. Furthermore, external axon stimulation is a common technique used by physicians to test reflex function and treat chronic neuropathic pain [19]. Little is known about the origin, control and functional effects of these additional APs. While postsynaptic effects of ectopic APs that propagate orthodromically have been shown, the effects of antidromic APs on information processing are mostly unknown [18].

In pseudounipolar neurons of the sensory system, such as pain fibers, proprioceptors, and somatosensory neurons, ectopic APs traveling towards the periphery may more easily invade the primary SIZ and the sensory dendrites. In these neurons, there is no soma between the axon and sensory dendrites, which is why in this case the latter are often referred to as receptive endings instead. Without a soma between the axon and the receptive endings, these neurons have fewer impedance changes [20] to stop antidromic AP propagation from reaching

the periphery. Despite ectopic APs typically having lower frequencies, AP collisions [21] and failures due to refractory membrane block [22] must be rare whenever the sensory neuron's primary SIZ is silent, giving ectopic APs ample opportunity to invade the receptive endings in the periphery.

We argue, using a 'simple' proprioceptor and computational modeling, that antidromic traveling ectopic APs modify sensory encoding by invading the primary SIZ in the periphery and modulating membrane excitability. This is a particularly intriguing concept, since the frequency of antidromic APs can be determined through external stimulation, or through neuromodulatory or synaptic actions on the axon trunk. These actions may allow humoral and neural influences to alter sensory information as it travels towards the central nervous system. To test our hypothesis, we utilized the experimentally advantageous anterior gastric receptor (AGR, [23, 24]). AGR is a single-cell muscle tendon organ in the crustacean stomatogastric ganglion [25] – a well-characterized system for the investigation of cellular and circuit neuromodulation [26]. AGR generates ectopic APs in its several centimeter-long axon trunk, spatially distant from the primary SIZ in the periphery [27]. To test whether changes in AGR's ectopic AP frequency determine peripheral information encoding, we elicited different ectopic frequencies using extracellular axon stimulations while we chemically elicited peripheral AP bursts.

Our data show that ectopic APs propagated without failures towards the periphery, where they invaded the primary SIZ and caused three distinct frequency-dependent actions on sensory encoding: (1) an increase burst onset latency, (2) a reduction AP number, and (3) a reduction the burst duration. These effects increased when ectopic APs continued throughout the encoding of sensory information and caused significant frequency-dependent decreases in the average and maximum frequency. Using computational models of generic neurons, we show that slow ionic conductances facilitate antidromic AP modification of sensory encoding. Slow ionic conductances, such as those elicited by persistent Sodium, hyperpolarization-activated (HCN), and slow Potassium channels are ubiquitous in neurons and axons [28–30], indicating that sensory modification by antidromic APs may be inherent to many other systems. We conclude that axonal neuromodulation provides a means to rapidly influence sensory encoding via ectopic APs that invade the periphery, without directly or locally affecting the sites of stimulus reception and AP initiation.

2. Materials and methods

2.1. Dissection

The stomatogastric nervous system (STNS) of adult male crabs (*Cancer borealis*) was isolated following standard procedures [31], and superfused with physiological saline (10–12°C, [32]) KCl was increased 10–20 fold for high Potassium (K^+) saline. To maintain osmolarity, NaCl was reduced appropriately. K^+ saline depolarizes the membrane, and its effective concentration was determined for each preparation. Octopamine hydrochloride (OA, Sigma Aldrich)

was diluted in saline to the desired concentration (0.1–100 μM). OA was cooled to 10–12°C and manually applied to the isolated STG in a petroleum jelly well. As a control, saline was applied at the same temperature 3 min before each neuromodulator application. Measurements were taken in steady-state (2–5 min after OA wash in). To prevent cumulative effects due to repeated modulator application, wash-outs were 5 min long with continuous superfusion of cooled saline. Peripheral bursts were elicited with a 0.1–0.5 s puff of K^+ saline to a continuously saline-superfused well around the *pdgn*. To prevent accumulation of modulator effects 60–90 s washout occurred between puffs.

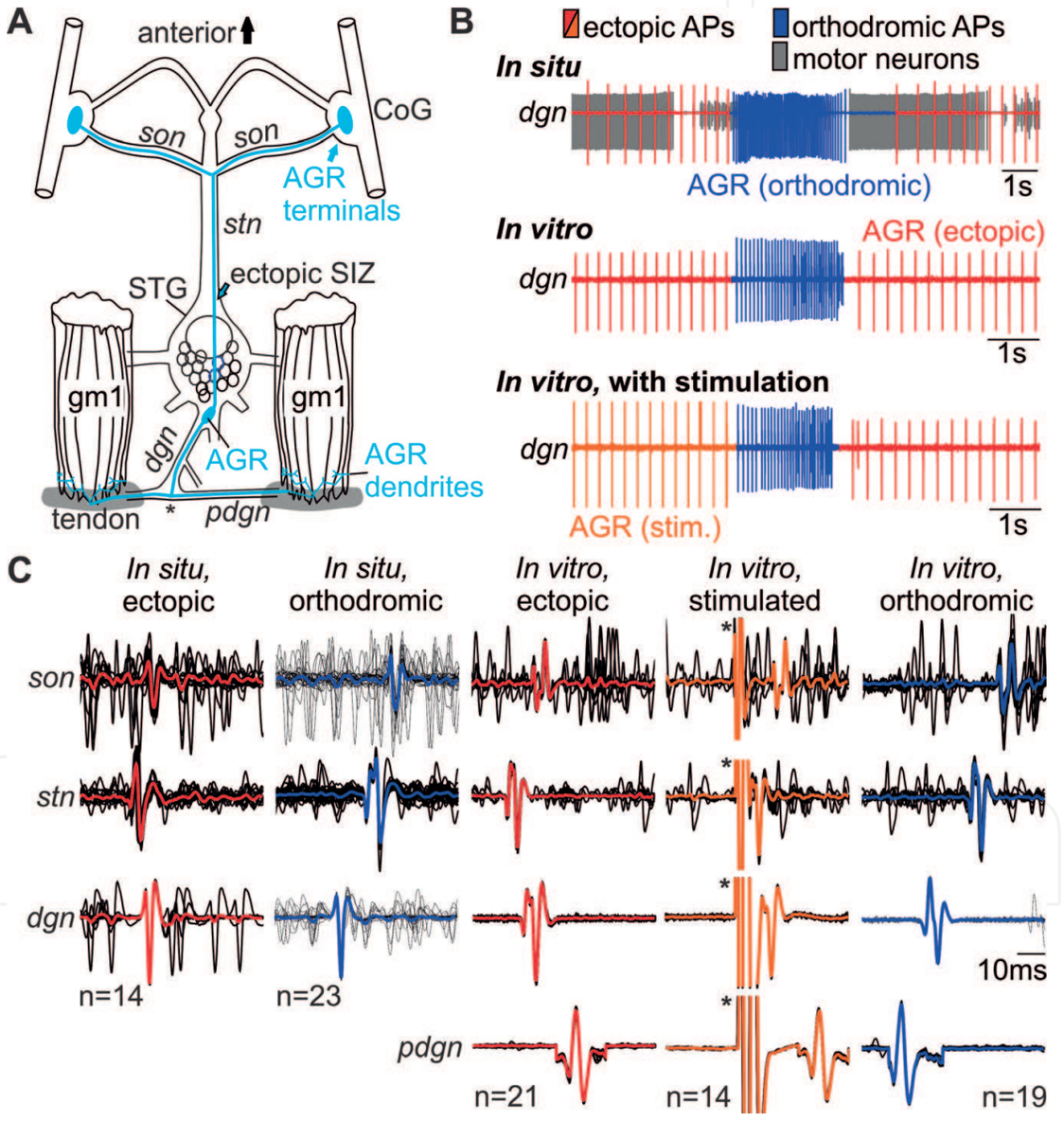

Figure 1. A. Schematic representation of STNS and AGR. AGR projects an axon to the gm1 muscles in the periphery, and to the premotor CoGs. The AGR ectopic SIZ is located in the *stn*, near the anterior end of the STG. The primary SIZ is near the gm1 muscles (*). B. AGR produces spontaneous ectopic APs *in situ* and *in vitro* (original recordings). Ectopic APs can be elicited with stimulation of AGR's central axon. APs have been color coded for clarity. C. Overlay ('multisweep') and average (colored) of nerve recordings containing the AGR axon used to track AP propagation from posterior (*dgn*) to anterior (*son*), in different conditions. Stimulation artifacts are labeled with (*).

2.2. Extracellular recordings, stimulations, and optical imaging

Standard techniques were used for extracellular recordings and data analysis [33]. The activity of AGR was monitored on multiple extracellular recordings simultaneously, namely on the stomatogastric nerve (*stn*), the dorsal gastric nerve (*dgn*), and the supraoesophageal nerve (*son*), see **Figure 1A**. To identify AGR, we used APs recorded on the *dgn* or *stn* and performed a time-correlation analysis (multisweep). Ectopic APs (1–10 Hz) were elicited with extracellular nerve stimulation [34] of the AGR axon trunk. The lipophilic voltage-sensitive dye Di-4-ANNEPDHQ [35] was used according to published protocols [36]. To facilitate access of the dye to the axons, the connective tissue sheath of the *pdgn* was manually removed. We used event-triggered averaging of APs to improve the signal-to-noise ratio of the optically recorded data (similar to [37]).

2.3. Data analysis, statistics and figure preparation

Data were analyzed using scripts for Spike2 (available at www.neurobiologie.de/spike2). To compute the EC_{50} of OA, instantaneous firing frequency. was normalized to control frequencies, then to the minimum and maximum frequency within each animal. Average responses to OA ± SD are plotted. To compare changes in burst parameters, results were normalized to the control bursts measured before stimulation and plotted as a function of the normalized difference. Mean normalized differences ± SD are plotted. Pearson correlation analyses were used to assess changes in AGR peripheral burst activities in response to forcing different AGR ectopic AP frequencies. Tests were computed in SigmaPlot (version 12 for Windows, Systat Software GmbH, Erkrath, Germany). Final figures were prepared with CorelDRAW Graphics Suite (version X7, Corel Corporation, Ottawa, ON, Canada).

2.4. Modeling

Computation models were designed using NEURON [38] using standard Hodgkin-Huxley ionic conductances in a cable model of an unmyelinated axon. The model length was set to 1.213 cm with 10 μm compartments and the axon diameter was 0.6 μm. Axial resistivity (28 Ω*cm) and membrane capacitance (1 $\mu F/cm^2$) were constant through the length of the neuron. The neuron had three sections, the axon (1012 μm), the peripheral SIZ (100 μm), and the dendritic terminal (101 μm). Active channel properties were conserved in the axon and peripheral SIZ, except only the peripheral SIZ had I_h or I_{Ks} (**Table 1**).

Ionic current	$\bar{g}_{max}$ (mS/cm^2)	Gating	Activation function	Tau (ms)	E_x (mV)
I_{Na}	0.4	m^3	1/(1 + exp.(−0.4(36 + v))	0.19exp(−0.05(v + 40)	50
		h	1/(1 + exp.(39.5 + v))	40exp(−0.025(v − 55))	
I_{Kd}	1.09	n^4	1/(1 + exp.(0.125(−33 − v)))	55exp(−0.015(v −28))	−77
I_{Ks}	0.1	n^4	1/(1 + exp.(0.125(−33 − v)))	4000/cosh((v + 73)/12)	−90
I_l	0.0016				−60
I_h	0.103	h	1/(1 + exp.((v + 70)/7)	300 or 3000	−10

Table 1. Parameters of ionic currents used in computational models.

3. Results

3.1. Antidromic ectopic action potentials invade the site of sensory encoding

The effects of antidromic APs on sensory encoding can be challenging to delineate. We use the anterior gastric receptor neuron (AGR) of the crab, *C. borealis* because it is experimentally advantageous. AGR is a bipolar single-cell muscle tendon organ that projects two axons from its cell body - one towards the peripheral gastric mill 1 (gm1) muscles, and one to the commissural ganglia (CoGs, **Figure 1A**), where it innervates premotor control neurons [39]. The CoGs are analogous to the vertebrate brainstem, and contain a set of descending projection neurons that modulate downstream motor circuits in the stomatogastric ganglion (STG) and promote appropriate behavioral responses. The primary function of AGR is to encode information about changes in gm1 muscle tension and to convey this to the CoG networks. Sensory information is encoded as bursts of APs with maximum frequencies between 20 and 30 Hz at the primary SIZ in close proximity to the peripheral gm1 muscles (**Figure 1B**). APs generated at this site are propagated unidirectionally towards the integrating centers in the upstream CoGs. **Figure 1C** shows an *in-situ* recording of AGR, using multiple extracellular recordings at different sites along its axons. AGR burst activity was elicited by isometric gm1 muscle contractions that increased muscle tension (similar to [23]). All APs in this burst were recorded first in the dorsal gastric nerve (*dgn*), through which AGR innervates the gm1 muscles. They then passed through the soma before reaching the stomatogastric (*stn*) and superior esophageal (*son*) nerves, through which the AGR axon innervates the CoGs. The soma lies posterior to the STG, and functionally and physically connects the two AGR axons. Unlike most neuronal somata, AGR's cell body possesses active properties and thus act as a continuation of the axon [40].

In addition to the peripheral SIZ, AGR generates APs at a second SIZ in its axon trunk whenever no sensory bursts are produced (**Figure 1B**, [23]). These APs first occurred in the *stn*, before appearing on the *dgn* and *son* (**Figure 1C**). These spontaneous APs thus traveled bidirectionally from the axon trunk towards the CoGs and the periphery, and were not elicited at the primary SIZ. Previous studies have estimated that these ectopic APs originate approximately 225 microns anterior to the STG neuropil, near the origin of the *stn* [32]. Thus, they were initiated spatially distant from the primary SIZ, at an approximate distance of 1 cm. AGR maintains its firing properties and SIZs even when isolated. In these *in vitro* conditions, the gm1 muscles are dissected away from the peripheral dendrites, removing the source of sensory stimuli, and only STG, CoGs, and the connecting nerves containing AGR's axons were retained (see **Figure 1A**). Sensory-like bursts can be generated when short puffs of K^+ physiological saline are applied locally to the peripheral dendrites. In the experiment shown in **Figure 1B**, a petroleum jelly well was placed around the *pdgn* containing the sensory dendrites of AGR (**Figure 1A**) and a puff of K^+ saline was applied (see Materials and Methods). The elicited APs first appeared on the *pdgn*, demonstrating that they were initiated at the peripheral application site (**Figure 1C**). In contrast, spontaneous APs that occurred in between peripheral bursts, were first recorded on the *stn* and simultaneously on the *dgn*. They continued bidirectionally towards the CoGs, appearing on the *son*, and towards the peripheral AGR dendrites, appearing on the

pdgn. This is consistent with previous results [27, 32] and the intact animal [23], and suggests that these *in vitro* spontaneous tonic APs are generated ectopically in AGR's axon trunk. We used our ability to generate uniform sensory bursts at controlled times and track AP direction to investigate the modulatory effects of antidromic APs on sensory encoding. To control the frequency of ectopic APs in the axon trunk, we elicited APs through extracellular stimulation of the AGR axon in the STG well (**Figure 1B**, [34]). Forced ectopic APs followed the same pattern of propagation as spontaneously generated ectopic APs: bidirectionally from the STG well to the *stn* and *dgn* (**Figure 1C**).

To confirm that ectopic APs traveled without failures throughout the entire length of the AGR axon, we first recorded spontaneous and stimulated APs on the *son*, near the terminal ends of AGR in the CoGs, as well as from the *pdgn*, i.e. the *dgn* branch that responded to K^+ stimulation and contained the primary SIZ. In all recordings (N = 14), ectopic APs reached the *son* and *pdgn* without ever failing.

This provided good evidence that ectopic APs propagated throughout the entire length of AGR. However, extracellular recordings have limited spatial resolution due to the space required to place electrodes. Therefore, it was difficult to determine if ectopic APs truly invaded the axon terminals in the CoG and the sensory encoding region, respectively. While we did not expect AGR's APs to fail when they enter the CoG axon terminals, we decided to intracellularly record from a known postsynaptic target neuron of AGR, the commissural projection neuron 2 (CPN2). **Figure 2A** shows intracellular somatic recordings of CPN2 and AGR. AGR was tonically active and APs were generated at the ectopic AP SIZ. Each AGR AP was followed by a time-locked EPSP in CPN2 (**Figure 2B**), demonstrating that ectopic APs propagated all the way to the axon terminals and elicited postsynaptic responses.

In the periphery, for ectopic APs to modulate sensory encoding, they must affect the primary SIZ. AGR encodes sensory stimuli pertaining to changes in muscle tension, and there are no postsynaptic structures to measure invading antidromic APs. In contrast to the output terminals in the CoGs, the AGR axon splits into several collaterals, with several branches innervating each of the two bilaterally symmetric gm1 muscles (**Figure 1A**). Axonal branching poses a problem for APs if they propagate from a single axon trunk towards a branch point, since the branching increases membrane impedance [20], and may decrease currents promoting AP propagation. This can lead to propagation failures, AP reflections, or both. Sensory APs from the AGR periphery propagate orthodromically from the branches into the main axon trunk, and are thus unlikely to be affected at these branch points. Antidromic APs, however, enter these branches coming from the main axon trunk, and may thus encounter non-permissive conditions. To test whether APs indeed invaded the primary SIZ without failure, we used the voltage sensitive dye, Di-4-ANNEPDHQ to record and identify the AGR axon in the periphery (see Materials and Methods). This dye has two major advantages: it changes fluorescence with membrane potential with high temporal and spatial acuity, which overcomes the limited spatial resolution of the extracellular recordings, and it selectively stains neuronal membranes, making it possible to visually identify and separate individual axons in a nerve bundle [32]. We applied the dye to the *pdgn* well used to isolate and activate the primary SIZ with K^+ saline. This locally stained all axon membranes in the *pdgn*. Besides AGR, the *dgn* contains the axons

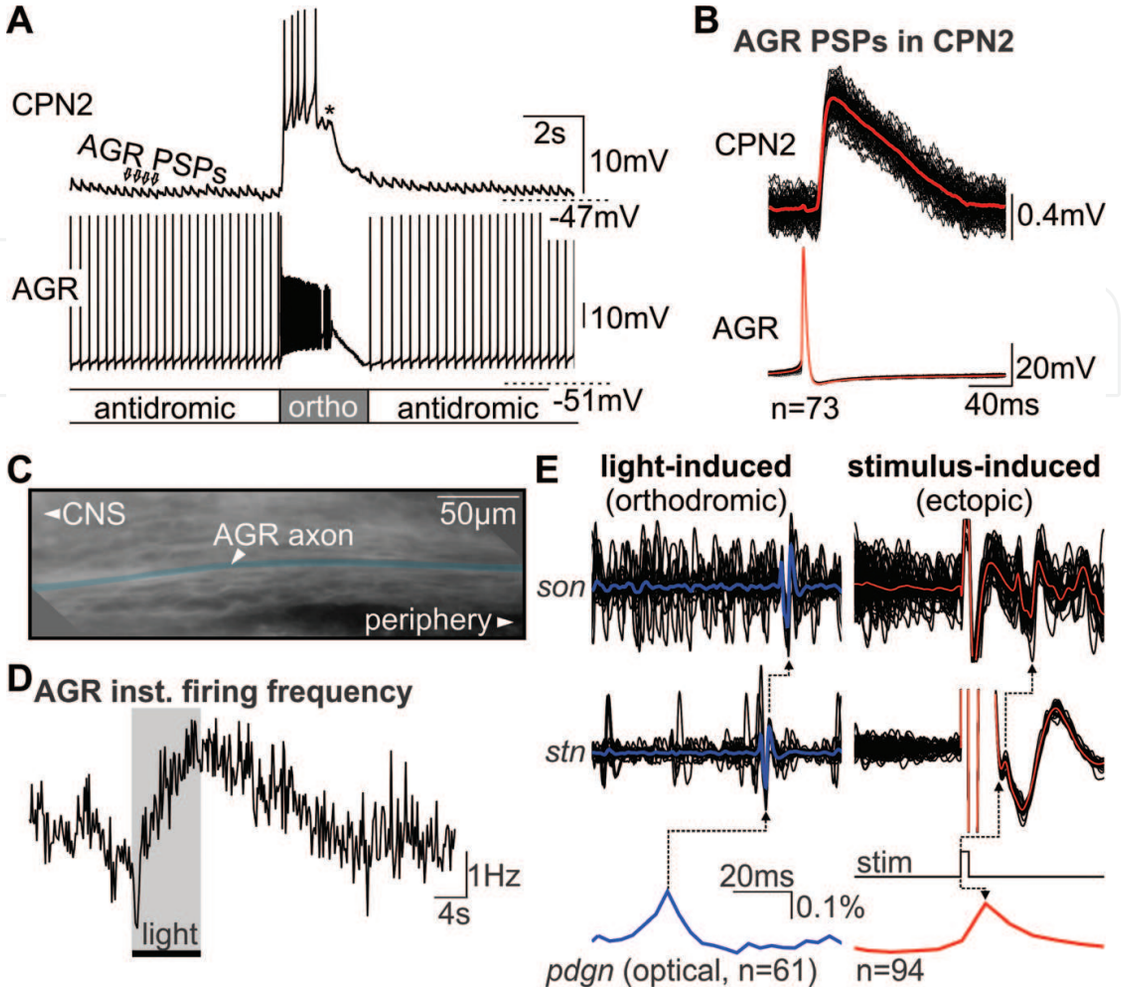

Figure 2. A. Intracellular recordings of AGR and its postsynaptic partner, CoG projection neuron CPN2. EPSPs in CPN2 were time locked to APs in AGR during spontaneous ectopic AP activity, while strong AGR firing elicited a burst of APs (*). B. Multisweep and average of EPSPs in CPN2, triggered by APs in AGR. C. High resolution photo of *pdgn* with AGR's peripheral axon (blue). D. AGR's firing frequency increases when the peripheral (primary) SIZ is illuminated with fluorescent excitation light. E. Optical recordings of primary SIZ. Left: light-induced APs traveled orthodromically towards the CoGs. Right: stimulus evoked ectopic APs traveled antidromically towards the periphery and invaded the primary SIZ.

of several STG motor neurons [41]. We identified the AGR axon by recording the optical signals of all stained axons, and aligning them to APs on the electrical recordings. Only optical signals from the AGR axon were consistently timed to extracellularly recorded AGR activity. We first used spontaneously generated APs to identify the AGR axon in the *dgn*, and then visually tracked the identified axon towards the periphery using the membrane staining. Lipophilic voltage-sensitive dyes such as the one we used here have excitatory side-effects with high-intensity fluorescence illumination [32, 42, 43]. We used this fact to our advantage: the excitation light was focused on a small area the nerve (225 µm, [32]). We found that when illuminated, APs originated in the periphery, i.e. they first appeared on the electrical recording of the *dgn*, and then propagated to the *stn* and *son*. As we moved illumination along the axon, AP frequencies varied substantially. SIZs are defined by an increased propensity to generate APs. Therefore, we determined that the region which generated the highest AP frequency

would be the approximate location of the primary SIZ. **Figure 2C** shows the location on AGR that resulted in the highest firing frequency with illumination in **Figure 2D**. When we optically tracked APs initiated there, we found that they started at the site of illumination, and propagated orthodromically along the AGR axon (**Figure 2E**).

To determine if ectopic APs could penetrate this peripheral area, we first forced ectopic APs by extracellular stimulation of the AGR axon in the STG (see **Figure 1C**) and optically recorded the primary SIZ. Because illumination elicited orthodromic APs, there was a potential for collisions between orthodromic APs and antidromic ectopic APs [21]. To ensure that ectopic APs would not fail to be recorded in the periphery due to collisions, we forced ectopic APs at a higher frequency than the spontaneous firing frequency (1–2 Hz higher than the peripheral frequency). We found that all stimulated APs elicited an optical signal in the periphery time-locked to the stimulus (**Figure 2E**, right). Thus, ectopic APs invaded AGR's stimulus encoding regions. Taken together, we find that AGR has two SIZs, one that spontaneously generates ectopic APs in the axon trunk, and one that generates APs in response to sensory stimuli. While APs encoding sensory stimuli travel unidirectionally in orthodromic direction, ectopic APs travel bidirectionally. Antidromic ectopic APs invade the primary SIZ of AGR.

3.2. Axonal amine modulation increases ectopic action potential frequency

Since AGR's ectopic APs invade the periphery, we hypothesized that these APs modulate sensory encoding occurring there. In the simplest case, ectopic APs will penetrate the sensory SIZ at a constant frequency, leading to a static, continuous effect on sensory encoding. However, AGR's spontaneous ectopic firing frequency is variable. *In vivo*, it varies between 0.7–9.4 Hz (3.66 $\pm$ 2.2 Hz, N = 17) between animals, but it can also change quickly within a given animal. Our *in vitro* recordings revealed an average ectopic firing frequency consistent with the *in vivo* data (3.36 $\pm$ 0.64 Hz, N = 14). However, the range of frequency changes *in vitro* is smaller in comparison to the *in vivo* range (2.31–4.74 Hz). The STG is subject to heavy neuromodulation from hormones in the blood stream and from peptide and amine modulators released from descending modulatory projection neurons [44–46]. *In vitro*, modulation is reduced, potentially leading to a much reduced variability in activity in comparison to *in vivo* [47]. The reduced modulation may account for the smaller range of AGR frequencies when compared to intact animals.

We have previously shown that the axon of AGR passes through the heavily modulated area in the STG as it projects from the periphery to the CoGs, and possesses receptors for the biogenic amine Octopamine (OA). OA is the invertebrate analog of norepinephrine and present in both the STG and *stn* [48]. We hypothesized that OA would affect the spontaneously generated ectopic APs, and increase their frequency. To test this, we locally applied OA at different concentrations to the recording well containing AGR's ectopic SIZ. We identified the recording well nearest to the ectopic SIZ as shown previously, using a multisweep of several extracellular recording wells. AGR firing frequencies were measured in a steady state for all concentrations of OA. We followed each measurement by washing out OA through superfusion of physiological saline until the ectopic AP firing frequency returned to baseline

frequency. First, we found that the ectopic firing frequency of AGR increased with the application of OA (**Figure 3A**), and it did so in a concentration dependent manner with an EC_{50} of 4.13 µM (**Figure 3B**, sigmoidal fit, R^2 = 0.988, SE of estimate 0.053, $p < 0.001$). The average maximum frequency elicited by OA application ranged from 3.35 ± 1.054 Hz at 0.1 µM OA to 5.09 ± 1.34 Hz at 100 µM OA (N = 6), which corresponded to an average increase of 64.7 ± 47.8% at 100 µM OA. We further found that the latency between OA application and the half-maximum frequency diminished with increasing OA concentration, with an EC_{50} value of 1.00 µM (four parameter logistic curve fit, R^2 0.994, SE of estimate 0.026, $p < 0.001$, **Figure 3B**, N = 6). Finally, the location of the ectopic SIZ remained unchanged and APs did not dislocate at any OA concentration, suggesting that OA exerted it actions directly at the axonal ectopic SIZ (**Figure 3C**). This demonstrates that there is OA concentration dependent ectopic firing frequency modulation in the AGR axon, enabling various frequencies at which ectopic APs will penetrate the periphery.

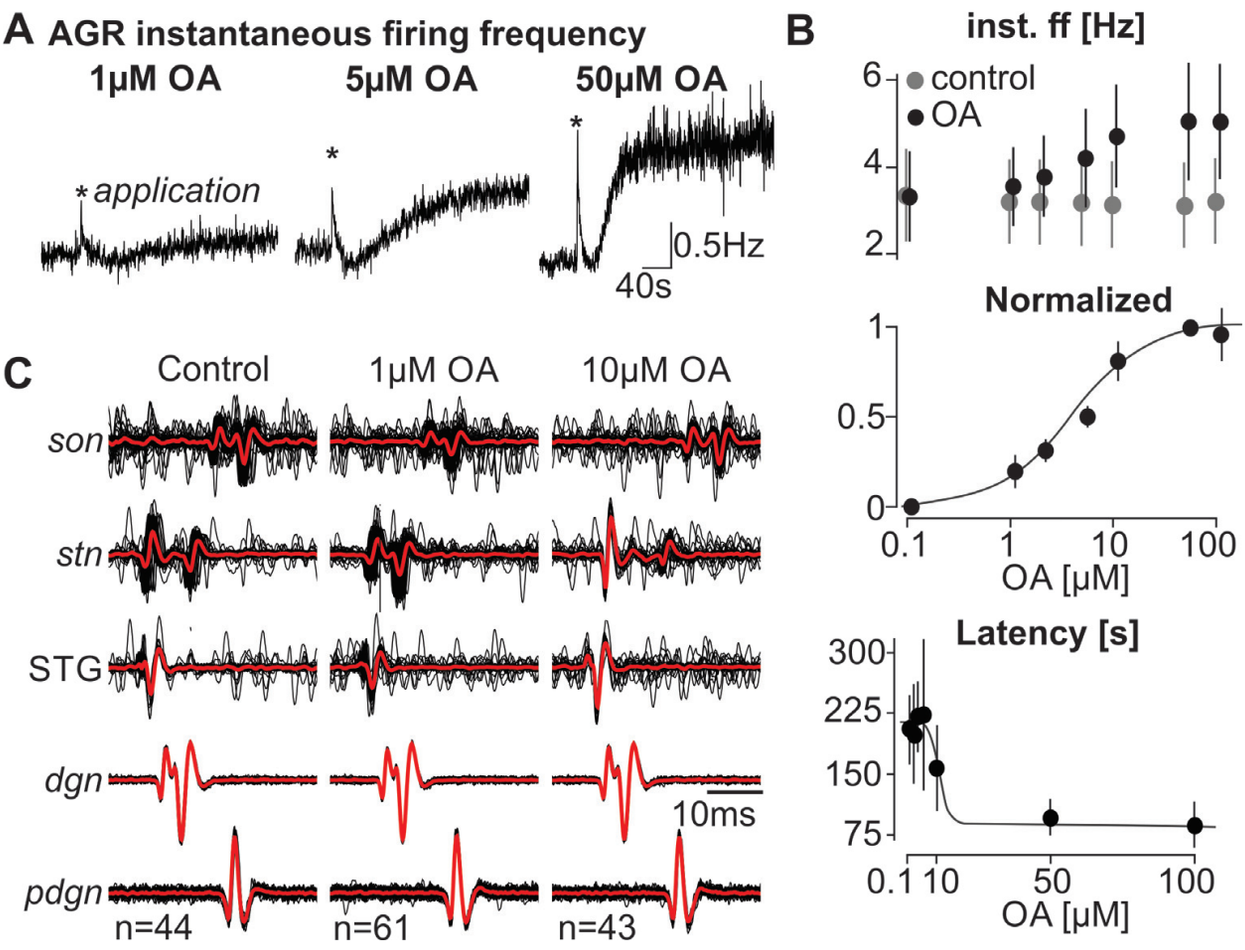

Figure 3. A. AGR firing frequency increases in a concentration dependent manner when OA is applied to the ectopic SIZ. Original recording of an individual animal at three concentrations. B. Dose response curves of AGR instantaneous firing frequency (top: raw; middle: normalized) and response latency (raw) after OA application. C. Multisweeps and averages of nerve recordings that contain the AGR axon to track AP propagation and the location of the ectopic SIZ at different concentrations of OA. OA did not displace the ectopic SIZ.

3.3. Antidromic action potentials have frequency-dependent effects on sensory encoding

Since AGR's ectopic APs invade the periphery at different frequencies, we hypothesized that there is frequency-dependent modulation of sensory encoding occurring there. To test this, we extracellularly forced ectopic APs to a set of fixed firing frequencies (1–10 Hz) and measured the effect on various parameters of stimulus encoding. Ectopic APs were continuously elicited (at least 20 APs at each ectopic frequency) before a local puff K^+ saline was applied to elicit a peripheral burst (**Figure 1B**). Ectopic AP stimulation continued until the first AP in the burst, mimicking the behavior of the spontaneous and modulated ectopic APs in AGR (**Figure 1B**). We then compared changes in the peripheral bursts in control (no ectopic stimulation) to experimental bursts with stimulated ectopic APs preceding the burst. Sensory stimuli can be encoded in the number of APs, the frequency, and the precise timing of APs. We thus measured the change in number of APs per burst, the average and maximum AP frequencies, the durations of peripheral bursts, and their onset latencies (the time between the K^+ saline puff and the first AP of the peripheral burst).

We found that invading ectopic APs had significant influences on several aspects of stimulus encoding. **Figure 4** shows a comparison of a control burst to a burst with forced ectopic frequency of 6 Hz. Burst delay, burst duration and the number of APs in burst were clearly diminished when ectopic APs are present. The smaller number of burst APs was not due to AP collisions, since we (1) were able to account for all ectopic APs in the periphery, and (2) AP collisions could be identified by missing APs on the multisweep recordings. Since ectopic AP stimulation stopped when the first burst spike was detected, we found collisions to be rare (less than 2%). In general, increasing ectopic AP frequencies caused stronger effects on the sensory burst. For example, burst onset latency significantly increased with ectopic AP frequency ($p = 0.01$, Pearson correlation coefficient $R^2 = 0.532$, **Figure 4**Aii), indicating that membrane excitability at the beginning of the burst decreased with higher ectopic AP frequencies. There was also a significant negative correlation between ectopic AP frequency and the number of APs in a burst ($p = 0.001$, Pearson correlation coefficient $R^2 = 0.729$; **Figure 4**Aiii). This resulted in a nearly 30% decrease in the number of APs in a burst at 10 Hz ectopic frequency. Concurrently, burst duration decreased significantly with ectopic AP frequency ($p < 0.001$, Pearson correlation coefficient $R^2 = 0.750$; **Figure 4**Aiv), reaching a nearly 30% decrease at 10 Hz. Neither average nor maximal burst frequency changed significantly with ectopic AP frequency ($R^2 = 0.122$ and 0.0696 respectively, **Figure 4**Av, vi), although both tended to be lower at higher ectopic AP frequencies.

Ectopic APs occur spontaneously and in response to modulator actions at the ectopic SIZ in AGR. However, ectopic APs can also be elicited by synaptic actions at axo-axonic synapses such that the ectopic firing frequency is determined by the occurrence of synaptic potentials in the axon [10, 11, 18]. In this case, ectopic firing would not cease when the sensory burst is elicited. While this is not the case for AGR, the effects of continuous ectopic spiking were tested by continuing the forced ectopic APs throughout the sensory burst. **Figure 4B** shows an example recording for continued ectopic spiking at 6 Hz, in comparison to a control burst without forced ectopic APs. As a consequence of the continued ectopic firing during the sensory burst, AP collisions were more prevalent, although still rare. We estimate less than

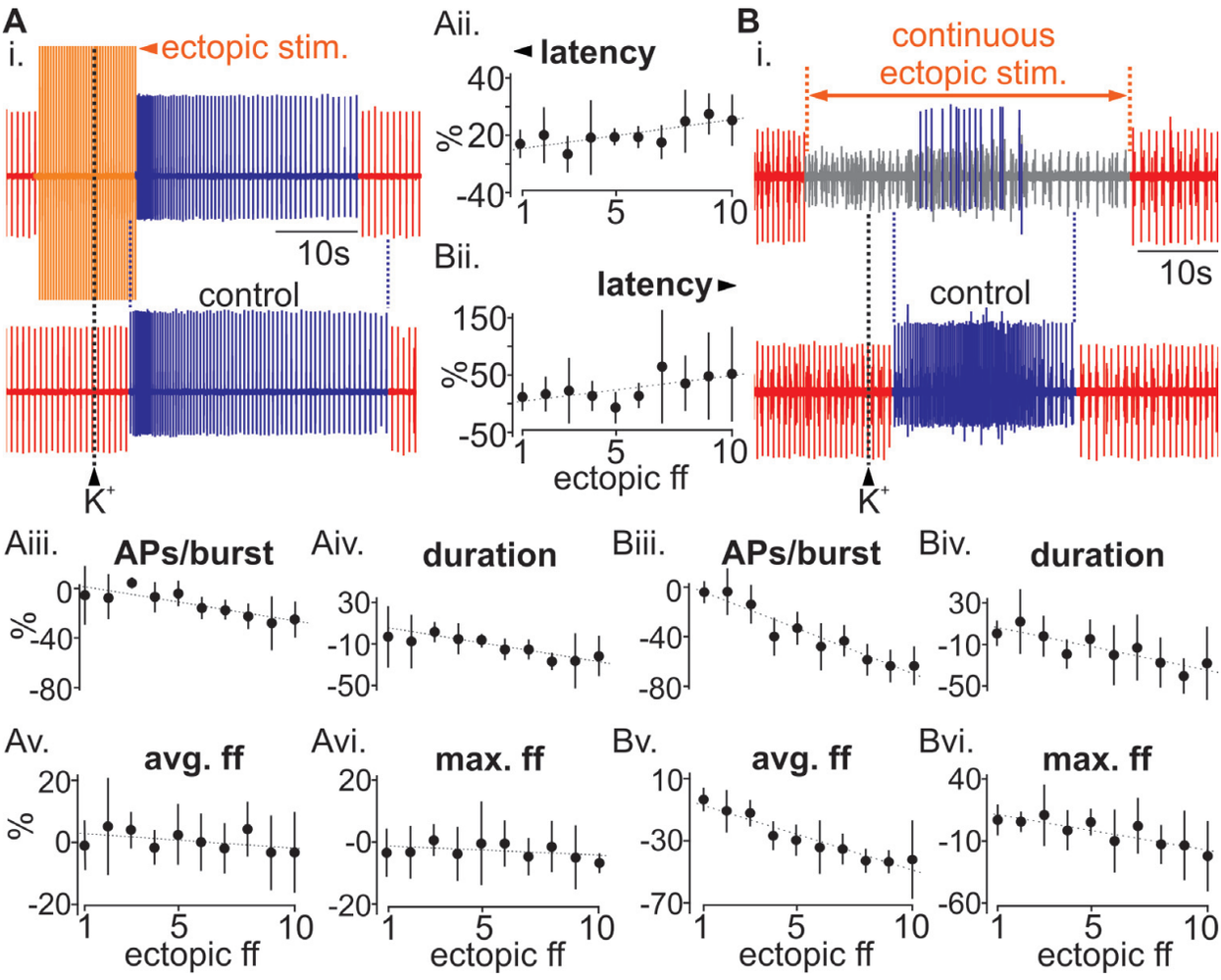

Figure 4. A. Original recordings of AGR ectopic and burst AP activities. i: Control sensory burst (bottom), elicited with high potassium (K^+) in the periphery. Top: With forced ectopic APs that ended at the beginning of the sensory burst. ii: Corresponding changes in latency of elicited sensory bursts at different ectopic AP frequencies (mean ± SD). iii: Number of spikes per burst. iv: Burst duration. v: Average intraburst frequency. vi: Maximum intraburst frequency. B. i: Control sensory burst and burst with ectopic APs that continued throughout. ii–vi: Like in A.

5% of all ectopic APs collided on their way to the periphery. This low number is mostly due to the small distance between ectopic and primary SIZs (about 1 cm), and that propagating APs 'occupy' the axon only for a short amount of time (around 10 ms given the propagation speed of AGR's APs of around 1 m/s). Consequently, even at ectopic frequencies of 10 Hz (interspike intervals of 100 ms), axons were non-refractory for 90% of the time.

Like in the previous experiments, the effects of ectopic spiking on the sensory burst were immediately obvious. In this case, they were more pronounced: burst latency increased significantly with ectopic AP frequency ($p = 0.03$, Pearson correlation coefficient $R^2 = 0.4643$, **Figure 4**Bii), further supporting the notion that membrane excitability at the burst start is lowered when ectopic APs enter the primary SIZ. Burst duration and the number of APs in a burst significantly decreased with ectopic AP frequency (AP number: $p < 0.001$, Pearson correlation coefficient $R^2 = 0.9152$; duration: $p = 0.001$, Pearson correlation coefficient $R^2 = 0.7512$, **Figure 4**Biii, iv), resulting in a greater than 40% reduction for both measurements

at 10 Hz ectopic frequency. In contrast to the previous experiments, average and maximal burst frequency now changed significantly with ectopic AP frequency (R^2 = 0.9192 and 0.7525 respectively, $p < 0.001$ and $p = 0.001$, Pearson correlation, **Figure 4**Bv, vi), following the same trend as already seen when ectopic APs did not penetrate the burst.

3.4. Modulation of sensory encoding requires slow ionic conductances in the periphery

Taken together, our data indicate that ectopic APs invade the periphery where they affect sensory bursts in a frequency-dependent manner. This leads to the question; does this axon or its SIZ possesses distinct properties that facilitate the actions of invading ectopic APs, and if so, which properties may these be? To address this question, we created a computational model axon using NEURON [38]. The details of the model are given in the Materials and Methods. Briefly, the model was a linear axon trunk primary SIZ, and a single sensory dendrite. The axon and SIZ possessed active properties and were able to generate APs. The dendritic compartments were passive, i.e. did not possess any voltage-gated ion channels. Ectopic APs were elicited with pulsed current injections (40 nA, 1 ms) at different frequencies into the axon trunk. Ectopic APs propagated from the axon trunk towards the primary SIZ. Sensory bursts were elicited with ramp-and-hold current stimuli into the peripheral dendrites (**Figure 5A**). This assembly allowed us to reproduce ectopic APs that either penetrated the sensory burst (like in the case of strong synaptic inputs via axo-axonal synapses) or stopped upon burst start (like for spontaneous and modulated ectopic APs).

The simplest axonal configuration is probably the one described by Hodgkin and Huxley (HH) in their groundbreaking work on the squid giant axon [49]. HH axons are limited in that they only possess voltage-gated Sodium and Potassium currents in addition to the passive membrane properties. Thus, they may not reflect more complex propagation dynamics and AP modulation reported more recently for a variety of axons [4]. Nevertheless, HH axons explain the properties of AP initiation and propagation observed in many axons. To test which neuronal properties would facilitate the effects ectopic APs have on sensory bursts, we thus first started out with a HH axon that approximated biological firing frequencies. We implemented gate kinetics according to previously published axon models [6], and adjusted them to produce robust firing. We elicited sensory bursts of 3 s duration and approximately 30 Hz frequency. **Figure 5A** shows an example of these bursts following a 6 Hz ectopic stimulation sequence at its arrival at the peripheral SIZ. The corresponding sensory burst and a control burst without ectopic APs are shown as well. The sensory burst was unaffected by the presence of the ectopic APs. To exclude that this was due to the specific ectopic AP frequency used, we varied frequency from 1 to 10 Hz. We found no obvious influences on any burst parameters (**Figure 5C, D, E**, circles), with the exception of a small, but consistent frequency-dependent increase of burst latency with higher ectopic AP frequency (**Figure 5C**, bottom).

Which conductances could enable ectopic APs to affect sensory encoding then? In addition to the standard HH properties, AGR possesses several ionic conductances that may affect sensory encoding [40]. In specific, a slow hyperpolarization-activated cation current (I_h) seems to affect AP frequency in the sensory burst, and a slow Calcium-dependent Potassium current (I_{Ks}) seems to affect burst timing and structure such that firing frequencies during the burst

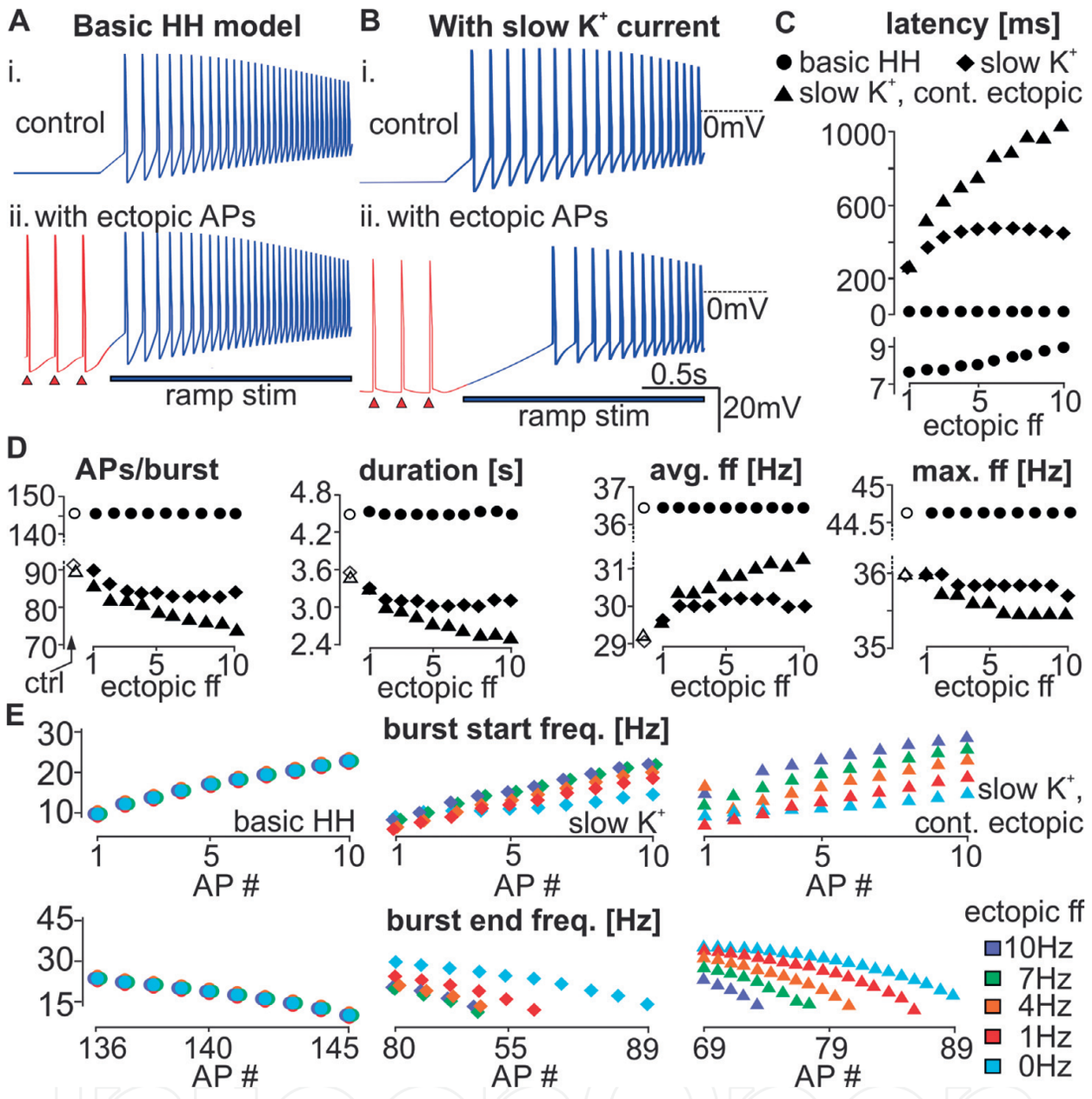

Figure 5. Comparison of membrane potential traces of AGR models without (A) and with (B) I_{Ks} at the primary SIZ. i: Truncated control sensory burst sensory burst, elicited by ramp-and-hold current in the peripheral dendrite. ii: With ectopic APs elicited in the axon trunk. Ectopic APs ended at the beginning of the sensory burst. Red arrowheads indicate ectopic stimulation. C. Corresponding changes in latency at different ectopic AP frequencies. Bottom: magnification of HH model burst latency. D. Changes in burst parameters with ectopic AP frequency. E. Comparison of burst shapes using instantaneous firing frequencies at burst start and end. AP number corresponds to the count of APs in the burst.

accommodate and diminish towards burst end [40]. Slow conductances are common to many sensory neurons, including pain fibers [28, 50], for example. To test whether these slow ionic conductances may enable ectopic APs to exert a frequency-dependent effect on the sensory burst, we added them individually to our axon model. We first implemented I_h, with a time constant of 300 ms, according to previously published data in the stomatogastric ganglion [51].

While the sensory burst was strongly affected by the presence of I_h, this was independent of any ectopic firing, and adding ectopic APs at any frequency had no further influence on the burst (data not shown). This was also the case when we increased the I_h time constant to achieve 10× slower kinetics, as suggested by previous measurements in AGR [40]. Since I_h had no frequency-dependent effects on the sensory burst, we did not consider it further.

In contrast to I_h, Calcium-activated Potassium currents are activated when neurons depolarize, and are slow enough to maintain hyperpolarizing currents. When we implemented I_{Ks} in the primary SIZ, we saw a clear influence when we changed ectopic AP frequencies. **Figure 5B** shows the results from a model with 6 Hz ectopic AP frequency. Changes in ectopic AP frequency had similar effects to the biological system: with increasing ectopic AP frequencies, burst latencies increased, AP numbers in the burst decreased, and burst duration decreased (**Figure 5C, D**, diamonds). There was a small increase in average intraburst firing frequency, and a small decrease in the maximum firing frequency (**Figure 5C, D**). These frequency-dependent effects were most obvious between 1 and 5 Hz ectopic frequencies, and saturated at higher ectopic frequencies.

To also determine the effects of strong synaptic input at the axon trunk, we modeled continuous ectopic firing through the burst. Similar to what we observed experimentally, the effects of changes in ectopic AP frequency were exaggerated in comparison to when ectopic APs stopped with the burst onset. Specifically, there was an increase in burst latency that did not plateau with higher ectopic frequencies (**Figure 5C**). The maximum latency observed at 10 Hz was more than twice that of previous model. The effects on AP number and burst duration were also strengthened, resulting in larger decreases. There were again small effects on the average and maximum intraburst firing frequencies (less than 2 Hz). We noted that these small changes contrasted to the biological experiments. The smaller influence on maximum frequency is likely due to the strength of the ramp-and-hold current used, which was designed to reach biological relevant frequencies (~30 Hz). However, these frequencies are close to maximum frequencies the model can sustain. Consequently, the dynamic range around the maximum frequency might be limited.

To address the difference in average frequency between model and physiology, we assessed burst shape by measuring instantaneous firing frequencies at burst start and burst end (**Figure 5E**). We again compared the basic HH model with the ones containing the I_{Ks}. Firing frequencies in the HH model burst were not different from the control burst at any ectopic AP frequency, both at the beginning or end of the burst. In contrast, both models with I_{KS} showed higher instantaneous firing frequencies at burst start when ectopic AP frequency was increased. Conversely, at the end of the burst, instantaneous firing frequencies were lower. These two effects were stronger in the model where ectopic APs continued through the burst. Together, these effects might explain why there are few changes in average intraburst firing frequency.

In conclusion, our experimental and model data demonstrate that antidromic APs can invade the primary SIZ of sensory neurons, and cause frequency-dependent modulation of sensory

encoding at this site. Our model results suggest that for ectopic APs to exert their effects, ionic conductances with slow kinetics must be present at the primary SIZ.

4. Discussion and conclusions

We demonstrate that modulation of the axon trunk of a proprioceptive neuron influences the encoding of sensory information in the distant periphery. The frequency of ectopic APs initiated in the axon is increased by the biogenic amine Octopamine in a concentration dependent manner, leading to a larger number and higher density of APs that propagate towards and invade the sensory encoding SIZ. We show three frequency-dependent actions on sensory encoding with ectopic APs that stop once an orthodromic burst begins: (1) an increase in the onset latency, (2) a reduction of AP number, and (3) a reduction in the duration of the sensory burst. These effects are strengthened when ectopic APs are elicited throughout the burst, and there is a significant frequency-dependent decrease in the average and maximum burst frequencies. Computational models demonstrate that antidromic APs modify sensory encoding in generic neurons when slow ionic conductances are present. Thus, axonal neuromodulation serves to rapidly influence sensory encoding distantly from the sites of stimulus reception and AP initiation.

4.1. Neuromodulation of sensory systems

Sensory neurons are dynamic and change their responses in a state- and context-dependent manner. Consequently, their AP trains do not solely depend on stimulus properties, but also on internal and external conditions of the neuron. In recent years, increasing evidence about the ability of the neuromodulatory system to influence sensory systems has accumulated. Neuromodulation has been shown to modify the response to identical sensory stimulus and cause significant functional changes in behavior and perception by acting on intrinsic and synaptic properties [5]. Modulators like monoamines, peptides, and opiates, for example, alter reflexes such as startle responses [52]. While initially thought to be related to optimal energy expenditure, it is now clear that altering sensory responses is a widely used phenomenon to allow dynamic adaptations. Thus, neuromodulation allows organisms to modify neuronal and circuit responses to changing external and internal conditions, and allows sensory systems to contribute to not just one, but many behaviors.

More recently, many non-reflex sensory responses have been added to the list of modulated systems, including social communication [53], taste [54], olfaction [55], hearing [56], and pain [57]. For instance, the AP responses of mammalian pain and itch receptors are differentially affected by a variety of immune molecules and neuromodulators that alter nociceptive TRP channel activation during injury, inflammatory, and other pathological conditions [58], including Parkinson disease [59]. Neuromodulators also convey history- and state-dependent sensory responses. The receptor thresholds in newt primary olfactory receptors which determines odor perception sensitivity, for example, are modulated by adrenaline [55]. In the crustacean STNS, the AP response of a muscle stretch receptor is modulated by at least six distinct

modulators, including monoamines, neuropeptides, and GABA [60–62]. These modulators switch how sensory information is encoded (from burst coding to AP coding), and encoding preciseness [63]. Neuromodulation enables the encoding process of both slow and fast processes to be largely plastic.

4.2. Modulation of axons

The actions and functions of neuromodulators on axons, as opposed to synaptic and dendritic regions, remain enigmatic, in part due to the common misconception that axons are only simple and robust carriers of information. Membranes of both myelinated and unmyelinated axon trunks are endowed with ionotropic and metabotropic receptors for transmitters and neuromodulators [4, 64–66], and several different types of ion channels (such as I_h [8, 67–76]; P, N and L type Ca channels [77–84]), providing compelling evidence for axonal neuromodulation. While the origins of axonal modulators are often unknown, it is reasonable to assume that modulation stems from synaptic, paracrine, and endocrine sources [5, 85] and is an intricately balanced process that defines axon excitability. This results in more flexibility of propagation dynamics including conduction velocity and APs number [66, 86–92], thus increasing the computational and processing capabilities of the neuron [93–97]. Conversely, several disorders and pathologies of the neuromodulatory system severely impair neuronal communication and axonal properties [98–100].

4.3. Axonal modulation modifies distant sensory encoding through ectopic action potentials

A lesser-studied phenomenon is how axon modulation affects frequency encoding in neurons. Recent studies have suggested that the size and location of SIZs help regulate neuron excitability and define responses to synaptic inputs and membrane potential changes [101–103]. Moreover, pathologies and modulators can shift SIZ location [11, 32], or generate entirely new SIZs in the axon trunk. For example, hyper-excitability of spinal pain fibers in the dorsal horn is suggested to underlie chronic pain and itch [57]. Similarly, chronic inflammation can hyper-excite proprioceptive sensory axons and lead to ectopic APs that are initiated far from the primary SIZ and travel bi-directionally [11]. While common in many systems and neurons [4], including sensory neurons, the effects ectopic APs that travel antidromically towards the site of sensory reception may have on sensory encoding remain poorly understood.

We argue that antidromic traveling ectopic APs modify sensory encoding by invading the primary SIZ and modulating membrane excitability. This is a particularly intriguing concept, since it allows the modulatory system to alter sensory information before and after it is transduced, and as it travels towards the central nervous system. This is especially true when the ectopic AP frequency changes in different modulatory conditions, as we show for Octopamine modulation of AGR. In the STNS, modulatory descending projection neurons are a major source of neuromodulation [44, 104, 105], and it is reasonable to assume that these neurons modulate the AGR axon [106]. Given that descending modulatory projection neurons are a hallmark of most sensorimotor systems [107], axonal neuromodulation may be common and allow the nervous system to control its own sensory encoding.

The idea that backpropagating APs influence information encoding is not new. Studies in neocortex and hippocampus demonstrate that locally (at the axon initial segment) generated APs backpropagate into the dendritic areas, and modify subsequent signal encoding [2, 108]. To our knowledge, we are the first ones to directly show antidromic ectopic APs can serve a similar function, i.e. that APs generated distantly from the dendritic structures can affect information encoding. A similar phenomenon has been suggested in dorsal root ganglion cells [11, 13, 109]. The implications of antidromic ectopic APs and backpropagating APs from the axon initial segment are distinct though: APs initiated at the axon initial segment will always be elicited by dendritic activity, and therefore backpropagation can only affect future events. It thus can never modify the entirety of the information encoded. In contrast, ectopic APs influenced by axonal neuromodulation are not dependent on incoming sensory or synaptic events, and can thus modulate the entirety of the incoming sensory information. This may be a potential mechanisms by which motor systems control information entering the central nervous system.

4.4. Antidromic action potentials allow more flexibility in sensory encoding

Our data indicate that the effects ectopic APs have on sensory encoding depends on whether ectopic APs continue through the sensory burst or stop when it begins. Specifically, frequency-dependent decreases in average and maximal burst frequency are only present when ectopic APs continue through the burst. Though there are frequency-dependent changes in the burst onset latency, AP number, and burst duration in both paradigms, these effects are stronger when ectopic APs continue through the burst. This may be in particular pertinent to the treatment of neuropathologies using continuous high frequency stimulation [19, 110]. While the high frequency will overrun all sensory information, this may not be necessary to provide the best treatment. Examples for this come from the treatment of chronic neuropathic pain with spinal cord stimulation, where continuous stimulation is used to block all peripheral sensations with a tonic train of stimuli [111]. This prevents the perception of pain, but can result in paresthesia [112]. We show ectopic AP frequencies lower than sensory burst frequencies can change sensory encoding, suggesting high frequency stimulation may not be a prerequisite for treatment. Nevertheless, it seems reasonable to speculate about the relationship of sensory burst frequency and the ectopic APs frequency range, which modulates it. Higher frequencies of either SIZ leads to more 'winner takes all' situations, where the higher frequency SIZ simply overruns the lower frequency SIZ. The potential for modulation is thus limited by the opportunity of the ectopic APs to reach the encoding regions. In other words, the sensory AP frequencies must at some point fall below the ectopic AP frequency to allow them to invade the primary SIZ.

Our computational models indicate that a prerequisite for these actions is the presence of slow ionic conductances. While the detailed biophysical mechanisms that affected particular spike parameters are beyond the scope of this study, slow ionic conductances greatly influence neuronal behavior and responses to synaptic input. For example, transitions between tonic and bursting states are mediated by changes in slow Potassium currents and their functional antagonists, such as I_h, and persistent Sodium or T-type Calcium currents [29, 113]. In axons, slow conductances and membrane potential changes have been implicated in affecting axonal

excitability and AP propagation. For example, slow potassium channels affect AP width and transmitter release in myelinated axons of cortex [30], and slow currents elicited by the Sodium-Potassium pump affect AP propagation in crustacean motor neurons [6]. It would not be surprising to find similar AP-induced, slow accumulating, currents in peripheral sensory axons or dendrites. For AGR, the main effect of the ectopic APs seems to stem from I_{Ks}, or a similar current that imposes a slow inhibitory action that accumulates as ectopic APs invade the primary SIZ. Axon modulation thus appears to give sensory neurons the opportunity to be more flexible, depending on the source and type of modulation, in their ability to encode stimuli. This may be particularly important for time critical processes, and behaviors that rely on time sensitive synaptic processes that require precise AP timing.

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