Dynamic dendritic compartmentalization underlies stimulus-specific adaptation in an insect neuron

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Prešern J, Triblehorn JD, Schul J. Dynamic dendritic compartmentalization underlies stimulus-specific adaptation in an insect neuron. J Neurophysiol 113: 3787–3797, 2015. First published April 15, 2015; doi:10.1152/jn.00945.2014.—In many neural systems, repeated stimulation leads to stimulus-specific adaptation (SSA), with responses to repeated signals being reduced while responses to novel stimuli remain unaffected. The underlying mechanisms of SSA remain mostly hypothetical. One hypothesis is that dendritic processes generate SSA. Evidence for such a mechanism was recently described in an insect auditory interneuron (TN-1 in Neoconocephalus triops). Afferents, tuned to different frequencies, connect with different parts of the TN-1 dendrite. The specific adaptation of these inputs relies on calcium and sodium accumulation within the dendrite, with calcium having a transient and sodium a tonic effect. Using imaging techniques, we tested here whether the accumulation of these ions remained limited to the stimulated parts of the dendrite. Stimulation with a fast pulse rate, which results in strong adaptation, elicited a transient dendritic calcium signal. In contrast, the sodium signal was tonic, remaining high during the fast pulse rate stimulus. These time courses followed the predictions from the previous pharmacological experiments. The peak positions of the calcium and sodium signals differed with the carrier frequency of the stimulus; at 15 kHz, peak locations were significantly more rostral than at 40 kHz. This matched the predictions made from neuroanatomical data. Our findings confirm that excitatory postsynaptic potentials rather than spiking cause the increase of dendritic calcium and sodium concentrations and that these increases remain limited to the stimulated parts of the dendrite. This supports the hypothesis of “dynamic dendritic compartmentalization” underlying SSA in this auditory interneuron.

THE REDUCTION OF NEURAL ACTIVITY to repetitive stimulation is a common feature of sensory systems. This response reduction is often stimulus specific in that activity to the repeated signals is suppressed while responsiveness to infrequent signals is maintained or increased (Ayala and Malmierca 2012; Grill-Spector et al. 2006). At the single-cell level, this process is commonly referred to as stimulus-specific adaptation (SSA; Ulanovsky et al. 2003) although the neural underpinnings remain elusive (Escara and Malmierca 2013).

Several mechanisms have been considered to underlie SSA expressed by individual neurons, such as synaptic depression of afferents (e.g., Wehr and Zador 2005), increased inhibition (e.g., Perez-Gonzales et al. 2012), or the change of excitatory/inhibitory balance (Jääskeläinen et al. 2007). Hyperpolarizing membrane currents activated by membrane depolarization, such as sodium- or calcium-gated potassium currents, which contribute to auditory adaptation (Abolafia et al. 2011), have previously been discounted as likely mechanisms underlying SSA (Nelken and Ulanovsky 2007). These currents are typically associated with spiking (i.e., spike-rate adaptation) and have therefore been considered independent of the origin of inputs (Nelken and Ulanovsky 2007; Ulanovsky et al. 2003, 2004; Wehr and Zador 2005). However, such currents may be activated even in the absence of spikes and modulate the shape of excitatory postsynaptic potential (EPSPs) (Gollisch and Herz 2004; Nanou et al. 2008). Therefore, such currents could lead to SSA if the inputs of common and rare signals are spatially separated and the currents remain limited to the part of the dendrite stimulated by the common signal (Triblehorn and Schul 2013).

Recent findings (Triblehorn and Schul 2013) suggest that dendritic currents contribute significantly to the SSA-like response properties of a well described insect auditory interneuron (Römer et al. 1988; Stumpner 1999; Triblehorn and Schul 2009). The Neoconocephalus (Orthoptera: Tettigoniidae) TN-1 neuron reliably responded to deviant pulses after responses to common standard pulses ceased, provided the standard and deviant pulses differed sufficiently in their carrier frequency (pitch) (Schul and Sheridan 2006; Schul et al. 2012). Extensive physiological testing (Schul et al. 2012) revealed that TN-1 adaptation to fast pulse rates did not result from 1) adaptation of its afferents, 2) synaptic depression of the afferent TN-1 excitatory synapses, or 3) known inhibitory inputs to TN-1. However, injection of a calcium chelator into TN-1 significantly delayed the adaptation to repeated stimulation; application of low-sodium saline significantly reduced adaptation to standard pulses (Triblehorn and Schul 2013). Thus sodium- and calcium-gated potassium currents (Bhattacharjee and Kaczmarek 2005; Sah and Faber 2002) are likely candidates to cause SSA in TN-1.

The stimulus specificity of the adaptation could result from the spatial organization of the afferents and dendrite of TN-1 (Triblehorn and Schul 2013). TN-1 receives direct excitatory inputs from the auditory receptor neurons, which are tonotopically arranged by their spectral sensitivity; each receptor neuron overlaps only with a limited region of the TN-1 dendrite (Römer et al. 1988; an overview of the gross anatomy of the katydid auditory system is provided in MATERIALS AND METHODS). A repeated stimulus would, depending on its carrier frequencies, stimulate parts of the dendrite and initially elicit action potentials (APs). Dendritic mechanisms (e.g., calcium- and/or sodium-activated hyperpolarizing currents) could then cause adaptation, potentially leading to the suppression of APs (Fig. 1). If the dendritic processes underlying adaptation re-
main spatially limited to the areas of the dendrite excited by repeated stimuli, this would create an adapted dendritic compartment where the afferents conveying the repeated signal terminate. Meanwhile, other regions of the dendrite with different spectral selectivity would remain sensitive and could transmit new/deviant stimuli (Fig. 1). We refer to this adaptation being spatially restricted to the stimulated regions of the dendrite as “dynamic dendritic compartmentalization” (DDC). DDC is our working hypothesis describing how SSA is generated at the level of a single neuron (Fig. 1). Two conditions must be met for DDC to underlie SSA: 1) the location of calcium and sodium accumulation, and thus the shape of the adapted compartment, should be dependent on the carrier frequency of the repeated stimulation; 2) the shape of the adapted compartment should change when the carrier frequencies of the repeated stimulus changes.

In the present study, we use ion imaging to investigate whether the accumulation of calcium and sodium 1) occurs in the dendritic regions stimulated by the carrier frequency of the repeated signal, 2) remains spatially limited to those regions, and 3) follows the same time course as predicted by Triblehorn and Schul (2013); calcium accumulation is fast and transient while sodium accumulation is slower and sustained through the stimulus duration. Positive results would indicate that the calcium- and sodium-mediated processes underlying adaptation would also be spatially limited to the stimulated regions of the dendrites with the same time course, providing further support for the DDC hypothesis as a mechanism underlying SSA.

MATERIALS AND METHODS

Overview of the Katydid Hearing System

The ears of katydids are located below the knees of the forelegs. Each ear contains about 35 receptor cells, each one tuned to a characteristic frequency that ranges approximately from 6 to 60 kHz (Höbel and Schul 2007). The afferent axons project through the leg nerve into the auditory neuropile within the prothoracic ganglion (PTG), where they connect with auditory interneurons, including TN-1. The projection areas of the receptor cells are tonotopically organized both along the anterior/posterior and dorsal/ventral axes (see Fig. 6C; Ebendt et al. 1994; Römer et al. 1988). In general, low frequencies project more anteriorly than higher frequencies. The dendritic field of TN-1 covers most of the auditory neuropile in the PTG. Axon collaterals ascend to the brain and descend toward the abdomen ipsilateral to the dendrites; the soma is located contralateral to axon and dendrite in the PTG (Fig. 2). TN-1 receives excitatory input from auditory receptor cells ipsilateral to the dendritic field. The projection area of each receptor cell overlaps only with a small part of the TN-1 dendrite so that the spatial pattern of synaptic input on the TN-1 dendrite resembles the tonotopic organization of the auditory neuropile. Contralateral inhibition on TN-1 is mediated by a local
interneuron. Detailed descriptions of TN-1 morphology and physiology are given elsewhere (Römer et al. 1988; Schül 1997; Stumpner 1999; Triblehorn and Schül 2009).

Animals

Adult individuals of *Neoconocephalus triops* (Linnaeus 1758) were either collected in Puerto Rico or reared from field-collected insects in the laboratory (F1 and F2 generations) under summer conditions (Beckers and Schül 2008). Animals were kept for at least 2 wk after collection or their final molt before being used in the experiments. We used both male and females and could not detect differences between them.

Preparation

Animals were anesthetized using CO₂ and fixed ventral side up with Kerr’s yellow sticky wax on a metal holder. The forelegs were fixed on a wire (1-mm diameter) perpendicular to the body axis; mid and hind legs were removed. The PTG was exposed by removing the prothoracic sternite and covered with saline modified after Fielden 1999; Triblehorn and Schül 2009).

Electrophysiology and Injection of Calcium and Sodium Indicators

Because of the large diameter of the ascending axon, TN-1 spikes can be readily identified in hook recordings from the cervical connectives (e.g., Faure and Hoy 2000). We monitored TN-1 activity extracellularly, using tungsten hook electrodes (70–100-μm diameter) applied to the cervical connectives. Hook electrode and the connective were covered with petroleum jelly to prevent desiccation. Extracellular recordings were amplified using a custom-made amplifier and band-pass filtered (100-4,000 Hz; Krohn Hite 3384). Intracellular recordings and dye injections were made using thick-walled borosilicate glass microelectrodes (20–50 MΩ resistance; WPI). Signals were low-pass filtered (5 kHz) and amplified (Warner Instruments IE 210). After penetration and identification of TN-1, we iontophoretically injected either a calcium or sodium indicator into either the axon or main dendrite using hyperpolarizing current (3–5 nA) for 5–15 min.

We used Oregon Green BAPTA-1 hexapotassium salt (OGB) (Life Technologies) as an indicator for the concentration of free intracellular calcium. The electrode tip was filled with 400 μM OGB in 400 μM potassium acetate (Baden and Hedwig 2009). OGB has high affinity to calcium (K<sub>d</sub> = 170 μM) and thus high sensitivity at low Ca<sup>2+</sup> concentrations (Baden and Hedwig 2006). To measure intracellular sodium concentrations, we used a 500 μM solution of sodium green tetrachelammonium salt (Life Technologies; K<sub>d</sub> = 6 mM) in 500 µM tetramethylammonium salt (Life Technologies; doi:10.1152/jn.00945.2014 • www.jn.org

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Fig. 2. Imaging of the TN-1 neuron in the prothoracic ganglion of *N. triops*. A: photograph of a Lucifer yellow staining of TN-1 after clearing the tissue with methyl salicylate. Dendritic tree (DT) is visible axonipsilateral near the midline of the ganglion. TN-1 has ascending and descending axon (AA and DA, respectively) collaterals. The stained TN-1 receives excitatory input from afferents entering the ganglion in the top leg nerve (LN). The ganglion is oriented rostral to the left. B: photograph of a TN-1 filled with Oregon Green BAPTA-1 calcium indicator. Soma and ascending and descending axons are visible without stimulation. The 2 regions of interest (ROIs) (ROIs are indicated; frame marks the area of acquisition (512 × 128 pixels)). Orientation of the ganglion as in A. C: comparison of the calcium fluorescence changes when stimulated with fast pulse rates (140 pulses/s) with 15-kHz (top) or 40-kHz (bottom) carrier frequency. TN-1 responses are shown 2 s before stimulus beginning (frame 1, top), at stimulus beginning (frame 9), and after the stimulus ended (frame 21). Background was removed using ImageJ. Arrowheads indicate the location of the peak fluorescence in the dendritic ROI, which differed between the 2 stimulus frequencies. Scale bars denote 200 μm. Note that B and C stem from the same preparation. However, in B, the static fluorescence was imaged, whereas, in C, the change of fluorescence (ΔF/F<sub>0</sub>) is shown.
μM potassium acetate. The electrode shafts were backfilled with 1 M potassium acetate.

To avoid unnecessary chelating effects of calcium and sodium indicators, we limited the injected amount of indicator to the minimum that we had determined during preliminary recordings. We allowed 60 min between injection and imaging for the indicator to distribute from the injection site to the dendrites.

TN-1 responses were also monitored through the extracellular recording from the connective before and after the indicator injection and after the imaging. We only included experiments where pre- and postimaging TN-1 responses did not differ by more than one spike per bin when comparing the average peri-stimulus time histograms (PSTHs) with 20-ms bin width. The pre- and postimaging responses were pooled and are shown as PSTHs together with the time course of fluorescence data.

Acoustic Stimulation

The acoustic stimuli for neuron identification and preinjection and postimaging testing were generated by 16-bit DA converter system (National Instruments PCI-6251) at a sampling rate of 250 kHz using custom-designed software developed in LabView (National Instruments). Stimuli were delivered via a loudspeaker (Technic 10TH400C) mounted 50 cm from the preparation, perpendicular to the insect’s body and ipsilateral to the recording side.

During imaging, stimuli were played back from a Marantz PMD-671 solid-state recorder (24 bit, 96-kHz sampling rate), amplified, and presented through a Motorola KSN1218C loudspeaker. The loudspeaker was mounted perpendicular to the insect’s body and ipsilateral to the recording side.

Signal amplitudes were adjusted to 80 dB sound pressure level (SPL) (peak, re 20 μPa) at the position of the insects using one-quarter-inch condenser microphone (G.R.A.S. 40 BF) and B&K 2231 sound level meter (Brüel and Kjær).

Stimuli consisted of pulses with 3.5-ms duration (including 0.5-ms rise and fall time). The pulses were repeated with a period of 7 ms (140 pulses/s, pps) or 140 ms (7 pps). The fast pulse rate reliably elicited adaptation and a burst of APs occurred only at the beginning of the stimulus. The slow pulse rate was faithfully encoded by TN-1 with one or several APs per pulse (Schul and Sheridan 2006; Schul et al. 2012). We used two different carrier frequencies (15, 40 kHz), which were sufficiently different to reliably result in SSA (Schul et al. 2012). Spatial differences between projection of sensory neurons tuned to 15 and 40 kHz (see above) should result in significant spatial separation of the activated regions of the TN-1 dendritic field, as predicted by our DDC hypothesis. We chose these two frequencies for consistency with previous work (Schul and Triblehorn 2013; Schul et al. 2012) and because of the spatial representation of these two frequencies in the auditory neuropile.

Each stimulus situation began with a 2-s-long silent period, followed by a pulse train of either 3.0- or 7.8-s duration. A silent period of 30 s was kept before the next stimulus was tested. We repeated each stimulus three to eight times; two to six different stimuli were tested in each preparation. The sequence of stimulation was randomized across preparations.

During calcium experiments, we tested the fast pulse rate with pulse trains of 3.0-s and 7.8-s duration and the slow pulse rate with 3.0-s durations. All stimuli were presented at 15 and 40 kHz. During sodium experiments, we tested both fast and slow pulse rates with only the 3.0-s pulse train duration because of strong bleaching effects of the sodium indicator dye during the 7.8-s pulse train (see below).

Imaging

The imaging experiments used an inverted Zeiss Live5 line-scan confocal microscope. Therefore, the animal had to be fixed ventral side down after injection of the indicator dyes. The holder with the insect was removed from the stand, flipped over, and placed ventral side down on a coverslip-bottom Petri dish. The Ni-Cr ganglion spoon embedded in the wax well wall positioned the PTG just above the coverslip. The preparation was fixed to the bottom of the Petri dish with a few drops of wax.

A 488-nm diode laser attenuated between 5 and 25% of maximum power and provided excitation of the sodium and calcium indicator dyes. A ×10 objective and a zoom setting of 0.5 were used during data acquisition. The focus depth was set approximately to the dorso-ventral middle of the TN-1 dendritic field. A pinhole setting of 100% resulted in a depth resolution ≤130 μm, covering the whole depth of the neuropile. Images (12-bit resolution, 512 × 128 pixels) were acquired in line-scan mode with the long axis parallel to the midline of the insect. The images covered a range of ~1,264 × 36 μm and included the complete auditory neuropile and parts of the TN-1 axons (Fig. 2). Image acquisition took place at a rate of 4 frames/s. This meant that, during stimulation with the slow pulse rate (7 pps), most frames covered two stimulus pulses. Therefore, the imaging data cannot resolve the temporal pattern of this stimulus at the pulse level. Image acquisition was synchronized with the sound stimulation using a custom-made interface; it started 2 s before the beginning of the sound stimulus, i.e., the stimulus started with the ninth frame of the image sequence.

After completion of the imaging experiment, the preparation was removed from the coverslip and mounted back on the stand ventral side up to check the condition of TN-1 with hook electrodes (see above).

Imaging Analysis

Preparation of the acquired image sequences. We analyzed the imaging data using ImageJ (National Institutes of Health). The 12-bit image sequences were converted to 8 bits, and all the acquired sequences were filtered using the Kalman filter of ImageJ. Pixel values were transformed to changes of fluorescence relative to the background intensity (∆F/∆F₀), using the average of frames 3–6 as the F₀ value. We defined two rectangular regions of interest (ROI), each 512 pixels long and 10–15 pixels wide (Fig. 2B). The first ROI (dendritic) covered the medial section of the TN-1 dendritic field. The second ROI (axonal) was more lateral and included the main dendrite, which contains the spike-generating zone and connects to the axon collaterals (Fig. 2B). We collapsed each ROI into an array of 512 × 1 pixels by averaging the values of the 10–15 pixels across the width of the ROI. To smooth these arrays, we applied a local regression-fitting function (Loess, span = 0.15) to the two arrays of each frame using a custom-written R program (R Core Team 2014). Finally, the data were averaged on a frame-by-frame basis for the different presentations of the same stimulus.

Bleach compensation. Because of low intracellular sodium concentrations, sodium imaging typically has a low signal-to-noise ratio and is therefore influenced by bleaching of the sodium indicator. To account for this, we used the built-in functions of the MacBiophotonics package of ImageJ (McMaster University, Hamilton, Ontario, Canada). Bleach compensation was calculated for each ROI separately.

We calculated the average intensity value of the last two frames before the start of the stimulation (i.e., frames 7 and 8) of all sequences acquired in a preparation. These average values were used to construct a calibration curve by arranging them in the temporal sequence in which they were acquired, with each acquired frame equivalent to one data point of the calibration curve. Thus we considered only the time when the laser was active and bleaching occurred. Time between data acquisition (i.e., when the laser was off) was not included in bleach correction. An exponential decay function was fitted through the average values using the ImageJ fitting function. This resulting curve served as calibration function for the bleach correction. The measured fluorescence was corrected for each frame by dividing each pixel value inside the ROI with the corresponding...
value on the calibration function. The data analyses described below were then performed on the bleach-corrected sodium data. The signal-to-noise ratio was much better for the calcium imaging, and, although bleaching obviously occurred, it did not cause effects large enough to camouflage the results relevant to our questions. For that reason, and also to avoid potentially introducing artifacts, we chose not to perform bleach correction of the calcium signals.

**Time course of the fluorescence.** To reduce the noise caused by areas of the ROI with weak or no calcium/sodium accumulation, we focused the analyses of the time course to the areas in the ROI with strong fluorescence. For each stimulus, we identified the frame with the strongest response and located the 10 adjacent pixels with the highest fluorescence within that frame. This was performed separately for the dendritic and axonal ROIs. We then measured the fluorescence intensity of these pixels in each frame and plotted them over time. Depending on the stimulus frequency, different pixels were evaluated in each preparation, as 15 and 40 kHz excited different areas of the dendritic field. We standardized the time courses of each experiment before averaging them among preparations. We used the three frames preceding stimulation (i.e., frames 6–8) as the 0 value.

The calcium and sodium signals differed in their time course. The calcium signals were transient and peaked during the first frame of stimulation (9th frame) and decayed from there. For these experiments, we normalized the calcium signal for each experimental parameter (acoustic frequency, pulse rate, and ROI) to a value of 0 using the baseline values (frames 6–8) and a maximum value of 1 using the calcium fluorescence in the first frame of stimulation (frame 9). Using the least-squares method, we fitted an exponential function to the descending part of the averaged calcium signal (3-s stimuli: frames 10–20; 8-s stimuli: frames 10–40) and estimated the time constant of the decay ($\tau_{\text{decay}}$).

The sodium signal was tonic with a slower rise time, and it remained high throughout the stimulation (see **RESULTS**). Here, we set the average value of frames 15–18 (1.5–2.5th second of the stimulation) to 1 for the standardization of the amplitude. Within each preparation, we standardized the signals in response to one carrier frequency to the amplitude of the fast pulse rate in the dendritic ROI. This was done to highlight the consistent amplitude differences between dendritic and axonal ROI and the absence of a signal at the slow pulse rate.

Using the least-squares method, we fitted an exponential function to the ascending part (the first 2 s of the pulse train, equivalent to frames 9–15) of the averaged sodium signal and estimated the time constant of the rise ($\tau_{\text{rise}}$).

**Determination of the fluorescent peak positions.** We determined the spatial positions of the fluorescence peak for stimulation with 15 and 40 kHz at 140 pps from the standardized data described in the previous section. Because calcium fluorescence peaked immediately after the beginning of the stimulation, we determined the spatial peak position in the first frame after the beginning of the stimulation (9th frame in the sequence). For sodium experiments, we averaged frames 15–18 (1.5–2.5th s of the stimulation) to determine the spatial pattern of the sodium signal.

For each individual signal, we found the range where the amplitude was $>95\%$ of the peak value. We calculated the peak position as the midpoint between the rostral and caudal limits of the $>95\%$ range. The spatial patterns were aligned across individuals by using the peak value during 40-kHz stimulation as a reference. We show the average pattern with standard deviations.

**RESULTS**

We found the response properties of TN-1 as in previous studies (Schul et al. 2012; Triblehorn and Schul 2013). The spectral sensitivity was broad with low thresholds ($<40$ dB SPL) between 6 and 60 kHz. Stimulation with the fast pulse rate (140 pps) at both carrier frequencies (15 and 40 kHz) caused rapid adaptation; spiking responses ceased after a burst of spikes or a single spike at the beginning of the pulse train. In contrast, the slow pulse rate (7 pps) elicited responses of one to three spikes to each pulse. These response characteristics did not change significantly after the injection of either calcium or sodium indicators or after the imaging procedures. PSTHs of typical TN-1 responses are shown below together with the temporal imaging data in Figs. 3–5.

**Time Course of the Calcium Response**

During the silent period at the beginning of the stimulus, TN-1 filled with the calcium dye (OGB) showed low fluorescence, indicating low levels of free cytosolic calcium in the dendritic field (Fig. 2B). Both soma and axon had detectable fluorescence without stimulation (Fig. 2B). Stimulation with 15 and 40 kHz at 140 pps led to a strong increase in calcium in both the axonal and dendritic ROI (Fig. 2C). After the end of the pulse train, calcium levels returned to starting levels (Fig. 2C).

During stimulation with the fast pulse rate at 15 and 40 kHz, we recorded a transient calcium response in both ROIs (Fig. 3A). The calcium signal peaked within the first frame after stimulus onset (i.e., within 250 ms) and dropped back to baseline levels over approximately the next 3 s, both for the 3.0-s and 7.8-s stimulus duration (Fig. 3). The $\tau_{\text{decay}}$ of individual measurements ranged between 1.9 and 2.5 s and did not differ significantly between the dendritic and axonal ROIs or stimulus durations (Wilcoxon signed-rank test, $P > 0.5$ in all tests, $n = 5–6$).

At the end of the fast pulse train, the dendritic calcium signal had a slight dip. A rebound of axonal and dendritic calcium signals occurred during the first second after the stimulus. Both these effects were most pronounced at the long stimulus duration.

When stimulated with the low pulse rate, the calcium signal increased rapidly during the first 250 ms after stimulus beginning and remained high throughout the stimulus duration. This sustained increase in calcium signal was observed at 15 and 40 kHz in both ROIs (Fig. 4). Note that the rate of image acquisition (4/s) was slower than the pulse rate of the stimulus (7/s), so that potential peaks in the calcium signal to each stimulus pulse were not resolved.

Pharmacological experiments (Triblehorn and Schul 2013) predicted a transient calcium signal during stimulation with fast pulse rates; our results are in agreement with that prediction.

**Time Course of the Sodium Response**

The intensity of the sodium signal was weaker and had a lower signal-to-noise ratio than the calcium signal. As with
calcium, stimulation with the fast pulse rate resulted in an increase in the sodium signal at both 15- and 40-kHz carrier frequencies. However, the increase in the sodium signal differed from the calcium signal in two ways. First, the sodium signal had a slower time constant of the rise (15 kHz: $\tau_{\text{rise}} = 1.4$ s; 40 kHz: $\tau_{\text{rise}} = 1.8$ s) and remained elevated for the stimulus duration for both carrier frequencies even though TN-1 spiking activity occurred only at the beginning of the stimulus (Fig. 5A). Second, the sodium signal in the axonal ROI was much weaker at 20–40% of the amplitudes in the dendritic ROI (Fig. 5A).

Stimulation with the slow pulse rate (7 pps), which elicited ongoing spiking, did not result in significant sodium signals in either the dendritic or axonal ROI (Fig. 5B). Thus sodium levels were correlated with pulse rate of the stimulus rather than TN-1 spiking; the fast pulse rate (140 pps) evoked a few spikes at the beginning of the pulse train but elicited a tonic sodium signal. In contrast, stimulation with 7 pps resulted in many more TN-1 spikes but little, if any, sodium accumulation.

**Pharmacological experiments (Triblehorn and Schul 2013)** predicted a tonic sodium signal during stimulation with fast pulse rates; our results were in agreement with that prediction.

**Location of Calcium and Sodium Peaks**

The DDC hypothesis predicts that the calcium- and sodium-based processes underlying TN-1 adaptation remain limited to the parts of the dendritic field that are stimulated by the carrier frequency of the fast pulse rate, whereas other parts of the dendritic field retain sensitivity. The peak location of the calcium and sodium signals in the dendritic field should be frequency dependent, with the 15-kHz peak being more rostral.
In the dendritic ROI, the peak signal for both calcium and sodium occurred more rostrally during 15-kHz stimulation compared with during 40-kHz stimulus (Fig. 6, A and C). The peak position of the averaged 15-kHz calcium pattern was 90.3 μm more rostral than that of the 40-kHz signal (Fig. 6A). The 15-kHz sodium pattern was 78.5 μm more rostral (Fig. 6C). For all individual preparations, the peak range (with amplitudes >95%) for 15-kHz stimulation was located more rostral than for 40-kHz stimulation (Fig. 6, A and C, insets). These differences in peak positions were highly significant for calcium and sodium (Wilcoxon signed-rank test for paired data, P = 0.02 for both calcium and sodium, n = 7 each, Fig. 6). These results are in agreement with the above stated predictions.

In the axonal ROI, the calcium signals during stimulation with 15 and 40 kHz peaked in the same location (Fig. 6B). The regions with peak ranges with amplitudes >95% largely overlapped between 15 and 40 kHz in all individual preparations. Peak positions did not differ between the two carrier frequencies (Wilcoxon signed-rank test for paired data, P = 0.31, n = 7, Fig. 6B). Because of its low amplitude, we could not reliably determine a peak location of the sodium signal in the axonal ROI.

**DISCUSSION**

SSA requires the storage of information about the preceding stimulation. Many neurophysiological mechanisms have been discussed to underlie this information storage (review in Escera and Malmierca 2013). Dendritic mechanisms involving hyperpolarizing potassium currents mediated by sodium and/or calcium could potentially underlie SSA if two requirements are met: 1) the inputs of repeated and rare stimuli are spatially separated on the dendrite; and 2) the hyperpolarizing potassium currents are activated only in the dendritic regions stimulated by the repeated signal. Thus ionic currents would have to be activated by local postsynaptic potentials rather than by spiking activity.

The *Neoconocephalus* auditory interneuron TN-1 exhibits SSA-like behavior in that its spiking responses to repeated signals are suppressed within tens of milliseconds, whereas rare signals differing in carrier frequency continue to elicit APs (Schul et al. 2012). This SSA takes place at rates that are one to two orders of magnitude faster than described in vertebrates; response suppression occurs in TN-1 at repetition rates >50 pps, whereas deviant pulses can have repetition rates as high as 7–10 pps (Schul and Sheridan 2006). This reflects the relevant auditory scene of these insects, with male calls having amplitude modulation rates up to 200 pps and rare predator (e.g., bat) signals in the range of 5–10 pps (Hartley and Suthers 1989; Schul et al. 2014).

SSA in TN-1 depended on the intracellular accumulation of calcium and sodium (Triblehorn and Schul 2013). Removal of intracellular calcium delayed adaptation to repeated signals by a few hundreds of milliseconds. Replacement of sodium in the bath with lithium or choline led to strongly reduced adaptation to repeated signals in the range of 5–10 pps (Hartley and Suthers 1989; Schul et al. 2014).
Fig. 6. The spatial pattern of the calcium (A, dendritic; B, axonal) and sodium (C, dendritic) fluorescence signals during stimulation with the fast pulse rate (140 pps). The curves show the averaged spatial fluorescence pattern (n = 7 each) during stimulation with the fast pulse rate at 15 kHz (dark gray) and 40 kHz (light gray). The shaded areas indicate the SD. Individual curves were aligned relative to the peak of the 40-kHz pattern. The panels above the curves in A–C show the ranges of each individual’s pattern with amplitudes >95% (15 kHz, dark gray; 40 kHz, light gray) to highlight the variability of the data across preparations; the reference number of each preparation is given. In the dendritic ROI (A and C), calcium- and sodium-related fluorescence at 15 kHz is located more rostral than at 40 kHz. In the axonal region (B), the calcium peaks do not differ significantly. D: sagittal section through the auditory neuropile of the tettigonid Tettigonia viridissima with the projections of 1 receptor cell shown in each panel; the characteristic frequency of each receptor cell is given. Receptor cells with characteristic frequencies close to 40 kHz project about 100 μm caudally from those with characteristic frequencies close to 15 kHz; the projection area of 15 kHz is also more dorsal than 40 kHz. Note that this sagittal section is orthogonal to the view of the neuropile in Fig. 2. It represents the slice of the ganglion imaged in our dendritic ROI. [Redrawn from Römer et al. (1988) with permission.]

1994; Römer et al. 1988). The projections are also at different levels in the dorso-ventral direction, as is shown in the sagittal sections of the auditory neuropile (Fig. 6D). Therefore, there is little, if any, overlap between the afferent projections onto the TN-1 dendrite stimulated by the 15- and 40-kHz frequencies. On the basis of these morphological data of the projection areas, the separation of the two fluorescence peaks was predicted to be about 100 μm, a remarkable match to our finding of 78.5- and 90.0-μm separation. The overlap of the spatial fluorescence plots in Fig. 6, A and C, was due to the dorso-ventral layering of the receptor cell projections, which our imaging could not resolve.

**Effect and Effectors of Calcium and Sodium**

We recorded strong calcium signals in the dendritic and axonal ROIs. During stimulation with the fast pulse rate, time signals in both ROIs were phasic, peaking at the beginning of the fast rate, and were tonic at the slow pulse rate (Figs. 3 and 4). In the dendritic ROI, the peak position depended on the carrier frequency (Fig. 6). As spike-triggered processes should affect the whole dendrite equally, they cannot account for the localized calcium signal in the dendritic ROI. Thus dendritic processes must account for a significant part of the calcium accumulation in the dendritic ROI. The most likely candidates are low-voltage-activated calcium channels (LVCCs). LVCCs are fast inactivating (Caterall 2011; Haag and Borst 2000). Stimulation with fast pulse rates elicited a tonic depolarization in TN-1 that persisted after cessation of APs (Triblehorn and Schul 2009). These EPSPs would elicit a transient calcium current at the stimulus beginning that quickly ceases as the LVCCs inactivate, resulting in the transient calcium signal observed.

The weak persistent calcium signal observed during the long stimulus (Fig. 3B) could be due to a weak persistent calcium current observed in some invertebrate LVCCs (Haag and Borst 2000; Haag et al. 1997). The rebound of the calcium signal after stimulus end (Fig. 3) can be explained by LVCCs deinactivating as the membrane repolarizes after the EPSP ends. The interpretation of these later parts of the calcium signal, however, is highly speculative without knowledge about the
characteristics of calcium currents and channels in this group of insects.

Stimulation with the slow pulse rate would result in a short EPSP (with AP riding on them) to each pulse, separated by depolarization of the membrane that allows the LVCCs to deinactivate. Thus each pulse would elicit a transient calcium current that adds up to the tonic calcium signal observed (Fig. 4). Because of the slow sampling rate (4 frames/s), stimulation with 7 pps was recorded as a tonic calcium signal, and peaks that might occur to each stimulus pulse would not be resolved. The high affinity of OGB for calcium results in slow release with falling concentrations of free calcium (Augustine et al. 2003; Baden and Hedwig 2006), further reducing the temporal resolution of the measurements. Thus is not clear whether the tonic calcium signal (Fig. 4) was an artifact of the high affinity of OGB for calcium, the slow sampling rate, or some combination of the two. Calcium-gated processes alone were not able to completely suppress TN-1 spiking even at fast pulse rates; during sodium-removal experiments, TN-1 fired throughout the stimulus duration. Spike rate decreased within about 100–200 ms, presumably caused by a calcium-dependent process, but never ceased (Triblehorn and Schuł 2013).

Our data are inconclusive whether a second, high-voltage-activated calcium current contributed to the strong calcium signal in the axonal ROI. As the absolute amplitude in the axonal ROI was typically larger than that in the dendritic ROI (data not shown), optical cross talk or calcium diffusion from the dendritic to the axonal ROI is unlikely to be the source of the signal in the axonal ROI. Whether LVCCs or high-voltage-gated channels generate the signal in the axonal ROI cannot be distinguished based on our data.

A significant sodium accumulation occurred only in the dendritic ROI and during stimulation with fast pulse rates (Fig. 5). Because a stimulus with fast rates elicits more EPSPs and fewer spikes compared with slow-rate stimulation, the sodium signal was correlated with the EPSP activity, not spikes. The spatial peak of the sodium signal was frequency dependent, confirming a dendritic origin of the sodium inflow. The rise time of the sodium signals was much slower than that for calcium, peaking after about 1 s, which parallels the results of our previous low-sodium experiments (Triblehorn and Schuł 2013).

**Dendritic Mechanisms Underlying SSA**

Physiological and pharmacological experiments established that the adaptation to a repeated stimulus was attributable to intrinsic processes in TN-1, mediated by sodium and calcium (Schul et al. 2012; Triblehorn and Schuł 2013). Here, we established that an accumulation of calcium and sodium occurs in the dendrite with temporal patterns predicted by the previous experiments. Furthermore, calcium and sodium accumulations were limited to the stimulated parts of the dendrite. By extension, the calcium and sodium mechanisms underlying adaptation would also be limited to the stimulated parts of the dendrite, thus allowing adaptation to be stimulus (i.e., frequency) specific.

Sodium-activated potassium currents contribute to adaptation in numerous systems (e.g., Kaczmarek 2005; Sah 1996; Vergara et al. 1998). Thus SSA based on DDC may also occur at longer time scales, for instance as bat echolocation calls from a specific background (i.e., male calls). For example, in a different tettigonid species, SSA of TN-1 functions to detect short conspecific calls in background noise (Siegert et al. 2013).

Although the central role of calcium- and sodium-dependent potassium currents for nonspecific neural adaptation to repeated stimuli has been recognized (e.g., Kuznetsova et al. 2008; Sanchez-Vives et al. 2000), they are not typically mentioned in the context of SSA because they seemingly affect the general responsiveness of a neuron independently of the input pattern. Adding a spatial dimension may, however, allow for SSA to be generated within a single neuron; if its inputs are spatially distributed on the dendrite, then sodium- and calcium-dependent potassium currents causing reduced sensitivity may remain limited to the stimulated regions of the dendrite, while others remain sensitive (Fig. 1). Activity-dependent regulation of potassium currents can remain effective for periods ranging from milliseconds to tens of seconds (Bhattacharjee and Kaczmarek 2005; Sah 1996; Vergara et al. 1998). Thus SSA based on DDC may also occur at longer time scales, for instance as in the auditory system of mammals (Ulanovsky et al. 2004).

One common explanation for SSA is synaptic depression or inhibition of the inputs carrying the repeated stimulus (Grill-Spector et al. 2006; Wehr and Zador 2005). Functionally, such SSA at the level of the afferent neurons would result in similar spiking patterns as SSA generated with DCC. However, dendritic SSA would be independent of the afferent properties and therefore allow afferents to drive novelty-detecting neurons, while at the same time faithfully transmitting the pattern of the repeated stimulus to other neuron populations. The simplicity of this mechanism and the universal occurrence of the underlying ion channels make DDC an appealing explanation for SSA, in particular at lower levels of vertebrate and invertebrate sensory systems. The DDC mechanism (Fig. 1) described in the tettigonid TN-1 neuron makes a plausible case that SSA...
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: J.P. and J.D.T. performed experiments; J.P. analyzed data; J.P. prepared figures; J.D.T., and J.S. edited and revised manuscript; J.P., J.D.T., and J.S. approved final version of manuscript; J.D.T. and J.S. conceived and design of research; J.S. interpreted results of experiments; J.S. drafted manuscript.

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