Artifactual hyperpolarization during extracellular electrical stimulation: Proposed mechanism of high-rate neuromodulation disproved

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Introduction

Extracellular electrical stimulation is widely used to activate neurons in experiments and for diverse clinical applications [1]. It is reasonably well understood how electric field stimulation (EFS) activates neurons [2–4]. Neurons can also be inhibited by EFS; specifically, EFS applied to peripheral nerves at frequencies between 10 and 100 kHz (and as low as 4 kHz in non-mammalian axons) has been shown to block spike propagation by causing depolarization and sodium channel inactivation [5–10]. Net inhibition can also occur indirectly if EFS-evoked spikes engage inhibitory neurons, as occurs in conventional low-frequency (40–60 Hz) spinal cord stimulation (SCS) where, consistent with the Gate Control Theory [11], spikes evoked in dorsal column fibers propagate antidromically into the spinal dorsal horn and engage inhibitory neurons that help block pain signals [12–14]. Recently, application of EFS at frequencies between 1 and 10 kHz (henceforth referred to as kEFS) to the spinal cord has emerged as a promising new form of SCS [15,16]. Unlike conventional SCS, which is associated with a buzzing sensation (paresthesia) caused by orthodromic
propagation of the spikes evoked in dorsal column fibers, high-frequency SCS is paresthesia-free, which suggests that dorsal column fibers are not activated [17,18]. But nor is high-frequency SCS associated with the sensory deficits that would occur if spike propagation is blocked. The mechanism of action of high-frequency SCS thus remains unclear and has become the focus of intense study given the clinical efficacy of this treatment [19,20].

Recent experiments suggested that kEFS hyperpolarizes spinal neurons [21]. Those results, if true, demonstrate a novel form of direction inhibition that could be harnessed to treat a multitude of neurological disorders and would represent a major breakthrough in elucidating how high-rate SCS relieves pain. However, the experimental data reported by Lee et al. are inconsistent with simulations [22] and with studies on nerve block (see above) that predict, if anything, that depolarization should occur. This discrepancy suggests that either the simulations or the experiments are flawed. With respect to potential experimental flaws, patch clamp amplifiers have been reported to exhibit recording artifacts when used in current clamp mode [23,24]. The effects on voltage measurement are usually quite minor, but, to the best of our knowledge, no one has tested whether EFS exacerbates this artifact in PCAs or causes some other aberration. Ruling out such artifacts and reconciling the experimental data with simulations is critical before moving forward.

We thus sought to verify the original report of kEFS-induced hyperpolarization [21] and elucidate how that hyperpolarization occurs using electrophysiological and optical methods. After reproducing the hyperpolarization, inconsistencies revealed by optical recording prompted deeper investigation that ultimately revealed the hyperpolarization to be caused by an artifactual current originating from the PCA during EFS. Refutation of kEFS-induced hyperpolarization as a novel form of direct inhibition is disappointing for neuromodulation applications, but awareness of the artifact we have uncovered is critical for anyone studying the cellular effects of extracellularly applied electric fields.

Materials and methods

Cell culture and slice preparation

All experimental procedures were approved by the Animal Care Committee at The Hospital for Sick Children. To culture hippocampal neurons, a pregnant Sprague-Dawley rat (Charles River, Montreal) was anesthetized with 4% isoflurane and killed by cervical dislocation. Hippocampi from 8 to 12 embryos (17–19 days gestation) were dissected, placed in sterile Hank’s solution at 4 °C, and mechanically dissociated by trituration. Hippocampal neurons were plated onto poly-D lysine-coated coverslips with Neurobasal™ media (Gibco 21103-049) supplemented with 1% fetal bovine serum (FBS), B-27™ supplement (Thermo Fisher 17504-044) and 0.5 mM L-glutamine (Gibco 25030-081) at a culture density of <1 × 10^6 cells/mL. Coverslips were maintained for up to 3 weeks in a 37 °C incubator at 7% CO₂ with maintenance media (plating media without FBS) changed every 3–4 days. Neurons were used for experiments at 14–21 days in vitro (DIV). Slice preparations were described as previously [25,26].

Electrophysiology

The recording chamber was perfused at ~2 ml/min with oxygenated (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (ACSF). To block fast synaptic transmission, ACSF was supplemented with (in μM) 10 bicuculline methiodide, 10 CNQX and 40 D-AP-5 (HelloBio, Princeton, NJ). We patched neurons visualized on coverslips (cultures) or in lamina I/II of spinal cord slices or in the CA1 region of hippocampal slices. Neurons were recorded in the whole-cell configuration with >70% series resistance compensation using an Axopatch 200B amplifier or an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA). Patch pipettes were pulled from borosilicate glass capillaries (WPI) and were filled with an intracellular solution composed of (in mM): 125 KMeSO₄, 5 KCl, 10 HEPES, 2 MgCl₂, 4 ATP, 0.4 GTP; pH was adjusted to 7.2 with KOH (pipette tip resistance 6–9 MΩ). All recordings were at room temperature (~22 °C). For most experiments, traces were low pass filtered at 100 kHz and digitized at 500 kHz using a CED 1401 computer interface (Cambridge Electronic Design, Cambridge, UK). To standardize across neurons, pre-stimulus membrane potential was set to −65 mV (after correction for a liquid junction potential of ~9 mV) using tonic current injection.

Voltage-sensitive dye (VSD) imaging

Cultured neurons 14–21 DIV were stained using a Fluovolt Membrane Potential Kit™ (Life Technologies, Carlsbad, CA) [27]. Fluovolt dye was diluted in the PowerLoad concentrate and Live Cell Imaging Solution (LCIS) (ThermoFisher Scientific), supplemented with 10 mM glucose to a final concentration 75% of that recommended by the manufacturer. Coverslips were washed 3x in room temperature LCIS before undergoing a 15 min incubation in the diluted Fluovolt solution followed by 3x glucose supplemented LCIS washes. The VSD was excited with a 505 nm LED (Thorlabs, Newton, NJ) at an intensity of 0.68 mW/mm² and was imaged with a 40x water immersion lens (0.75 NA) and Zeiss filter set 46HE (excitation, 500/25; emission, 535/30). Neurons were imaged during 500 ms-long light exposures (separated by 700 ms-long dark periods) using a NeuroCCD-SM256 camera at an acquisition rate of 50 Hz on maximal gain (30x) (RedShirt Imaging, Decatur, GA). Image analysis was performed with Neuroplex software (RedShirt Imaging). Calibration curves were generated by measuring fluorescence intensity changes (ΔF) during current injection steps (in the absence of kEFS) while recording membrane voltage in current clamp thus allowing ΔF measurements (in arbitrary units reflecting pixel intensities) to be converted directly to ΔV on a cell-by-cell basis.

Electric field stimulation

Extracellular electrical stimulation was delivered through a pair of preclinical leads (Boston Scientific, Valencia, CA) placed 10 mm apart in the recording chamber (Fig. 1A). One lead served as the anode and the other as the cathode. Lead impedance was tested before each experiment and was consistent across the study. Constant current was distributed equally to the four platinum contacts on each lead using a therapy-grade Precision Spectra™ External Stimulator (ETS) from Boston Scientific™ that had undergone a non-commercial firmware change to enable the delivery of waveforms with frequencies up to 10 kHz. The ETS is battery powered and remote controlled by the Precision Spectra™ Clinician Programmer tablet and meets IEC60601 standards for electrical isolation, which means that, except for the stimulator leads in the recording chamber, the entire stimulation system is electrically isolated from the recording equipment. The ETS was further shielded with a grounded tinfoil sheet; grounding the sheet to the isolation table (to which other equipment was grounded) or to a separate ground made no difference. Indeed, ETS shielding served only to reduce transients near the onset and offset of EFS that originated from commands sent via remote control from the programmer. Simulations predicted (Sup. Fig. S1) and measurements of bath voltage confirmed (Fig. 1B) that equal distribution of constant current to the four contacts on each of the two, parallel...
oriented leads produced a uniform electric field. A biphasic charge-balanced waveform with 40 μs-long pulses separated by a 10 or 100 μs-long interpulse interval (for EFS < or > 1.2 kHz, respectively) was applied at 1, 5 or 10 kHz. The waveform shape and charge balance were confirmed with recordings of the bath voltage (see inset on Fig. 1B). To measure the unitary hyperpolarization, we studied the first response to the same waveform (40 μs pulses with 100 μs interpulse intervals) applied at 150 Hz.

**Statistical analysis**

Statistical analysis was conducted using Sigmaplot v11. Distributions were tested for normality using the Kolmogorov-Smirnov test. When neurons were re-tested under different conditions (e.g. different EFS frequencies or amplitudes), a paired t-test or repeated measures ANOVA was applied. For comparing between groups (e.g. between amplifiers or pipette shielding conditions), an unpaired t-test was applied. Convolutions were carried out in Matlab.

**Results**

**EFS-induced hyperpolarization is observed in current clamp recordings**

Current clamp recordings in cultured hippocampal neurons revealed robust hyperpolarization during kEFS (Fig. 1C); spinal neurons behave the same way (see below). Data from 11 neurons tested with all EFS parameter combinations — frequencies of 1, 5, and 10 kHz with amplitudes from 0.5 to 5 mA — are summarized in Fig. 1D. Group averages (±SEM) are shown in black and data from individual cells are shown in gray. Stimulus amplitude had a significant effect ($F_{9,10} = 8.724, p < .001$) whereas frequency did not ($F_{2,10} = 0.897, p = .424$), although there was a significant interaction between amplitude and frequency ($F_{18,10} = 1.795, p = .029$, two-way repeated measures ANOVA). Since 1 kHz EFS evoked the most consistent responses, most subsequent testing focused on this frequency.

To demonstrate the inhibitory effect of this hyperpolarization, we tested whether kEFS-induced hyperpolarization increased rheobase, the minimum injected current required to evoke spiking. Rheobase was measured before and during kEFS by injecting a series of current steps with increasing amplitudes into the neuron (Fig. 2A). Rheobase was significantly increased by 5 mA, 1 kHz kEFS ($t = 9.114, p < .001$, paired t-test), thus confirming that spiking is inhibited by kEFS-induced hyperpolarization. This confirmed that kEFS-induced hyperpolarization is not illusory, but the possibility remained that the act of measuring voltage might introduce the hyperpolarization (i.e. an observer effect); in other words, the voltage change is measured correctly but might arise because of how it is measured (see below).

**kEFS-induced hyperpolarization occurs only in patched neurons**

To quantify kEFS-induced hyperpolarization using a method independent of the PCA, neurons were loaded with a voltage-sensitive dye (VSD) so that voltage could be measured optically (Fig. 3A and B). VSD-based measurements confirmed that 5 mA, 1 kHz EFS caused significant hyperpolarization in patch clamped

Please cite this article in press as: Lesperance LS, et al., Artifactual hyperpolarization during extracellular electrical stimulation: Proposed mechanism of high-rate neuromodulation disproved, Brain Stimulation (2017), https://doi.org/10.1016/j.brs.2017.12.004
neurons (Fig. 3C left panel; \( t = 7.345, p < .001 \), one-sample \( t \)-test). Electrophysiological and VSD-based voltage measurements were tightly correlated (\( r^2 = 0.82 \)) and the slope of the regression line (0.90 ± 0.17) did not differ significantly from the expected slope of 1 (\( t = 0.59, p = .58 \), one-sample \( t \)-test) (Fig. 3C right panel).

Unlike the kEFS-induced change in fluorescence illustrated in Fig. 3A, VSD imaging conducted in the same neuron, but before patching (with the patch pipette positioned in the bath, near but not on the neuron), revealed no kEFS-induced fluorescence change (Fig. 4A). Comparing VSD-measured hyperpolarization induced by 5 mA, 1 kHz EFS applied before patching, during current clamp recording, and again after removal of the patch pipette revealed hyperpolarization only when neurons were patched (Fig. 4B, left panel). This led us to hypothesize that the patch pipette acts as an antenna that converts the varying electric field into current that flows into the neuron. From this, we predicted that shielding the patch pipette with Sylgard would attenuate kEFS-induced hyperpolarization. As predicted, there was a significant interaction between shielding status and patching status on VSD-measured hyperpolarization (\( F_{2,13} = 10.433, p < .001 \); two-way repeated measures ANOVA), with significantly greater kEFS-induced hyperpolarization occurring with unshielded pipettes (\( -21.8 ± 1.4 \) mV; mean ± SEM) than with shielded pipettes (\( -10.0 ± 1.5 \) mV) (\( p < .001 \), Student-Newman-Keuls test) (Fig. 4B). Electrophysiological measurements using a PCA corroborated these findings (Fig. 4C, \( t = 3.622, p = .003 \), \( t \)-test).

To ensure that the artifactual nature of kEFS-induced hyperpolarization was not limited to 1 kHz EFS, VSD measurements were conducted in additional cells tested with 3.5 mA, 10 kHz EFS. As with 1 kHz EFS, patched neurons exhibited kEFS-induced voltage changes (Fig. 4D). The effect varied across neurons in a manner consistent with variability reported in Fig. 1D, but VSD and electrophysiological measurements were tightly correlated within each neuron (Fig. 4E). VSD imaging in an additional ten unpatched neurons showed no significant fluorescence changes induced by 10 kHz EFS applied at any intensity (Fig. 4F, \( p > .05 \) for all one-sample \( t \)-tests even before correction for multiple comparisons). Fluorescence changes were not converted to voltage changes because calibration curves require patching, but based on typical calibration curves, deviations in Fig. 4F correspond to <1 mV. The

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Fig. 2. kEFS-induced hyperpolarization inhibits spiking. (A) Sample traces show determination of rheobase at baseline (top) and during kEFS (bottom) based on the weakest current injection step able to evoke spiking. (B) Summary of rheobase at baseline and during 5 mA, 1 kHz EFS for 9 neurons. Rheobase was significantly increased by kEFS (\( t = 9.114, p < .001 \), paired \( t \)-test).

Fig. 3. VSD imaging confirms kEFS-induced hyperpolarization of patched neurons. (A) Sample electrophysiology trace (top) and simultaneous VSD measurements (in arbitrary units, AU) from 500 ms-long epochs before, during, and after EFS (bottom). Imaging was conducted in epochs to help minimize bleaching and phototoxicity. The kEFS-induced fluorescence change was quantified as the difference between fluorescence averaged across epochs during kEFS vs fluorescence averaged across the two epochs before and two epochs after kEFS. Insets show differential interference contrast (DIC) image (top) and pseudo-colored VSD image (bottom). (B) Sample calibration curve based on fluorescence measurements during simultaneous current clamp recordings, with voltage changes induced by current injection steps. (C) Summary of VSD-measured hyperpolarization during 5 mA, 1 kHz EFS in 8 neurons (left); hyperpolarization was significant (\( t = 7.345, p < .001 \), one-sample \( t \)-test). Right panel shows correlation between VSD- and electrophysiology-measured voltage changes during kEFS.
lack of kEFS-induced hyperpolarization in unpatched neurons is also consistent with computer simulations (Sup. Fig. S2).

kEFS-induced hyperpolarization depends on the type of amplifier and recording mode

Reports that patch-clamp amplifiers (PCAs) can cause artifacts during current clamp recordings [23,24] led us to suspect that our Axopatch 200B PCA was implicated in converting the electric field into a hyperpolarizing current. We therefore repeated electrophysiology-based measurements of EFS-induced hyperpolarization using an Axoclamp 2B, which operates as a true voltage-follower amplifier (VFA). Fig. 5A shows the response of a typical neuron recorded with this VFA during 5 mA, 1 kHz EFS. Measurements conducted with the VFA revealed no kEFS-induced hyperpolarization (Fig. 5B; 0.1 ± 0.1 mV, t = 0.574, p = .584, one sample t-test) and no change in rheobase (Fig. 5C; t = 0.0, p = 1.0, paired t-test). To verify that kEFS-induced hyperpolarization was not unique to the particular PCA we had used, we measured the effects of 5 mA, 1 kHz EFS between 1 and 5 mA. Fluorescence intensity was not significantly altered from baseline at any intensity (p > .05, one-sample t-tests even before correction for multiple comparisons).
Finally, to assess if the recording mode and associated circuitry were critical, we used a PCA to conduct back-to-back voltage clamp and current clamp recordings in three additional neurons during 2 mA, 1 kHz EFS; weaker EFS was used to avoid saturating the voltage clamp signal. Using each neuron’s input resistance to calculate the current responsible for hyperpolarization, current clamp recordings revealed hyperpolarization consistent with a 44.7 ± 6.4 pA outward current, which is significantly different from 0 (t = 12.0, p = .007, one-sample t-test). Voltage clamp recordings revealed a 14.4 ± 8.5 pA inward current, which is not significantly different from 0 (t = 1.7, p = .23). The discrepancy between the two measurements is significant (t = 5.339, p = .033, paired t-test), demonstrating that the artifact is limited to the current clamp mode of PCAs.

This last observation, namely that recordings conducted with the same Axopatch 200B amplifier (PCA) did or did not exhibit the artifact depending on the recording mode (current clamp vs. voltage clamp, respectively), argues against there being any defect in the stimulator (e.g. DC leak). The proper functioning of the stimulator is further supported by the lack of hyperpolarization in unpatched neurons (see Fig. 4) or in neurons recorded with an Axoclamp 2B amplifier (VFA) (see Fig. 5). Instead, all of the data point to an artifactual hyperpolarization introduced by the PCA when operating in current clamp mode.

**kEFS-induced hyperpolarization of neurons in acute slices**

Like in cultured neurons, pyramidal neurons tested in acute hippocampal slices exhibited kEFS-induced hyperpolarization (Fig. 6A) and the associated change in rheobase (Fig. 6B) when recorded with a PCA but not when recorded with a VFA. Likewise, amongst dorsal horn neurons recorded from lamina I and II of the spinal cord (Fig. 6C, D) the kEFS-induced hyperpolarization was observed only when recording with a PCA but not with a VFA. The proper functioning of the stimulator is further supported by the lack of hyperpolarization in unpatched neurons (see Fig. 4). This last observation, namely that recordings conducted with the same Axopatch 200B amplifier (PCA) did or did not exhibit the artifact depending on the recording mode (current clamp vs. voltage clamp, respectively), argues against there being any defect in the stimulator.
spinal cord slices, kEFS-induced hyperpolarization (Fig. 6C) and the associated change in rheobase (Fig. 6D) were observed with the PCA, but not with the VFA. These results confirm that kEFS-induced hyperpolarization is not limited to a particular cell type or experimental preparation but, rather, is linked to the electrophysiological equipment.

Data presented above demonstrate that kEFS-induced hyperpolarization is an artifact rather than a novel basis for direct inhibition. The true mechanism of action of kEFS remains to be unravelled. To that end, understanding this EFS-induced artifact is critical for reassessing past studies whose data may have been compromised by it and for designing future studies and instrumentation to avoid it. This is relevant not only for neuromodulation research, but for all fields in which extracellular electrical stimulation is used. Thus, the remainder of our study was dedicated to uncovering the basis for the PCA-associated artifact.

**Mechanism of kEFS-induced hyperpolarization**

Inspecting the voltage trajectory at the onset of kEFS reveals that each biphasic EFS pulse evokes a transient hyperpolarization (arrows in bottom panel of Fig. 7A) and that these "unitary" hyperpolarizations appear to summate when EFS pulses are repeated at high rate (red curve in right panel). To characterize the unitary hyperpolarization, we measured hyperpolarization following the first EFS pulse (3 mA) delivered at 150 Hz—the lowest rate at which the stimulator could deliver therapy-grade biphasic rectangular pulses and found that the return of voltage to its baseline was well fit by a double exponential (Fig. 7B). For neurons tested with unshielded pipettes (n = 6), the fast time constant of the polarization decay was 215 ± 38 µs (mean ± SEM) with amplitude of 3.09 ± 0.74 mV and the slow time constant was 9.19 ± 1.86 ms with amplitude of 1.52 ± 0.10 mV. For neurons tested with shielded pipettes (n = 5), the fast time constant was 293 ± 88 µs with amplitude of 3.27 ± 0.48 mV and the slow time constant was 9.00 ± 2.22 ms with amplitude of 0.92 ± 0.26 mV. From here, we focussed on the slow component since the fast component was too brief to summate across the 1 ms interstimulus interval during 1 kHz EFS, and we found that the amplitude (t = 2.318, p = .046, t-test) but not the time constant (t = 0.063, p = .951, t-test) of the slow component was significantly reduced by pipette shielding.

To test whether the slow component of the unitary hyperpolarization summates to yield the cumulative hyperpolarization ascribed to kEFS, we convolved the unitary response with a 1 kHz event train. The voltage trajectory thus modeled (Fig. 7C) closely resembled experimental data (e.g. Fig. 7A). Furthermore, consistent with linear summation, the rate of cumulative hyperpolarization matched the decay time constant of the unitary hyperpolarization. Comparing cumulative hyperpolarization induced by 3 mA, 1 kHz EFS with hyperpolarization predicted from the summation of the unitary response revealed a strong linear correlation (r² = 0.85) whose slope of 0.77 ± 0.14 was not significantly different from the expected slope of 1 (t = 1.66, p = .14, one sample t-test) (Fig. 7D). This result demonstrates that the summation of unitary hyperpolarizations is sufficient to account for sustained hyperpolarization, which again argues against a contribution from other possible factors such as DC leak [29].

Next, we sought to explain how the unitary hyperpolarization arises from the PCA since kEFS-induced hyperpolarization occurs only in PCA-based current clamp recordings (see above). PCAs are designed around an I-V converter optimized for voltage clamp; current clamp is achieved by adding a positive feedback loop [30,31] (Fig. 8A left). Magistretti et al. showed that artifacts can arise in current clamp because of this circuitry [23,24], but they never tested the effects of extracellular EFS. Following their lead in monitoring output current (Iout), we observed that EFS induced nanoampere fluctuations in Itot (Fig. 8B bottom). The fast positive and negative components of Iout were equivalent, consistent with the symmetric EFS waveform and the symmetric fluctuation in bath voltage (Vb), again ruling out DC leak from the stimulator. A few

![Fig. 7. Transient hyperpolarization after individual EFS pulses summate during kEFS.](image)
nanoamperes of current delivered to the bath via the recording pipette (when the pipette is not patched on a neuron) will not affect bath voltage ($V_b$) — orders of magnitude stronger current (i.e. milliamperes) are delivered by the stimulator to the bath. On the other hand, a few nanoamperes delivered directly to the inside of a neuron (during whole cell recording) can dramatically alter membrane voltage ($V_m$). Changes in $V_m$ are slowed by the membrane capacitance, which is notable since the 40 μs-long EFS pulses are very short relative to the membrane time constant, which is tens of milliseconds. Together these factors predict that voltage changes recorded in whole-cell mode (where pipette voltage $V_p = V_b + V_m$) will deviate slowly from the otherwise abrupt voltage changes recorded in the bath (where $V_p = V_b$) because, in the former case, $I_{out}$ will affect $V_m$ and vice versa, thus creating a feedback loop through which $V_m$ is unrecoverably altered (see box in Fig. 8B).

Sample traces in Fig. 8B confirm this prediction. In all neurons tested ($n = 6$), voltage deviations indicative of altered $V_m$ (see *s on Fig. 8B) appeared after an upward voltage deflection and were greater when the upward deflection preceded the downward deflection during biphasic EFS. This difference in unitary hyperpolarization predicts that cumulative hyperpolarization will differ depending on whether EFS evokes up-down or down-up voltage deflections, which depends on the anode-cathode designation on EFS leads and the neuron’s position in the electric field. The prediction was confirmed (Fig. 8C, $t = 4.26$, $p = .008$, paired t-test).

**Discussion**

Our results demonstrate that kEFS does not induce hyperpolarization that could account for the analgesic effects of high-rate SCS. Specifically, kEFS hyperpolarizes neurons only when they are recorded with a PCA, which is obviously not the conditions under which neurons normally operate. According to VSD imaging, unpatched neurons were not hyperpolarized during kEFS (Fig. 4), and nor was any kEFS-induced hyperpolarization observed in neurons recorded with a VFA (Fig. 5 and Fig. 6). Further testing uncovered an artifact linked to the current clamp mode of PCAs. The lack of kEFS-induced hyperpolarization — after excluding the artifact — is consistent with past studies (see Introduction) showing that kEFS is more likely to cause depolarization, and only when applied at frequencies and amplitudes higher than those used here or by Lee et al. [21]. Beyond disproving kEFS-based direct hyperpolarization as a novel mode of action for high-rate SCS, our results raise important concerns about artifacts that arise during extracellular EFS.

Please cite this article in press as: Lesperance LS, et al., Artifactual hyperpolarization during extracellular electrical stimulation: Proposed mechanism of high-rate neuromodulation disproved, Brain Stimulation (2017), https://doi.org/10.1016/j.brs.2017.12.004
Magistretti and colleagues previously raised concerns about current clamp recordings conducted with PCAs. Their first report emphasized the mismeasurement of membrane voltage [23], but subsequent work clarified that voltage was actually perturbed during the measurement process [24]. This constitutes an observer effect: Whereas a simple mismeasurement caused by the quasi-static superposition of fluctuations in $V_m$ on top of an otherwise accurate signal representing $V_m$ could be corrected post-recording (e.g. by subtracting changes in $V_m$ from $V_p$ to isolate changes in $V_m$), the introduction of an artifactual current during the recording process introduces uncorrectable changes in the voltage since nonlinearities arise from the numerous voltage-gated ion channels in the cell membrane. The problem occurs because PCAs are optimized for low-noise voltage clamp recording. Current clamping is achieved in PCAs in a rather convoluted way, which, as we have shown, can be compromised by extracellular EFS. Specifically, when EFS-induced changes in $I_{out}$ feed back onto $V_m$, voltage measurement is compromised in a subtle yet irreversible way (unlike the simple superposition of fluctuations in $V_m$ on top of an otherwise accurate signal representing $V_m$).

The impact on $V_m$ of ultra-short fluctuations in $I_{out}$ is small (Fig. 8) and has, therefore, likely gone unrecognized in past experiments that utilized EFS in conjunction with PCA-based current clamp recordings. However, if $I_{out}$ fluctuations recur before the unitary hyperpolarization has dissipated, the effects summate and cause substantial cumulative hyperpolarization (Fig. 7). When EFS is applied at kilohertz frequencies, the cumulative hyperpolarization became obvious, and suspicious. We focused on summation occurring at 1 kHz EFS but, at higher rates, even faster voltage fluctuations may summate, which likely explains the strong but highly variable voltage responses observed during 5 and 10 kHz EFS. But even when single pulses are used, PCA-based voltage measurements during or shortly after the pulse are likely to be compromised. Separate from measuring voltage, other measurements like rheobase and chronaxie would also be affected by the introduction of artifactual current from the PCA during extracellular stimulation. It is difficult to judge the degree to which past studies may have been affected because there are numerous factors (e.g. pipette shielding) that impact the degree to which this artifact is manifest, but preventive steps should be taken to avoid this artifact in future studies.

Conclusions

The biophysical mechanism underlying the analgesic effect of high-rate SCS remains unclear. According to simulations, nerve block during high-rate SCS required exceedingly strong stimulus intensities [22] and transient neuronal activation typically precedes the nerve block induced by kEFS [5], yet there is no clinical evidence for transient activation during high-rate SCS [20]. Furthermore, results from the Evaluation of Spinal Cord Stimulation Pulse Rate On Clinical Outcomes (PROCOST) clinical trial suggest that SCS at rates between 1 and 10 kHz provides equivalent pain relief [32], yet the bottom end of that range falls well outside the frequency range associated with nerve block. Beyond the presence or absence of paresthesia, other differences between low- and high-rate SCS such as optimal lead placement [33] hint at the differences in mechanism but do not point to obvious candidate mechanisms [20]. The rise and fall of direct inhibition via kEFS-induced hyperpolarization as a completely novel mode of action should serve as a cautionary tale about artifacts caused by electric fields. Vigilance against such artifacts is crucial for neuromodulation research and other fields utilizing electrical stimulation. At the very least, PCAs should be avoided (or used with caution) when conducting current clamp recordings when EFS is involved, especially if the goal is to measure the effects of that stimulation. More generally, independent approaches (e.g. VSD imaging) and technology (e.g. amplifiers with different designs) should be used to cross-validate results.

Financial support

This research was funded by Boston Scientific(R_2016_013533). SAP is supported by a Canadian Institutes of Health Research (CIHR) New Investigator Award and an Ontario Early Career Award. ML is supported by a fellowship from Fonds de recherche du Québec - Santé (FRQS).

Authorship statement

LSL and SR conducted experiments. ML and TZ conducted simulations. LSL, ML, TZ, SR and SAP analyzed data. All authors contributed to the study design and manuscript preparation.

Conflicts of interest

SAP is on the Scientific Advisory Board and TZ and RE are paid employees of Boston Scientific.

Acknowledgements

We wish to thank Brad Hershey (Boston Scientific) and Marom Bikson (The City University of New York) for helpful input and Russell Smith (The Hospital for Sick Children) for expert technical assistance.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.brs.2017.12.004.

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Please cite this article in press as: Lesperance LS, et al., Artifactual hyperpolarization during extracellular electrical stimulation: Proposed mechanism of high-rate neuromodulation disproved, Brain Stimulation (2017), https://doi.org/10.1016/j.brs.2017.12.004