Examining protection from anoxic depolarization by the drugs dibucaine and carbetapentane using whole cell recording from CA1 neurons

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White SH, Brisson CD, Andrew RD. Examining protection from anoxic depolarization by the drugs dibucaine and carbetapentane using whole cell recording from CA1 neurons. J Neurophysiol 107: 2083–2095, 2012. First published January 25, 2012; doi:10.1152/jn.00701.2011.—As an immediate consequence of stroke onset, failure of the Na⁺-K⁺-ATPase pump evokes a propagating anoxic depolarization (AD) across gray matter. Acute neuronal swelling and dendritic beading arise within seconds in the future ischemic core, imaged as changes in light transmittance (ΔLT). AD is itself not a target for drug-based reduction of stroke injury because it is generated in the 1st min of stroke onset. Peri-infarct depolarizations (PIDs) are milder AD-like events that recur during the hours following AD and contribute to infarct expansion. Inhibiting PIDs with drugs could limit expansion. Two types of drugs, “caines” and σ₁-receptor ligands, have been found to inhibit AD onset (and may also oppose PID initiation), yet their underlying actions have not been examined. Imaging ΔLT in the CA1 region simultaneously with whole cell current-clamp recording from CA1 pyramidal neurons reveal that the elevated LT front and onset of the AD are coincident. Either dibucaine or carbetapentane pretreatment significantly delays AD onset without affecting resting membrane potential or neuronal input resistance. Dibucaine decreases excitability by raising spike threshold and decreasing action potential (AP) frequency, whereas carbetapentane eliminates the fast afterhyperpolarization while accentuating the slow afterhyperpolarization to reduce AP frequency. Orthodromic and antidromic APs are eliminated by dibucaine within 15 min but not by carbetapentane. Thus both drugs reduce cortical excitability at the level of the single pyramidal neuron but through strikingly different mechanisms. In vivo, both drugs would likely inhibit recurring PIDs in the expanding penumbra and so potentially could reduce developing neuronal damage over many hours post-stroke when PIDs occur.

stroke; pyramidal; neuroprotection

CORTICAL NEURONS UNDERGO a propagating wave of depolarization within 1–2 min following focal stroke onset, leading to formation of an ischemic core over hours. The extent of this ischemic or “anoxic” depolarization (AD) represents the most reliable determinant of ensuing brain damage (Kaminogo et al. 1998). As the earliest upstream event leading to necrosis in the core, AD blockade is not considered a clinical target for improving stroke outcome because AD is over long before the patient reaches the endoplasmic reticulum (ER) and treatment can commence. However, in the hours following stroke onset, recurrent peri-infarct depolarizations (PIDs) spread from the edge of the ischemic core out into the penumbra (Nedergaard and Astrup 1986; Nedergaard and Hansen 1993). Here, ATP levels fall ~50% compared with 75–90% in the core (Folbergrova et al. 1992), so these events are AD-like but are able to repolarize. Their recurrence further depletes energy reserves of penumbral neurons over many hours and likely promotes dysfunction. PIDs increase both growth rate (Hartings et al. 2003) and volume of the core in rodent models (Back et al. 1996; Busch et al. 1996; Takano et al. 1996). They can be imaged coursing along the surface of mouse neocortex following focal stroke (Farkas et al. 2008). Clinically, PIDs are recorded from the cortical surface of patients in the hours and days following head trauma (Fabricius et al. 2006) or stroke (Dohnen et al. 2008). Thus a surprisingly prolonged “window of opportunity” is available for suppressing these recurring events.

During AD, extracellular K⁺ suddenly climbs to 50–60 mM, whereas extracellular Na⁺ (from 130 to ~50 mM) and Ca²⁺ (from ~2 to 0.1 mM) decrease precipitously (Lipton 1999). These ionic movements toward ionic equilibrium are initially through voltage-gated channels and possibly through pannexin-1 hemichannels (Thompson et al. 2006). Rupturing of neurons would also support such change over the longer term. Reducing extracellular Na⁺ from ~145 to 28 mM does not significantly alter AD onset (Tanaka et al. 1997), although AD amplitude is reduced in low extracellular Na⁺ (Yamamoto et al. 1997). Glutamate receptor activation is not required for the generation or propagation of cortical AD in adult rodent slices (Anderson et al. 2005; Jarvis et al. 2001; Muller and Somjen 2000; Tanaka et al. 1997) and in vivo (Hernandez-Caceres et al. 1987; Lipton 1999; Murphy et al. 2008; Nellgard and Wieloch 1992). There is no evidence that any transmitter agonist or antagonist affects AD onset, probably because synapses fail before AD onset. AD is also not stopped by blocking of synaptic transmission with low calcium (Jarvis et al. 2001; Obeidat et al. 2000; Obrenovitch et al. 2000) or with K⁺ or Ca²⁺ channel blockers (Yamamoto et al. 1997). On the other hand, numerous studies have found that the “caine” family of Na⁺ channel blockers significantly delay AD onset (Taylor et al. 1999; Urenjak and Obrenovitch 1996; Weber and Taylor 1994). This is also true for some σ-receptor (σR) ligands (Anderson et al. 2005). Like the caines, certain σR ligands have been proposed to be neuroprotective in models of stroke and Alzheimer’s disease (Maurice and Su 2009). Neither type of drug has been examined with intracellular recording to observe drug effects at the level of the cortical neuron.

A reasonable assumption is that a drug blocking or delaying AD onset will also inhibit PID onset in vivo. However, PIDs are difficult to distinguish from spreading depression (SD) in vivo. A PID that propagates into uncompromised tissue is essentially an SD event. Although not measured to date, small amounts of both drugs likely cross the blood-brain barrier because dibucaine is neurotoxic at higher concentrations in...
patients, whereas carbamapentane (CP) is quite lipophilic (log $P$ value of 4.2). Here, we examine how dibucaine, a potent Na$^+$ channel blocker (APPENDIX) and AD inhibitor, works at the level of the single pyramidal neuron to reduce susceptibility to AD (and probably to PIDs as well). We compare the effects of dibucaine with CP (a $\sigma$R ligand that also delays AD onset) to try to identify a common mechanism of AD inhibition. (For more information on both compounds, see APPENDIX.)

**METHODS**

**Hippocampal Slice Preparation**

A protocol including these studies was approved by the Queen’s University Animal Care Committee. Male Sprague-Dawley rats, 21–28 days old (Charles River, Saint-Constant, Quebec, Canada), were cared for in accordance with the Canadian Council on Animal Care. They were housed in a controlled environment (22 ± 1°C, 12:12-h light-dark cycle) with Purina rat chow and water supplied ad libitum. A rat was placed in a rodent restrainer and guillotined. The brain was excised within 1 min and placed in ice-cold oxygenated (95% $O_2$–5% $CO_2$) high-sucrose artificial cerebrospinal fluid (aCSF).

Hippocampal slices (Fig. 1A) were cut in the coronal plane (400 μm) using a vibrating blade microtome (Leica VT1000 S; Wetzlar, Germany). Slices were transferred to a net submerged in a beaker of regular aCSF gassed with $O_2$-CO$_2$, at 22°C and slowly warmed to 31°C over 1 h for electrophysiological recording and imaging. Experiments involving AD were performed at 35°C.

**Experimental Solutions**

Control aCSF contained (in mM): 120 NaCl, 3.3 KCl, 26 NaHCO$_3$, 1.3 MgSO$_4$, 1.2 NaH$_2$PO$_4$, 11 d-glucose, and 1.8 CaCl$_2$ (pH 7.3–7.4, 295 mosmol/kgH$_2$O). High-sucrose aCSF included 240 mM sucrose. The pH was adjusted to 7.3 with KOH, and osmolality was 270 mosmol/kgH$_2$O. Series resistance was compensated by a Bessel filter (LPF 202a; Molecular Devices) provided low-pass filter- ing at 10 Hz. Pipette junction potentials were corrected just before achieving whole cell mode. Orthodromic responses by CA1 neurons were evoked by stimulating the alveus. Single-cell recording in the granule cell layer (GC) was in the upper blade. Sch. collat., Schaffer collaterals; PYR, pyramidal neurons; OR, stratum orions; RAD, stratum radiatum; LM, lacunosum molecular. B: individual components of the anoxic depolarization (AD) trace recorded under current-clamp in the single cell. AD initiation (1) is the point where the initial slow depolarization transitions to a more rapid depolarization in minutes and seconds. The peak potential (2) of the AD is the point where the slope of the fast AD transitions to the slope of the late depolarization (in millivolts). The fast AD amplitude (3) was measured between AD initiation and the peak potential (in millivolts). The duration of the late depolarization (4) was measured between the peak potential and the maximal depolarization (in millivolts). $I_{Na^+}, Na^+$ currents; OGD, oxygen-glucose deprivation.

**Single-Cell Electrophysiology**

The micropipette solution contained (in mM): 130 K$^+$-glucuronate, 10 KCl, 1.1 MgCl$_2$, 5 EGTA, 10 HEPES, 2 Na$^+$-ATP, and 0.1 CaCl$_2$. The pH was adjusted to 7.3 with KOH, and osmolality was 270 mosmol/kgH$_2$O. In one group of experiments, 10 or 100 μM dibucaine was added to the micropipette solution, replacing equimolar K$^+$-glucuronate.

CA1+ pyramidal cells or granule cell neurons (Fig. 1A) were “blind” patch-clamp recorded from the CA1 hippocampal neurons were made in current-clamp mode. Orthodromic (ortho.) action potentials (APs) of CA1 neurons were activated by stimulating (stim.) the stratum radiatum (RAD) near the CA3 region, and antidromic (anti.) activation involved stimulating the alveus. Single-cell recording in the granule cell layer (GC) was in the upper blade. Sch. collat., Schaffer collaterals; PYR, pyramidal neurons; OR, stratum orions; RAD, stratum radiatum; LM, lacunosum molecular. B: individual components of the anoxic depolarization (AD) trace recorded under current-clamp in the single cell. AD initiation (1) is the point where the initial slow depolarization transitions to a more rapid depolarization in minutes and seconds. The peak potential (2) of the AD is the point where the slope of the fast AD transitions to the slope of the late depolarization (in millivolts). The fast AD amplitude (3) was measured between AD initiation and the peak potential (in millivolts). The duration of the late depolarization (4) was measured between the peak potential and the maximal depolarization (in seconds). $I_{Na^+}, Na^+$ currents; OGD, oxygen-glucose deprivation.

Orthodromic responses by CA1 neurons were evoked by stimulating Schaffer collaterals of CA3 axons in stratum radiatum (Fig. 1A) using a concentric bipolar electrode (Rhodes Electronics, Woodland Hills, CA) with a shaft diameter of 0.25 mm. An antidromic response was initiated only after a stable membrane potential was obtained and <5 min had passed to allow for dialyzing the pipette solution into the cell. Neuronal recordings were acquired before and following 30 min of drug administration by bath at 31°C. Initial control values of several electrophysiological parameters were pooled and compared with the post-30-min values. Data were analyzed offline with Clampex software (version 10.1). Then, temperature was slowly increased to 35°C over 10 min for a total of 40 min of drug pretreatment before inducing simulated ischemia with OGD. Several properties of the induced AD were measured (Fig. 1B).

The current pulse injection protocol was nine sequential 1-s current steps of 100 pA ranging between −500 and 300 pA and occurring every 6.5 s. Trains of action potentials (APs) were elicited at depolarizing current levels, and estimates of whole cell input resistance were obtained from the slope of the current-voltage relationship (I-V) curve at negative current pulse levels using Clampfit software.

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was elicited by stimulating the alveus adjacent to the recorded CA1 neuron. Stimulation (0.5 ms in duration, 0.1 Hz) was at the minimal strength to evoke a single AP, which was monitored until failure or until the 30-min drug treatment elapsed. After orthodromic failure, excitatory postsynaptic potential (EPSP) amplitude continued to be monitored at the same stimulus strength and frequency.

**Imaging $\Delta LT$**

$\Delta LT$ of the slice were monitored in real-time during OGD. Cell swelling was imaged as an increase in LT during AD initiation/propagation in cortical brain slices. Dendritic damage was imaged as decreased LT in the wake of the AD front that followed the LT increase (Obeidat and Andrew 1998). This LT reduction results from light scattering caused by dendritic beads forming within minutes of AD onset in brain slices (Andrew et al. 2002; Jarvis et al. 1999; Polischuk et al. 1998). This coincides with comparable beading during AD in the intact mouse neocortex detected as a reduction in light reflectance (Murphy et al. 2008). The slice was illuminated using a broadband, voltage-regulated halogen light source (Fig. 2A) on an upright light microscope. The light traversed a band-pass filter that transmitted red and near-infrared light (690–1,000 nm). Video frames were acquired using a 12-bit digital camera (Hamamatsu C4742-95) set to an exposure time of 0.05 s. Images were acquired at 32 frames/s and averaged using Imaging Workbench (IW6) software (INDEC BioSystems, Santa Clara, CA). To synchronize imaging with electrophysiology, Clampex software triggered the IW6 image acquisition. The first averaged image in a series served as a control ($T_0$), which was subtracted from each subsequent experimental image of that series ($T$). The resulting series of subtracted images revealed changes in LT over time [using the formula $\Delta T/T_0 = (T - T_0)/T_0$], which was displayed using a pseudocolor intensity scale (Fig. 2A). Zones of interest were selected to quantify and display graphically the $\Delta T$ data offline. Graphing and statistical analyses of data were carried out using SigmaPlot for Windows (Jandel Scientific) or Microsoft Excel. Images were imported, and figures were prepared using CorelDRAW Graphics.

**Statistical Analysis**

Neurons were analyzed if they displayed stable resting membrane potentials of at least −55 mV and if the series resistance and capacitive currents could be sufficiently compensated. Recordings were terminated if access resistance increased to where it could not be compensated using the bridge balance. Statistical significance was determined by unpaired $t$-tests, and all data are presented as means ± standard deviation. Control data were pooled before drug application and compared with a 30-min pretreatment of each concentration and drug type. The significance criterion was set at $P < 0.05$.

**RESULTS**

**Simultaneous Current-Clamping and $\Delta LT$ Imaging During OGD**

Within 6 min of OGD, a front of elevated LT coursed through the gray matter of the CA1 region (Fig. 2A), propagating at a rate of 2–4 mm/min. AD onset time in the CA1 region was measured as the time point when the LT front...
passed by the recording electrode (Fig. 2, B and C; 4:37 min), an average of 5.3 ± 1.3 min after switching to OGD (n = 41 slices). Under whole cell current-clamp, the recorded AD waveform was subdivided into 3 components: a small but prolonged early depolarization, an ensuing rapid depolarization (the fast AD) coincident with the LT front, and a later slow depolarization that approached 0 mV (Fig. 1B). CA1 cells consistently underwent fast AD (mean amplitude 28.8 ± 6.2 mV), coinciding with the LT front passing the micropipette (n = 13; Table 1) with a slope of 2.7 ± 1.5 mV/s and a peak potential of −9.4 ± 4.6 mV. The slower, late depolarization (also called the persistent depolarization) had a slope of 0.28 ± 0.16 mV/s and took an average of 46.4 ± 31.3 s before reaching 0 mV. Six additional recordings were obtained without ATP in the pipette (to eliminate the possibility of this additional energy source interfering with the AD waveform).

The absence of ATP in the pipette had no discernible effect on AD (data not shown).

Following AD, membrane potential remained near 0 mV while the slice evolved optical signs of damage (Fig. 2B, bottom right). There was no evidence of repolarization as monitored for up to 30 min post-OGD. There was also a continued decrease in LT within CA1 dendritic regions over the minutes following AD propagation with no sign of recovery after return to control aCSF. The reduction in LT in these regions is partly the result of dendritic beading that scatters light despite maintained slice swelling (see DISCUSSION). Once AD propagated, the LT decrease was inevitable, although some neurons could return to near baseline membrane potential if OGD was immediately terminated on AD initiation (data not shown).

### Bath Pretreatment with Dibucaine or CP

An important initial component of AD is a massive influx of Na⁺ into neurons, so various attempts to inhibit AD have involved the use of sodium channel blockers. Dibucaine has proven to be the most effective caine tested in our laboratory based on imaging ΔLT (Douglas et al. 2011). As demonstrated with other caines (Creveling et al. 1983), bath-applied dibucaine is thought to enter neurons slowly through briefly opened Na⁺ channels and then bind to the channel on the intracellular side to block the pore. In the current study, hippocampal slices preexposed to 40 min of 10 μM dibucaine displayed significantly delayed AD onset from 5.3 ± 1.3 to 9.3 ± 1.9 min (P < 0.001) in nine slices tested (Fig. 3, A and C). Pretreatment with 1 μM dibucaine caused no delay in this CA1 study (Fig. 3C) but was significantly effective on AD onset in neocortical slices (Douglas et al. 2011).

### Dibucaine Introduction Intraneuronally

#### Single CA1 pyramidal cells.

The primary pharmacological effects of dibucaine have been attributed to its binding within the voltage-gated sodium channel, but it also has poorly defined effects on the membrane itself. As an alternative to bath exposure, we directly introduced 10 or 100 μM dibucaine through the recording pipette. This tested whether the AD recorded in the single neuron exposed to the drug could be altered as the LT front passed through neighboring neurons and glia not exposed to the drug. Dibucaine acting within the recorded cell alone could not slow or stop the fast AD as measured either electrophysiologically or as the LT wave front. This is not surprising because AD is a population event. However, either concentration of dibucaine clearly slowed depolarization of the recorded cell itself, particularly during the late AD (Fig. 4, A–C). Ten micromolar dibucaine increased the late AD duration by >50% to 73.2 ± 37.2 s (P < 0.05; n = 11), whereas 100 μM dibucaine increased it by >250% to 124.5 ± 50.9 s (P < 0.001; n = 14; Fig. 4, B and C, and Table 1). Dibucaine at 100 μM also reduced the mean amplitude, peak potential, and slope of the fast AD as well as the slope of the late AD (Table 1). However, inevitably, the neuron completely depolarized.

#### Single-granule cells.

AD propagation is also imaged in the dentate gyrus (data not shown). To examine whether the intracellular effects of dibucaine on AD were specific to CA1 pyramidal cells, granule cells of the dentate gyrus were recorded without (Fig. 5A) and with (Fig. 5B) 10 μM dibucaine in the pipette. This concentration reduced the fast AD amplitude from 23.3 ± 8.2 mV in 10 untreated granule cells to 15.8 ± 5.3 mV (P < 0.01; n = 13; Table 2). Moreover, it reduced the peak potential of the fast AD from −11.4 ± 6.0 mV in untreated granule cells to −23.3 ± 6.3 mV (P < 0.01). Importantly, the latency to reach 0 mV following AD increased to 132.7 ± 63.7 s compared with 44.2 ± 27.1 s (P < 0.001) in

### Table 1. Anoxic depolarization properties recorded in CA1 pyramidal cells evoked by oxygen/glucose deprivation

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 17)</th>
<th>10 μM Dib (n = 11)</th>
<th>100 μM Dib (n = 14)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast AD ampl, mV</td>
<td>28.8 ± 6.2</td>
<td>26.1 ± 10.2</td>
<td>18.9 ± 6.9*</td>
<td>Reduced</td>
</tr>
<tr>
<td>Peak potential, mV</td>
<td>−9.4 ± 4.6</td>
<td>−15.4 ± 11.0</td>
<td>−23.4 ± 11.2†</td>
<td>Reduced</td>
</tr>
<tr>
<td>Slope of fast AD, mV/s</td>
<td>2.7 ± 1.5</td>
<td>2.1 ± 1.6</td>
<td>1.5 ± 0.4*</td>
<td>Reduced</td>
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<tr>
<td>Slope of slow depol, mV</td>
<td>0.28 ± 0.16</td>
<td>0.18 ± 0.06</td>
<td>0.16 ± 0.04*</td>
<td>Reduced</td>
</tr>
<tr>
<td>Late AD duration, s</td>
<td>46.4 ± 31.3</td>
<td>73.2 ± 37.2*</td>
<td>124.5 ± 50.9†</td>
<td>Increased</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05; †P < 0.01. Dib, dibucaine; fast AD ampl, amplitude of fast component of anoxic depolarization (AD); peak potential, peak amplitude of the depolarization; slope of fast AD, slope of the fast component of AD; slope of slow depol, slope of slow component of depolarization; late AD duration, duration of the late or persistent depolarization.

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untreated granule cells (Table 2). Thus 10 μM dibucaine pretreatment slowed AD progression within individual granule cells in a manner similar to CA1 neurons. As expected, simply attempting to block AD in the single neuron did not affect AD within the population. The remnant of the fast AD recorded in these neurons (and CA1 cells) may actually be driven by the rise in extracellular K⁺ concentration from AD generated in neighboring neurons.

Dibucaine and Ouabain-Induced AD

Adding 100 μM ouabain to aCSF initiated an event similar to OGD-induced AD (Fig. 6A). Ouabain directly inhibits the Na⁺-K⁺-ATPase by binding to the pump on the extracellular side of the plasma membrane. Latency to AD onset induced by ouabain (6.3 ± 0.1 min, n = 17) was not statistically different from slices exposed to OGD (5.3 ± 1.3 min, n = 11). Ouabain induced a slightly steeper fast AD (slope of 3.9 ± 1.3 mV/s) compared with OGD (2.7 ± 1.5 mV/s). As well, ouabain increased the peak potential (~4.0 ± 1.8 mV) compared with OGD (~9.4 ± 4.6 mV; P < 0.05; Table 3, Fig. 6A). Therefore, ouabain-induced AD was qualitatively similar to that induced by OGD but progressed slightly faster and was marginally more depolarizing.

Ten micromolar dibucaine pretreatment for 40 min prevented AD in all six slices exposed to 10 min of 100 μM ouabain. This was more dramatic than the simple delay in onset induced by OGD. However, with ouabain, the membrane potential did slowly depolarize from a resting level of around ~65 mV to a mean level of ~26.0 ± 5.50 mV by 10 min of ouabain (Table 3, Fig. 6B). So, compared with
Intracellular Sodium Channel Blocker Effects on CA1 AD

<table>
<thead>
<tr>
<th>Control</th>
<th>0 mV</th>
<th>Late AD</th>
<th>Fast AD</th>
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<tr>
<td>10 μM Dibucaine</td>
<td>0 mV</td>
<td>Late AD</td>
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<tr>
<td>100 μM Dibucaine</td>
<td>0 mV</td>
<td>Late AD</td>
<td>Fast AD</td>
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</table>

Fig. 4. CA1 recordings of AD onset during intracellular introduction of Na+ channel blockers compared with control. A: no drug was included in the patch pipette. B and C: 10 and 100 μM Dib, respectively, reduced the fast AD (but not its onset time), thereby prolonging the late AD. Such a delay in reaching complete depolarization is considered neuroprotective.

complete depolarization induced by ouabain alone (Fig. 6A), 10 μM dibucaine inhibited the fast AD such that the CA1 neuron only partially depolarized and then remained at about −40 mV for the duration of the recording, essentially blocking a full-blown AD.

Drug Effects on CA1 Intrinsic and Synaptic Properties

Bath-applied dibucaine effects. To help characterize how dibucaine may delay AD onset and progression, single CA1 neurons were whole cell-patched under current-clamp mode to measure changes in their intrinsic and synaptic electrophysiological properties. An AP train evoked by a depolarizing pulse lasting 1 s revealed changes in AP threshold and waveform depending on the drug and its concentration (Fig. 7, A and B).

Intracellular Effects of Dibucaine on Granule Cell AD

<table>
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<tr>
<th>Control</th>
<th>0 mV</th>
<th>Late AD</th>
<th>Fast AD</th>
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<tbody>
<tr>
<td>10 μM Dibucaine</td>
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<td>Fast AD</td>
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Fig. 5. Granule cell recordings and AD. A: control recording. B: intracellular introduction of 10 μM Dib. As in CA1 neurons, the AD onset time was unaffected, but the fast AD was attenuated, prolonging the late AD and delaying complete depolarization.

Table 2. AD properties recorded in granule cells evoked by oxygen/glucose deprivation

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 13)</th>
<th>10 μM Dib (n = 10)</th>
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<tr>
<td>Fast AD ampl, mV</td>
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<tr>
<td>Slope of fast AD, mV/s</td>
<td>1.0 ± 0.6</td>
<td>0.75 ± 0.3</td>
<td>No effect</td>
</tr>
<tr>
<td>Slope of slow depol, mV/s</td>
<td>0.29 ± 0.20</td>
<td>0.22 ± 0.10</td>
<td>No effect</td>
</tr>
<tr>
<td>Late AD duration, s</td>
<td>44.2 ± 27.1</td>
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Values are means ± SD. *P < 0.05; †P < 0.01.

Neither resting membrane potential nor whole cell input resistance were altered after 30 min of bath pretreatment with either 1 or 10 μM dibucaine (Table 4). During depolarizing current injection, 1 μM dibucaine did not alter the fast afterhyperpolarization (fAHP), AP threshold, AP frequency, or AP duration. A surprisingly small reduction in AP amplitude (P < 0.05) was noted, however, in nine CA1 cells examined. Increasing dibucaine concentration to 10 μM enhanced the fAHP amplitude (measured relative to the AP threshold) from −3.4 ± 1.9 mV (n = 45, control aCSF) to −10.8 ± 3.7 mV (n = 20, 10 μM dibucaine; P < 0.001; Fig. 8A, Table 4). The slow afterhyperpolarization (sAHP) of the last AP in the train increased from −5.8 ± 1.8 to −15.8 ± 5.0 mV (P < 0.01; Fig. 8B). Ten micromolar dibucaine pretreatment also increased AP threshold, shifting it from −47.5 ± 4.4 mV (n = 45, control aCSF) to −37.7 ± 4.5 mV (n = 20; P < 0.001; Figs. 7B and 9A, Table 4). Corresponding reductions in AP frequency at all three current injection levels (Fig. 9B, Table 4) were likely caused by the increase in AP threshold as well as the increase in both the fast and slow AHP amplitudes.

AP duration measured at half-AP amplitude was unchanged by both 1- and 10 μM dibucaine after the first AP in the train (Table 4). However, after the last AP in the train (measured at

Ouabain-Induced AD in CA1 Neurons

A: Control | 0 mV | 20 mV | 1 min | -62 mV | Ouabain

B: 10 μM Dibucaine | 0 mV | 20 mV | 1 min | -61 mV | Ouabain

Fig. 6. Ouabain-induced AD (100 μM, 10 min) was elicited in all control CA1 neurons (A), whereas a 40-min pretreatment with 10 μM Dib (B) attenuated AD despite ouabain treatment for the full 10 min. The resting potential remained at about −40 mV.
Table 3. AD properties evoked by oxygen/glucose deprivation or ouabain recorded in CA1 neurons

<table>
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Values are means ± SD. *P < 0.05; †P < 0.01. OGD, oxygen/glucose deprivation.

the highest current injection level), AP duration (measured at half-AP amplitude) increased from 1.2 ± 0.2 to 2.0 ± 1.7 ms (n = 9; P < 0.01; Table 5). This was perhaps facilitated by Na+ channel inactivation by the drug, as dibucaine is known to induce a use-dependent block by binding inactivated Na+ channels.

To investigate drug actions further, single APs were syngaptically evoked in CA1 neurons by orthodromic stimulation of the CA3 Schaffer collaterals. Antidromic activation was also evoked by stimulating the alveus region. One micromolar dibucaine was not tested further as it was ineffective at this concentration. Orthodromic APs were lost at an average of 7.4 ± 2.4 min (n = 8) after 10 μM dibucaine exposure measured at 0.5 V above the initial voltage strength required to evoke a typical response (P < 0.01; Fig. 10A, left). AP threshold continued to increase over the following 5–10 min until the evoked spike failed. Evoked EPSPs continually decreased in amplitude over the 30-min treatment, from a maximum of 14.8 ± 5.0 mV at initial AP loss to 6.3 ± 5.0 mV (P < 0.05; n = 8). Antidromic spikes were more resistant to block, taking an average of 15.0 ± 14.6 min to fail at the initial stimulus strength (P < 0.01; Fig. 10A, right). It remained possible to evoke an antidromic AP by increasing the stimulus voltage (data not shown).

After 30 min of pretreatment with 10 μM dibucaine, antidromic APs displayed slightly enhanced depolarizing afterpotentials (DAP) at 3 and 5 ms (Fig. 10B). With 30 and 100 μM CP, the intrinsic electrophysiological properties of 14 CA1 cells (Table 4) were not significantly different from pretreatment with 10 μM CP alone. The exception was the fAHP, which was preserved in 8 of 14 cells (Table 4). The inclusion of 3 μM dibucaine with 30 μM CP

Combined Bath-Applied Effects of Dibucaine and CP

Our observations that dibucaine and CP each reduced the excitability of CA1 neurons through different mechanisms prompted us to search for additive effects of both drugs on the excitability of single neurons. Following 30-min pretreatment with 1 μM dibucaine and 10 μM CP, the intrinsic electrophysiological properties of 14 CA1 cells (Table 4) were not significantly different from pretreatment with 10 μM CP alone. The exception was the fAHP, which was preserved in 8 of 14 cells (Table 4). The inclusion of 3 μM dibucaine with 30 μM CP

The last AP in the train was not measured with 100 μM CP because only a single AP or AP doublet could be evoked.

When single APs were evoked by either antidromic or orthodromic stimulation, DAP amplitude (measured at 3 and 5 ms) was enhanced following pretreatment with 30 μM CP to 18.7 ± 5.3 mV and 15.5 ± 2.8 mV (P < 0.01), respectively, from control values of 11.3 ± 2.5 and 10.9 ± 2.5 mV (Fig. 10, B and C). One hundred micromolar CP enhanced the DAP to 31.9 ± 7.7 and 21.8 ± 3.9 mV as measured in six CA1 cells (P < 0.01; Fig. 10C). At 100 μM CP, orthodromic APs failed at −30 min. Unlike dibucaine, APs could still be evoked at higher voltages, as tested in three CA1 cells (data not shown).

Furthermore, antidromic stimulation (at baseline strength) could generate APs for the entire 30 min of CP exposure in five of six cells with 30 μM CP and one of three cells with 100 μM CP (data not shown). This suggests that the CP was altering spike-related currents more in the soma-dendritic region than in the axon.

![Fig. 7. AP discharge by CA1 neurons is attenuated by both Dib and CP but by different mechanisms. All recordings had resting potentials between −61 and −64 mV. A: an AP train in control aCSF during a 100-pA depolarizing current pulse lasting 1 s (initiated at the arrow). B: a 30-min pretreatment with 10 μM Dib required a 200-pA injection to fire a maximum 2 APs. Dib reduced AP frequency by raising the AP threshold (Table 4). C: a 30-min pretreatment with 100 μM CP also reduced AP frequency but without a corresponding increase in threshold (Table 4). Rather, CP increased post-AP afterhyperpolarizations and slowed AP discharge rates.](image-url)
RMP, mV  

<table>
<thead>
<tr>
<th>Control (n = 45)</th>
<th>3 μM Dib (n = 9)</th>
<th>10 μM CP (n = 12)</th>
<th>30 μM CP (n = 11)</th>
<th>100 μM CP (n = 5)</th>
<th>3 μM Dib and 30 μM CP (n = 11)</th>
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<tr>
<td>RMP, mV</td>
<td>62.5 ± 3.0</td>
<td>59.7 ± 3.0</td>
<td>62.4 ± 3.2</td>
<td>65.6 ± 3.1</td>
<td>64.0 ± 2.6</td>
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<tr>
<td>fAHP, mV</td>
<td>−3.4 ± 1.9</td>
<td>2.7 ± 1.7</td>
<td>Absent* (11/12)</td>
<td>Absent*</td>
<td>Absent*</td>
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<tr>
<td>AP threshold, mV</td>
<td>47.5 ± 4.4</td>
<td>46.0 ± 3.8</td>
<td>47.7 ± 2.7</td>
<td>49.1 ± 2.8</td>
<td>47.0 ± 3.4</td>
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<tr>
<td>AP freq (0.1 nA), Hz</td>
<td>12.7 ± 9.5</td>
<td>12.3 ± 9.5</td>
<td>6.8 ± 4.4*</td>
<td>6.0 ± 2.1*</td>
<td>1.2 ± 1.3†</td>
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<tr>
<td>AP freq (0.2 nA), Hz</td>
<td>24.3 ± 9.5</td>
<td>34.2 ± 16.4</td>
<td>13.2 ± 4.2*</td>
<td>9.4 ± 2.5*</td>
<td>1.6 ± 0.9†</td>
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<tr>
<td>AP freq (0.3 nA), Hz</td>
<td>31.5 ± 8.7</td>
<td>N/A</td>
<td>16.8 ± 5.1†</td>
<td>11.4 ± 2.8†</td>
<td>2.2 ± 1.1↑</td>
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<td>AP ampl, mV</td>
<td>89.6 ± 10.0</td>
<td>70 ± 21.5*</td>
<td>82.3 ± 14.4</td>
<td>74.9 ± 15.4*</td>
<td>62.5 ± 15.0↑</td>
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<td>AP dur (½ ampl), ms</td>
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<td>1.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>1.4 ± 0.9*</td>
<td>2.0 ± 2.6†</td>
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<tr>
<td>R_{in}, MΩ</td>
<td>47.4 ± 8.4</td>
<td>46.6 ± 1.7</td>
<td>45.8 ± 6.3</td>
<td>66.8 ± 17.2*</td>
<td>80.9 ± 16.9↑</td>
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</table>

Values are means ± SD. *P < 0.05; †P < 0.01. CP, carbetapentane; RMP, resting membrane potential; fAHP, fast afterhyperpolarization; AP, action potential; freq, frequency; dur (½ ampl), AP duration at ½ of AP amplitude; R_{in}, input resistance; N/A, not applicable.

Raised the AP threshold of the 1st AP in the train to −38.8 ± 4.6 mV compared with −49.1 ± 2.8 mV with 30 μM alone (P < 0.01; Fig. 9A, Table 4). The increased AP threshold observed at these drug concentrations caused an expected reduction in AP frequency compared with 30 μM CP alone (P < 0.001; Fig. 9B, Table 4). The effect of pretreatment with dibucaine and CP on intrinsic CA1 properties therefore appeared to be additive.

**DISCUSSION**

AD is imaged as a focally initiating, and then propagating, increase in LT (Anderson et al. 2005; Jarvis et al. 2001; Muller and Somjen 1999). We recorded AD in single pyramidal neurons while simultaneously imaging LT within the coronal cerebral slice to study AD initiation and potential recovery. Combining these techniques also helps clarify previous experiments concerning AD delay induced by either dibucaine or CP. Either drug that delayed AD at lower concentrations also preserved synaptic function based on evoked population responses, suggesting that AD could be inhibited without undermining normal cortical function (Douglas et al. 2011). Here, we looked at the level of the single neuron to determine how these drugs may be protective by delaying AD onset and, by extension, inhibiting recurring PIDs. Delaying AD or PID onset protects because gray matter spends less time postdepolarization exposed to OGD, which is when neurons become irreversibly damaged.

**Drug Effects on AD Initiation**

**Bath pretreatment with dibucaine.** Previous experiments in our laboratory showed that 1 μM dibucaine pretreatment delayed AD and at the same time preserved evoked synaptic function in both cortical and hippocampal neurons (Anderson et al. 2005). The present experiment did not detect a delay in AD with 1 μM dibucaine, probably due to the shorter pretreatment time of 30 min. Ten micromolar dibucaine pretreatment, however, was found to delay AD in the present experiment significantly.

With 10 μM dibucaine, the evoked CA1 field potential was reduced by 80% (Douglas et al. 2011). Our current intracellular work revealed that both the orthodromic and antidromic APs were blocked and that evoked EPSP amplitude was significantly reduced during the 30-min pretreatment. It is likely that the inhibition of AD onset by dibucaine at 10 μM results in part from suppression of AP firing by blocking both the sustained and the slow Na^+ currents (I_{NaS}). OGD itself blocks the fast Na^+ current, thereby damping AP discharge and thus synaptic transmission. Additional intracellular parameters were measured to delineate better how reducing pyramidal cell excitability may delay AD, thus providing neuroprotection.

The best documented effect of dibucaine is its action on the local anesthetic binding site within voltage-gated Na^+ channels (Kuroda et al. 2000; Ragsdale et al. 1994), although direct...
Inhibiting Anoxic Depolarization

A Drug Effects Upon AP Threshold

B Drug Effects on AP Firing Frequency

Fig. 9. A: Drug effects on AP threshold in CA1 neurons. Pretreatment with 10 μM Dib for 30 min significantly raised AP threshold in both the 1st and last AP of the train. The threshold of the 1st AP increased to −37.7 ± 4.5 mV compared with −47.5 ± 4.4 mV in control CA1 neurons. The last AP threshold increased to −28.3 ± 4.2 mV from −41.2 ± 4.0 mV. CP failed to raise the threshold of the 1st AP. However, by the last AP in the train, threshold significantly increased in 10 or 30 μM CP. This helps explain why spiking slowed during the spike train (Fig. 8). Addition of 3 μM Dib to 30 μM significantly raised threshold in both the 1st and the last AP. Numbers within bars represent cells tested. B: drug effects on AP firing frequency. Firing was significantly reduced by exposure to 10 or 100 μM CP at all 3 levels of current injection (0.1, 0.2, and 0.3 nA). Likewise, 10 μM Dib reduced firing rate. Adding 3 μM Dib to 30 μM CP significantly reduced frequency compared with 30 μM alone.

measurements of the various Na⁺ currents reduced by dibucaine have not been carried out. Lidocaine is a similar (but less potent) caine that reduces the voltage-activated Na⁺ current in isolated pyramidal neurons (Kaneda et al. 1989).

Intracellular introduction of dibucaine. Dibucaine slowed AD progression when introduced into the single pyramidal neuron or granule cell, even as surrounding neurons go through the standard, faster AD. The fact that dibucaine was able to reduce the fast AD amplitude while delaying the late depolarization indicates that the single neuron itself can dictate much of the intracellular AD waveform. However, a remnant of the fast AD was always recorded. Note that a fast AD is also recorded in astrocytes (data not shown), suggesting that the fast AD is partly generated by sudden efflux of K⁺ by the surrounding neurons synchronously undergoing AD. The current responsible for the fast AD involves Na⁺, Cl⁻, and Ca²⁺ influx and K⁺ efflux (Czeh et al. 1993) but is also driven passively by the sudden increase in K⁺ to ~50 mM. The late depolarization has been attributed to inward Na⁺ and Ca²⁺ currents (Yamamoto et al. 1997).

Bath pretreatment with CP. The mode of action on central nervous system (CNS) neurons of σR ligands had not been examined in detail. Our study supports previous limited data showing no change in CA1 membrane potential, cell input resistance, or AP threshold during 50 min of exposure to 100 μM dextromethorphan (Wong et al. 1988) or to the σR ligand OPC-24439 (Ishihara et al. 1999). Our CP pretreatment reduced CA1 excitability, but, unlike dibucaine, CP did not increase the AP threshold. It did increase the sAHP, which likely helped slow the firing rate. σRs have been localized to plasma membrane, mitochondrial membrane, and dendrites (Alonso et al. 2000), and no staining has been observed on the axons of presynaptic neurons (Alonso et al. 2000). SO, AD delay by CP likely involves the CA1 soma-dendritic region. The unchanged antidromic AP evoked in our study confirms the lack of a σR ligand effect at the level of the axon.

σR ligands have been shown to inhibit voltage-activated K⁺ current in tumor cells (Wilke et al. 1999) and in oocytes expressing voltage-gated K⁺ channels and σR (Aydar et al. 2002). Specific potassium channel effects observed in cardiac cells include decreased delayed outwardly rectifying potassium current, large-conductance (BK) current, and M-current (Zhang and Cuevas 2005). Calcium-activated potassium channels such as BK, small conductances (SK), and intermediate conductance (IK) underlie the AHP in neurons. CP enhanced the sAHP in our study, which would also explain the spike frequency adaptation that we observed (Sah 1996). The spike broadening by CP is also suggestive of BK channel block through its role in spike repolarization.

Some σR ligands (including CP) are neuroprotective in cortical neurons (Hayashi et al. 2000; Katnik et al. 2006), where the authors measured Ca²⁺ increase under simulated ischemia. CP attenuated the increase in intracellular calcium caused by sodium azide (which blocks aerobic metabolism) and glucose deprivation in cultured cortical neurons. This was attributed to direct preservation of intracellular Ca²⁺ homeostasis. The IC₅₀ value (13 μM CP) required to attenuate Ca²⁺ increases caused by simulated ischemia in cultured neurons corresponds to values obtained in our experiments showing AD delay in brain slices.

Dibucaine effects on ouabain-induced AD. Low concentrations of dibucaine have been shown to block AD during 10 min of ouabain exposure (Douglas et al. 2011), but it was unclear whether the drug maintains neurons at a normal resting potential. Our current-clamp recordings with dibucaine show that there is a small but steady depolarization that would not be detected with extracellular DC recording. Thus the lack of a DC shift makes it appear that AD was fully blocked (Douglas et al. 2011) when in fact a slow depolarization was ongoing, which would reduce the evoked population spike. Ouabain-induced AD is more easily blocked with equivalent drug concentrations than OGD-induced AD (Anderson et al. 2005).
Properties of the last AP in the train

Comparing the role of dibucaine in AD delay as a reduction in metabolic load seems to contradict its ability to delay AD induced by ouabain, which is independent of tissue ATP levels (Anderson et al. 2005). It seems likely that dibucaine protects from ouabain and OGD in a similar fashion by inhibiting currents associated with the AD that open as a result of Na\(^+\)-K\(^+\) pump failure.

**Actions of Dibucaine and CP**

Our findings show a reduced neuronal excitability induced by both dibucaine and CP as indicated by the reduction in AP frequency in response to current injection. Identifying the exact underlying mechanism, however, requires voltage-clamp work using various channel blockers. Clearly, dibucaine reduces excitability by increasing AP threshold through the blockade of AP-associated sodium channels, and CP reduces excitability by increasing the sAHP. Subtle calcium or potassium channel effects, however, cannot be excluded as both can be inhibited by local anesthetics (Hirota et al. 1997; Oda et al. 1992). Although both drugs delayed AD through different mechanisms of reduced cortical excitability, it is unlikely that combining both drugs would delay AD any further without compromising slice health.

**CA1 Neuron Recovery from OGD**

The ability of single CA1 neuron to recover membrane potential after AD depends on several factors. As in our study, Tanaka et al. (1997) did not observe recovery by adult CA1 neurons in submerged slices. However, Czeh et al. (1993) demonstrated repeatable “hypoxic spreading depression” events within slices in an interface chamber. Interface slices have the advantage of a rapid diffusion rate from the oxygen-rich atmosphere compared with submerged slices, which draw oxygen from the aCSF. More importantly, by lowering O\(_2\) but not glucose, neurons still have a residual energy source and are thus not truly ischemic. We could quickly reintroduce aCSF immediately following the fast AD by observing the approaching AD front. Such neurons were able to recover full membrane potential after AD had passed the recording electrode, if the aCSF was immediately returned. Accurate recording of whole cell input resistance measured during the recovery period was difficult due to movement of the slice as it swells. However, in the cells that did not display a large increase in access resistance, whole cell input resistance levels were similar to levels measured before OGD exposure (data not shown). This indicates that an additional period of tens of seconds is required for permanent depolarization and dysfunction.

In our experiments that tested recovery of the orthodromic response in CA1 neurons, full APs could not be evoked at any time within the recovery interval (up to 40 min). EPSPs could eventually be evoked but at reduced amplitude compared with pre-OGD. In contrast, antidromic APs could be reactivated once the neuron repolarized enough to restore Na\(^+\) channel activation. Perhaps the lasting effects of AD cause irreparable harm to dendritic spines while preserving AP discharge. In support, no slice showed a return to LT baseline in dendritic regions in recovered slices, further indicating synaptic damage.

![Fig. 10. A: on either orthodromic (left) or antidromic (right) stimulation, 10 \(\mu\)M Dib induced spike failure. The orthodromically evoked spike failed after an average of 7.4 ± 2.4 min, whereas it took about twice as long for the antidromic spike to fail (15 ± 4.6 min). This may be because presynaptic axonal APs in CA3 axon collaterals were more easily blocked than the CA1 axonal AP. B: drug effects on the DAP. Representative traces of antidromically evoked APs in control, 10 \(\mu\)M Dib, and 100 \(\mu\)M CP are shown. The DAP was measured as the voltage amplitude relative to resting membrane potential at 3 and 5 ms after AP threshold. C: CP significantly increased the DAP amplitude at both 3 and 5 ms in a concentration-dependent manner at 30 and 100 \(\mu\)M, whereas 10 \(\mu\)M Dib did not (*\(P < 0.05\)). V, voltage.](http://jn.physiology.org/doi/10.1152/jn.00701.2011)
from continuing dendritic beading. Since the current space clamp is incomplete past the proximal dendrites (Brown et al. 1990), it is possible that distal dendrites remain susceptible to OGD and continue to bead, whereas the cell body recovers to some degree. Also, whereas dendritic beading has shown recovery following brief ischemia (Murphy et al. 2008), CA1 population recovery following AD in slices is more problematic.

Conclusions

This study shows that at low millimolar concentrations, dibucaine (a Na⁺ channel blocker) and CP (a σR ligand) maintain whole cell input resistance and membrane potential of CA1 pyramidal neurons. Therefore, the drugs do not seem to affect the baseline excitability of the neurons. However, they both reduce AP discharge, albeit in distinctly different ways, which helps explain their ability to inhibit AD onset. Reducing spike firing means lowered synaptic communication. Synaptic release/reuptake is a major expenditure of energy in the brain, yet preservation of ATP levels does not seem to be the key protective mechanism because both drugs also delay AD induced by ouabain where the ATP levels do not drop. There are likely other ways that both drug types slow AD onset, but we are still limited in our understanding of the exact mechanisms driving AD (and thus PIDs). For example, we know that the caines block the fast Na⁺ channel driving the AP, but it is not clear how effectively they bind Na⁺ channels driving the sustained and slow Na⁺ channels during OGD (APPENDIX). Indeed, it is not possible to isolate pharmacologically these channels to ascertain their contribution to the AD.

Nevertheless, our results show that the two drugs affect single pyramidal cells in distinctly different ways. It is likely that the specific pharmacophore for the caines that elicits AD inhibition and the pharmacophore for σR ligands are different. Theoretically, drug design techniques can identify these pharmacophores and incorporate their properties into a single drug that might inhibit AD in the submicromolar range.

APPENDIX

Dibucaine is one of the most potent local anesthetics of the caine family based on its binding to the batrachotoxin (BTX) binding site within the pore of the voltage-gated sodium channel (Creveling et al. 1983). IC⁵₀ values are 1.4 μM for dibucaine, 5.4 μM for bupivacaine, 240 μM for lidocaine, and 940 μM for benzocaine (Creveling et al. 1983). No binding occurs at the TTX binding site nor within the Na⁺ channel (Hille 2001). Intracellular location includes both the plasma membrane, hypothalamus, and hippocampus, but not axon fibers or terminals (Hanner et al. 1996). Intracellular location includes both the plasma membrane and intracellular compartments such as mitochondria, ER (Alonso et al. 2000). Activation of σ1R increases delayed outwardly rectifying K⁺ currents (Wilke et al. 1999), BK channels, and the M-current within cardiac neurons (Zhang and Cuevas 2005).

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
**AUTHOR CONTRIBUTIONS**


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