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Extracellular sodium modulates the excitability of cultured hippocampal pyramidal cells

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Abstract

Recent studies demonstrated a photophobia mechanism with modulation of nociceptive, cortico-thalamic neurons by retinal ganglion cell projections, however, little is known about how their neuronal homeostasis is disrupted. Since we have found that lumbar cerebrospinal fluid (CSF) sodium increases during migraine and that cranial sodium increases in a rat migraine model, the purpose of this study was to examine the effects of extracellular sodium ($[Na^+]_o$) on the intrinsic excitability of hippocampal pyramidal neurons. We monitored excitability by whole cell patch using a multiplex micropipette with a common outlet to change artificial CSF (ACSF) $[Na^+]_o$ at cultured neurons accurately ($SD < 7$ mM) and rapidly (< 5 s) as determined by a sodium selective micro-electrode of the same size and at the same location as a neuronal soma. Changing $[Na^+]_o$ in ACSF from 100 to 160 mM, choline-balanced at 310 – 320 mOsm, increased the action potential (AP) amplitude, decreased AP width, and augmented firing rate by 28%. These effects were reversed on returning the ACSF $[Na^+]_o$ to 100 mM. Testing up to 180 mM $[Na^+]_o$ required ACSF with higher osmolarity (345 – 355 mOsm), at which the firing rate increased by 36% between 100 to 180 mM $[Na^+]_o$, with higher amplitude and narrower APs. In voltage clamp mode, the sodium and potassium currents increased significantly at higher $[Na^+]_o$. These results demonstrate that fluctuations in $[Na^+]_o$ modulate neuronal excitability by a sodium current mechanism, and that excessively altered neuronal excitability may contribute to hypersensitivity symptoms.

Keywords

Photophobia; neuronal excitability; extracellular sodium; sodium selective micro-electrode; action potentiation; current clamp; voltage clamp

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1. Introduction

Fluctuating excitability is a fundamental characteristic of neurons and its homeostatic regulation depends on the intrinsic properties of the neuronal membrane, its many synaptic inputs, and the local environment. Failed homeostasis may result in symptoms from excessive excitability, such as epilepsy and the sensory disturbances of migraine, or from decreased excitability, such as depression. For example, the recently reported neuronal pathway for migraine photophobia (Nosedá et al., 2010) must be more excitable to relay the discomfort induced by normal ambient light, though the basis for this excess excitability is not known. Regaining neuronal excitability homeostasis is central to the treatment of these disorders and this may only be achieved with a more complete understanding of how homeostasis fails.

A study of relevant cations that may affect neuronal excitability in the failed homeostasis of migraine highlighted sodium deviation (Harrington et al., 2006b): of lumbar cerebrospinal fluid (CSF) levels of potassium, calcium, sodium, and magnesium, only sodium changed (increased) during migraine. This change was confined to the central nervous system (blood plasma levels were unchanged) and CSF and blood osmolarity did not change. We recently performed ^{23}Na MRI in a rat migraine model (Harrington et al., 2011): cranial sodium increased by 7 – 17% in synchrony with behavioral manifestations of central sensitization and brainstem cFos activation. Furthermore, mathematical simulations revealed that an increase in extracellular sodium, $[\text{Na}^+]_o$, equivalent to that observed in the rat model increases the firing frequency of spontaneous action potentials (APs): To build a cell model, we constructed a simple soma from a representative cylinder (diameter: 20 μm ; length: 20 μm) and a 50 μs time step. The model cell soma includes sodium current, potassium currents (delayed rectifier, Kdr, and A-type, KA), leak current, Na,K-ATPase, and sodium diffusion. The intracellular resistance, was defined as 150 $\Omega\cdot\text{cm}$; membrane capacitance as 1 $\mu\text{F}\cdot\text{cm}^{-2}$, and the resting membrane potential was defined as -65 mV. At 145 mM $[\text{Na}^+]_o$, the model fired at 3.25 Hz and increased by 17 % at 165 mM $[\text{Na}^+]_o$ to 6.38 Hz (Harrington et al., 2011). This simulation supports the notion that rising $[\text{Na}^+]_o$ might increase neuronal excitability.

Sodium homeostasis has long been recognized as essential for neuronal excitability, and sodium permeability increases during the AP (Hodgkin and Katz, 1949; Hodgkin, 1964; Huxley, 1964). Thus, the AP potential peak (where sodium permeability is greater than for other ions) will be close to the sodium reversal potential according to the Nernst equation. This relationship predicts that altered extracellular sodium concentration ($[\text{Na}^+]_o$) will change the AP amplitude, a result confirmed experimentally in squid axon (Hodgkin and Katz, 1949; Hodgkin, 1964). Similar results were obtained in myelinated nerve (Huxley and Stampfli, 1951) and skate heart (Seyama and Irisawa, 1967). Furthermore, lower $[\text{Na}^+]_o$ decreased the height and rising rate of APs in the smooth muscles of the cat ureter (Kobayashi and Irisawa, 1964), and lowered amplitude and increased the width of APs in rat dorsal root ganglion neurons (Amir et al., 1999). Thus, $[\text{Na}^+]_o$ affects AP shape and excitability.

When a neuron is at rest, the Na^+ influx through voltage-gated Na^+ channels is low, as these channels are usually closed or inactivated. However, the channel gate is displaced when $[\text{Na}^+]_o$ increases (Kuo and Liao, 2000). Higher $[\text{Na}^+]_o$ speeds recovery from the inactivation state, enabling an earlier action potential and leading to hyperexcitability (Kuo and Liao, 2000). Higher sodium induces more sub-threshold oscillations in addition to AP changes, which might play a role in neuropathic pain (Amir et al., 1999).

It would be helpful to extend these observations with experiments to examine more details of the effects of increased $[\text{Na}^+]_o$ on the excitability of brain neurons. Hippocampal neuronal excitability is of interest because this region is vulnerable to spreading depression, epilepsy, ischemia, and anoxia (Johnston et al., 1991; Kunkler and Kraig, 2003; Pantoni et al., 2000; Stracciari et al., 2008). The goal of this study was to investigate the effects of $[\text{Na}^+]_o$ on the excitability of cultured hippocampal pyramidal neurons.

2. Results

Primary pyramidal cell culture

The cultured cells were heterogeneous since they were dissociated and cultured from the whole hippocampus. Most are pyramidal cells based on morphology: the cells were phase-bright, with smooth membranes, pyramidal-shaped soma, with one or several thin basal dendrites and one large-diameter apical dendrite, sometimes with branching dendrites (Figure 1A). Since Na^+ , K^+ , ATPase is a key regulator of $[\text{Na}^+]_o$, we studied Na^+ , K^+ , ATPase expression in these cultured neurons. The cultured cells expressed Na^+ , K^+ , ATPase subunits alpha -1 and -3, but not alpha -2, with molecular weight around 112 kDa (arrowed in Figure 1B) and sodium channels with molecular weight around 230 kDa (data not shown). The sodium channels and Na^+ , K^+ , ATPase isoforms were visualized by phase shift and specific immunostaining (Figure 1C; alpha 3 data not shown). Thus, the neurons we studied have pyramidal morphology and express alpha -1 and -3 Na^+ , K^+ , ATPase and sodium channels.

Rapid and accurate switching of $[\text{Na}^+]_o$ for electrophysiology experiments

Figure 1E shows that the sodium selective micro-electrode (SSME) recording of $[\text{Na}^+]$ at the neuronal site changed to each intended value within 5 seconds after each switch. For our series of ACSFs ($n = 3$) at normal osmolarity with a source of ACSF $[\text{Na}^+]$ of 100, 120, 140, 160 mM, the mean $[\text{Na}^+]$ (SD) detected at the soma position was 105.85 (4.02), 119.03 (2.87), 141.75 (0.02), and 156.98 (2.30) mM, respectively. In experiments at higher osmolarity ($n = 3$), the source ACSF $[\text{Na}^+]$ of 100, 140, 180 mM was detected as a mean of 109.70 (5.03), 141.75 (0.02), and 185.07 (6.54) mM, respectively. Thus, the measured $[\text{Na}^+]$ that reached the position of the neuronal soma was close to the $[\text{Na}^+]$ in the delivery pipette, and the change was complete and stable within 5 seconds. The calibration of the SSME from a log scale of the sodium concentration indicated a linear relationship (Figure 1D).

Excitability of cultured neurons and $[\text{Na}^+]_o$

The hippocampal pyramidal neurons we recorded had a resting membrane potential of -64.52 ± 4.54 mV at 100 mM $[\text{Na}^+]_o$, and -63.20 ± 5.37 mV at 160 mM $[\text{Na}^+]_o$ ($P > 0.05$; $n = 7$). Their input resistance was 449.99 ± 312.91 M Ω at a $[\text{Na}^+]_o$ of 100 mM and 425.16 ± 304.25 M Ω at a $[\text{Na}^+]_o$ of 160 mM ($P > 0.05$; $n = 7$). These cells did not fire spontaneously, however, a positive current injection induced a train of APs characteristic of hippocampal pyramidal cells (Figure 2A).

A 3 – 4 mM increase in $[\text{Na}^+]$ in cerebrospinal fluid was reported in migraine (Harrington et al, 2006) that presumably was diluted from a larger shift in intracranial $[\text{Na}^+]_o$, as reflected in the 17% increase in intracranial $[\text{Na}^+]$ in the rat migraine model (Harrington et al, 2011) that is equivalent to a change from 145 ~ 165 mM $[\text{Na}^+]$. Since Na MRI resolution is pushing the limits of sensitivity and a local increase of $[\text{Na}^+]$ may be greater than these reported measures, we recorded the effect on APs of altering $[\text{Na}^+]_o$ between 100 and 180 mM. Figure 2A represents data from a typical cell when exposed to 100 mM, followed by 160 mM, and then returning to 100 mM $[\text{Na}^+]$. When the $[\text{Na}^+]_o$ changed from 100 to 160 mM, the neuron fired faster with the same current injection (left and middle panel in Figure

2A and Table 1a). The averaged increase rate for 7 neurons was 27.9% (32% in this particular neuron) with 60 pA injected. After the $[\text{Na}^+]_o$ was changed back to 100 mM, the neuronal firing returned to the lower frequency for the same current injection (right panel in Figure 2A). The first AP recorded with 160 mM $[\text{Na}^+]_o$ had a significantly larger amplitude and smaller width than with 100 mM $[\text{Na}^+]_o$ (Figure 2B and Table 1b). Since the individual APs in a train are not identical, we compared the averaged AP peak amplitude and AP width at the threshold level in the AP train. The results confirm a higher AP amplitude (SD) in 160 mM $[\text{Na}^+]_o$ of 28.51 (7.16) mV vs 23.53 (6.86) mV in 100 mM $[\text{Na}^+]_o$ ($P < 0.01$); and a narrower AP (SD) in 160 mM $[\text{Na}^+]_o$ of 3.43 (1.07) ms vs 4.74 (1.19) ms in 100 mM $[\text{Na}^+]_o$ ($P < 0.001$) (Figure 2, C1 and C2) ($n=7$). The neuronal resting membrane potential (RMP), and hyperpolarization properties were not changed (Table 1a and 1c).

ACSFs with $[\text{Na}^+]$ of 100, 140, 180 mM (all at 345 – 355 mOsm) allowed us to study an even higher $[\text{Na}^+]_o$. Neurons again fired faster in the higher $[\text{Na}^+]_o$ (Figure 3A), an increase of 36% from 100 – 180 mM with 60 pA injection. The first AP showed a larger amplitude and narrower width (Figure 3B). The averaged APs in a train had a larger amplitude: the peak voltage (SD) was 28.15 (10.39) mV in 180 mM $[\text{Na}^+]_o$ vs 19.41 (6.94) mV in 100 mM $[\text{Na}^+]_o$ ($P < 0.05$); the width was 3.64 (1.08) ms in 180 mM $[\text{Na}^+]_o$ vs 6.91 (2.65) ms in 100 mM $[\text{Na}^+]_o$ ($P < 0.05$) ($n=4$) (Figure 3, C1 and C2). When ACSFs changed from normal to higher osmolarity, our recordings indicated a lower firing frequency (Figures 2A & 3A), consistent with the literature (Azouz et al., 1997).

Sodium and potassium current changes with higher $[\text{Na}^+]_o$

Cultured neurons were voltage-clamped. Depolarizing voltage, stepped from a holding potential of -65 mV, induced transient inward current followed by stable outward current. The transient inward (sodium) currents and delayed rectifier potassium current (Kdr) were recorded simultaneously and their I–V curves were plotted (Figure 4A–C). The inward sodium current was abolished by tetrodotoxin (TTX) (date not shown) and was significantly greater in ACSFs with 180 compared to that with 100 mM $[\text{Na}^+]_o$ (Figure 4A & B). The outward Kdr was significantly greater at the voltage of $+30 \sim +50$ mV in 180 mM sodium ACSF compared to that in 100 mM sodium ACSF (Figure 4A & C; the error bars are SDs). Thus, the potassium current at the depolarization state was greater at higher $[\text{Na}^+]_o$.

3. Discussion

Neurons in primary culture maintain typical hippocampal pyramidal cell excitability

The serum-free growth medium Neurobasal/B27 has been reported to support survival of primary cultured neurons with pyramidal morphology and characteristic electrophysiological phenotype (Azouz et al., 1997; Evans et al., 1998; Xu et al., 2006; Xu et al., 2009). Our cultured cells also had pyramidal morphology and prominent pyramidal neuronal electrophysiological properties: resting membrane potential of $-60 \sim -70$ mV; cells did not fire spontaneously; when negative current was injected, cells showed an obvious anomalous rectifier hyperpolarization-activated cation current (h current) (data not shown); when positive current was injected, cells had trains of APs, after which there was an after-hyperpolarization (AHP) (Evans et al., 1998). Voltage clamping demonstrated a fast-sodium current and delayed rectifier potassium current in these recorded neurons. Pyramidal cells also have other types of potassium current (I_A) and calcium current (Alshuaib et al., 2001; Evans et al., 1998; Xing et al., 2006). A calcium-activated potassium current was also present in these cells, since obvious AHPs were seen in the cells after an AP train (Evans et al., 1998). In conclusion, our primary cultured cells are hippocampal pyramidal neurons both in morphological and electrophysiological properties.

Extracellular sodium affects the electrophysiological properties of pyramidal neurons

Our studies show that hippocampal pyramidal neurons fire faster, and their AP amplitude is larger and their width is smaller in higher $[Na^+]_o$. Our finding that the resting membrane potential and input resistance was not significantly affected by sodium change might be because the sodium permeability has been shown to be very low during the resting potential in the squid axon (Hodgkin and Katz, 1949) and in isolated rat spinal cord neurons (Forsythe and Redman, 1988). However, extracellular sodium has been shown to gate the potassium permeability around the resting membrane potential in the hippocampus (Filippov and Krishtal, 1999).

The effects of $[Na^+]_o$ on AP peak amplitude are consistent with previous studies (Hodgkin and Katz, 1949; Huxley and Stampfli, 1951; Seyama and Irisawa, 1967). The effects of $[Na^+]_o$ on AP width have not been consistent in previous studies. Lower $[Na^+]_o$ increases the width of APs in rat dorsal root ganglion neurons (Amir et al., 1999). Higher $[Na^+]_o$ increased the AP width in skate heart (Seyama and Irisawa, 1967), probably because heart cells have larger calcium conductance. AP width is also affected by other factors: Older rats had lower potassium current (including delayed rectifier and A-type currents) than young rats, which might contribute to a decreased AP width (Alshuaib et al., 2001). A wider AP allows more calcium influx and could induce more neurotransmitter release (Shah et al., 2006), which may affect neuronal excitability or synaptic plasticity. Thus the consistent changes in AP width from changing $[Na^+]_o$ are likely to have functional consequences.

The AP changes were observed almost immediately following the changes in $[Na^+]_o$ and were stable for 5 ~ 10 minutes during our recordings. The change of intracranial sodium reported in a rat migraine model (Harrington et al., 2011) occurred within 15 minutes after intra-peritoneal nitroglycerin, as fast as the sodium MRI could be obtained, but most of the dynamics of *in vivo* sodium fluctuations remain to be determined. Neurons *in vivo* are likely to be exposed to elevated $[Na^+]_o$ for more than 10 minutes and, therefore, longer duration experiments would be required to investigate the prolonged effect from elevated $[Na^+]_o$.

Sodium current contributes to the $[Na^+]_o$ -induced changes in APs

We recorded sodium currents consistent with previous published data (Xing et al., 2006; Xu et al., 2006). Our experiments demonstrate that the maximum sodium current was greater in ACSF at higher $[Na^+]_o$ (Figure 4B). This data is consistent with the Nernst equation, from which higher E_{Na} will increase sodium conductance (increasing the AP amplitude), and is also consistent with prior studies: Matzner and Devor (Matzner and Devor, 1992) reported that increased maximal sodium conductance reduced the threshold for repetitive neuronal firing, based on modeling studies. They also demonstrated that neurons with increased maximal sodium conductance have a lower minimum sustainable rhythmic firing frequency as well as a higher maximal sustainable firing frequency. Thus, the neuron with increased maximal sodium conductance will have a bigger dynamic range for stimulus encoding, resulting in abnormally greater firing and consequent neuropathic sensations (Matzner and Devor, 1992). Their experimental data indicated that an increased sodium current was responsible for hyper-excitability at the site of nerve injury (Matzner and Devor, 1994). Thus, a $[Na^+]_o$ -induced, larger sodium conductance would contribute to increased neuronal excitability. Moreover, increased $[Na^+]_o$ would facilitate sodium current recovery from the inactivation state (Kuo and Liao, 2000) that might also contribute to faster APs.

Potassium current contributes to the $[Na^+]_o$ -induced changes in APs

We recorded potassium (delayed rectifier) current that is consistent with previous published data (Xing et al., 2006; Xu et al., 2006). The increase in potassium current induced by higher $[Na^+]_o$ might contribute to the narrower AP width we observed. The mechanism for the

increased potassium current is not yet known. It might be directly from a sodium-activated potassium current (Hess et al., 2007) or through another messenger-mediated effect. Alternatively, it may be indirect: higher $[\text{Na}^+]_o$ would raise the sodium current reversal potential according to the Nernst equation, resulting in greater sodium current and increasing intracellular $[\text{Na}^+]$. Higher intracellular $[\text{Na}^+]$ would activate Na^+ , K^+ , ATPase (Figure 1B & C), resulting in a higher intracellular $[\text{K}^+]$ and a lower potassium current reversal potential, based on the Nernst equation. A lower potassium reversal potential would lead to a larger driving force for potassium current, which would contribute to larger potassium current. Another example of potassium current affecting AP width could be seen in aging rats (Alshuaib et al., 2001).

The voltage clamp is not perfect in our cultured neurons because they could have dendrites as long as 200 μm , as reported (Xu et al., 2009). Thus we could not exclude the space clamp problem yet. Experiments in acutely dissociated neurons might overcome this issue.

Sodium substitution effects

When changing the $[\text{Na}^+]_o$, it is essential to balance the osmolarity, and any component selected for this role may itself influence the electrophysiology. The choline we used is a common replacement, yet it has been shown to affect neurons directly through muscarinic M1 receptor or nicotinic receptors (Alkondon et al., 1997; Carriere and El-Fakahany, 2000; Costa and Murphy, 1984). Extracellular sodium replacement by sucrose (10% or 100%) caused neurotrophin secretion in hippocampal neurons, while even 100% sodium substitution by choline did not lead to neurotrophin secretion in a slower time scale (mins) (Hoener, 2000). Choline induces glutamate and adenosine release in rat hippocampal slices in a slower time scale (mins) (Fowler, 1995). Thus, sodium substitution might cause effects independent from sodium changes. In a more related study of cat ureter smooth muscle, AP width increased when sodium was substituted with choline or sucrose, while the AP width decreased when Tris was substituted; AP amplitude responses were all the same (Kobayashi and Irisawa, 1964). Thus the AP amplitude might be determined by $[\text{Na}^+]_o$, while AP width was affected by choline chloride as well as $[\text{Na}^+]_o$ (Kobayashi and Irisawa, 1964).

Conclusions

Consistent with previous studies of the $[\text{Na}^+]_o$ effect on cellular excitability, we have demonstrated a similar effect on pyramidal cells. Elevated $[\text{Na}^+]_o$ induces larger sodium and potassium currents with earlier, more frequent, taller, and narrower APs. The faster sodium recovery from inactivation induced by higher $[\text{Na}^+]_o$ (Kuo and Liao, 2000) and the larger sodium and potassium current contribute to the augmented firing frequency when neurons are injected with the same amount of current. We propose that altered brain $[\text{Na}^+]_o$ will cause functional changes in hippocampal pyramidal neuronal excitability that might play an important role in migraine. Since Na^+ , K^+ , ATPase is a key regulator of $[\text{Na}^+]_o$ homeostasis, further experiments with different Na^+ , K^+ , ATPase isoforms will be explored.

4. Methods

Cell culture

Experimental protocols were approved by the local animal care and use committee. 69 Sprague-Dawley (SD) rat pups between P0 and P2 were used in our experiments. Pups were iced and euthanized by decapitation. The brain was removed into Hank's balanced salt solution in a 35 mm culture dish. Hippocampi were removed and transferred to 15 ml tubes with 2 ml B27/Hibernate (Invitrogen, Carlsbad, CA/Brainbits, Springfield, IL). Cell dissociation was similar to that described, modified from BrainBits cell culture protocol.

Briefly, hippocampi were incubated at 37°C in 1 mg.ml⁻¹ papain (Worthington, Lakewood, NJ) in HibernateE-Calcium (BrainBits, Springfield, IL) without B27 for 20 minutes. After incubation, hippocampi were triturated in Hibernate/B27. Once undispersed pieces settled by gravity after 1 minute, the supernatant was transferred to new sterile 15 ml tubes and centrifuged 200 g for 2 minutes. The cell pellet was resuspended in 1~3 ml B27/Neurobasal (Invitrogen, Carlsbad, CA) + 0.5 mM glutamax media (Invitrogen, Carlsbad, CA) + 25 µM glutamate. Cell aliquots were counted in a hemocytometer with trypan blue. Cells were diluted in media (B27/Neurobasal + 0.5 mM glutamax + 25 µM glutamate) and plated in poly-D-lysine coated 35 mm culture dishes (BD, Franklin Lakes, NJ) at a density of 30×10^3 cells.cm⁻², and incubated in 5% CO₂ at 37°C. After 4 days *in vitro* (DIV), the entire culture media was changed to B27/Neurobasal + 0.5 mM glutamax, without glutamate. Half of the media volume was then changed every 4 – 7 days. Neurons after 4 – 14 DIV were used for experiments.

Antibodies

For both western blotting and cell immunostaining, the following primary antibodies were used: Na⁺, K⁺, ATPase monoclonal mouse anti- alpha -1, -2, & -3 chains (Santa Cruz Biotechnology, Santa Cruz, CA); Pan sodium channel SP16 segment from (Chemicon/Millipore, Temecula, CA). Secondary antibodies for western blotting were all goat anti-mouse, alkaline phosphatase-conjugated (Santa Cruz Biotechnology, Santa Cruz, CA); for fluorescent staining, the secondary antibodies were Alexa Fluor 488 and 594, anti-mouse (Molecular Probes/Invitrogen, Sunnyvale, CA).

Western Blotting

Western blots were performed as before (Harrington et al., 2006a), with modifications. Ten to twenty thousand cells were disrupted in 50 µL of Tris buffer, pH 6.8. An aliquot was taken for protein assay (QUANT-IT, Invitrogen, Carlsbad, CA); the remainder was sonicated in a cup-horn (Branson, Danbury, CT) dissolved in 50 µL of a solution of 2% SDS, 5% β-mercaptoethanol, 10% sucrose, and 0.002% bromophenol blue, in 50 mM Tris buffer, pH 6.8, and total protein of 1 – 3 µg was transferred to the well of a gradient 4 – 20% T Criterion SDS gel calibrated by Precision Plus molecular weight markers (both from BioRad, Hercules, CA). Proteins were separated by electrophoresis and transferred to polyvinylidene difluoride membrane, as recommended by the manufacturer. Membranes were rinsed 3 times in Tris-buffered saline with 0.05% Tween-20 (TBST), incubated in TBST with 1: 2000 primary antibody for 2 hours, rinsed three times in TBST, then incubated with 1: 2000 secondary antibody in TBST for 45 minutes. Membranes were then washed in TBS 3 times, and shaken in a solution of NBT/BCIP (Thermo Scientific, Rockford, IL) for 10 – 20 minutes until the specific band appearance was optimal. The membrane was washed with water, dried at room temperature, and digitized on an Epson Expression 1680.

Immunofluorescence microscopy

Each well of an 8 chamber BD BioCoat Poly-D-lysine CultureSlide (VWR, Brisbane, CA) was seeded with approximately 20,000 hippocampal pyramidal-enriched cells, and cultured as described above. Cells attached within 24 hours and were fixed with a solution containing 10% formaldehyde in 10 mM TBS for 10 minutes, then rinsed quickly with TBS five times. Cells were then treated with 0.25% Triton X100 in 10 mM TBS for 5 minutes, washed in TBS, then incubated with primary antibody for one hour. The optimal dilution for all antibodies was 1: 400 in TBS containing 1% bovine serum albumin (Sigma, St. Louis, MO). After 2 quick TBS washes, the cells were incubated with 1: 1000 dilution of fluorescent antibody in 10 mM TBS for 30 minutes. Cells were then rinsed five times in TBS, chamber walls were removed, the slide was drained and covered with ProLong Gold antifade reagent

(Invitrogen, Sunnyvale, CA), and a cover slide was attached and sealed with clear fingernail polish. Slides were cured in darkness, usually for > 24 hours.

Cells were viewed and images were acquired with a Retiga 2000R (Qimaging, Surrey, Canada) controlled by a PC laptop running QCapture Pro software. The camera was coupled to a Nikon Diaphot-TMD microscope with epi-fluorescence attachment. A custom made slider with Nikon cubes allowed excitation/emissions of 360/460 for DAPI, 485/520 for Alexa Fluor 488, and 575/630 for Alexa Fluor 594, viewed with 10, 20, and 40 X objectives. Three to six areas of each well were photographed under each wavelength and with phase shift optics.

Sodium Selective Micro-electrode Fabrication

SSMEs were prepared according to a literature procedure (Stinner et al., 1989). Briefly, clean glass micropipettes with an opening of approximately 10 – 20 μm were drawn from borosilicate glass tubing (1.5 mm OD World Precision Instrument, Sarasota, FL) using a micropipette puller (P-97, Sutter Instruments Co., Novato, CA). Pipette tips with internal diameter of 10 – 20 μm were used to mimic the size of the pyramidal cells used in these experiments. The interior walls of the micropipettes were silanized by filling with a solution (5 % w/w) of dichlorodimethylsilane (Aldrich, Milwaukee, WI) in chloroform and heating in an oven to 140 $^{\circ}\text{C}$ for 1 h. After rinsing with chloroform and subsequent drying in oven, the micropipettes were dipped, sharp end first, into the sodium selective membrane mixture for several minutes (sodium ionophore I, cocktail A, Fluka, St. Louis, MO) in order to introduce a liquid membrane via capillary action. The electrodes were then back-filled with an aqueous electrolyte solution of 0.1 M NaCl, and air bubbles were removed by gentle tapping. Each SSME was completed by inserting a silver wire (Alpha Aesar, Ward Hill, MA), coated with silver chloride into the pipette barrel and secured with PTFE tape.

The SSME was mounted to the headstage (Molecular Devices, Sunnyvale, CA). Voltage signal recordings were obtained by an Axopatch 700B amplifier (Molecular Devices, Union City, CA) and Digidata 1440 (Molecular Devices, Sunnyvale, CA), the same devices used in our electrophysiology experiment. Sample rate was 10 kHz. Recorded traces were filtered online by 50 kHz. The SSME was calibrated with standards before and after experiments. In order to study neuronal excitability in different $[\text{Na}^+]_o$, we determined the $[\text{Na}^+]$ that was delivered by a micropipette directed towards a SSME with an orifice the size of a pyramidal neuron. The SSME was first calibrated: the measured voltage (mV) of a series of different $[\text{Na}^+]$ was linearly proportional to the logarithm of $[\text{Na}^+]$, with an R^2 of 0.96 (Figure 2A), allowing us to measure the $[\text{Na}^+]$ in ACSF from the measured voltage. Next, a barrel with multiple capillaries, each containing ACSF with a different $[\text{Na}^+]$ was connected to a common pipette submerged in the 4 mL of ACSF in the chamber with a constant $[\text{Na}^+]$ of 140 mM continuously circulating at 1–2 $\text{mL}\cdot\text{min}^{-1}$. To determine the effect of dilution from the constant $[\text{Na}^+]$ in the ACSF of the culture bath and its rate of change when a new solution was directed at the patched cell from the capillary barrel, the SSME was tested in the same configuration as if it were the cell undergoing study. The barrel pipette tip was positioned around 200 – 500 μm away from the soma or SSME on the floor of the chamber. When ACSF with a different $[\text{Na}^+]$ was delivered from the barrel tip, the SSME voltage was measured. An electronic switch controlled the timing for delivery of ACSF with each different $[\text{Na}^+]$.

Control of cell culture environment during physiology experiments

All experiments were performed in 35 mm culture dishes containing around 4 mL of circulating ACSF with a fixed $[\text{Na}^+]$ of 140 mM, flowed in continuously at one end of the dish at 1 – 2 $\text{mL}\cdot\text{min}^{-1}$ and removed at the other end. To change $[\text{Na}^+]_o$ rapidly, a custom-

made barrel of micropipettes connected to a common pipette tip with a 10 – 20 μm tip was positioned around 200 – 500 μm away from the neuronal soma or SSME, to direct flow of ACSF solutions that differed only in $[\text{Na}^+]$ to the patched cell. These pipettes were fed from reservoirs from a cF-8VS valve assembly with a cFLOW controller (Cell MicroControls, Norfolk, VA) and delivered a change of solution at 1 mL min^{-1} .

Electrophysiology

Cultured hippocampal pyramidal neurons were visually identified based on their pyramidal shaped soma and one major apical dendrite. Whole-cell current- and voltage-clamp recordings were obtained from a MultiClamp 700B patch amplifier, Digidata 1440 digitizer, on a PC running pCLAMP software (all from Molecular Devices, Sunnyvale, CA). Recording borosilicate glass pipettes had resistances between 4 – 8 $\text{M}\Omega$ in the following intracellular solution (in mM): 140 K-Gluconate, 5 KCl, 4 NaCl, 10 HEPES, 0.3 Na_3GTP , 2 MgATP, at pH 7.3 – 7.4, osmolality between 280 – 300 mOsm. Osmolality was measured by depression of freezing point (Micro-Osmette, Precision Systems, Inc., Natick, MA). To record electrophysiological properties, a culture dish containing neurons was changed from the culture media to ACSF. The ACSF contained the following (in mM): 140 NaCl, 20 choline Cl, 2.5 KCl, 1 MgCl_2 , 1 CaCl_2 , 5 HEPES, 10 glucose. When 100 and 160 mM NaCl were used in ACSF, the concentration of choline Cl was adjusted to keep the same osmolality of 310 – 320 mOsm. For the 100 – 180 mM NaCl ACSFs, it was necessary to increase to 345 – 355 mOsm, again by adjusting with choline Cl. Electrical records were digitized at 10 kHz. The junctional potentials were not corrected. Only neurons with access resistance $< 30 \text{ M}\Omega$ were included in this study.

In current-clamp experiments, the voltage was recorded while cells were rest and injected with 20, 40, and 60 pA for 1 second. Neuronal electrical activities during steps of depolarization and hyperpolarization (1 s) were recorded from cells. RMP, AP numbers with injected current, first AP threshold time and amplitude, peak time and amplitude, AP width, hyperpolarization peak time and amplitude, and hyperpolarization steady-state voltage were determined from current-clamp recordings. The action potential threshold was identified as the voltage corresponding to the peak of the second derivative of the action potential waveform.

In voltage-clamp experiments, cells were held at -65 mV and were depolarized from -60 mV to $+70 \text{ mV}$ with 10 mV increments, for 50 ms. To compensate for linear capacitance and leak current, a voltage step response from -65 mV to -60 mV in front of the test potential was scaled proportionately and subtracted from each current recording.

To test the role of TTX-sensitive sodium channels in rates of action potential firing, TTX (Sigma-Aldrich, St. Louis, MO) was delivered directly to the cell in a short pulse using the Picospritzer® III (Parker Hannafin Corp, Cleveland, OH).

Statistical Analyses

Unless otherwise noted, significance was assessed at $P < 0.05$ with paired Student's t-test when comparing effects at different $[\text{Na}^+]_o$.

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Abbreviations

ACSF	artificial CSF
AHP	after-hyperpolarization
AP	action potential
CSF	cerebrospinal fluid
DIV	days <i>in vitro</i>
E_{Na}	sodium reversal potential
h current	hyperpolarization-activated cation current
Kdr	delayed rectifier potassium current
[Na⁺]_o	extracellular sodium concentration
RMP	resting membrane potential
SD	standard deviation
SSME	sodium selective micro-electrode
TBST	Tris-buffered saline with 0.05% Tween-20
TTX	tetrodotoxin

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Research highlights

Extracellular sodium modulates neuronal excitability; current and voltage clamp of cultured pyramidal neurons; increased neuronal excitability from sodium may contribute to photophobia.

Figure 1.

Primary cell culture model and the sodium-selective micro-electrode (SSME). A: The phase contrast image of rat hippocampal primary cultured neurons. Most of the cells were pyramidal neurons based on morphology: triangular soma, one apical dendrite, with more thin basal dendrites. B: Western blot of cultured cells express alpha -1 and -3, but not -2, Na^+ , K^+ , ATPase subunits at 112 KD (arrow). C: Double-labeled fluorescence imaging and phase contrast image indicated the expression of pan-sodium channels and Na^+ , K^+ , ATPase alpha -1 chain subunits in cultured cells. D: SSME voltage was linearly related to the log scale of $[\text{Na}^+]$, in this case with an R^2 of 0.96. E: Four different $[\text{Na}^+]$ of 100, 120, 140, and 160 mM were delivered to an SSME placed in the cell chamber at the same distance from the ACSF delivery barrel as the cultured neurons. Spikes in the upper panel indicate the start of switching the solution, as marked.

Figure 2.

The effect of $[Na^+]_o$ on firing of cultured neurons at normal ACSF osmolarity. Higher $[Na^+]_o$ increased neuronal firing recorded in current-clamped mode. A: Representative firing from one cell taken from one of five recordings every minute. $[Na^+]_o$ was altered as indicated. Neuronal firing increased in 160 mM $[Na^+]_o$ at all three current injections compared to that in 100 mM. This higher firing rate was reversed after returning to 100 mM $[Na^+]_o$. B: Comparing the first AP of each recording, the higher $[Na^+]_o$ induced taller and narrower APs, and this AP shape change was reversible (a, b, c as indicated in panel A). C: The averaged AP peak amplitude (C1) from the threshold level in a train was significantly larger in 160 mM $[Na^+]_o$ with a mean (SD) of 28.51 (7.16) mV compared to that in 100 mM $[Na^+]_o$ of 23.53 (6.86) mV (** $p < 0.01$, $n = 7$). The averaged AP width (C2) at the threshold level in a train was significantly smaller in 160 mM $[Na^+]_o$ with a mean (SD) of 3.43 (1.07) ms compared to that in 100 mM $[Na^+]_o$ of 4.74 (1.19) ms (*** $p < 0.001$, $n = 7$).

Figure 3.

The effect of $[Na^+]_o$ on firing of cultured neurons at higher ACSF osmolarity. A: Neurons fired progressively faster in 100, 140, and 180 mM $[Na^+]_o$ with current injection. B: Higher $[Na^+]_o$ induced the first APs to be taller and narrower (a, b, c as indicated in panel A). C: The averaged AP peak amplitude (C1) from the threshold level was also significantly larger in 180 mM $[Na^+]_o$ with a mean (SD) of 28.15 (10.39) mV compared to that in 100 mM $[Na^+]_o$ of 19.41 (6.94) mV ($*p < 0.05$, $n = 4$). The averaged AP width (C2) in a train was significantly smaller in 180 mM $[Na^+]_o$ with a mean (SD) of 3.64 (1.08) ms compared to that in 100 mM $[Na^+]_o$ of 6.91 (2.65) ms ($*p < 0.05$, $n = 4$).

Figure 4.

$[Na^+]_o$ elevation augments neuronal Na^+ and K^+ currents. A: TTX-sensitive transient inward current peaks were measured and plotted with voltage as the I-V curve for the fast sodium current. At the same voltage level (between $-20 \sim +70$ mV), the fast sodium current was greater in higher $[Na^+]_o$ compared to that in lower $[Na^+]_o$, (paired t-test, $*p < 0.05$; $**p < 0.01$, $n=6$). B: Steady state (ss) outward current (Ω) was measured and plotted with voltage as the I-V curve for K_{dr} current. At the voltage of $+30$ mV to $+50$ mV, outward current was greater in 180 mM $[Na^+]_o$ compared to that in 100 mM $[Na^+]_o$ (paired t-test, $*p < 0.05$; $**p < 0.01$, $n=6$).

Table 1

Table 1a: Basic membrane properties					
[Na ⁺] _o (mM)	RMP (mV)	AP#20	AP#40	AP#60	
100	-64.52(4.54)	3.71(4.50)	10.71(5.94)	14.86(6.54)	
160	-63.20(5.37)	4.57(5.03)	12.43(6.43)	19.00(6.63)*	

Table 1b: First action potential properties					
[Na ⁺] _o (mM)	API_thresT (ms)	API_thresA (mV)	API_peakT (ms)	API_peakA (mV)	API_width (ms)
100	350.21(74.31)	-27.16(5.04)	351.53(74.26)	23.35(6.95)	4.34(0.97)
160	334.76(65.26)	-27.43(6.43)	335.87(65.04)	29.16(6.37)**	3.14(0.87)***

Table 1c: Hyperpolarization properties			
[Na ⁺] _o (mM)	hypT (ms)	hypA (mV)	hyp_ssA (mV)
100	500.53(80.14)	-88.53(12.17)	-82.22(9.27)
160	485.13(40.34)	-86.29(10.87)	-80.31(9.60)

Data shown as mean (± SEM), n = 7.

* p < 0.05,

** p < 0.01,

p < 0.001. Abbreviations: the number of APs with current injection of 20 pA (AP#20), 40 pA (AP#40), or 60 pA (AP#60); first AP threshold time from the start of current injection and threshold amplitude from RMP (API_thresT in ms and API_thresA in mV); first AP peak time from the start of current injection and peak amplitude from RMP (API_peakT in ms and API_peakA in mV); first AP width at the threshold level (API_wid in ms); hyperpolarization peak time from the start of current injection and hyperpolarization peak amplitude from RMP (hypT in ms and hypA in mV), hyperpolarization steady state amplitude (hyp_ssA in mV).