Neuronal Sodium Leak Channel Is Responsible for the Detection of Sodium in the Rat Median Preoptic Nucleus

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INTRODUCTION

In mammals, the volume of the extracellular fluid (ECF) compartment is primarily dependent on sodium (Na⁺). Sodium, together with its accompanying anions, is a major determinant of the ECF osmolality. Consequently, an increase in the Na⁺ concentration ([Na⁺]ECF) and osmotic pressure in the ECF will result in cellular dehydration and a rise in ECF volume. In contrast, a reduction in ECF [Na⁺] and osmotic pressure due to the presence of pathological conditions (e.g., diarrhea and/or vomiting) will result in extracellular dehydration and a decrease in ECF volume. Osmotic perturbations of the ECF therefore are a threat to the integrity of the body, and powerful regulatory responses are available to restore hydromineral homeostasis (Bourque 2008; Denton et al. 1996). A prerequisite for these responses is the monitoring of ECF osmolality and Na⁺ levels. Osmoreceptors have been identified in the hypothalamus, and these neurons transduce the changes in ECF osmolality into action potential firing patterns via cellular mechanisms involving stretch-inactivating and TRPV channels (Bourque 2008; Ciura and Bourque 2006; Oliet and Bourque 1993; Voisin et al. 1999). The existence of specific cerebral Na⁺ sensors for the detection and correction of hydromineral imbalance has long been hypothesized (Andersson et al. 1975; Cox et al. 1987; Olsson and Kolmodin 1974; Weissinger et al. 1979), and a concentration-sensitive Na⁺ channel, called the NaX channel, has been reported to control sodium ingestion in mice (Noda 2006; Watanabe et al. 2000). In these rodents, the NaX channel is specifically expressed by glial cells of the circumventricular organs (Watanabe et al. 2006). In addition, the transduction of the ECF [Na⁺] (>150 mM) into neuronal action potentials involves a complex mechanism and results in part from lactate release by glial cells under hypernatriuric conditions (Shimizu et al. 2007). Thus glial cells appear to be the main effectors for the detection of extracellular Na⁺ levels in the subfornical organ (SFO) in mice.

Interestingly, a subset of neurons in the median preoptic nucleus (MnPO), a hypothalamic nucleus involved in osmoregulation and sodium ingestion (McKinley et al. 1999), has been shown to specifically respond to local variations in [Na⁺]ECF by changes in neuronal membrane potential and firing rate (Grob et al. 2004). In the context of a neuronal network in the hypothalamus that integrates both ECF osmolality and [Na⁺], this finding places the MnPO in a strategic position to monitor CSF Na⁺ levels. This initial study reporting the presence of Na⁺ sensors in the MnPO did not, however, reveal the cellular mechanism underlying [Na⁺] detection in this nucleus. Indeed the results were obtained using a brain slice preparation, and this methodological approach suggests two possible mechanisms for the Na⁺ sensitivity of MnPO neurons. On the one hand, these neurons could be intrinsically able to directly transduce a change in external [Na⁺] ([Na⁺]OUT) into changes in membrane potential. On the other hand, changes in the electrical activity of these neurons could be an indirect result of the release of a modulator from neighboring glial cells in response to a change in [Na⁺]OUT. Here we used electrophysiological recordings from dissociated MnPO neurons, as well as from brain slices, to investigate whether these neurons function as...
specific Na\(^+\) sensors and to identify the cellular mechanism underlying the detection of CSF Na\(^+\) levels.

METHODS

Ethical approval

The experimental protocol used in the present study was performed in accordance with the guidelines established by the Canadian Council on Animal Care and was duly approved by the Laval University Animal Care Committee (CPAQ).

Tissue preparation

Wistar rats (100–150 g) were deeply anesthetized by intraperitoneal injection of a ketamine-xylasine mixture (87.5 and 12.5 ml/kg, respectively) and decapitated. The brain was immersed in oxygenated (95% O\(_2\)-5% CO\(_2\)) ice-cold (2°C) artificial cerebrospinal fluid (aCSF) containing (in mM) 120 K-gluconate, 6 NaCl, 10 EGTA, 0.3 Na\(^+\)-GTP, and 4 Na\(^+\)-ATP, pH 7.4, osmolality: 295–305 mosm·kg\(^{-1}\). Micropipettes had a final tip resistance of 4–5 MΩ. The extracellular solution (control aCSF) had a composition identical to aCSF used for the storage of the slice (see tissue preparation). Hyponeutric-acidiotic aCSF was obtained by lowering NaCl concentration to 100 mM (300 mosm·kg\(^{-1}\)), and hypertonic aCSF was obtained by increasing NaCl concentration to 170 mM (350 mosm·kg\(^{-1}\)). Hyponeutric-acidiotic aCSF was obtained by substituting NaCl by 90 mM Na\(_2\)SO\(_4\) (300 mosm·kg\(^{-1}\)). Temperature of all the extracellular solutions was stabilized at 24°C. Hypo- and hypernatriuric aCSF were transiently and locally applied over the ventral region of the MnPO using a fast solution exchanger.

Calculation of the cation permeability ratio

Calculation was obtained from a modified Goldman-Hodgkin-Katz (GHK) equation, which is presented in the following text. Determination of \(E_{Na^+}\) was achieved for hypernatriuric aCSF ([Na\(^+\)]\(_{out}\): 150 mM NaCl)

\[
E_{Na^+} = \frac{RT}{zF} \ln \left( \frac{[Na^+]_{out}}{[Na^+]_{in}} \right)
\]  

(1)

Determination of \(E_{Li^+}\), or \(E_{CH6N3^+}\) (\(E_X\)) for aCSF containing Na\(^+\), Li\(^+\) or CH\(_3\)N\(_3^+\)

\[
E_X = \frac{RT}{zF} \ln \left( \frac{P_{Na}[Na]_{out} + P_{X}[X]_{out}}{P_{Na}[Na]_{in}} \right) - \frac{RT}{zF} \ln \left( \frac{[Na]_{out}}{[Na]_{in}} \right)
\]  

(2)

\([Na^+]_{out:} 100 \text{ mM, } [X]_{out:} [Li^+]_{out} \text{ or } [CH_3N_3^+]_{out:} 50 \text{ mM, } [Na^+]_{in:} 100 \text{ mM, } P_{X} \text{ is the channel permeability for the cation } X \text{ (Li}^+ \text{ or CH}_3\text{N}_3^+\text{).}

Subtraction of Eq. 1 from Eq. 2

\[
E_X - E_{Na^+} = \frac{RT}{zF} \ln \left( \frac{P_{Na}[Na]_{out} + P_{X}[X]_{out}}{P_{Na}[Na]_{in}} \right)
\]  

(3)

Due to the property of logarithm subtraction [ln(a+b) = ln(a) − ln(b)] and because [Na\(^+\)]\(_{in}\) is equal in the two conditions (100 mM), Eq. 3 is simplified to

\[
E_X - E_{Na^+} = \frac{RT}{zF} \ln \left( \frac{P_{Na}[Na]_{out} + P_{X}[X]_{out}}{P_{Na}[Na]_{out1}} \right)
\]  

(4)

Isolation of \(P_{X}/P_{Na}\)

\[
e_X - E_{Na^+} = \frac{P_{Na}[Na]_{out} + P_{X}[X]_{out}}{P_{Na}[Na]_{out1}} - \frac{P_{Na}[Na]_{out} + P_{X}[X]_{out}}{P_{Na}[Na]_{out2}}
\]  

(5)

\[
\frac{P_{X}}{P_{Na}} = \frac{[X]_{out} e^{(\frac{F}{RT}) (e_X - E_{Na^+})} - [Na^+]_{out2}}{[X]_{out}}
\]  

(6)

Replacing the known variables in Eq. 7 leads to the modified GHK equation

\[
\frac{P_{X}}{P_{Na}} = 3 e^{\frac{e_X - E_{Na^+}}{RT}} - 2
\]  

(7)

\(E_X\) and \(E_{Na^+}\) were determined experimentally. \(R = 8.31447 \text{ J/K·mol, } F = 96485 \text{ C/mol}, z = +1 \text{ (for Na}^+, \text{ Li}^+ \text{ and CH}_3\text{N}_3^+\text{), } T = 298.15 \text{ K.}

Immunocytochemistry

After tissue dissociation, drops containing cells (<1 μl) were directly plated on a microscope glass slide for 20–30 min. at 37°C.
The dissociated cells were fixed for 60 min in a paraformaldehyde (PFA)/borax solution and immediately used for immunocytochemistry. The fixed cells were incubated overnight at 4°C in PBS containing 5% native donkey serum, 1% BSA with rabbit anti-NaX antibody (1/250, kind gift of Pr. M. Tamkun, Colorado State University, tamkunmm@colstate.edu) and mouse anti-NeuN antibody (1/500, Clone A60, Millipore MA), or mouse anti-glial fibrillary acidic protein (GFAP) antibody (1/500, clone G-A-5, Sigma-Aldrich MO). The slides were first washed in PBS and incubated for 2 h at room temperature in PBS containing 5% native donkey serum, 1% BSA with Alexafluor-488 donkey anti-rabbit (1/100, green, Invitrogen, Ontario, ON, Canada), and Alexafluor-555 donkey anti-mouse (1/250, red, Invitrogen) as secondary fluorescent antibodies to visualize the NaX, NeuN, and GFAP proteins, respectively.

Immunohistochemistry

The animals (rats and mice) were deeply anesthetized with a ketamine-xylasine solution and killed with a transcardiac perfusion of a 4% PFA/borax solution (pH 9). Brains were removed, postfixed at 4°C for 2 h, and immersed in a 20% sucrose-containing PFA buffer for 48 h. Coronal brain sections of the lamina terminalis were cut with a microtome (20 μm thick) and incubated for 40 h at 4°C in PBS containing 5% native donkey serum, 1% BSA with anti-NaX antibody (1/1,000, and anti-NeuN antibody (1/1,000). The sections were first washed in PBS and incubated in PBS containing 5% native donkey serum, 1% BSA with Alexafluor-488 donkey anti-rabbit (1/1000, green), and Alexafluor-555 donkey anti-mouse (1/1000, red) secondary antibodies for 2 h at room temperature to visualize NaX and NeuN, respectively. Specificity of the anti-NaX antibody was verified on the one hand by preabsorbing the sera with a recombinant NaX fusion protein (Supplementary Fig. 1A). On the other hand, the rat anti-NaX antibody directed against the NaX protein sequence (Supplementary Fig. 1B) gave positive NaX immunostaining in the mouse SFO (Supplementary Fig. 1C), as previously reported (Watanabe et al. 2006). Note the absence of NaX immunostaining in various regions, including the brain, when the anti-NaX antibody was tested in tissue sections obtained from NaX knocked out mice (Hiyama et al. 2002).

Construction and purification of the rat NaX fusion protein

The rat polypeptide sequence from the interdomain 2–3 (amino acids 753–917) was targeted because it contains the peptide sequence previously used to prepare the anti-NaX antibody (NH2–SV–SETVOIASGESDIE–COOH) (Hiyama et al. 2002). The corresponding rat DNA coding sequence (nucleotide number 2267–2761 from Genebank No. NM_031686) was cloned in glutathione S transferase (GST) fusion protein expression vector pGEX-4T-1. Rat NaX fusion protein was produced in Escherichia coli BL21-Gold (DE3) and purified on glutathione sepharose 4B column.

Statistical analysis

Results obtained from electrophysiological recordings were expressed as means ± SE. Electrophysiological variables were tested for normality using both the Kolmogorov-Smirnov and D'Agostino-Pearson omnibus tests. Both current and voltage amplitudes were normally distributed and analyzed using parametric statistical tests. Comparison of repeated measures was performed using a paired Student’s t test. Other comparisons of the means were performed with one- or two-way ANOVA as indicated in the text. Further post hoc test was done when applicable. Statistical significance was defined as P < 0.05.

RESULTS

Neurons were dissociated from the ventral part of the MnPO to determine the cellular mechanism underlying the Na+ sensitivity of this strategic region of the hypothalamus. Whole cell voltage-clamp recordings were conducted on MnPO neurons under conditions of isotonic [Na+]i ([Na+]i,i; 100 mM) and in the presence of intra- and extracellular pharmacological agents to block Na+, K+, and Ca2+ voltage-operated channels. Neurons were held at −20 mV and were transiently bathed in aCSF with various extracellular pharmacological agents to block Na+, K+, and Ca2+ voltage-operated channels. Neurons were held at −20 mV and were transiently bathed in aCSF with various concentrations of agents, including 100 mM aCSF as control. The aCSF contained (in mM) 120 NaCl, 20 KCl, 2 CaCl2, 10 NaHCO3, 1 MgCl2, and 5 glucose.

FIG. 1. Acutely dissociated median preoptic nucleus (MnPO) neurons respond to a change in [Na+]o: A: the neuron is maintained in isosmotic control condition (100 mM NaCl). Bath application of hypernatriuric artificial cerebrospinal fluid (aCSF, 150 mM NaCl) triggers an inward shift of the holding current, whereas bath application of hyponatriuric aCSF (50 mM NaCl) triggers an outward shift of the current (B). Note that lower traces in A and B illustrate the holding current (HC) presented at a higher time scale in control (a) and during the change in aCSF [Na+]o (b). C: application of hyperosmotic aCSF (350 mosm.L−1 with mannitol) does not affect the HC, indicating that the change in the current amplitude is solely due to the change in [Na+]o.
[\text{Na}^+]_{\text{out}}$. A high [\text{Na}^+]_{\text{out}} (150 mM NaCl or 180 mM NaCl) triggered an inward shift of the holding current (HC) of $-11.8 \pm 1.2$ pA ($n = 21$) and $-14.4 \pm 1.4$ pA ($n = 18$), respectively (Fig. 1A). A low [\text{Na}^+]_{\text{out}} (50 mM NaCl) triggered an outward shift of the HC of $11.6 \pm 2$ pA ($n = 14$; Fig. 1B). However, application of mannitol, which raised the extracellular osmolality from 300 to 350 mosm.kg$^{-1}$, did not change the holding current ($n = 12$, Fig. 1C). Interestingly, a single neuron responded to both hypo- and hypernatriuric challenges, indicating that detection in extracellular Na$^+$ levels, but not in osmolality, is an intrinsic property of a single neuronal population of the MnPO. This unique feature was shared by 79% of the neurons tested (44 of 56 neurons).

Our results strongly suggest that a change in [\text{Na}^+]_{\text{out}} resulted in modification of the amplitude of a tonic current likely carried by Na$^+$ ions. To further test this hypothesis, depolarizing voltage ramps from $-30$ to $+20$ mV (16 mV/s) were applied to the same neuron in isonatriuric (control), hypernatriuric, or hyponatriuric aCSF to estimate the reversal potential of the current. The reversal potential of the ramp-activated current triggered by the application of aCSF containing 50 mM ($n = 14$), 150 mM ($n = 21$), or 180 mM NaCl ($n = 18$) was identical to the theoretical $E_{\text{Na}}$ determined with the Nernst equation (Fig. 2), thereby identifying the current as a Na$^+$ current. The current conductance ($g_{\text{Na}}$) was calculated with the current-voltage law at a depolarized and hyperpolarized membrane potential ($-20$ and $-60$ mV, respectively). Thus the estimated $g_{\text{Na}}$ was $0.82 \pm 0.12$ and $0.81 \pm 0.11$ nS at $-20$ and $-60$ mV, respectively ($n = 10$) in hypernatriuric aCSF (50 mM NaCl). In hypernatriuric aCSF (150 mM NaCl), $g_{\text{Na}}$ was $1.0 \pm 0.11$ and $0.98 \pm 0.12$ nS at $-20$ and $-60$ mV, respectively ($n = 13$). Finally, in hypernatriuric aCSF (180 mM NaCl), $g_{\text{Na}}$ was $0.97 \pm 0.12$ nS at $-20$ mV and $0.99 \pm 0.12$ nS at $-60$ mV ($n = 16$). These results show that $g_{\text{Na}}$ was not affected either by [\text{Na}^+]_{\text{out}} [2-way ANOVA, $F(2,72) = 0$, $P = 0.999$] or by the membrane potential [2-way ANOVA, $F(6,142) = 0$, $P = 0.993$].

This finding indicates that changes in [\text{Na}^+]_{\text{out}} modified the flux of Na$^+$ through a leak channel.

To further characterize this channel, we evaluated its permeability to different cations. Permeability to lithium (Li$^+$) and guanidinium (CH$_6$N$_3$Cl) was first tested at a steady-state membrane potential ($-20$ mV). Bath application of Li$^+$-containing aCSF (100 mM NaCl + 50 mM LiCl) triggered an inward shift of the HC similar in amplitude to the shift generated by a previous application of hypernatriuric aCSF ($-14.9 \pm 1.5$ pA, $n = 8$, paired $t$-test, $P = 0.32$; Fig. 3A). In contrast, application of guanidinium-containing aCSF (100 mM NaCl + 50 mM CH$_6$N$_3$Cl) only triggered a weak shift of the HC compared with the application of hypernatriuric aCSF ($-3.1 \pm 1.2$ pA, $n = 5$, paired $t$-test, $P = 0.004$; Fig. 3B). The high permeability of the leak channel for Li$^+$, combined with its poor permeability to guanidinium, ruled out the implication of a nonselective cation channel in this effect and further identified this channel as a putative Na$^+$ channel. Furthermore, the reversal potential of the current carried by Li$^+$ ions matched the $E_{\text{Na}}$ calculated for aCSF containing 150 mM NaCl (paired $t$-test, $P = 0.51$; Fig. 3C). By contrast, the reversal potential of the current carried by guanidinium was different from the $E_{\text{Na}}$ observed for aCSF containing 150 mM NaCl (paired $t$-test, $P = 0.0005$; Fig. 3D); this finding supports the weak permeability to guanidinium ions. The relative permeability of the channel for Li$^+$ and guanidinium ions was estimated using the Goldman-Hodgkin-Katz voltage equation. The ionic permeability ratios $P_{\text{Li}^+}/P_{\text{Na}^+}$ and $P_{\text{CH}_6\text{N}_3^+}/P_{\text{Na}^+}$ were $0.98 \pm 0.08$ ($n = 8$) and $0.2 \pm 0.14$ ($n = 5$), respectively.

We next tested the selectivity of the channel for rubidium
(Rb⁺). Surprisingly, the application of Rb⁺-containing aCSF (100 mM NaCl + 50 mM RbCl) prior to the application of hypernatriuric aCSF (150 mM NaCl) prevented the changes in the HC (-0.5 ± 0.32 pA; n = 6) commonly seen with the latter solution (Fig. 4). Moreover, the HC necessary to maintain the neuron at -20 mV under isonatriuric [Na⁺] was also shifted to the outward direction after Rb⁺ exposure. These data indicate that Rb⁺ obstructed the Na⁺ leak channel, thereby impeding Na⁺ influx. The biophysical properties of the channel reported here indicate that dissociated MnPO neurons respond directly to modifications in [Na⁺]out via changes in Na⁺ flux through a Na⁺ leak channel. Interestingly, the functionality of the Na⁺ leak channel is impaired by Rb⁺ ions.

It has been reported that the atypical Na⁺ channel (NaX channel) mediates Na⁺ influx in both neurons and glia in response to hypernatriuric aCSF (Hiyama et al. 2002; Watanabe et al. 2006). The Na⁺ leak channel characterized in dissociated MnPO neurons could thus be the NaX channel. Immunocytochemical staining using antibodies against the neuronal NeuN protein or the GFAP indicated that almost all dissociated MnPO cells used for the electrophysiological recordings were neurons (94%, 5 rats; Fig. 5A). Moreover, 90% of the dissociated NeuN-positive cells were co-labeled with an affinity-purified rabbit polyclonal antibody against the NaX channel that had been previously validated in the dorsal rot ganglion and SFO (Hiyama et al. 2002) (Fig. 5B). The specificity of the antibody was verified in the MnPO using brain slices (Supplementary Fig. 1A). Interestingly, the percentage of double-labeled cells (NeuN + NaX) was comparable to the percentage of dissociated cells that responded to a change in [Na⁺]out (79%; Fig. 5C). Further immunohistochemical labeling performed on brain slices showed that a majority

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**FIG. 3.** Na⁺ influx in dissociated MnPO neurons is achieved through a leak channel showing a cation permeability ratio similar to that of a Na⁺ channel. A: bath application of Li⁺-containing aCSF (100 mM NaCl + 50 mM LiCl) triggers a shift of the HC of similar amplitude than the hypernatriuric aCSF (150 mM NaCl). B: in contrast, guanidinium-containing aCSF (100 mM NaCl + 50 mM CH₃CN,CI) only generates a weak inward current. C1: typical representation of the ramp-activated current generated under Li⁺-containing aCSF (100 mM NaCl + 50 mM LiCl), This current is superimposed to the ramp-activated current generated by hypernatriuric aCSF. C2: bar graphs show the mean reversal potential of the ramp-activated current generated under Na⁺- and Li⁺-containing aCSF. D1: the ramp-activated current generated by guanidinium-containing aCSF is superimposed to the control current. D2: bar graphs show the mean reversal potential of the ramp-activated current generated under Na⁺ and guanidinium-containing aCSF. Mean comparison was tested with a paired t-test (**P < 0.01).
of the NeuN-positive cells in the MnPO was co-labeled for the NaX channel (9 rats; Fig. 6A). Thus the NaX channel is strongly expressed in a large neuronal population of the rat MnPO. Because the NaX channel had been previously found in mouse glial cells, expression of the NaX channel was also examined with the same antibody in mouse brain slices (from 6 C57BL/6 mice) containing the MnPO. The immunohistochemical data showed an absence of NaX labeling in the MnPO of mice (Fig. 6B). It is noteworthy that the discrepancy in NaX labeling between rats and mice is likely not attributable to a marked species difference in the sequence of the targeted peptide (Supplementary Fig. 1B). These anatomical data thus suggest that Na+ sensing might not be a common feature of rodent MnPO neurons. Moreover we tested whether neurons lacking the NaX channel can sense changes in [Na+]out. Electrophysiological recordings ([Na+]out: 150 mM; HP: −60 mV) were carried out in dissociated MnPO neurons (n = 7) and in dissociated cortical neurons (n = 9) from C57BL/6 mice and were compared with similar recordings performed in the rat (n = 14 and n = 9, respectively). Transient application of hypernatriuric aCSF (30 s, 170 mM NaCl) triggered an inward shift of the HC in a majority of the neurons tested regardless of
the brain region or species from which the neurons were recorded (Fig. 7A). The amplitude of the evoked current, however, showed discrepancies between the neuronal populations [1-way ANOVA, $F(3,28) = 8.43, P = 0.0004$]. The amplitude of the Na\textsuperscript{+}-induced current was significantly higher in the rat MnPO neurons compared with the other neuronal populations tested (Dunnett's test, $P < 0.05$; Fig. 7B). The current amplitude was not significantly different among the rat and mouse cortical neurons and the mouse MnPO neurons. Taken together, the high correlation between the electrophysiological recordings (Na\textsuperscript{+} leak current) and the immunofluorescent labeling (immunoreactivity for the Na\textsubscript{X} channel) strongly supports the identification of the Na\textsubscript{X} channel as the Na\textsuperscript{+} leak channel responsible for the Na\textsuperscript{+}-sensing ability of dissociated rat MnPO neurons. Moreover Na\textsuperscript{+} sensing was not observed in either rat cortical neurons or mouse cortical and MnPO neurons, which emphasizes the uniqueness of this feature of the rat MnPO.

A dual system of Na\textsuperscript{+}-sensing cells in the rat MnPO, consisting of both neurons and glial cells, is an attractive hypothesis. To test this possibility, whole cell recordings were carried out in brain slices containing the MnPO. Transient application (1 min) of hyper- and hyponatriuric aCSF (isosmotic) over the MnPO area rapidly depolarized and hyperpolarized the neurons, respectively ($n = 8$; Fig. 8A and C). These data, combined with the absence of a specific response to a change in extracellular osmolality, confirmed the ability of in situ MnPO neurons to change their excitability as a function of the [Na\textsuperscript{+}]\textsubscript{out}. This change in neuronal excitability might be partially mediated by lactate, a modulator released by glial cells in response to a hypernatriuric stimulus (Shimizu et al. 2007). The effects of

FIG. 6. Expression of the Na\textsubscript{X} channel in the MnPO neurons is specific to the rat. A1: combined immunofluorescent labeling of the Na\textsubscript{X} channel and of the NeuN protein in a rat brain slice reveals the presence of double-labeled cells in the MnPO. Note that the Na\textsubscript{X} labeling (green) was restricted to the MnPO neurons and to the cell layer lining the 3rd ventricle. Scale bar: 150 μm. A2: microphotographs illustrating high magnification ($\times$63) pictures of the previous staining. Scale bar: 20 μm. White arrows indicate typical examples of double-labeled cells in the MnPO. 3V, 3rd ventricle; ac, anterior commissure. B1: combined immunohistochemical labeling of the Na\textsubscript{X} channel and of the NeuN protein in a mouse brain slice. Note the absence of Na\textsubscript{X} labeling in the MnPO. Scale bar: 150 μm. B2: typical microphotographs illustrating high magnification ($\times$40) pictures of the previous staining. Scale bar: 20 μm. White arrows point NeuN positive cells in the MnPO.
local application of lactate (1 mM, 5–8 min) were tested in neurons that displayed the characteristic Na\textsuperscript{+}\text/-induced depolarization (n = 10). Lactate had no effect on the membrane potential of any of the neurons tested. This finding excludes the participation of the gliotransmitter lactate in Na\textsuperscript{+}\text/-induced depolarization of MnPO neurons (Fig. 8, B and C).

**DISCUSSION**

The present study indicates that neurons of the rat MnPO display real Na\textsuperscript{+}-sensing ability. In addition, we demonstrate the cellular mechanism of neuronal Na\textsuperscript{+}-sensing, i.e., the modification of the amplitude of a tonic Na\textsuperscript{+} influx through a specific Na\textsuperscript{+} leak channel. A strong similarity between the electrophysiological and immunofluorescent data supports the identification of this Na\textsuperscript{+} leak channel as the Na\textsubscript{X} channel in the MnPO. Interestingly we show here that detection of extracellular Na\textsuperscript{+} is not a common feature of rodent MnPO neurons. Indeed, neurons of the mouse MnPO do not specifically respond to hypernatriuric aCSF nor are they immunopositive for the Na\textsubscript{X} channel.

The present results obtained in dissociated MnPO neurons thus unravel the cellular mechanism of Na\textsuperscript{+} sensitivity that was first reported in MnPO neurons recorded from a tissue slice preparation (Grob et al. 2004).

**MnPO neurons act as genuine sodium sensors**

A previous study reported that a large neuronal population in the MnPO responded to modifications in extracellular [Na\textsuperscript{+}] by changes in membrane potential, thereby permitting these neurons to adjust their firing rate as a function of the environmental Na\textsuperscript{+} level (Grob et al. 2004). The exact role of MnPO neurons in the process of Na\textsuperscript{+} detection in the hypothalamus was later questioned, however, when the identity of the Na\textsuperscript{+} sensor in the SFO was assigned to glial cells (Watanabe et al. 2006) and when the ability of glia to release the gliotransmitter lactate during a hypernatremic challenge was demonstrated (Shimizu et al. 2007). This concept thus required a careful investigation of the cellular mechanism underlying the Na\textsuperscript{+} sensitivity of MnPO neurons. The present study demonstrates that the detection of extracellular Na\textsuperscript{+} levels in the MnPO is achieved by neurons, and this conclusion is supported by the methodology of the present study. Indeed the electrophysiological recordings were conducted in acutely dissociated cells—an appropriate preparation to unravel the intrinsic properties of the cells under study. Here application of hypo- or hypernatriuric aCSF to isolated cells triggers an out- or inward current, respectively, compared with isonatriuric conditions. Despite the use of an isonatriuric aCSF that did not permit spike recording, the neuronal phenotype of the dissociated cells was validated with immunocytochemistry. Indeed almost all the dissociated cells obtained from enzymatic and mechanical dissociation of MnPO tissue were immunopositive for the neuronal marker NeuN. In addition, some cells were recorded under regular aCSF at 60 mV, and they all displayed spikes in response to depolarizing pulses (data not shown). These anatomical and electrophysiological data identify the dissociated cells as neurons and demonstrate that Na\textsuperscript{+} sensitivity is an intrinsic property of the MnPO neurons. The possibility that stimulated astrocytes might partially drive the neuronal response induced by a change in [Na\textsuperscript{+}] in the MnPO was investigated using a hypothalamic slice. This type of preparation has been previously demon-
strated to maintain the modulatory action of glial cells on neuronal functions (Gordon et al. 2005; Saleh et al. 1996). Here we tested the action of the gliotransmitter lactate on the electrical activity of MnPO neurons that are depolarized by application of hypernatriuric ACSF. However, unlike hypernatriuric ACSF, lactate did not increase the firing activity of the neurons tested. This result indicates that the release of lactate by astrocytes in the MnPO under local hypernatriemic conditions is unable to influence neural activity and thus to contribute to the Na⁺-sensing mechanism in this nucleus. This result did not, however, rule out a role for glial cells in the complex cellular responses triggered by an alteration of the electrolyte balance in the MnPO. Such a modulatory role on neural activity could take place via the tonic release of angiotensin II that was reported in this nucleus (Henry et al. 2009). Astrocytes might also contribute to the acute regulation of the membrane potential of MnPO neurons by releasing the gliotransmitter taurine under hypoosmotic conditions. The latter regulatory action has been shown to take place in the supraoptic nucleus (Hussy et al. 2009). Astrocytes might also contribute to the Na⁺-sensing mechanism in this nucleus. This result did not, however, rule out a role for glial cells in the complex cellular responses triggered by an alteration of the electrolyte balance in the MnPO. Such a modulatory role on neural activity could take place via the tonic release of angiotensin II that was reported in this nucleus (Henry et al. 2009). Astrocytes might also contribute to the acute regulation of the membrane potential of MnPO neurons by releasing the gliotransmitter taurine under hypoosmotic conditions. The latter regulatory action has been shown to take place in the supraoptic nucleus (Hussy et al. 1997), a crucial hypothalamic nucleus for osmoregulation.

**Na⁺ leak channel is the molecular entity for Na⁺ detection**

In our previous study, electrophysiological recordings demonstrated that a change in extracellular Na⁺ levels triggered a current, and the direction of the current was dependent on the [Na⁺] with respect to the physiological [Na⁺] (Grob et al. 2004). However, the chemical nature of the current triggered by the change in [Na⁺] had not been identified. Here we manipulated the electrochemical gradient for the Na⁺ ions and to estimate the reversal potential of the current generated. Our results clearly show that the current was strictly carried by Na⁺ ions. The identity of the channel was determined by estimating the permeability of the pore for specific ions. The high permeability of the channel to Li⁺, combined with its poor permeability to guanidinium, ruled out the implication of a nonselective cation channel in this effect (Bevan and Yeats 1991; Cook et al. 1990). Moreover the permeability of these ions calculated with the Goldman-Hodgkin-Katz equation using the reversal potential of each individual current further identified this channel as a Na⁺ channel (Hille 2001). The stability of the Na⁺ conductance observed at various membrane potentials, combined with the voltage independency of the Na⁺-induced inward current, indicates that Na⁺ influx resulting from a change in [Na⁺]_out was achieved through a Na⁺ leak channel. The present data therefore identify both the ionic species and the ion channel that underlie the Na⁺-induced response previously reported in in situ MnPO neurons (slice preparation) (Grob et al. 2004).

Interestingly, testing for Rb⁺ permeability revealed a specific characteristic of the Na⁺ channel under study. On the one hand, Rb⁺ shifted the holding current in the outward direction with a concomitant reduction of the current noise. On the other hand, Rb⁺ blocked the Na⁺ current triggered by hypernatriuric ACSF. These data indicate that Rb⁺ in the extracellular solution impedes Na⁺ influx, suggesting that Rb⁺ acts as a blocking cation. The data with Rb⁺ provide the first evidence of the pharmacological profile of this channel. Taken together, the TTX insensitivity, the Rb⁺-induced channel block, and the voltage-independency constitute three specific properties that distinguish this channel from the voltage-gated Na⁺ channel family.

**FIG. 8.** Na⁺-sensing is not indirectly mediated by the release of the glial neuromodulator, lactate. A: typical illustration of the change in membrane potential of a neuron in response to various ACSF compositions. Note that hypernatriuric ACSF (170 mM NaCl, 350 mosm.l⁻¹) triggered a membrane depolarization similar in amplitude to hypernatriuric-isoosmotic ACSF (90 mM Na₂SO₄, 300 mosm.l⁻¹). However, isonatriuric-hyperosmotic ACSF (mannitol, 350 mosm.l⁻¹) had no effect, showing that the change in the membrane potential was mediated by a change in [Na⁺]_out and not by osmolality. Interestingly, hypernatriuric-isoosmotic ACSF triggered membrane hyperpolarization, indicating that the same neurons respond to both hypo and hypernatriuric ACSF. B: local application of hypernatriuric ACSF (170 mM NaCl) depolarized the MnPO neuron, generating a burst of action potentials. This depolarizing effect was not reproduced by lactate (1 mM). Spikes were truncated for illustration. C: bar graph summarizing the direction and mean changes in membrane potential in response to ACSF of various compositions.
Identification of the Na\textsuperscript{+} leak channel

Imaging analysis of the intracellular [Na\textsuperscript{+}] had identified the Na\textsubscript{X} channel as a concentration-sensitive Na\textsuperscript{+} channel, and the presence of this atypical Na\textsuperscript{+} channel was reported in glial cells of the SFO, in DRG neurons, and in neurons of the MnPO (Grob et al. 2004; Hiyama et al. 2002; Watanabe et al. 2006). The present study used dissociated MnPO neurons and a specific antibody against the Na\textsubscript{X} channel to investigate whether the Na\textsuperscript{+} leak channel is the Na\textsubscript{X} channel. In the absence of a specific pharmacological agent that blocks or efficiently reduces the amplitude of the Na\textsubscript{X}-dependent electrophysiological response, this conclusion is supported on the one hand by the high specificity of the antibody directed against the Na\textsubscript{X} channel. In addition to the fact that this antibody had previously been used to localize this channel in several cell types (Hiyama et al. 2002; Knittle et al. 1996), the specificity of the anti-Na\textsubscript{X} antibody was further verified with a preabsorption test using a recombinant Na\textsubscript{X} fusion protein containing the rat polypeptide sequence targeted to prepare the anti-Na\textsubscript{X} antibody. On the other hand, the percentage of double-labeled dissociated cells (NeuN- and Na\textsubscript{X}-immunoreactive cells) closely matched the percentage of dissociated cells displaying a Na\textsuperscript{+}-induced change in their holding current. Interestingly in cortical neurons that lack the Na\textsubscript{X} channel, the amplitude of the Na\textsuperscript{+}-induced response was dramatically smaller compared with the response measured in MnPO neurons. Finally the immunocytochemical data were validated in a brain slice preparation where the Na\textsubscript{X} staining was clearly present in a large population of NeuN-positive cells. This result agrees well with previous data showing the presence of Na\textsubscript{X} mRNA in the MnPO (Grob et al. 2004). Interestingly the latter study also indicated that neurons recorded in a brain region lacking Na\textsubscript{X} mRNA (median septum) did not display a hypotonic aCSF-induced membrane response. Taken together, all the data obtained from dissociated neurons, as well as from neurons in tissue preparations, strongly indicate that Na\textsuperscript{+} sensing is a unique feature of MnPO neurons. Moreover, our present and previous data favor the identification of the Na\textsuperscript{+} leak channel as the Na\textsubscript{X} channel in the MnPO.

Does Na\textsuperscript{+} sensing in the hypothalamus involve distinct mechanisms?

Sodium detection in the hypothalamus takes place in several nuclei. This fact highlights the complexity of the system, or systems working in concert, that maintains hydromineral homeostasis. In the magnocellular neurons of the supraoptic nucleus that synthesizes the antidiuretic and natriuretic hormones, local osmoreceptors have been shown to integrate both the Na\textsuperscript{+} and osmotic signals. This integration is achieved via mechanosensitive cation channels, the relative permeability of which to Na\textsuperscript{+} is increased with respect to the [Na\textsuperscript{+}]\textsubscript{out} (Voisin et al. 1999). In addition to these mechanosensitive channels, Na\textsubscript{X} channels contribute to [Na\textsuperscript{+}]\textsuperscript{i} detection in the SFO and in the MnPO. Despite the apparent similarity of the Na\textsuperscript{+} sensors in these two nuclei, the phenotype of the cells expressing the Na\textsubscript{X} channels, and thus the cellular mechanism of Na\textsuperscript{+} detection seems different in the brain structures monitoring sensory information mainly from the blood (SFO) and from the CSF (MnPO). In the SFO, glial cells express the Na\textsubscript{X} channel, and they release lactate in response to Na\textsuperscript{+}-induced enhancement of glucose uptake. Lactate in turn alters the excitability of local inhibitory neurons (Shimizu et al. 2007). In the MnPO, our results demonstrate that the Na\textsubscript{X} channels are expressed by neurons that have the intrinsic ability to transduce changes in [Na\textsuperscript{+}]\textsubscript{out} into modifications of Na\textsuperscript{+} influx. Therefore the output of the MnPO Na\textsuperscript{+}-sensor neurons is a function of the incoming Na\textsuperscript{+} information because their activity is modulated by both hyponatriuric CSF (outward current inducing a membrane hyperpolarization) and hypernatriuric CSF (inward current inducing a membrane depolarization).

Our findings also suggest that the mechanisms for brain sodium sensing might be species specific. In the rat, Na\textsuperscript{+} sensors are located in the MnPO to detect changes in CSF Na\textsuperscript{+} levels. In addition, Na\textsuperscript{+} sensors might also be present in the SFO to detect variations in plasma Na\textsuperscript{+} levels. This latter hypothesis is supported by the detection of Na\textsubscript{X} mRNA (Grob et al. 2004) and Na\textsubscript{X} protein in SFO neurons (data not shown). Interestingly functional osmoreceptors are also present in the rat SFO. Indeed electrophysiological recordings have shown that the activity of SFO neurons is significantly altered by changes in extracellular osmolality specifically achieved without changing the [Na\textsuperscript{+}]\textsubscript{out} (Anderson et al. 2000). However, in mice, Na\textsuperscript{+} detection in the brain seems restricted to the circumventricular organs because the gene encoding the Na\textsubscript{X} channel (Na 2 cDNA) does not appear to be expressed in the MnPO (Watanabe et al. 2000). This conclusion is further supported by the present data showing the absence of immunoreactivity for the Na\textsubscript{X} channel in the mouse MnPO. It should be noted that a change in [Na\textsuperscript{+}]\textsubscript{out} triggered a weak inward current in mouse MnPO neurons. This current, however, might represent a fairly general feature of many neurons rather than a specific response of the MnPO neurons because this small-amplitude Na\textsuperscript{+}-induced current was also recorded in cortical neurons of both rats and mice.

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