Regulation of Central Na^+ Detection Requires the Cooperative Action of the Na_x Channel and $\alpha 1$ Isoform of Na^+/K^+ -ATPase in the Na^+ -Sensor Neuronal Population

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The median preoptic nucleus (MnPO) holds a strategic position in the hypothalamus. It is adjacent to the third ventricle; hence, it can directly access the ionic composition of the CSF. MnPO neurons play a critical role in hydromineral homeostasis regulation by acting as central sensors of extracellular Na^+ concentration ($[\text{Na}^+]_{\text{ext}}$). The mechanism underlying Na^+ sensing involves the atypical Na^+ channel, Na_x . Here we sought to determine whether Na^+ influx in Na^+ sensors is actively regulated via interaction with other membrane proteins involved in cellular Na^+ homeostasis, such as Na^+/K^+ -ATPase.

The Na^+/K^+ -ATPase role was investigated using patch-clamp recordings in rat MnPO dissociated neurons. Na^+ current evoked with hypernatremic solution was diminished in the absence of ATP/GTP, indicating that Na^+/K^+ -ATPase play a central role in $[\text{Na}^+]_{\text{ext}}$ detection. Specific blockers of $\alpha 1$ and $\alpha 3$ isoforms of Na^+/K^+ -ATPase, ouabain or strophanthidin, inhibited this Na^+ current. However, strophanthidin, which selectively blocks the $\alpha 1$ isoform, was more effective in blocking Na^+ current, suggesting that the Na^+/K^+ -ATPase- $\alpha 1$ isoform is specifically involved in $[\text{Na}^+]_{\text{ext}}$ detection. Although strophanthidin did not alter either the membrane resistance or the Na^+ reversal potential, the conductance and the permeability of the Na_x channel decreased significantly. Our results suggest that Na^+/K^+ -ATPase interacts with the Na_x channel and regulates the high $[\text{Na}^+]_{\text{ext}}$ -evoked Na^+ current via influencing the Na^+ influx rate. This study describes a novel intracellular regulatory pathway of $[\text{Na}^+]_{\text{ext}}$ detection in MnPO neurons. The $\alpha 1$ isoform of Na^+/K^+ -ATPase acts as a direct regulatory partner of the Na_x channel and influences Na^+ influx via controlling the Na^+ permeability of the channel.

Introduction

Fluid homeostasis requires mechanisms controlling sodium (Na^+) concentration and water retention in intracellular and extracellular fluid compartments of the body. Na^+ sensing in body fluids is a critical step for hydromineral homeostasis, and sensors have been located in the systemic viscera and in the brain. The presence of osmoreceptors that detect hyperosmolality in the brain has been localized in the nuclei of the anterior hypothalamus (for review, see Orlov and Mongin, 2007). Mechanisms underlying osmodetection were well described in the magnocellular cells of the supraoptic nucleus, where extracellular osmolality changes influenced cell volume, leading to the activation of mechanosensitive channels (Oliet and Bourque, 1994). Moreover, osmoreceptors also have been identified in the vascular organ of the lamina terminalis (OVLt). OVLt neurons express transient receptor potential vanilloid channels, a cationic channel sensitive to osmotic variations. Changes in extracellular osmolar-

ity are translated to altered action potential firing rates in these cells (Zhang and Bourque, 2006). In addition to osmoreceptors, the presence of Na^+ sensors in the CNS was also demonstrated (Osborne et al., 1987). The median preoptic nucleus (MnPO) is located in the lamina terminalis along the third ventricle, hence it can directly access the ionic composition of CSF (Fitzsimons et al., 1998; Hussy et al., 2000). Direct access to the CSF places MnPO neurons in a key position to detect and regulate changes in the osmolarity-sodium concentration (McKinley et al., 1999). Indeed, a previous study showed that MnPO specifically responds to local variations in Na^+ concentration ($[\text{Na}^+]$) by changes in neuronal membrane potential and firing rate (Grob et al., 2004). This ability to detect $[\text{Na}^+]_{\text{ext}}$ variations is attributable to the presence of the Na_x channel, an atypical Na^+ channel described as a specific Na^+ sensor (Hiyama et al., 2002, 2004; Noda, 2006). The Na_x channel has recently been characterized as a Na^+ leak channel passing Na^+ ions through the membrane and detecting changes in $[\text{Na}^+]_{\text{ext}}$ (Tremblay et al., 2011). However, the mechanism by which the Na_x channel controls Na^+ accumulation remains unclear. Maintenance of a balanced intracellular sodium homeostasis is important to avoid cellular toxicity, and especially to maintain a low intracellular $[\text{Na}^+]$ for an accurate detection of $[\text{Na}^+]_{\text{ext}}$ changes.

The Na^+/K^+ -ATPase pump is the main extrusion system of intracellular Na^+ . This enzyme needs ATP to function and generates Na^+ ion efflux to maintain a low cytosolic $[\text{Na}^+]$. The

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catalytic α subunit contains a selective inhibitory binding site for cardiac glycosides (CGs) (Sweadner, 1989; Jewell and Lingrel, 1992). In the context of “local” regulation of Na⁺ homeostasis, functional interaction between the Na_x channel and Na⁺/K⁺-ATPase would be an important pathway of membrane–Na⁺-exchange regulation, which could be implicated in the detection of osmotic changes.

This study aims to determine the mechanism involved in the regulation of Na⁺ influx in MnPO neurons after changes in intracellular [Na⁺] and to identify the molecular partners of the Na_x channel involved in this regulation. Using MnPO dissociated neurons, we investigated the possibility that the Na_x channel is functionally regulated by the Na⁺/K⁺-ATPase pump and the underlying cellular mechanisms of [Na⁺]_{ext} detection.

Materials and Methods

Cell preparation

Wistar rats (3 weeks old, male) were deeply anesthetized by intraperitoneal injection of a ketamine–xylazine mixture (87.5 and 12.5 ml/kg, respectively) and decapitated. The brain was immersed in oxygenated (95% O₂–5% CO₂) ice-cold (4°C) dissection solution containing (in mM) 200 sucrose, 10 D-glucose, 2 KCl, 1 CaCl₂, 3 MgCl₂, 26 NaHCO₃, and 1.25 NaH₂PO₄, and a sagittal slice of 350 μ m containing the MnPO was prepared. The ventral region of the MnPO was then punched out and placed in oxygenated artificial CSF (aCSF) containing (in mM) 140 NaCl, 3.1 KCl, 2.4 CaCl₂, 1.3 MgCl₂, 10 HEPES, and 10 D-glucose, pH 7.4 (osmolality, 295–300 mosm $\cdot$ kg⁻¹). Enzymatic dissociation of the MnPO micropunches was obtained by successively incubating the pieces of tissue in aCSF containing Pronase (0.1 mg/ml) for 10 min at 37°C, then in aCSF containing bovine serum albumin (BSA; 2 mg/ml) for 15 min at 37°C, and finally in aCSF containing thermolysin (0.1 mg/ml) for 10 min at 37°C. The pieces of tissue were then mechanically dissociated by trituration using glass Pasteur pipettes with reduced diameter. Centrifugation (3500 rpm for 3 min at room temperature) was performed, and neurons were resuspended in aCSF solution. A total of 50 μ l of aCSF containing dissociated cells was directly plated on laminin-coated microcover glasses. The seeded microcover plate was incubated for 1 h at 37°C, in a 95% O₂–5% CO₂ humidified atmosphere, before being used for patch-clamp recordings or immunocytochemistry.

Electrophysiological recordings

Whole-cell voltage-clamp recordings were performed on dissociated MnPO neurons visualized under the Hoffman modulation contrast. Micropipettes were filled with a solution containing (in mM) 130 K-gluconate, 10 HEPES, 6 NaCl, 0.3 Na⁺-GTP, 4 Na⁺-ATP, and 10 EGTA, pH 7.2 (osmolality, 295–300 mosm $\cdot$ kg⁻¹). Micropipettes had a resistance of 4–5 M Ω . The extracellular solution (control aCSF) had the following composition (in mM): 140 NaCl, 3.1 KCl, 2.4 CaCl₂, 1.3 MgCl₂, 10 HEPES, and 10 D-glucose, pH 7.4 (osmolality, 295–300 mosm $\cdot$ kg⁻¹). Hypernatrionic aCSF was obtained by increasing NaCl concentration to 170 mM. Note that Na⁺ sensor neurons are defined as ones responding to a hypernatrionic application with a minimal depolarization of 4 pA. The condition allows separating the Na⁺ response of the membrane background noise. Please note also that MnPO neurons do not respond to osmolality changes (Grob et al., 2004; Tremblay et al., 2011). To test the Na⁺/K⁺-ATPase implication in Na⁺ regulation, pharmacological tests were performed, and stock solution of ouabain (10 mM) and strophanthidin (50 mM) were directly diluted in control aCSF. For recordings made under ATP- and GTP-free conditions, Na⁺-GTP and Na⁺-ATP were not added to the pipette solution.

For recordings designed to test the Na⁺ conductance and Na⁺ permeability of the Na_x channel, the micropipettes were filled with a solution containing (in mM) 100 NaCl, 25 CsCl, 5 HEPES, 5 TEACl, 0.3 Na⁺-GTP, and 4 Na⁺-ATP, pH 7.3 (osmolality, 295–305 mosm $\cdot$ kg⁻¹).

Isonatrionic aCSF required to test Na⁺ permeability and Na⁺ conductance of the channel had the following composition (in mM): 100 NaCl, 3.5 CsCl, 3.1 KCl, 2.4 CaCl₂, 1.3MgCl₂, 0.3 CdCl₂, 10 HEPES, 10 TEACl, 5

D-glucose, 1 4-aminopyridine, and 0.0005 TTX, pH 7.4 (osmolality, 295–300 mosm $\cdot$ kg⁻¹). Hypernatrionic aCSF was obtained by raising NaCl concentration to 170 mM.

All the electrophysiological recordings performed in dissociated neurons were performed at room temperature (21–23°C).

Calculation of the input resistance, the Na⁺ conductance, and the Na⁺ permeability

Calculation of the current resistance was obtained from a modified Ohm's law equation: $r = \Delta V_m / I_{\text{Test}}$.

I_{Test} was determined experimentally, and ΔV_m (–6 mV) was imposed by experimentation.

Calculation of the Na⁺ conductance (g_{Na}) was obtained from a modified Hodgkin and Huxley equation:

$$g_{\text{Na}} = \frac{I_{\text{Na}}}{V_m - E_{\text{Na}}} \quad (1)$$

The Na⁺ current (I_{Na}), the membrane potential (V_m), and the reversal potential of the Na⁺ current (E_{Na}) were experimentally determined. To determine the experimental reversal potential of the [Na⁺]-induced current (E_{Na}), all the current traces were subtracted from the total leak current by scaling the zero-current potential of the ramp-activated current to 0 mV under the isonatrionic condition (control).

Determination of theoretical E_{Na} was achieved for hypernatrionic aCSF ([Na⁺]_{out}: 170 mM NaCl) and was obtained from the following Goldman-Hodgkin-Katz (GHK) equation:

$$E_{\text{Na}} = \frac{RT}{zF} \ln \frac{[\text{Na}]_{\text{out}}}{[\text{Na}]_{\text{in}}} \quad (2)$$

Theoretical $E_{\text{Na}} = 13.6$ mV.

Calculation of the permeability was obtained from the following modified GHK Equation 1:

$$P_{\text{Na}} = \frac{I_{\text{Na}}}{z_{\text{Na}} \cdot V_m \cdot [\text{Na}]_{\text{in}}} \quad (3)$$

In this equation, the driving concentration

$$V_{\text{Na}} = z_{\text{Na}} \left(\frac{E_{\text{Na}} \cdot F}{RT} \right), \quad (4)$$

replacing V_{Na} in Equation 1.

$$P_{\text{Na}} = \frac{I_{\text{Na}}}{z_{\text{Na}} \cdot F \left(\frac{E_{\text{Na}} F}{RT} \right) \cdot [\text{Na}]_{\text{in}}} \quad (5)$$

I_{Na} and E_{Na} were experimentally determined ($r = 8.31447$ J/K $\cdot$ mol; $F = 96485$ C/mol; $z_{\text{Na}} = +1$; $T = 298.15$ K).

Immunocytochemistry

After tissue dissociation of the MnPO, 50 μ l of suspended cells was directly plated on a microscope coverslip for 20–30 min at 37°C. The dissociated cells were fixed for 60 min in a paraformaldehyde solution (pH 7.4) and immediately used for immunocytochemistry. The fixed cells were incubated overnight at 4°C in PBS containing 5% native goat serum, 1% BSA with rabbit anti-Na_x antibody (1:250; for details, see Tremblay et al., 2011), and mouse anti-NeuN antibody (1:500, clone A60; Millipore) or mouse anti-Na⁺/K⁺-ATPase $\alpha 3$ subunit (1:10, clone XVIF9-G10; Sigma-Aldrich) or mouse anti-Na⁺/K⁺-ATPase $\alpha 1$ subunit (1:10, clone C464.6; Millipore). The slides were first washed in PBS and then incubated for 2 h at room temperature in PBS containing Alexa Fluor-488 goat anti-rabbit (1:500, green; Invitrogen) and Alexa Fluor-555 goat anti-mouse (1:500, red; Invitrogen) as secondary fluorescent antibodies to visualize the Na_x and NeuN or Na⁺/K⁺-ATPase $\alpha 3$ or $\alpha 1$ proteins, respectively.

Immunocytochemical acquisition and analysis

Acquisition. Confocal laser-scanning microscopy was performed with an IX81-ZDC microscope equipped with a FV1000 scanning head and an Olympus 60 $\times$ OSC, NA 1.4 objective lens. Confocal images were acquired by sequential scanning with the 405, 488, and 546 nm laser lines, and the variable bandwidth filters were set optimally for the spectral properties of DAPI, Alexa Fluor 488, and Alexa Fluor 555 dyes. The Fluoview imaging software ASW3.01a (Olympus) was used to acquire and export z-stacks. Maximum intensity projections and volume rendering were calculated using the Surpass module in Bitplane Imaris 7.5.1.

Analysis. Colocalization analysis was performed with the Bitplane Imaris 7.5.1 colocalization module using the Costes' estimation for automatic threshold, which compares the Pearson's coefficient for nonrandomized versus randomized images and calculates the significance (Costes et al., 2004). The colocalization channel of Na_x with $\alpha 1$ Na^+/K^+ -ATPase was generated for visual representation, and Pearson's coefficients were calculated.

Immunocytochemistry quantification. The percentage of $\alpha 1$ and $\alpha 3$ isoform expression in the dissociated cell was obtained by cell counting and normalized with DAPI expression: three representative areas by slide, and three slides (one slide by animal) have been counted.

Statistical analysis

Results obtained from electrophysiological recordings were expressed as means $\pm$ SE. Current amplitude was normally distributed and analyzed using parametric statistical tests. Comparison of current amplitude during pharmacological tests (multiple application test, ouabain test, strophanthidin test, occlusion test), as well as comparisons of action potential characteristics, were performed using ANOVA with repeated measure (ANOVA_{rm}) as indicated in the text. We used the Newman-Keuls comparison test as a *post hoc* test for follow-up analysis. Comparisons of membrane characteristics (reversal potential, conductance, and permeability) were performed using a paired Student's *t* test. Statistical significance was defined as $p < 0.05$.

Results

A majority of neurons in the MnPO of adult rats have been demonstrated to specifically respond to changes in extracellular Na^+ concentration (Grob et al., 2004; Tremblay et al., 2011). These neurons are Na^+ sensors as they respond to changes in extracellular Na^+ concentration but not to changes in osmolality. The leak Na^+ channel Na_x has been demonstrated to be responsible for Na^+ sensing in this neural population (Tremblay et al., 2011). To characterize the response of MnPO neurons to altered extracellular $[\text{Na}^+]$, we recorded from dissociated MnPO neurons in voltage-clamp mode a repeatedly increased $[\text{Na}^+]_{\text{ext}}$. Cells were held around their resting membrane potential (range, -63 to -60 mV) for 2 min to allow the stabilization of the membrane holding current. Transient rise in aCSF $[\text{Na}^+]$ (170 mM NaCl; 20–30 s) triggered an inward current of 21.3 ± 3.3 pA ($n = 14$) (Fig. 1). These changes in membrane current were fully reversible after reexposure to isotonic aCSF (185.88 ± 21.4 s). This evoked current carried by Na^+ ions is the electrophysiological fingerprint of Na^+ -sensing neurons (Tremblay et al., 2011). The evoked Na^+ current did not show desensitization during repetitive application of hypernatric aCSF (ANOVA_{rm}; $F_{(2,39)} = 0.023$, $p = 0.5359$; $n = 14$; Fig. 1A). Maintenance of the evoked Na^+ current was, however, highly sensitive to cell energy depletion because the amplitude of the inward Na^+ current collapsed when repetitive application of transient hypernatric aCSF was performed in the absence of ATP and GTP in the patch pipette (ANOVA_{rm}; $F_{(4,44)} = 78.46$, $p < 0.0001$; $n = 16$; Fig. 1B1,B2). Rundown of the evoked Na^+ current caused by energy deprivation indicates that the system of Na^+ sensing depends on ATP and GTP presence and that suppression of ATP/GTP rapidly impairs Na^+ influx in Na^+ -sensing neurons.

Pharmacological impairment of Na^+/K^+ -ATPase activity reduces Na^+ influx in individual Na^+ sensor neurons

Our data showing that energy deprivation impairs Na^+ influx as the Na_x channel rapidly desensitizes suggest that Na^+/K^+ -ATPase could be involved in Na^+ current impairment. To test this possibility, first the expression pattern of various Na^+/K^+ -ATPase isoforms in MnPO neurons was analyzed. Double immunocytochemistry labeling of dissociated MnPO neurons was performed with an anti- Na_x antibody and a specific antibody directed against either the $\alpha 1$ or the $\alpha 3$ isoform of Na^+/K^+ -ATPase. Almost all the Na_x -expressing cells (90.4%) were immunopositive for the $\alpha 1$ isoform and also for the $\alpha 3$ isoform (90.9%) of Na^+/K^+ -ATPase (Fig. 1C). These double-labeling results demonstrate that both isoforms of the Na^+ pump are expressed in a single Na^+ -sensing neuron and that they might directly or indirectly interact with the Na_x channel. Next, the direct functional interaction between Na^+/K^+ -ATPase and the Na_x channel hypothesis was tested. Toward this aim, the evoked Na^+ currents in MnPO neurons were recorded in whole-cell configuration, and the effect of Na^+/K^+ -ATPase inhibitors was assayed. Two Na^+/K^+ -ATPase inhibitors, ouabain and strophanthidin were used, because they showed different degrees of affinity for the $\alpha 1$ and $\alpha 3$ isoforms of the Na^+ pump. Ouabain exhibits higher affinity to the $\alpha 3$ isoform, whereas strophanthidin inhibits more effectively the $\alpha 1$ isoform. The effect of hypernatric aCSF application (170 mM NaCl, 20 s) on the evoked Na^+ current was compared in the absence or in the presence of 50 μM ouabain (1 min). Ouabain significantly reduced the amplitude of evoked Na^+ current from 18.8 ± 8.7 to 8.8 ± 3.8 pA ($n = 11$; Fig. 2A). Ouabain-mediated inhibition was reversible. The evoked Na^+ current was partially recovered after 10 min of drug washout (ANOVA_{rm}; $F_{(2,20)} = 18.27$, $p = 0.0003$; $n = 11$). To test whether the two isoforms are equally involved, 50 μM strophanthidin (1 min) was applied, and the resulting evoked Na^+ current was measured. Strophanthidin also decreased the amplitude of the evoked Na^+ current from 23.5 ± 4.6 pA to 9.5 ± 5.1 pA ($n = 13$; Fig. 2B). Strophanthidin-mediated inhibition partially recovered after 10 min of drug washout (ANOVA_{rm}; $F_{(2,24)} = 31.38$, $p \leq 0.0001$; $n = 13$). Inhibition of the evoked Na^+ current was apparently more effective with strophanthidin than ouabain (50 μM ; $59.6 \pm 6.2\%$ vs $48.61 \pm 5.1\%$, respectively; Fig. 2); thus, we tested other concentrations to obtain dose-response curves and measure the half-maximal inhibitory concentrations (IC_{50}) of each drug. The IC_{50} values allowed us to further characterize which α isoform ($\alpha 1$ or $\alpha 3$) could be specifically involved in Na^+ current inhibition. The dose-response curves of the two Na^+ pump inhibitors showed that ouabain IC_{50} was 23.2 μM , whereas strophanthidin IC_{50} was lower (9.6 μM) (Fig. 3A). These pharmacological results strongly suggested that inactivation of the $\alpha 1$ isoform of Na^+/K^+ -ATPase plays a dominant role in the reduction of evoked Na^+ currents. Next, the relative involvement of $\alpha 1$ and $\alpha 3$ isoforms of Na^+/K^+ -ATPase in the Na^+ current impairment was determined with concomitant application of strophanthidin and ouabain on evoked Na^+ currents. After control application of hypernatric aCSF, strophanthidin (40 μM ; 1 min) decreased the Na^+ current amplitude from 23.5 ± 4.5 pA to 10.5 ± 2.9 pA ($n = 12$; Fig. 3B). Consecutive and concomitant application of ouabain (40 μM ; 1 min) did not decrease the Na^+ current amplitude any further, indicating that inhibition of the $\alpha 1$ isoform produced the maximal inhibition of the evoked Na^+ current (ANOVA_{rm}; $F_{(2,78)} = 9.8$, $p = 0.0002$; $n = 15$).

To confirm our previous results obtained with strophanthidin and test the putative physiological relevance of this inhibition, we

Figure 1. Desensitization of the Na_x channel, in the absence and presence of ATP/GTP. **A1**, Representative example of the inward Na^+ current observed during repeated application of hypernatremic aCSF (170 mM NaCl). **A2**, Mean Na^+ current amplitudes triggered by repeated application of hypernatremic aCSF. **B1**, Inward Na^+ current triggered by repeated applications of hypernatremic aCSF recorded with ATP/GTP-free intracellular solution. **B2**, Mean normalized Na^+ current amplitude triggered by each application of hypernatremic aCSF recorded with ATP/GTP-free intracellular solution. **C**, Typical microphotographs of immunocytochemical staining for DAPI, the Na_x channel, and the $\text{Na}^+/\text{K}^+-\text{ATPase}$ $\alpha 1$ or $\alpha 3$ subunit (top and bottom, respectively) in dissociated MnPO neurons ($***p < 0.01$). Stim, Stimulation.

tested the effect of an endogenous pharmacological agent, cinobufotalin. This agent, similarly to strophanthidin, exhibits higher affinity to the $\alpha 1$ isoform of $\text{Na}^+/\text{K}^+-\text{ATPase}$. Cinobufotalin is an endogenous mammalian bufadienolide released from the hypothalamus; hence, it could potentially act as regulator of $\text{Na}^+/\text{K}^+-\text{ATPase}$ activity *in vivo*. Local application of cinobufotalin (10 μM ; 1 min; Fig. 3C) also reduced the amplitude of the evoked Na^+ current from 20.4 ± 4.4 pA to 15.1 ± 2.2 pA (*t* test: $t = 0.047$; $p = 0.0005$; $n = 6$). The inhibitory effect of strophanthidin and cinobufotalin on evoked Na^+ current was similar (10 μM cinobufotalin induced $27.5 \pm 5.8\%$ inhibition, and 10 μM

strophanthidin induced $29.6 \pm 8.1\%$). In contrast, 10 μM ouabain only reduced the Na^+ current amplitude by $6.1 \pm 3.4\%$.

Overall, the present pharmacological results demonstrated that the decreased activity of the $\alpha 1$ isoform of $\text{Na}^+/\text{K}^+-\text{ATPase}$ leads to decreased Na^+ influx during hypernatremic aCSF application. The strong inhibitory effect of strophanthidin could be mimicked by the endogenously released cinobufotalin, which is released on acute plasma expansion and by chronic administration of the high- Na^+ diet (Fedorova et al., 1998, 2001). This finding suggests that physiologically relevant endogenous agents could regulate the Na^+ influx triggered by an increase in the

Figure 2. Na^+/K^+ -ATPase pump modulates inward Na^+ current through Na_x channel. **A1**, Inward Na^+ current triggered by hypernatremic aCSF under the control condition and during ouabain application ($50 \mu\text{M}$, 1 min). **A2**, Scatterplot graph illustrating the amplitude of the Na^+ currents triggered by application of hypernatremic aCSF under the control condition, during ouabain application, and after recovery. **A3**, Bar graph shows the mean normalized Na^+ current amplitude triggered by application of hypernatremic aCSF under these three conditions. **B1**, Typical inward Na^+ current triggered by hypernatremic aCSF under the control condition and during application of strophanthidin ($50 \mu\text{M}$, 1 min). **B2**, Scatterplot graph shows the amplitude of the Na^+ current triggered by application of hypernatremic aCSF under the control condition, during strophanthidin application, and under the recovery condition. **B3**, Mean normalized Na^+ current amplitudes triggered by application of hypernatremic aCSF under the three previous conditions ($*p < 0.5$; $**p < 0.1$; $***p < 0.001$). OUAB, Ouabain; STRO, strophanthidin.

extracellular Na^+ concentration via the control of the α isoform of Na^+/K^+ -ATPase.

Our results showed that cells expressing the Na_x channel also express both $\alpha 1$ and $\alpha 3$ isoforms of Na^+/K^+ -ATPase. Using confocal images aimed to determine whether these elements are localized into the same subcellular compartments. Double immunostaining of the Na_x channel and $\alpha 1$ or $\alpha 3$ isoforms of Na^+/K^+ -ATPase were performed on dissociated MnPO neurons and analyzed with a confocal microscope (Fig. 4). Microphotographs showed that although the $\alpha 1$ isoform was highly colocalized in several well defined spots with the Na_x channel, the $\alpha 3$ isoform showed almost no subcellular colocalization. The colocalization rate observed between the Na_x channel and the $\alpha 1$ isoform of Na^+/K^+ -ATPase was 43.54%, representing specific “hot spots.” In contrast, only 5.57% colocalization was observed between the Na_x channel and the $\alpha 3$ isoform of Na^+/K^+ -

ATPase. These results strongly support the hypothesis that the Na_x channel and the $\alpha 1$ isoform of Na^+/K^+ -ATPase are functionally linked and form physiological units in localized subcellular hot spots. These physiological units would be specialized to detect changes in $[\text{Na}^+]$ in MnPO neurons.

Inhibition of the $\alpha 1$ isoform of Na^+/K^+ -ATPase reduces the sensor permeability to Na^+

The reduced Na^+ influx in the presence of Na^+/K^+ -ATPase inhibitors could be explained by either the reduced driving force of Na^+ ions or an active functional interaction between the $\alpha 1$ isoform of Na^+/K^+ -ATPase and the Na^+ leak channel. In order to differentiate between these possibilities, the functional impact of Na^+/K^+ -ATPase activity on Na^+ influx was tested by investigating whether the mechanism of the blockade of the $\alpha 1$ -isoform reduced the inward Na^+ current. As previous results showed, ATP-GTP deprivation altered the basal Na^+ current (e.g., Na^+ leak current; Fig. 1*B*). Therefore, it was investigated whether changes in $\alpha 1$ isoform activity lead to the inhibition of the inward Na^+ current through an influence on the basal Na^+ current, or via a functional interaction with the channel. The effect of three different pharmacological conditions was tested: ATP/GTP-free intracellular medium, ouabain application ($50 \mu\text{M}$), and strophanthidin application ($40 \mu\text{M}$) on basal Na^+ current and on the kinetics of action potentials (APs) evoked with a voltage-step depolarization (Fig. 5). Variations in basal Na^+ current and action potential kinetics would indicate that the altered Na^+ driving force played a role in Na^+ current impairment when Na^+/K^+ -ATPase activity was inhibited. ATP/GTP deprivation induced membrane hyperpolarization ($15.25 \pm 3.387 \text{ pA}$; $n = 11$), corre-

sponding to a diminution of Na^+ leak current (Fig. 5*A1,D*). This variation in the basal current is matched with a significant reduction in AP amplitude from 819.1 ± 55.41 to $85.93 \pm 19.55 \text{ pA}$ (ANOVA_{rm}: $F_{(4,20)} = 68.84$, $p \leq 0.0001$; $n = 6$) and with increased AP half-width ($t_{1/2}$ width) measurements (from 1.282 ± 0.273 to $3.667 \pm 1.447 \text{ ms}$; ANOVA_{rm}: $F_{(4,20)} = 19.84$, $p < 0.0001$; $n = 6$; Fig. 5*A2–A4*). Ouabain application also induced membrane hyperpolarization ($8.514 \pm 4.054 \text{ pA}$; $n = 12$; Fig. 5*B1,D*). Similarly to the changes observed under the ATP/GTP-free condition, ouabain application lead to altered basal Na^+ current and significantly reduced the amplitude of APs (from 775.5 ± 227.4 to $178.7 \pm 55.11 \text{ pA}$; ANOVA_{rm}: $F_{(4,20)} = 6.36$, $p = 0.0018$; $n = 6$), whereas AP half-width was only moderately increased (from 1.558 ± 0.459 to $1.857 \pm 0.408 \text{ ms}$; ANOVA_{rm}: $F_{(4,20)} = 2.74$, $p = 0.0574$; Dunnett's *post hoc* test, $p = 0.0021$; $n = 6$) (Fig.

5B2–B4). Our data showed that ATP/GTP-free medium and ouabain affect both the basal Na^+ current and the Na^+ gradient, suggesting that they can effectively alter the Na^+ driving force.

In contrast, strophanthidin did not hyperpolarize the membrane significantly (0.423 ± 2.132 pA; $n = 9$) (Fig. 5C1,D), and it had no effect on the properties of APs [AP amplitude (ANOVA_{rm}: $F_{(4,15)} = 0.0027$, $p = 1.0$; $n = 5$); $t_{1/2}$ width (ANOVA_{rm}: $F_{(4,15)} = 0.158$, $p = 0.956$; $n = 5$)] (Fig. 5C2–C4). The lack of effect of strophanthidin application on basal Na^+ current or on the properties of APs indicated that this pharmacological agent does not have a significant effect on the membrane current gradient. These results together strongly suggest that Na^+ current inhibition by the Na^+/K^+ -ATPase $\alpha 1$ isoform did not occur via a change in Na^+ driving force.

To further test this hypothesis, the reversal potential (or equilibrium potential) of Na^+ ions was directly measured. Depolarizing voltage ramps from -10 to $+20$ mV (16 mV/s) were applied to the same neuron in isonatriuric (control), hypernatriuric aCSF or in the presence of strophanthidin to estimate the reversal potential of the Na^+ current. The reversal potential of the ramp-activated current in the hypernatriuric solution (11.85 ± 0.880 mV, $n = 8$) or in the presence of strophanthidin (11.74 ± 0.956 mV, $n = 8$) was identical (t test, $t = 0.347$; $p = 0.74$; $n = 8$) (Fig. 6B). These results showed that under the hypernatriuric condition and in the presence of strophanthidin, the ion concentration and gradient direction remains unchanged. Results obtained between these two conditions showed no significant differences in Na^+ reversal potential, suggesting that the inhibition of the inward Na^+ current by the $\alpha 1$ isoform did not affect the Na^+ driving force. Therefore, the mechanism of Na^+ current influx inhibition involved a functional interaction between the $\alpha 1$ isoform of Na^+/K^+ -ATPase and the Na^+ leak channel.

Our results showed that alteration in sodium driving force was not involved in the Na^+ current modulation by the $\alpha 1$ isoform of Na^+/K^+ -ATPase. Next, we aimed to identify the mechanism by which inward Na^+ current inhibition occurs. The inhibition of Na^+ current could be achieved by alterations of other membrane parameters, such as membrane resistance and Na^+ conductance and/or permeability. The membrane resistance applying hyperpolarizing voltage steps (-6 mV) to the same neuron in the control, hypernatriuric condition or in the presence of strophanthidin was measured ($40 \mu\text{M}/1$ min) (Fig. 6C). Membrane resistance was not significantly different under any of these conditions (control, 643.0 ± 102.7 M Ω ; hypernatriuric, 605.6 ± 76.89 M Ω , strophanthidin, 657.8 ± 127.2 M Ω) (ANOVA_{rm}: $F_{(2,8)} = 0.826$, $p = 0.471$; $n = 6$).

Figure 3. The $\alpha 1$ isoform of Na^+/K^+ -ATPase pump specifically regulates the inward Na^+ current via Na_x channel. **A1, A2**, Dose–response curves illustrating the inhibitory effect of ouabain (0 – $100 \mu\text{M}$; **A1**) and strophanthidin (0 – $100 \mu\text{M}$; **A2**) on the inward Na^+ current and indicating the IC_{50} value. **B1**, Typical representation of the inward Na^+ current triggered by hypernatriuric aCSF under the control condition, during strophanthidin application ($50 \mu\text{M}$, 1 min), and during concomitant application of strophanthidin and ouabain ($50 \mu\text{M}/1$ min). **B2**, Scatterplot graph showing the amplitude of the Na^+ current triggered by the application of hypernatriuric aCSF under these three conditions. **B3**, Bar graph illustrates the mean normalized Na^+ current amplitude triggered by application of hypernatriuric aCSF under these three conditions. **C1**, Inward Na^+ current triggered by hypernatriuric aCSF under the control condition and during application of cinobufotalin ($10 \mu\text{M}$, 1 min). **C2**, Bar graph shows the mean normalized Na^+ current amplitude triggered by application of hypernatriuric aCSF under these two conditions ($*p < 0.5$; $***p < 0.01$). OUAB, Ouabain; STRO, strophanthidin; CINO, cinobufotalin.

These experiments showed that altered membrane resistance is not involved in Na^+ current inhibition during strophanthidin application. Next, depolarizing voltage ramps were used to identify potential changes in sodium channel conductance (g_{Na}) and permeability (p_{Na}) during strophanthidin application. We estimated that g_{Na} was 0.95 ± 0.065 during hypernatriuric aCSF application and 0.64 ± 0.66 nS in the presence of strophanthidin (Fig. 6D1), demonstrating that the blockade of $\alpha 1$ isoform activity can significantly decrease g_{Na} (t test, $t = 6.32$; $p = 0.0002$; $n = 9$). Strophanthidin application had a similar effect on p_{Na} ; it was significantly decreased from 26.57 ± 7.544 to 20.95 ± 6.222 nm/s (t test, $t = 3.38$; $p = 0.0096$; $n = 9$) (Fig. 6D2). Our data demonstrated that strophanthidin application reduced both g_{Na} and p_{Na} . These two membrane parameters reflect the dynamics of Na^+ ion flux through the channel. Thus, these results indicated that strophanthidin binding on the $\alpha 1$ isoform of Na^+/K^+ -ATPase reduces Na^+ ion flow through the

Figure 4. Na_x channel and the $\alpha 1$ isoform of $\text{Na}^+/\text{K}^+-\text{ATPase}$ form specific microdomains involved in Na^+ detection and regulation. **A**, Top, Microphotographs of immunocytochemical staining of dissociated MnPO neurons for DAPI, the Na_x channel, and the $\text{Na}^+/\text{K}^+-\text{ATPase}$ $\alpha 1$ isoform and the merge of all channels. Bottom, Merged signals and colocalization analysis of Na_x channel and $\alpha 1$ isoform staining using Imaris software. **B**, Similar analysis visualizing the $\alpha 3$ isoform of the colocalization between the Na_x channel and the $\text{Na}^+/\text{K}^+-\text{ATPase}$ $\alpha 3$ isoform was so low that the image depicting 3D microdomains could not be generated.

Na_x channel, and this reduction leads to an inhibition of the inward Na^+ current during $[\text{Na}^+]_{\text{ext}}$ variations (Fig. 7).

Discussion

The present study demonstrated a new mechanism involved in the functional regulation of sodium homeostasis in MnPO neurons. Our results showed that the $\text{Na}^+/\text{K}^+-\text{ATPase}$ $\alpha 1$ isoform regulates Na^+ influx mediated by the Na_x channel during $[\text{Na}^+]_{\text{ext}}$ variations. This regulation is the result of a reduction of channel permeability and could be carried by a change of Na_x channel

conformation. This functional partnership between the Na_x channel and the $\alpha 1$ isoform of $\text{Na}^+/\text{K}^+-\text{ATPase}$ provides a new process of cellular regulation and detection of Na^+ changes at the central level. Thus, this new mechanism could constitute another control system for the maintenance of hydromineral homeostasis.

Un-desensitization of Na_x channel is essential for a well Na^+ detection in MnPO neurons

Variations in sodium and water in the environment require the development of a mechanism designed to ensure a constant os-

Figure 5. The reduction of evoked Na^+ current by Na^+/K^+ -ATPase $\alpha 1$ isoform through Na_x channel does not affect the driving force. **A1**, Amplitude of the sodium leak current and AP characteristics during energy deprivation (ATP/GTP-free pipette solution; **A**), ouabain application (OUAB; $50 \mu\text{M}$, 4 min; **B**), and strophanthidin application (STRO; $40 \mu\text{M}$, 4 min; **C**). **A1**, **B1**, **C1**, Typical basal Na^+ current under the three conditions. Note deflections materialize a series of spikes induced by depolarizing voltage step (1 step by 10 mV from -60 to 10 mV). **A2**, **B2**, **C2**, Typical representations of APs induced by depolarizing voltage along the time. **A3**, **B3**, **C3**, Bar graphs showing the mean amplitude for each AP along the time. **A4**, **B4**, **C4**, Bar graphs showing the mean half-width value for each AP along the time. **D**, Time plot representing the slope of the Na^+ leak current under these three conditions. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 6. The $\text{Na}^+/\text{K}^+-\text{ATPase } \alpha_1$ isoform induces a diminution of Na^+ ion flow through Na_x channel. **A**, Inward Na^+ current triggered by hypernatric aCSF under the control condition and during strophanthidin application ($40 \mu\text{M}$, 1 min). Note that surges and truncated traces before and during each application of hypernatric aCSF materialize a ramp-activated current (voltage was steadily injected from -10 to 20 mV) and series of resistance current induced by hyperpolarization voltage (-6 mV, fivefold). **B1**, Representative example of the ramp-activated current (-10 to $+20$ mV/ms) generated under the control condition and during application of hypernatric aCSF under the control condition and during strophanthidin application. **B2**, Scatterplot graph showing the reversal potential values obtained from ramp-activated current during application of hypernatric aCSF under the control condition and during strophanthidin application. **C1**, Representative example of resistance current generated under the isonatriuric (control) condition, during application of hypernatric aCSF under the control condition, and during strophanthidin application. **C2**, **D1**, **D2**, Scatterplots demonstrating resistance (**C2**), conductance (**D1**), and permeability (**D2**) values under the control condition, during application of hypernatric aCSF, and during strophanthidin application (** $p < 0.01$, *** $p < 0.001$). STRO, Strophanthidin.

molality of body fluids around an equilibrium value (Johnson and Gross, 1993). Anterior hypothalamus nuclei, more particularly in the lamina terminalis (LT), play a key role in the detection and regulation of osmolarity where specific osmoreceptor-expressing neurons will detect plasmatic and CSF changes in osmolarity (Lobo et al., 2004). The MnPO, one of the LT nuclei, plays a central role in Na^+ detection and osmoregulation (Fitzsimons, 1998; McKinley et al., 1999; Grob et al., 2004). In our previous study, electrophysiological recording demonstrated that the Na_x channel is a Na^+ leak channel involved in Na^+ sensing, specifically in rat MnPO neurons (Tremblay et al., 2011). Here we report that another characteristic of the Na_x channel is to be not desensitized after repeated stimulations. This additional property allows this channel to remain operational, even after several changes in $[\text{Na}^+]_{\text{ext}}$. These combined results demonstrate the essential role of the Na_x channel in $[\text{Na}^+]$ detection and regulation in MnPO neurons.

To maintain a steady level of $[\text{Na}^+]$ in the cell, Na^+ ions have to be continuously extruded from the cell. Na^+ is mainly extruded by the Na^+/K^+ -ATPase pump, which is also expressed in MnPO neurons. Na^+/K^+ -ATPase is a membrane enzyme catalyzing the active transport of Na^+ and K^+ across the cell membrane, allowing the maintenance of a low cytosolic $[\text{Na}^+]$ (Sweadner, 1989). Therefore, the presence of the Na_x channel and Na^+/K^+ -ATPase pump, seems necessary for an efficient regulation of Na^+ homeostasis in MnPO neurons.

Thus, from its strategic localization along the third ventricle, its connection to other LT nuclei involved in osmoregulation, and the ability to exhibit the functional Na_x channel and Na^+/K^+ -ATPase pump in its neurons, the MnPO has a predominant role in Na^+ homeostasis regulation and osmoregulation.

Cooperative action between Na_x channel and $\alpha 1$ isoform of Na^+/K^+ -ATPase: complex formation

This study demonstrated a functional interaction between the Na_x channel and the $\alpha 1$ isoform of Na^+/K^+ -ATPase. In this partnership, inhibition of the $\alpha 1$ isoform induces a reduction of the inward Na^+ current during hypernatremic application. This inhibitory effect observed on the Na^+ current is certainly attributable to indirect interaction via $\alpha 1$ isoform inhibition. To ensure that this modulation is not caused by a direct antagonist binding on the Na_x channel, sequences of the CG-binding sites and the α subunit of the Na_x channel were aligned. No sequence alignment between the CG binding site and the regulatory α subunit of the Na_x channel was found; together with previous

Control

Hypernatremic

Hypernatremic + STRO

Figure 7. Suggested mechanism by which the Na^+/K^+ -ATPase regulates the Na_x channel. Direct interaction between Na^+/K^+ -ATPase and the Na_x channel allows the tight, localized control of sodium concentration in the MnPO cell. The summary diagram illustrates the proposed mechanism of sodium concentration regulation by the complex formed by the Na_x channel and the $\alpha 1$ isoform of Na^+/K^+ -ATPase, under various conditions. STRO, Strophanthidin; ext, exterior; int, interior.

pharmacological results, this observation suggests the formation of a complex between the Na_x channel and the Na^+/K^+ -ATPase $\alpha 1$ isoform. This possibility is supported by the demonstration of the physical link between the channel and the Na^+/K^+ -ATPase $\alpha 1$ isoform (Shimizu et al., 2007). However, based on this evidence, we cannot rule out the possibility that homology in the tertiary structure still enables interaction between CGs and the Na_x channel. Besides, the study by Shimizu et al. (2007) demonstrated a physical link between the C-terminal region of the Na_x channel (amino acid residues 1489–1681) and the catalytic domain of the $\alpha 1$ isoform of Na^+/K^+ -ATPase (amino acid residues 596–717). Thus, the functional interaction demonstrated by our pharmacological results, combined with sequence analysis and

the presence of a physical link between the two proteins, strongly suggests the existence of a functional complex between the Na_x channel and the $\alpha 1$ isoform of $\text{Na}^+/\text{K}^+-\text{ATPase}$ in the MnPO neurons. This protein–protein interaction plays a key role in the detection of $[\text{Na}^+]_{\text{ext}}$ changes and in the regulation of sodium exchanges at the neuronal membrane.

Similar interaction exists between NMDA receptors and various kinases/phosphatases that regulate its activity. Several intracellular processes, such as receptor trafficking and synaptic stabilization, are influenced by NMDA receptor interacting partners. These different functional interactions modulate synaptic transmission and plasticity (for review, see Bard and Groc, 2011).

Relevance of $\text{Na}_x/\text{Na}^+/\text{K}^+-\text{ATPase}$ complex in cellular context

Our results demonstrated a functional and physical link between the Na_x channel and the $\alpha 1$ isoform of $\text{Na}^+/\text{K}^+-\text{ATPase}$; this interaction enables the local integration of Na^+ inputs and outputs. This physical proximity ensures rapid and local cellular responses to changes in $[\text{Na}^+]_{\text{ext}}$. Such a local regulation of $[\text{Na}^+]_{\text{ext}}$ would allow us to characterize the $\text{Na}_x/\text{Na}^+/\text{K}^+-\text{ATPase}$ complex as a new type of “microdomain” specialized in Na^+ detection and regulation. With the precise regulation of $[\text{Na}^+]_{\text{ext}}$, Na^+ microdomains could prevent Na^+ toxic rises in $[\text{Na}^+]_{\text{ext}}$. Results obtained from double immunostaining of the Na_x channel and the α isoform strongly support the hypothesis that the Na_x channel and the $\alpha 1$ isoform of $\text{Na}^+/\text{K}^+-\text{ATPase}$ would form microdomains regrouped in hot spots dedicated to Na^+ detection and regulation in MnPO neurons.

The regulation of intracellular $[\text{Ca}^{2+}]$ is under similar tight regulation. Ca^{2+} microdomains are involved in both presynaptic and postsynaptic events controlled by highly localized pulses of Ca^{2+} . Microdomains can rapidly respond to local Ca^{2+} changes and have influence on a spatially restricted intracellular compartment. Moreover, the ability of single neurons to process enormous amounts of information depends on such spatial restriction of the Ca^{2+} signaling system (Berridge et al., 2006). Like the Ca^{2+} microdomain, the Na^+ microdomain formed by the Na_x channel and the $\alpha 1$ isoform of $\text{Na}^+/\text{K}^+-\text{ATPase}$ would be a small-scale system of Na^+ detection and regulation.

Our data showed that $\alpha 1$ isoform inhibition does not change the Na^+ driving force, whereas blockade of the $\alpha 3$ isoform diminished the driving force and decreased the AP amplitude. We suggested that each α isoform could have a specific role within neurons. The $\alpha 1$ isoform, forming a complex with the Na_x channel, would establish a Na^+ microdomain engaged in the precise local regulation of $[\text{Na}^+]_{\text{ext}}$. This α isoform of $\text{Na}^+/\text{K}^+-\text{ATPase}$ would then be involved in Na^+ exchange to regulate the cellular osmotic balance via a local control of osmolality. In contrast, the $\alpha 3$ isoform could be responsible for a more global regulation of $[\text{Na}^+]_{\text{ext}}$, which could impact cellular excitability. Indeed, via changes in driving force and action potential firing pattern, MnPO neurons could transmit information about osmotic change to their targets. Moreover, MnPO has direct access to CSF ionic composition, thus osmotic changes in its neurons reflect by extension osmotic change in the CSF. The principal MnPO targets are magnocellular cells of the supraoptic nucleus (SON) and paraventricular nucleus (PVN). These nuclei are involved in the secretion and release of vasopressin and oxytocin, two antidiuretic and natriuretic hormones, respectively, which act peripherally on Na^+ and water reabsorption (Antunes-Rodrigues et al., 2004).

Cellular excitability is a unique property of neurons, and the $\alpha 3$ isoform is exclusively expressed in neuronal cells (Antonelli et al., 1995). Therefore, the $\alpha 3$ isoform could be involved in the regulation or modulation of the cellular excitability mechanism and hence contribute to information transfer from the MnPO to its targets.

During Na^+ challenge, the $\text{Na}^+/\text{K}^+-\text{ATPase}$ $\alpha 1$ isoform, coupled with the Na_x channel, would regulate Na^+ locally to avoid cellular toxicity and regulate cellular osmolality in MnPO neurons, whereas the $\alpha 3$ isoform would be responsible for the maintenance of cellular excitability to transfer osmolality information about CSF to the PVN and SON.

Relevance of the $\text{Na}_x/\text{Na}^+/\text{K}^+-\text{ATPase}$ complex in physiological context

In a physiological context, many previous studies on Na^+ variations and hypertension showed that ouabain-like compounds (OLCs), an endogenous class of cardiotonic steroids like bufadienolides, are released by acute plasma expansion and by chronic administration of high- Na^+ diet in mammals. These OLCs are able, like strophanthidin and ouabain, to inhibit $\text{Na}^+/\text{K}^+-\text{ATPase}$ activity and to bind the CG-binding site, and their structure is similar to those of CGs (Hamlyn et al., 1982, 1991; Kawamura et al., 1999). Endogenous release of OLCs mainly takes place at the lateral, paraventricular, and anterior hypothalamus and in the level of preoptic areas (de Wardener et al., 1987; Takahashi et al., 1988; Yamada et al., 1992). Interestingly, more recent studies showed that intracerebroventricular infusion of high- $[\text{Na}^+]_{\text{ext}}$ solution induces an increase in OLC release in these hypothalamus areas (Wang and Leenen, 2002, 2003).

Here we observed that bufadienolide applications induced an inhibition of $\text{Na}^+/\text{K}^+-\text{ATPase}$ activity, resulting in a reduction of Na^+ current in MnPO neurons. In addition to structural similarities between OLCs and strophanthidin, they both effectively inhibited the hypernatremic aCSF-evoked Na^+ current. Bufadienolides could act as endogenous regulators of the $\alpha 1$ isoform of $\text{Na}^+/\text{K}^+-\text{ATPase}$ and therefore influence Na^+ permeability in MnPO neurons. The $\text{Na}_x/\text{Na}^+/\text{K}^+-\text{ATPase}$ channel complex is functionally active and expressed in MnPO neurons located in hypothalamic areas where OLCs are also released in physiological conditions. Thus, this new $\text{Na}_x/\text{Na}^+/\text{K}^+-\text{ATPase}-\alpha 1$ isoform complex described in this study plays a central role in the control of Na^+ influx under physiological conditions.

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