Intrinsic properties of the sodium sensor neurons in the rat median preoptic nucleus

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Authors contribution: Aurore Voisin realized all the patch-clamp experiments, analyzed the data, designed the figures and wrote the manuscript draft. Guy Drolet and Didier Mouginot designed the experimental project, were involved in the data analysis and reviewed the manuscript.

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†Unfortunately, Dr. Mouginot passed away June 23rd, 2012. This paper was written while he was still alive and under his supervision.
Abstract

The essential role of the median preoptic nucleus (MnPO) in the integration of chemosensory information associated with the hydromineral state of the rat relies on the presence of a unique population of sodium (Na\(^+\)) sensor neurons. Little is known about the intrinsic properties of these neurons; therefore, we used whole-cell recordings in acute brain slices to determine the electrical fingerprints of this specific neural population of rat MnPO. The data collected from a large sample of neurons (115) indicated that the Na\(^+\) sensor neurons represent a majority of the MnPO neurons in situ (83%). These neurons displayed great diversity in both firing patterns induced by transient depolarizing current steps and rectifying properties activated by hyperpolarizing current steps. This diversity of electrical properties was also present in non-Na\(^+\) sensor neurons. Subpopulations of Na\(^+\) sensor neurons could be distinguished, however, from the non-Na\(^+\) sensor neurons. The firing frequency was higher in Na\(^+\) sensor neurons, showing irregular spike discharges, and the amplitude of the time-dependent rectification was weaker in the Na\(^+\) sensor neurons than in non-Na\(^+\) sensor neurons. The diversity among the electrical properties of the MnPO neurons contrasts with the relative function homogeneity (Na\(^+\) sensing). However, this diversity might be correlated with a variety of direct synaptic connections linking the MnPO to different brain areas involved in various aspects of the restoration and conservation of the body fluid homeostasis.

Keywords: lamina terminalis; sodium homeostasis; hydromineral balance; Na\(_X\) channel
INTRODUCTION

Osmoregulation, thirst and salt appetite are considered regulatory feedback responses that are engaged to adequately confront acute and sustained hydromineral perturbations. The median preoptic nucleus (MnPO) is located in the mid-lamina terminalis (LT) and is considered as a critical brain center for regulating these responses. Indeed, the role of the MnPO has been functionally established using tissue lesions of the ventral preoptic area. On the one hand, the chemical lesions produced adipsia in response to acute dehydration, hypertonic stimulus or angiotensin II (AngII) injection (8, 24). On the other hand, a tissue lesion that encompasses the MnPO and the vascular organ of the lamina terminalis (OVLT) reduced Na⁺ ingestion evoked by systemic sodium (Na⁺) deficiency (6, 11, 12, 22). Finally, lesions of the MnPO attenuated vasopressin secretion at the neurohypophysis in response to hyperosmolality (24, 25).

The essential role of the MnPO in the osmoregulation and initiation of motivated behaviors is primarily associated with the integration and processing of chemosensory and humoral signals in response to perturbations in hydromineral homeostasis. However, a single specific sensory signal rarely produces a homogenous cellular response throughout MnPO neurons, suggesting the complex organization of this nucleus and its heterogeneity in terms of the neuronal populations involved in the hydromineral homeostasis. Accordingly, in vivo recordings showed that plasma hyperosmolality increases the spike discharges in one subpopulation of the MnPO neurons projecting to the paraventricular nucleus of the hypothalamus (PVH), but it has no effect in other subpopulations projecting to that same area (37). In vitro recordings of MnPO neurons without identified projections revealed that hyperosmotic and hypoosmotic extracellular solutions differentially affect firing activity ranging from a reduction to an increase in firing activity (39). Overall, these studies illustrate the heterogeneity of neuronal populations that dynamically respond to a change in systemic or local osmolality. The response to humoral signals also
highlights the existence of several populations of MnPO neurons. AngII plays a primary role in the induction of thirst and salt appetite and has been reported to increase the excitability of a restricted population of MnPO neurons (2, 35, 39). However, endogenous AngII has been shown to facilitate GABAergic neurotransmission afferent to MnPO neurons responding to changes in extracellular Na⁺ levels (18). The inhibitory action of endogenous AngII is consistent with the AngII-mediated reduction of MnPO excitability observed in Na⁺-depleted rats, a condition associated with the enhanced production and release of AngII (21). These apparently opposite actions of AngII on the excitability of MnPO neurons indicate that the peptide exerts distinct postsynaptic actions on MnPO neurons, illustrating the complex organization of this nucleus and possibly, the presence of neuronal populations with distinct electrophysiological properties. Overwhelming physiological evidence has shown that endogenous opioid peptides regulate fluid consumption (5, 9, 28, 30, 34, 41) and spontaneous salt intake (4, 13, 31). The MnPO might be an important target of endogenous opioid peptides in regulating fluid and electrolyte consumption. Indeed, an electrophysiological study clearly demonstrated that MnPO neurons are characterized by the functional expression of mu-opioid receptors, whose activation induces membrane hyperpolarization and thus, the inhibition of the electrical activity (17). Interestingly, a single episode of Na⁺ depletion / repletion selectively modified the proportion of neurons responding to the specific agonist of the mu-opioid receptors DAMGO. Indeed, Na⁺ depletion was correlated with the sustained overexpression of functional mu-opioid receptors, which was restricted to the Na⁺-responsive neuronal population. Moreover, Na⁺ repletion was correlated with a diminution of the population of Na⁺-insensitive MnPO neurons that were hyperpolarized by DAMGO application (17). This study unveiled the plasticity of neuronal populations involved in the opioid-responsive network of the MnPO.
The data summarized above indicate that a large population of the MnPO could be identified according to its sensitivity to extracellular Na⁺ levels. This unique feature characterizes the ability of these neurons to transduce a change in extracellular Na⁺ concentrations into a change in membrane potential, thereby adjusting the firing rate as a function of the environmental Na⁺ level (14). Recently, an electrophysiological study performed on dissociated MnPO neurons demonstrated that Na⁺ sensing is an intrinsic property of these neurons, i.e., the expression of specific Na⁺ leak channels, such as the NaX channel (40). These neurons constitute a population of Na⁺ sensor neurons within the MnPO, and this specific neuronal population integrates CSF Na⁺ concentration with AngII and opioid signals to deliver the appropriate command to downstream effectors, such as magnocellular neuroendocrine cells. The active electrical properties of these neurons are important for understanding how this specific neuronal population encodes and transduces sensory information, i.e., the variation of Na⁺ levels. Using electrophysiological recordings in hypothalamic slices that preserve the integrity of the neurons, the goal of this study is to identify the electrophysiological fingerprints of the Na⁺ sensor neurons of the MnPO. The characterization of the intrinsic electrical properties of the Na⁺ and non-Na⁺ sensor neurons will increase our knowledge concerning MnPO physiology, particularly hydromineral homeostasis.

MATERIALS AND METHODS

The experiments performed in the present study were carried out in accordance with the guidelines established by the Canadian Council on Animal Care, and were duly approved by our institutional Animal Care Committee (CPAC-Université Laval). Fifty two young male Wistar rats (21 to 25 days old) were obtained from Charles River Canada (St-Constant, Québec) and housed
in plastic cages (two rats per cage) for one week acclimatization to standard laboratory conditions before use for experimentation (14h of light and 10h of dark cycle at 23°C).

**Brain slice preparation**

Rats were deeply anesthetized with intraperitoneal injection of a ketamine–xylazine mixture (87.5 and 12.5 mg/kg, respectively) and then decapitated. The brain was quickly removed and immersed in oxygenated (95% O2- 5% CO2) ice-cold (2 °C) artificial cerebrospinal fluid (aCSF) containing (in mm): sucrose, 200; D-glucose, 10; KCl, 2; CaCl2, 1; MgCl2, 3; NaHCO3, 26; NaHPO4, 1.25. A block of tissue including the forebrain region was mounted on a vibratome stage (Leica VT 1000S, Nussloch, Germany) and immersed in the oxygenated ice-cold aCSF for slicing. One sagittal slice (350 µm thick) containing the MnPO was collected and immediately transferred to a submersion-type recording chamber (Warner Instruments, Hamden, CT, USA) in which the slice was continuously perfused at a rate of 2 mL/min with oxygenated (100% O2) aCSF containing (in mM): NaCl, 135; KCl, 3.1; CaCl2, 2.4; MgCl2, 1.3; HEPES, 10; D-glucose, 10. aCSF pH was titrated to 7.4 with NaOH (10 N), and osmolality was adjusted to 298–300 mOsm/L.

**Whole-cell recordings**

The electrophysiological recordings were performed on individual MnPO neurons visualized with the near infrared differential interference contrast principle. Current-clamp recordings were obtained using thin-walled (1.5 mm o.d.; 1.1 mm i.d.) borosilicate glass capillaries (G75150T-4; Warner Instruments) pulled on a two-stage vertical PIP5 puller (HEKA electronics, Mahone Bay, NS, Canada). Micropipettes were filled with an intracellular solution containing (in mM): K-gluconate, 135; NaCl, 6; HEPES, 10; Na+-GTP, 0.3; Na+-ATP, 4. The intracellular solution was
titrated to pH 7.2 with KOH (1 mM), and osmolality was adjusted to 295 mOsm/L with sorbitol. Micropipettes had a final tip resistance of 4–6 MΩ. The extracellular solution was identical to the aCSF used for the recovery of the slice (see above). Hypernatriuric aCSF (170 mM NaCl, 330 mOsm/L) was transiently (1 min) and locally applied over the MnPO to identify the recorded neuron as a Na⁺ sensor or a non-Na⁺ sensor neuron. We determined that a neuron was responsive to hypernatriuric solution when the response amplitude was higher than 3 mV. This threshold was chosen to avoid the amplitude variations due to fluctuations of rest membrane potential. It has been determined in reducing the amplitude of the background to the amplitude of hypernatriuric-induced response. Temperature of regular (150 mM NaCl) and hypernatriuric (170 mM) aCSF was fixed at 24°C using a thermostated recording chamber and a syringe system heat controller (Warner Instruments). Whole-cell current-clamp recordings were obtained using an EPC 9 patch-clamp amplifier (HEKA electronics). The fast capacitance was compensated after forming the gigaseal, and appropriate whole-cell and series resistance compensations were applied after rupture of the cell membrane (series resistance 12.3 ± 0.28 MΩ). Liquid junction potential was calculated at 12.4 mV, and membrane potential was corrected accordingly.

Data acquisition and analysis

For long-lasting recordings (> 1 min), the analog DC signals (current and voltage) were sampled at 2 kHz (PowerLab4SP, ADInstruments, Colorado Springs, CO, USA), visualized with Chart5 (version 5.4.1, ADInstruments), and stored on the computer hard disc for further analysis. For short recordings (< 1 min), DC signals were acquired using PULSE (HEKA electronics) at a sampling rate of 2 kHz.

The spike frequency adaptation has been determined when the last interspike interval was doubled compared to the first interspike interval. Note that no MnPO neurons showed a regular
tonic firing but displayed a pronounced frequency adaptation. Rectification current was determined by subtracting the amplitude at the peak of rectification minus the amplitude at steady state and should be superior to 3 mV. Note that MnPO neurons displaying a time-dependent rectification showed a very distinct sag of amplitude without any doubt.

Membrane time constant (τₘ), resistance (Rₘ), conductance (gₘ) and capacitance (Cₘ) were calculated from the averaged membrane response (Vₘ) to five identical and consecutive hyperpolarizing current pulses (-5 pA; Iₚₑₛₜ) elicited from -60 mV. Rₘ was calculated from the Ohm law (Rₘ= Vₘ/Iₚₑₛₜ) and τₘ was found to correspond to the time required to reach 63% of the steady-state Vₘ in response to the current pulses. gₘ was calculated from Rₘ (gₘ=1/Rₘ) and Cₘ was calculated from the equation Cₘ= τₘ/ Rₘ.

Statistical analysis

All the results are expressed as mean ± SEM. Dependent variables were tested for normality using the Kolmogorov–Smirnov test. All the variables were normally distributed and further analyzed using parametric tests. The compared distribution of the rectifying and spiking properties among the populations of Na⁺ sensor and non-Na⁺ sensor neurons was analyzed using the Chi-squared test. The comparison of the amplitude of the Na⁺-evoked depolarization and membrane capacitance of the Na⁺ sensor neurons distributed among subpopulations (according to their spiking activity) was performed with one-way ANOVA. The comparison of the spike amplitude, spike width and spike frequency in response to depolarizing current steps and the comparison of the hyperpolarization amplitude in response to negative current steps in Na⁺ sensor and non-Na⁺ sensor neurons were performed using two-way ANOVA (main group effect: Na⁺ sensor neurons vs. non-Na⁺ sensor neurons; main current step effect: from -5 to -45 pA for the negative current steps and from +5 to +20 pA for the depolarizing current steps). All the
statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $P < 0.05$ (*) and $P < 0.01$ (**).

RESULTS

To obtain an accurate characterization of the Na$^+$ sensor neurons of the MnPO, whole-cell patch-clamp recordings were obtained from a large population of neurons ($n=115$) distributed throughout the ventral region of the MnPO (vMnPO). This unique neuronal population was identified through the transient application (1 min) of hypernatriuric aCSF (170 mM). In 83% of the neurons tested (96 out of 115 neurons), hypernatriuric aCSF triggered a membrane depolarization of 7.9 +/- 0.5 mV (Figure 1A), while no change in the membrane potential was observed in the remaining neurons (19 out of 115 neurons; Figure 1B). The hypernatriuric aCSF-induced change in the membrane potential result only from a change in the extracellular Na$^+$ concentration and not in osmolality. Indeed, our laboratory previously showed that local application of a hyperosmotic-isonatriuric stimulus (350 mOsm.l$^{-1}$ with mannitol; 150 mM NaCl) had no apparent effect on the excitability of the vMnPO neurons (14, 40). The typical Na$^+$-induced depolarization was considered to be the electrophysiological fingerprint of Na$^+$ sensor neurons.

Characterization of the firing properties of the Na$^+$ sensor neurons

The firing pattern of the Na$^+$ sensor neurons was determined using a series of transient depolarizing current steps (800 ms). The experimental protocol was applied following a short stabilization period of the membrane potential (1-2 min) after rupturing the membrane. Based on the specific firing patterns illustrated in Figure 2A, three distinct neuronal populations were observed in the vMnPO. One neuronal population was characterized by irregular spike discharges
with a gradual decrease in firing (Type A neuron), whereas a second population displayed a very strong spike frequency adaptation defined by one initial spike followed by a plateau phase (Type C neurons). A third neuronal population was characterized by one initial spike followed by a short burst of spikes superimposed onto a robust calcium (Ca$^{2+}$) spike (Type B neurons). Under our experimental conditions, the large sample of vMnPO neurons (n=115) was primarily distributed among type A (46%; n=53) and type C (43%; n=50) neurons. Type B neurons represent a weak population of vMnPO neurons (10%; n=12). However, none of these firing patterns was specific to the Na$^+$ sensor neurons. Moreover, the distribution of each firing pattern was almost identical between Na$^+$ sensor neurons (n=96) and other neurons (n=19; Figure 2B). The type A neurons represented 47% (n=45) of the Na$^+$ sensor neurons and 42% (n=8) of the non-Na$^+$ sensor neurons, whereas type C neurons represented 42% (n=40) and 53% (n=10) of the Na$^+$ sensor and non-Na$^+$ sensor neurons, respectively. Despite being fewer in number, type B neurons were also found in the Na$^+$ (11%; n = 11) and non-Na$^+$ (5%; n = 1) sensor neurons. Our recordings indicated however, a noticeable difference between the Na$^+$ and non-Na$^+$ sensor neurons displaying irregular spike discharges (type A neurons, 47% and 42% of the population, respectively). The spontaneous firing frequency of these two populations was analyzed in response to incremented depolarizing current steps (5, 10, 15 and 20 pA; 800 ms; Figure 3A). The firing frequency was significantly higher in the Na$^+$ sensor neurons compared with the non-Na$^+$ sensor neurons (two-way ANOVA; main effect of group: $F_{1,180} = 9.58, P = 0.002$ and current step intensity: $F_{3,180} = 2.87, P = 0.04$; no interaction: $F_{3,180} = 0.14, P = 0.93$; Figure 3A and 3B). The ionic component of the spikes elicited by a large membrane depolarization was further determined and depolarizing current steps above 10 pA (15 and 20 pA) elicited high-threshold spikes that were abolished with the bath application of cadmium, (Cd$^{2+}$; 10 mM; Figure 3C), a broad-spectrum pharmacologic blocker of high-threshold voltage-gated Ca$^{2+}$ channels. However,
an analysis of the Ca\textsuperscript{2+} spike characteristics did not reveal a significant difference in the properties of these spikes in the Na\textsuperscript{+} and non-Na\textsuperscript{+} sensor neurons. Both the amplitude and width of the Ca\textsuperscript{2+} spikes (Figure 3D) were identical in the two populations (two-way ANOVA; spike amplitude: effect of group: F\textsubscript{1,46} = 1.16, P = 0.29 and current step intensity: F\textsubscript{1,46} = 0.18, P = 0.68; no interaction: F\textsubscript{1,46} = 0.00, P = 0.98; spike width: no effect of group: F\textsubscript{1,46} = 0.04, P = 0.84 and current step intensity: F\textsubscript{1,46} = 0.65, P = 0.42; no interaction: F\textsubscript{1,46} = 0.02, P = 0.89).

The amplitude of the depolarization triggered by hypernatriuric aCSF was similar in the type A, type B and type C Na\textsuperscript{+} sensor neurons, as illustrated in Figure 4A and 4B. The similarity of the response could result from a non-uniform current density and thus, from a different size among the Na\textsuperscript{+} sensor neurons. However, the membrane capacitance (Cm) was not different between type A, type B and type C Na\textsuperscript{+} sensor neurons (one-way ANOVA, F\textsubscript{2,93} = 0.24, P = 0.785; Figure 4C).

**Characterization of the rectifying properties of the Na\textsuperscript{+} sensor neurons**

MnPO neurons have also been characterized by the presence of distinct membrane rectifications in response to membrane hyperpolarization (Bai and Renaud, 1998; Grob et al., 2005). Here, we performed recordings on a large number of neurons (n=115), and the results revealed the distribution of these neuronal populations in the vMnPO (Figure 5). Neurons displaying time-dependent rectification (type 2 neuron) represented 28% (n = 32) of the total population, whereas neurons showing time-independent rectification (type 3 neuron) represented 43% (n = 50) of the vMnPO neurons. The remaining neuronal population did not display rectifying properties (type 1 neuron) and represented 29% (n = 33) of the total population.

The data did not show an association of a specific rectifying pattern with the Na\textsuperscript{+} sensing ability of the vMnPO neurons. However, the distribution of each rectifying pattern was different in Na\textsuperscript{+}
sensor neurons (n=96) compared with non-Na\(^+\) sensor neurons (n=19) (Chi-squared test, \(P = 0.0003\); Figure 5B). Type 1 neurons represented a larger population (47%) in the non-Na\(^+\) sensor neurons compared with the Na\(^+\) sensor neurons (25%). By contrast, type 2 neurons represented a weaker population in the non-Na\(^+\) sensor neurons (11%) than in the Na\(^+\) sensor neurons (31%). The weight of the third population (type 3 neurons) remained identical in the Na\(^+\) sensor (44%) and non-Na\(^+\) sensor (42%) neurons.

Because the distribution of type 2 neurons was different in the Na\(^+\) sensor and non-Na\(^+\) sensor neurons whereas the distribution of type 3 neurons remained identical in the Na\(^+\) sensor and the non-Na\(^+\) sensor neurons, we examined the characteristics of the membrane rectification in these two neuronal populations (Figure 6). The amplitude of the steady-state hyperpolarization triggered by negative current steps (from -5 to -45 pA; 1200 ms) was measured in neurons showing either time-dependent (type 2 neuron) or time-independent membrane rectification (type 3 neuron; Figure 6). The intensity-to-voltage relationship (I/V curve), characterizing type 2 neurons, indicates that the membrane rectification was weaker in the Na\(^+\) sensor neurons compared with the non-Na\(^+\) sensor neurons (two-way ANOVA; main effect of group: \(F_{1,243} = 7.33, P = 0.007\) and current step intensity: \(F_{8,243} = 14.31, P < 0.0001\); no interaction: \(F_{8,243} = 0.04, P = 1.00\); Figure 6A). In contrast, the I/V curve displayed by the type 3 neurons revealed no difference in the amplitude of the membrane rectification in Na\(^+\) sensor and non-Na\(^+\) sensor neurons (two-way ANOVA; effect of group: \(F_{1,403} = 0.31, P = 0.58\) and current step intensity: \(F_{8,403} = 22.63, P = 0.58\); no interaction: \(F_{8,403} = 0.13, P = 0.99\); Figure 6B). The possibility that the discrepancy in the amplitude of the time-dependent membrane rectification might be attributed to a non-uniform neuron size and thus, to the heterogeneous density of the current underlying the rectification, was investigated in the Na\(^+\) sensor and non-Na\(^+\) sensor neurons. However, the membrane capacitance, \(C_m\), measured in type 2 neurons was not different between
the Na\(^+\) sensor and the non-Na\(^+\)-sensor neurons (55.78 ± 3.04 vs. 58.01 ± 4.62; unpaired t-test, \(P = 0.85\)). Notably, the input resistance, \(R_m\), of the type 2 neurons was not different between the Na\(^+\) sensor and non-Na\(^+\) sensor neurons (unpaired t-test, \(P = 0.12\); data not shown). These results indicate that the difference observed in membrane rectification did not reflect differences in the size of the neurons or the ionic shunt in one of the two subpopulations.

**DISCUSSION**

The MnPO is an essential structure in the physiology of rat hydromineral homeostasis. In addition to the strategic location of the MnPO in the rostral wall closing the third ventricle, the key role of the MnPO likely relies on the presence of a specific neuronal population, which behaves as a genuine Na\(^+\) sensor. This study utilized a large sample of Na\(^+\)-sensor neurons, and the data indicate that this class of MnPO neurons did not constitute a homogenous neuronal population in terms of the basic electrophysiological properties. Indeed, neurons that respond to a variation in extracellular [Na\(^+\)] through a change in their membrane potential belong to three distinct neuronal populations, according to either their firing pattern, or their rectifying properties. However, specific subpopulations of the Na\(^+\) sensor neurons can be distinguished from non-Na\(^+\) sensor neurons according to the ionic currents underlying spike frequency adaptation and time-dependent rectification. The discrepancy in their basic electrophysiological properties might imply subtle characteristics of Na\(^+\) sensor neurons in terms of specific responses to depolarizing or hyperpolarizing membrane potentials.

*Three distinct patterns of firing characterize the Na\(^+\) sensor neurons.*
The *in situ* electrophysiological characterization of the Na\(^+\) sensor neurons indicated that these neurons represent a large population of ventral MnPO, ranging from 72% (14) to 83% (the present study) of the recorded neurons. The high proportion of the Na\(^+\) sensor neurons was also obvious from the data obtained from electrophysiological recordings in acutely dissociated MnPO neurons identifying 79% of the neurons as Na\(^+\) sensor neurons (40). Therefore, the Na\(^+\) sensor neurons constitute the major population of ventral MnPO neurons. The homogeneity in function (brain Na\(^+\) sensing) might be correlated with morphological homogeneity. The anatomical characteristics of the ventral MnPO neurons were obtained using Lucifer Yellow tracing that revealed small ovoid soma and two to three main dendrites lacking spines in most of the cells (2). The high proportion of Na\(^+\) sensor neurons in the MnPO and the relative homogeneity in the neuron morphology invalidates the latter criteria used to identify Na\(^+\) sensor neurons. We thus, sought to determine whether the Na\(^+\) sensor neurons might be distinguished from other neuronal populations of the MnPO by the presence of specific electrophysiological properties.

The Na\(^+\) sensor neurons displayed three distinct firing patterns in response to positive current steps. Almost half of the recorded Na\(^+\) sensor neurons were characterized by a light spike frequency adaptation characterized by a gradual reduction in firing (type A neuron; 47% of the neurons), whereas a second population displayed a very strong spike frequency adaptation leading to a cessation of the firing activity (type C neuron; 42% of the neurons). A weaker third population of neurons was characterized by the presence of one initial spike followed by a robust burst of calcium spikes (type B neurons; 11% of the neurons). The heterogeneity in the firing pattern of the Na\(^+\) sensor neurons of the MnPO might be relevant to the recording condition. The MnPO neurons were recorded using a whole-cell patch-clamp technique, which results in the rapid dialysis of the intracellular medium by the pipette solution. This experimental condition
might contribute to the collapse of intracellular factors required to optimize the activity of ionic channels that are critical for the firing pattern. However, the characterization of the electrical activity was achieved in a similar way for all the MnPO neurons, i.e., immediately after the rupture of the patch membrane and stabilization of the membrane potential. The rapid characterization of the spiking activity lowers the probability of an experimental bias and supports the presence of a heterogeneous population of Na\(^+\) sensor neurons based on distinct firing patterns. The presence in a specific nucleus of neuronal cell populations displaying various electrical fingerprints is not unique and was observed in several other regions of the central nervous system. For example, in the superior colliculus, neurons displaying a marked spike frequency adaptation (similar to the MnPO type A neurons) were intermingled with neurons showing a rapid and complete spike inactivation (similar to the MnPO type C neurons) and neurons characterized by a burst-spiking activity (similar to the MnPO type B neurons) (33). Neocortical neurons displayed either regular-spiking activity similar to the firing pattern of type A neurons, or a strong burst of spikes, similar to type B neurons (7). This heterogeneous electrical activity might suggest that the integration and encoding of a specific sensory message (extracellular Na\(^+\) levels) differed from one neuronal population to another. This variety might characterize the functional organization of the network in which distinct electrical activities identify specific projection populations.

Despite similarities in the firing pattern, the main neuronal population (type A MnPO neurons) showed a higher firing frequency in the Na\(^+\) sensor neurons than in the non-Na\(^+\) sensor neurons, suggesting that the spike frequency adaptation is slightly reduced in type A Na\(^+\) sensor neurons. Spike frequency adaptation characterized by both a gradually reduced firing and an increased interspike interval has been well established in several regions of the brain and is attributed to Ca\(^{2+}\)-activated K\(^+\) channels (3, 10, 19, 32, 36, 42, 43). Action potential-mediated depolarization
triggers Ca\(^{2+}\) influx via voltage-dependent Ca\(^{2+}\) channels that subsequently activate Ca\(^{2+}\)-activated K\(^+\) channels, such as the SK channels, thereby generating afterhyperpolarization (AHP) that slows down action potential discharges. The SK channels are involved in spike frequency adaptation and irregular firing pattern induced by a long depolarizing current (43). Three subtypes of SK channels have been cloned (20), and the distribution of the mRNA of each isoform of the SK channel has been established in the rat CNS (36). Interestingly, this study reported a different expression level of the SK2 and SK3 isoforms in preoptic nuclei. Therefore, it is reasonable to conclude that the Na\(^+\) sensor and non-Na\(^+\) sensor neurons express a different type of SK channel, which could explain the slight difference in spike frequency adaptation observed in these two subpopulations of ventral MnPO neurons.

**The Na\(^+\) sensor neurons display three distinct patterns of rectifying properties**

The activation of rectifying properties in response to membrane hyperpolarization represents a critical aspect of the neuron physiology to avoid severe fluctuations in the resting membrane potential. Our data show that the Na\(^+\) sensor neurons form a heterogeneous population in terms of rectifying properties in response to negative current pulses. Approximately half of the population was characterized by the presence of a time-independent membrane rectification (type 2 neuron; 44% of the neurons), whereas a second population displayed time-dependent membrane rectification (type 1 neuron; 25% of the neurons). The last population remained unresponsive to hyperpolarizing current steps (type 3 neuron; 31% of the neurons). As for the firing pattern, the presence of distinct neuronal populations in the same nucleus based on different rectifying properties is not unique. For example, the anterior bed nucleus of the stria terminalis contains type II neurons that exhibit a depolarizing sag in response to hyperpolarizing current injection and type III neurons that display fast anomalous rectification without a depolarizing sag (16). The
superior colliculus also groups neurons eliciting a rapid hyperpolarization followed by a slow repolarization, or voltage sag, with neurons displaying time-independent membrane hyperpolarization (33). In the ventral MnPO, these classes of neurons highlighted populations that are all endowed with brain Na\(^+\) sensing, but they present varying degrees of dynamic responses to membrane hyperpolarization. These classes of neurons might characterize distinct components of the hydromineral network involved in either osmoregulatory, autonomic, or behavioral regulatory responses.

Although the vMnPO neurons displayed varying degrees of rectifying properties, the percentage of rectifying neurons was higher in the Na\(^+\) sensor neurons than in the non-Na\(^+\) sensor neurons. Moreover, the distribution of type 1 neurons was three times higher in the Na\(^+\)-sensor neurons (31% vs. 11% in the non-Na\(^+\) sensor neurons, respectively). However, the amplitude of the time-dependent rectification was slightly weaker in the Na\(^+\) sensor neurons. The time-dependent membrane rectification is characterized by a membrane repolarization, or voltage sag, following the initial hyperpolarization, and this rectification is usually attributed to the activation of a Cs\(^+\)-sensitive hyperpolarization-activated cation current (2, 15, 29). Moreover, the use of ZD7228, a specific I\(_h\) channel blocker, indicates that the time-dependent membrane rectification was mediated through the activation of the I\(_h\) channels (16). The higher amplitude of the time-dependent rectification observed in the non-Na\(^+\) sensor neurons might likely be attributable to a higher current density in these neurons and, thus, a higher density of I\(_h\) channels. Alternatively, the molecular structure of the channel might be different in the two neuronal populations. Four genes (HCN1-HCN4) encode distinct isoforms of the hyperpolarization-activated I\(_h\) current, and the distribution of the individual pattern of HCN mRNA revealed the presence of HCN isoforms with varying degrees of expression in the hypothalamus (26). It is therefore possible that the
specific expression of HCN isoforms underlies the difference observed in the $I_h$ current amplitude in Na$^+$ sensor and non-Na$^+$ sensor neurons.

**Perspectives and significance**

This study showed that a majority of neurons in the ventral MnPO respond to variations in extracellular Na$^+$ levels. This unique characteristic reveals the relative function homogeneity (Na$^+$ sensing), which contrasts with the heterogeneity of the electrical properties displayed by this neuronal population. Indeed, the Na$^+$ sensor neurons display three types of distinct firing patterns and three types of membrane rectification in response to hyperpolarization. Unfortunately, no correlation could be made between the firing discharges and the rectification, demonstrating the considerable diversity among these unique neurons. The diversity among the electrical properties of the Na$^+$ sensor neurons might be correlated with the diversity of direct synaptic connections established between the MnPO and brain regions involved in various aspects of the restoration and conservation of body fluid homeostasis (38). A rise in extracellular Na$^+$ levels might generate patterned responses in Na$^+$ sensor neurons that could be specific to the network target, e.g. the PVH magnocellular and parvocellular cells for neuroendocrine and autonomic regulation, or higher integrative structures for behavioral regulation (1, 27, 37, 44). The diversity of the rectifying properties might have a significant impact on the excitability of the Na$^+$ sensor neurons. Indeed, $I_h$ has been correlated with the control of the resting membrane potential and the membrane potential in response to hyperpolarization, particularly during intense inhibitory synaptic barrage (23). Similar to the variety of the firing pattern, the heterogeneity of the rectifying properties in the Na$^+$ sensor neurons might also be correlated with the efferent targets of these subpopulations. The characterization of the electrophysiological fingerprints of the Na$^+$ sensor neurons with the concomitant identification of their projecting areas requires further
investigation to characterize the considerable diversity of Na\(^{+}\) sensor population in the hydromineral homeostasis network.

The Na\(^{+}\) sensor and non-Na\(^{+}\) sensor neurons, which represent approximately 80% and 20% of the vMnPO neurons, respectively, can be distinguished by the subtle characteristics of their firing and rectifying properties. Indeed, our data show that the Na\(^{+}\) sensor neurons that displayed regular firing presented an increased spiking frequency and, consequently, a reduced spike frequency adaptation than the non-Na\(^{+}\) sensor neurons. The amplitude of the time-dependent rectification was also weak in the Na\(^{+}\) sensor neurons. These discrepancies suggest that the Na\(^{+}\) sensor and non-Na\(^{+}\) sensor neuron subpopulations might express distinct ionic conductances that highlight neuronal excitability and shape electrical activity. However, the impact of such diversity from a physiological viewpoint remains unclear.

**GRANTS**

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REFERENCE LIST


**Figure 1.** Functional identification of the Na⁺ sensor neurons in the vMnPO. Transient application of hypernatriuric aCSF (170 mM) induced either a membrane depolarization (A) or no change in the membrane potential (B). The reversible membrane depolarization triggered by the application of hypernatriuric aCSF identifies the Na⁺ sensor neurons.

**Figure 2.** The Na⁺ sensor neurons display distinct firing patterns in acute brain slices. A: Typical illustration of the three distinct firing patterns observed in the vMnPO neurons in response to depolarizing current steps of 800 ms. Type A neurons were characterized by irregular spike discharges (upper trace). Type B neurons displayed a robust Ca²⁺ spike (middle trace) and type C neurons showed a strong spike adaptation (lower trace). B: Bar graph illustrating the distribution of the firing patterns in the Na⁺ sensor and non-Na⁺ sensor neurons.

**Figure 3.** Type A Na⁺ sensor neurons fire at higher frequency than the non-Na⁺ sensor neurons. A: Illustration of the firing pattern of the type A neurons in the two populations of MnPO neurons. B: Intensity-to-firing frequency plot indicates that the Na⁺ sensor neurons discharge at higher frequency that the non-Na⁺ sensor neurons. C: Typical illustration of high-threshold Ca²⁺ spikes in MnPO neurons. Local application of cadmium (10 mM) abolished the high threshold spikes elicited by depolarizing current steps (> 10 pA). D: Bar graph histograms indicating that the Ca²⁺ spike characteristics were identical in the Na⁺ sensor and in the non-Na⁺ sensor neurons. The duration of each depolarizing current step was 800 ms. Results were expressed as means ± SEM. ** Statistical significance at P < 0.01.
**Figure 4.** The amplitude of the Na\(^+\)-evoked depolarization is not a valid criteria to distinguish between the populations of Na\(^+\) sensor neurons.

A: Typical illustration of the depolarization triggered by hypernatriuric aCSF (170 mM) recorded in individual Na\(^+\)-sensor neurons characterized by a distinct firing pattern (type A, B and C neuron). Neurons were held around -60 mV and four depolarizing steps of 800 ms were applied (5 pA increment). B: Bar graph histogram indicating that the Na\(^+\)-evoked depolarization was similar in the three populations of Na\(^+\) sensor neurons. C: Bar graph histogram indicating that membrane capacitance (neuronal size indicator) was identical in the three populations of Na\(^+\) sensor neurons.

**Figure 5.** The Na\(^+\) sensor neurons display various patterns of membrane rectification in acute brain slice.

A: Two distinct rectifying patterns were observed in the MnPO neurons in response to hyperpolarizing current steps: neurons showing a time-dependent membrane rectification (Type 2 neuron) and neurons displaying a time-independent membrane rectification (Type 3 neuron). In addition, neurons without evoked membrane rectification were also present in the MnPO (type 1 neuron). B: Bar graph histogram representing the distribution of the three types of neurons in the Na\(^+\) sensor and in the non-Na\(^+\) sensor neurons. The hyperpolarizing current steps were applied during 1200 ms. Results were expressed as means ± SEM. * Statistical significance at \( P < 0.05 \).

**Figure 6.** Type 2 Na\(^+\) sensor neurons are characterized by a weaker membrane rectification than the non-Na\(^+\) sensor neurons.

A: The amplitude of the time-dependant hyperpolarization evoked by negative current steps of 1200 ms duration, was measured at a steady-state membrane potential (a). The current-voltage
relationship (I/V curve) indicated that the amplitude of the hyperpolarization was higher in the Na⁺ sensor neurons, than in the non-Na⁺ sensor neurons. **B:** The amplitude of the time independent hyperpolarization was similar in the Na⁺ sensor and in the non-Na⁺ sensor neurons. Results were expressed as means ± SEM. ** Statistical significance at $P < 0.01$. 
A

Type A neuron

-60 mV

Type B neuron

-60 mV

Type C neuron

-60 mV

B

neuronal population (%)

Na+ sensor neurons

Na+ non-sensor neurons

Type A

Type B

Type C
A

non-Na⁺ sensor neuron

-60 mV

Na⁺ sensor neuron

-60 mV

20 mV

100 ms

B

Action potential frequency (Hz)

Current pulse (pA)

Na⁺ sensors
non-Na⁺ sensors

C

Control

Cadmium (10 mM)

-60 mV

5 pA

100 ms

D: Ca²⁺ spikes

Current pulse (pA)

Na⁺ sensor neurons
non-Na⁺ sensor neurons

Action potential width (ms)

Action potential amplitude (mV)
A

Type A neuron

Type B neuron

Type C neuron

Hypernatriuric aCSF

-60 mV

Hypernatriuric aCSF

Hypernatriuric aCSF

10 sec

10 mV

B

Type A neuron

Type B neuron

Type C neuron

Hypernatriuric aCSF

Hypernatriuric aCSF

Membrane capacitance (pF)

C

Hypernatriuric aCSF-induced depolarization (mV)

Type A

Type B

Type C

Membrane capacitance (pF)

Type A

Type B

Type C
A

Type 1 neuron
-60 mV

Type 2 neuron
-60 mV

Type 3 neuron
-60 mV

B

Neuronal population (%)

Type 1
Type 2
Type 3

Na⁺ sensor neurons
non Na⁺ sensor neurons

*
A: Type 2 neuron

-60 mV

20 mV

200 ms

5 pA

B: Type 3 neuron

-60 mV

20 mV

200 ms

5 pA