Endogenous angiotensin II facilitates GABAergic neurotransmission afferent to the Na⁺-responsive neurons of the rat median preoptic nucleus

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ABSTRACT
The median preoptic nucleus (MnPO) is densely innervated by efferent projections from the subfornical organ (SFO) and, therefore, is an important relay for peripherals chemosensory and humoral information (osmolality and serum levels angiotensin II). In this context, controlling the excitability of MnPO neuronal populations is a major determinant of body fluid homeostasis and cardiovascular regulation. Using a brain slice preparation and patch-clamp recordings, our study sought to determine whether endogenous angiotensin II (AngII) modulates the strength of the SFO-derived GABAergic inputs to the MnPO. Our results showed that the amplitude of the IPSCs were progressively reduced by 44 ± 2.3% by (Sar\textsuperscript{1}, Ile\textsuperscript{8})-AngII, a competitive angiotensin type 1 receptor (AT\textsubscript{1}R) antagonist. Similarly, losartan, a non-peptidergic AT\textsubscript{1}R antagonist decreased the IPSC amplitude by 40.4 ± 5.6%. The facilitating effect of endogenous AngII on the GABAergic input to the MnPO was not attributed to a change in GABA release probability and was mimicked by exogenous AngII which potentiated the amplitude of the muscimol-activated GABA\textsubscript{A}/Cl\textsuperscript{−} current by 53.1 ± 11.4%. These results demonstrate a postsynaptic locus of action of AngII. Further analysis reveals that AngII did not affect the reversal potential of the synaptic inhibitory response, thus privileging a cross-talk between postsynaptic AT\textsubscript{1} and GABA\textsubscript{A} receptors. Interestingly, facilitation of GABAergic neurotransmission by endogenous AngII was specific to neurons responding to changes in the ambient Na\textsuperscript{+} level. This finding, combined with the AngII-mediated depolarization of non-Na\textsuperscript{+}-responsive neurons reveals the dual actions of AngII to modulate the excitability of MnPO neurons.

Key words: hydromineral homeostasis; sodium homeostasis; neuropeptides; hypothalamus
INTRODUCTION

The median preoptic nucleus (MnPO) is the midline structure of the lamina terminalis (LT) and a pivotal brain site for the hydromineral and cardiovascular homeostasis (See (18, 30) for detailed reviews). Chemical lesions of the MnPO produce deficits in both osmotically- and angiotensin II-stimulated water intake and vasopressin secretion (7, 13, 27) and increase need-free sodium intake (12). Similar lesions impaired cardiovascular reflex activity (28) and blocked pressor responses elicited by sodium hyperosmolality or intracerebroventricular injection of angiotensin II (AngII) (5, 17, 44).

In the context of a functional hypothalamic neuronal network, the MnPO is considered an integrator of chemosensory (osmolality) and humoral (AngII) signals relevant to hydromineral and cardiovascular homeostasis. Changes in plasma osmolality have been shown to alter the spiking activity of MnPO neurons (1, 29, 37, 38) and our laboratory discovered that these changes in electrical activity of MnPO neurons were driven by changes in the CSF Na⁺ level, rather than CSF osmolality (14). Behavioral studies showed that AngII infused into the OVLT or adjacent MnPO caused an increase in Na⁺ intake (8, 10, 11). Electrophysiological studies reported that the spiking activity of neurons within the median preoptic area (mainly the MnPO) was transiently enhanced after iontophoretic application of AngII (38, 40) or following an increase in systemic AngII (37). Finally, in vitro recordings revealed that AngII increased spike frequency in MnPO neurons (41) via postsynaptic depolarization (3). All these modulatory actions likely result from AngII that is released from the axonal projections of angiotensinergic neurons located in the subfornical organ (SFO). This is supported by the identification of many nerve cell bodies in this structure with Ang-like immunoreactivity (26) in addition to Ang-like immunoreactive fibers invading the MnPO (25).

As a putative mechanism for hydromineral and cardiovascular regulation, AngII has also been shown to alter the excitability of hypothalamic neurons by modulating their afferent synaptic neurotransmission. In the paraventricular nucleus (PVN), AngII increases the excitability of spinally projecting PVN neurons by reducing their GABAergic inputs. This action is mediated by activation of the angiotensin type 1 receptor (AT₁R) (22, 23). In contrast, AngII signaling through presynaptic AT₁R increases the excitability of magnocellular neurons by potentiating excitatory synaptic neurotransmission afferent to the supraoptic nucleus (33).
Although these previous studies identified the effects and related mechanisms of action of AngII on brain regions involved in hydromineral and cardiovascular regulation, unraveling the action(s) of endogenous angiotensin II on neuronal excitability would constitute an important breakthrough in the physiology of central homeostatic mechanisms. In this context, we used an electrophysiological approach applied to an in vitro hypothalamic slice preparation to investigate the putative effects of an AT₁R antagonist on the excitability of MnPO neuronal populations, particularly those that specifically responded to a change in the ambient Na⁺ level.

**MATERIALS AND METHODS**

The experiments described in the present study were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were duly approved by the Animal Care Committee of the Centre Hospitalier de l’Université Laval.

**Hypothalamic slices**

Acute hypothalamus slices were prepared from male Wistar rats (4-5 weeks old). The animals were at first anesthetized with a ketamine-xylasine solution (87.5 and 12.5 mg/kg, respectively) injected intraperitoneally and decapitated. Brains were quickly removed from the skull and submerged in ice-cold (2°C) artificial cerebrospinal fluid (aCSF) continuously bubbled with a gas mixture (95% O₂-5% CO₂) and containing in mM: 2 KCl, 1 CaCl₂, 3 MgCl₂, 26NaHCO₃, 1.2 NaHPO₄, 10 D-Glucose, 200 sucrose, pH 7.4. Osmolality was adjusted to 298-300 mOsm/l with mannitol. One sagittal hypothalamic slice (350-μm-thick) containing both the SFO and MnPO was obtained with a vibratome (VT1000S; Leica, Nusloch, Germany) and then transferred to a submersion-type recording chamber (Warner Instruments, inc.; Hamden, CT) mounted on a Gibraltar plateform (Burleigh Instruments, inc.; Fishers, NY). The slice was continuously bathed at 2-3 ml/min with oxygenated aCSF containing in mM: 123 NaCl, 3.1 KCl, 2.9 CaCl₂, 20 Na-gluconate, 10 HEPES, 5 D-glucose, pH 7.4; osmolality 298-300 mOsm/l. Bath temperature in the recording chamber and drug reservoirs was maintained at 25°C using a heater controller (TC-344B, Warner Instruments).
Electrophysiology

Whole-cell patch recordings were mainly performed in neurons located in the ventral part of the MnPO, in a region immediately adjacent to the anterior commissure. A tight gigaohm seal was obtained on individual neurons under visual control using the near infrared differential interference contrast principle. Patch pipettes were made from borosilicate glass capillaries (G75150T-4, Warner Instruments) with a resistance of about 4 to 5.5 MΩ. Pipettes were filled with a solution containing in mM: 124 K-gluconate, 12 KCl, 6 NaCl, 2 Na⁺-ATP, 0.1 Na⁺-GTP, 10 HEPES. pH was adjusted to 7.2 with KOH and osmolarity to 298-300 mOsm/l with sorbitol. Recordings were performed with an EPC9 amplifier (Heka Electronics, Inc., Mahone Bay, NS, Canada). The fast capacitance electrode was first compensated and appropriate whole-cell and series-resistance (RS) compensation were applied after rupture of the cell membrane (RS < 15 MΩ). Cells showing a change in RS more than 15% during recording were rejected from the analysis. Liquid junction potential was evaluated at 12.6 mV and membrane potential was corrected accordingly. Electrophysiological signals were filtered at 3kHz, digitalized at 2 kHz and stored on the computer hard drive for further analysis. Data analysis was performed using the Pulsefit software (Heka Electronics).

Synaptic currents were evoked with a concentric bipolar tungsten electrode placed within the subfornical organ, or in the fiber track coursing in front of the fornix. To record pharmacologically isolated inhibitory postsynaptic currents (IPSCs), electrical stimulation was carried out in the presence of 1 mM kynurenic acid, a broad spectrum blocker of the ionotropic excitatory amino-acid receptors. In addition, CGP52432 (10 µM) was added to the extracellular solution to rule out inhibition of GABA release by the activation of presynaptic GABAB receptors, which have been reported to modulate IPSC amplitude at the MnPO inhibitory synapse (20). Contribution of HCO3⁻ anions to the GABA_A receptor-mediated anion currents was almost null, as pH of the extracellular solution was balanced with HEPES. Therefore, under nominally HCO3⁻-free conditions, the reversal potential of IPSCs (E_{IPSC}) was approaching the reversal potential of chloride ions (E_{Cl⁻}). Theoretical E_{Cl⁻} calculated from the Nernst equation was of -51 mV at a recording temperature of 25°C.

In most recordings, the MnPO neurons were voltage clamped at -60 mV and IPSCs were evoked with repetitive stimulation at 0.2Hz. Regarding the direction of stable eIPSCs, neurons were transiently clamped at -70 mV (inward eIPSCs), -60 and/or -50 mV (outward eIPSCs) to
increase the driving force for Cl ions. Low frequency stimulation (0.2 Hz) of the SFO, or of the fibertrack linking the SFO to the MnPO was chosen in order to avoid depletion of the activated synapses and thus, insure constant synaptic responses all along the recordings. A series of 10 consecutive eIPSCs was sampled every 4 minutes after stabilization of the IPSC amplitude and three series of eIPSCs served as base line before drug application ($t_{-8 \text{ min.}}$, $t_{-4 \text{ min.}}$, $t_0 \text{ min.}$: control). The change in eIPSC amplitude resulting from drug application was assessed on three to four series of eIPSCs ($t_{4 \text{ min.}}$, $t_{8 \text{ min.}}$, $t_{12 \text{ min.}}$, and $t_{16 \text{ min.}}$: test) and expressed as a percentage of control eIPSCs.

A current-to-voltage relationship of IPSCs was built by varying the holding potential in 10 mV increments from -110 to -60 mV and measuring the resulting IPSC amplitude ($I$). The reversal potential of IPSCs ($E_{\text{IPSC}}$) was determined by using linear regression to calculate the best-fit line for $I/I_{\text{max}}$, $I_{\text{max}}$ being the IPSC amplitude recorded at -110 mV under the control condition. The intercept of this line with the abscissa was taken as $E_{\text{IPSC}}$. The reversal potential of the muscimol-activated GABA$_A$/Cl$^-$ current ($E_{\text{GABA}_A}$) was evaluated by subtracting the current resulting from a depolarizing voltage ramp (-90 to -60 mV; 16 mV/sec.) applied at the peak of the muscimol response (1 μM, 30 sec.) from a similar current elicited before the muscimol application. $E_{\text{GABA}_A}$ was then determined with the intercept of the $\Delta$ current with the 0 current line.

**Drugs and application**

Muscimol (Tocris Cokson, Inc., Ellisville, MO) was applied on the ventral region of the MnPO using a fast solution changer and manifold (model RSC-160; Bio-Logic, Grenoble, France). Angiotensin II, [Sar$^1$, Ile$^5$, Ile$^8$]-angiotensin II, Losartan-K$^+$ (Sigma-Aldrich Canada Ltd., Oakville, ON), PD 123,319 diflurane (RBI/Sigma, Natik, MA) were added to the extracellular solution at the concentration indicated in the text. These drugs were diluted to their final concentration just before being bath applied. Extracellular solution containing Angiotensin II, [Sar$^1$, Ile$^5$, Ile$^8$]-angiotensin II, PD 123,319, was added with BSA 0.1% to avoid peptide sticking to the perfusion line.

**Statistical analysis**
Raw data are expressed as means ± SEM. The Gaussian distribution of the dependent variables was first tested using the Kolmogorov-Smirnov normality test. Comparison of means for repeated measures was performed with a one-way ANOVA. When appropriate, post-hoc Tukey’s multiple comparison test was applied and statistical significance was determined at P < 0.05. Comparison of means obtained before and after drug application was performed using a paired t-test and p < 0.05 was considered significant.

RESULTS

All the patch-clamp recordings were carried out in the voltage-clamp mode at a holding potential of −60 mV. Fast GABAergic postsynaptic response elicited at that membrane potential was characterized by an outward current (IPSC) resulting from a hyperpolarized reversal potential of the IPSCs (E_{IPSC}, figure 1A). This is in agreement with our previous demonstration of a highly regulated Cl⁻ homeostasis by a K⁺/Cl⁻ cotransporter in vMnPO neurons (15). As expected, the outward IPSC was abolished by bath application of bicuculline (25 μM), a selective antagonist for the GABAA receptor subtype (figure 1A). The reliability of the fast inhibition of vMnPO neurons during whole-cell recording was tested by eliciting several series of 10 consecutive IPSCs evoked at 0.2Hz. As illustrated in figure 1B, repetition of low frequency stimulation of inhibitory input evoked outward IPSCs of stable amplitude over a period of at least 20 minutes. This result likely reveals that the efficiency of the Cl⁻ gradient was maintained during low frequency stimulation enabling stable inhibition of vMnPO neurons.

The fast SFO-mediated inhibition of the MnPO is regulated by endogenous angiotensin II.

The present study examined a possible regulatory action of endogenous angiotensin II on the fast inhibitory synaptic transmission between the SFO and the vMnPO. After stabilization of the eIPSC amplitude, three series of 10 consecutive IPSCs were sampled every 4 minutes as control IPSCs. Four series of IPSCs were then recorded in the presence of a specific peptide angiotensin type I receptor (AT₁R) antagonist, (Sar¹, Ile⁸)-AngII (10 μM). Our data showed that the eIPSCs were progressively reduced in the presence of (Sar¹, Ile⁸)-Ang II compared to control (One-way ANOVA, F_{6,54} = 100, p < 0.0001, n = 10, figure 2A). The reduction in eIPSC amplitude was stable after 12 min. of drug incubation and was 44 ± 2.3%
compared to control (Tukey’s test t_{12\text{min.}} \text{ vs. } t_{-8\text{min.}}, t_{12\text{min.}} \text{ vs. } t_{-4\text{min.}}, t_{12\text{min.}} \text{ vs. } t_{0\text{min.}}, p < 0.001, n = 10). Interestingly, bath application of 10\mu M losartan, a non-competitive AT_1R antagonist, reduced the amplitude of eIPSCs in the same range with an expected faster time course (One-way ANOVA, F_{6,24} = 4.4, p < 0.01, n = 5, figure 2B). The reduction in eIPSC was maximal after 4 min. of drug incubation and was 40.4 \pm 5.6\% compared to control (Tukey’s test t_{4\text{min.}} \text{ vs. } t_{-8\text{min.}}, t_{4\text{min.}} \text{ vs. } t_{-4\text{min.}}, t_{4\text{min.}} \text{ vs. } t_{0\text{min.}}, p < 0.001, n = 5). It has to be noted that recovery from (Sar^1, Ile^8)-AngII was not observed, at least in a time window of 10 to 20 min. after drug washout. Similar sustained depression of the eIPSCs was also observed in 3 out of 5 neurons tested with losartan. In the 2 remaining cells, recovery was 62\% and 80\% compared to control after a washout period of 16 min. In contrast to what was observed with the AT_1R antagonists, bath application of a selective antagonist at the angiotensin type 2 receptor, the spinacine derivative PD 123319 (10\mu M), had no effect on the amplitude of eIPSCs (One-way ANOVA, F_{4,24} = 0.53, p = 0.71, n = 7, figure 2C). These results demonstrated that the amplitude of the eIPSCs was specifically modulated by tonic activation of the AT_1R. The amplitude of the GABA_A response was efficiently regulated by a functional K^+/Cl^- cotransporter in this nucleus (15). It was thus possible that AngII might control the strength of the GABAergic synaptic events by regulating the activity of the K^+/Cl^- cotransporter. This possibility is unlikely, however, since the action of (Sar^1, Ile^8)-AngII was not accompanied by a shift in the reversal potential of the eIPSCs. E_IPSC was -75.2 \pm 6.5 mV under control conditions and -75.9 \pm 6.7 mV in the presence of (Sar^1, Ile^8)-AngII (paired t-test, p = 0.8; n = 5; figure 3).

**Angiotensin II facilitates GABA_A receptor-mediated responses via the activation of postsynaptic AT_1 receptors**

The next series of experiments was designed to investigate the locus of action of AngII. The presence of presynaptic AT_1R in controlling GABA release was first tested by using a paired-pulse paradigm, commonly used to measure changes in release probability. A series (10) of two consecutive IPSCs with an interstimulus interval of 150 ms was elicited under control condition and after a 12-16 minute incubation with (Sar^1, Ile^8)-AngII. The amplitude of both the first and second synaptic events (eIPSC1, eIPSC2) was decreased by 36 \pm 4 \% and 33 \pm 3.6 \%, respectively (eIPSC1: paired t-test, p = 0.004; eIPSC2: paired t-test, p =
The paired-pulse ratio (PPR: eIPSC2 / eIPSC1) was found to be similar under the two conditions (PPR: 0.92 ± 0.07 in control vs. 0.95 ± 0.06 in the presence of (Sar\(^1\), Ile\(^8\))-AngII; paired \(t\)-test, \(p = 0.422\); \(n = 5\), figure 4A3). Similarly, steady-state application of losartan reduced the amplitude of two consecutive eIPSCs (150 ms) by 50.5 ± 5.4 % and 45.7 ± 5.7 %, respectively (eIPSCs1: paired \(t\)-test, \(p = 0.002\); eIPSC2: paired-\(t\)-test, \(p = 0.015\); \(n = 4\); figure 4B1-2). The paired-pulse ratio between eIPSC2 and eIPSC1 was not affected by losartan (PPR: 0.67 ± 0.05 in control vs. 0.7 ± 0.06 in the presence of losartan; paired \(t\)-test, \(p = 0.404\); \(n = 4\); figure 4B3). All these results make a presynaptic location of AT\(_1\)R on GABAergic terminals unlikely, and to further validate the postsynaptic location of the AT\(_1\)R, we investigated the effect of exogenous AngII on the amplitude of the muscimol-activated GABA\(_A/Cl^–\) current recorded at -60 mV. Our results showed that the outward muscimol-activated GABA\(_A/Cl^–\) current was increased by 53.1 ± 11.4 % in the presence of 1 \(\mu\)M AngII (paired \(t\)-test, \(p = 0.01\); \(n = 6\); figure 5A1, 5B1), whereas the amplitude of the GABA\(_A/Cl^–\) current elicited by two consecutive applications of muscimol remained unchanged (-7.3 ± 9 %; paired \(t\)-test, \(p = 0.455\); \(n = 6\); figure 5A2). As for the eIPSCs, the AngII-mediated increase in the amplitude of the GABA\(_A/Cl^–\) current was not associated with a change in the reversal potential of the current. The estimated \(E_{GABA_A}\) determined from slow depolarizing ramps ranging from -90 to -60 mV (16 mV.s\(^{-1}\)) was -72.1 ± 1.1 mV under control conditions and -73.6 ± 0.6 mV in the presence of AngII (paired \(t\)-test, \(p = 0.11\); \(n = 6\); figure 5B2). As expected, \(E_{GABA_A}\) measured from two consecutive applications of muscimol remained stable (-71.6 ± 2.15 mV for muscimol application 1 and –70.9 ± 2.4 mV for muscimol application 2).

The AngII-mediated regulation of the GABA\(_A\) responses reported above was observed in MnPO neurons displaying outward eIPSCs or muscimol-activated GABA\(_A/Cl^–\) current recorded at -50 or -60 mV. Here, we investigated possible modulation of the GABA\(_A\) response in MnPO neurons displaying an inward inhibitory response when held at -60 mV. In these rare cells, local application of muscimol triggered an inward GABA\(_A/Cl^–\) current and the amplitude of the inward current was then examined at -90 mV in order to increase the driving force for Cl\(^–\) ions. At this membrane potential, bath application of 1 \(\mu\)M AngII enhanced the amplitude of the GABA\(_A/Cl^–\) current by 86.5 ± 9.9 % (paired \(t\)-test, \(p= 0.016\); \(n = 4\); figure 5C1). This facilitation of the GABA\(_A/Cl^–\) current was not accompanied by a change in the reversal
potential of the current, as $E_{\text{GABA}_A}$ was estimated at $-58.3 \pm 2$ mV under control conditions and at $-57 \pm 3.7$ mV in the presence of AngII (paired $t$-test, $p = 0.72$, $n = 4$; figure 5C2). Taken together, the data reported above indicate that activation of postsynaptic AT$_1$R modified the activity of GABA$_A$ receptors expressed in the neurons of the vMnPO.

**AngII has a dual postsynaptic actions in the vMnPO neurons**

MnPO neurons have previously been shown to respond to exogenous AngII by a sustained depolarization (3, 36). In order to reconcile these data with the present results, we introduced an identification criterion for neurons displaying AngII-mediated modulation of the GABA$_A$ response, i.e. sensitivity to a change in ambient Na$^+$ level (14). Our results indicated that all neurons displaying either the (Sar$_1$, Ile$_8$)-AngII-induced reduction in eIPSCs ($n = 5$) or the AngII-induced facilitation of the muscimol-activated GABA$_A$/Cl$^-$ current ($n = 6$) also responded to local application of iso-osmotic-hyponatriuric aCSF (300 mOsm.l$^{-1}$, 100 mM NaCl; 1 min) with a membrane hyperpolarization of $7 \pm 0.9$ mV (figure 6A, lower trace). In these neurons, the AngII-mediated effect on the GABA$_A$ response was never combined with a change in the holding potential (figure 6A, middle trace). In a few cells tested for this Na$^+$-specific sensitivity, the hyponatriuric aCSF did not generate a change in the membrane potential (7 out of 41 neurons, 17%), indicating that these neurons were not responsive to variation in extracellular Na$^+$. Interestingly in these cells, local application of AngII (1 µM; 3-4 min) triggered a sustained depolarization ($11 \pm 3.5$ mV) as illustrated in figure 6B.

These results indicated that AngII either facilitated the fast inhibitory transmission afferent to the Na$^+$-responsive neurons or increased the excitability of a non-identified neuronal population in the vMnPO by depolarizing these neurons (figure 6C).
DISCUSSION

The present study revealed the tonic control of the inhibitory synaptic transmission afferent to the median preoptic nucleus by angiotensin II. This peptide-mediated facilitation of the GABA\textsubscript{A} response was based on a postsynaptic cellular mechanism involving crosstalk between the angiotensin type 1 receptor and the GABA\textsubscript{A} receptor. The present study showed that the AngII-mediated modulation of the inhibitory neurotransmission was restricted to the neuronal population of the MnPO that specifically responded to variations in ambient Na\textsuperscript{+}. Interestingly, we also found that AngII had a postsynaptic depolarizing action on MnPO neurons that were not sensitive to the variation of extracellular Na\textsuperscript{+} ions, indicating that AngII could differentially modulate the excitability of two distinct neuronal subpopulations of the MnPO.

*The strength of the fast inhibitory response is modulated by endogenous angiotensin II*

Our data using specific AT\textsubscript{1}R antagonists demonstrated that the amplitude of evoked IPSCs was under tonic control mediated by angiotensin II. This direct evidence for the control of inhibitory synaptic events was confirmed by the facilitating effect of exogenous AngII on muscimol-activated GABA\textsubscript{A} currents. However, local application of AngII rarely increased the IPSC amplitude (potentiation of eIPSCs was only observed in 2 neurons, MG and MH, personal communication), suggesting that the tonic release of endogenous AngII occluded the action of bath applied AngII on eIPSCs. Endogenous AngII enhanced the efficacy of inhibitory neurotransmission via the activation of postsynaptic AT\textsubscript{1}R. Since a majority of MnPO neurons express a functional Cl\textsuperscript{-} extrusion system (15), the mechanism of action of AngII might be indirect, altering Cl\textsuperscript{-} homeostasis. Our demonstration that both the (Sar\textsuperscript{1}, Ile\textsuperscript{8})-AngII-induced reduction in IPSC amplitude and the AngII-induced increase in the GABA\textsubscript{A}/Cl\textsuperscript{-} current were not accompanied by a change in $E_{\text{GABA}_A}$ was of great importance to rule out direct coupling between the AT\textsubscript{1}R and the K\textsuperscript{+}/Cl\textsuperscript{-} cotransport system, suggesting an AngII-mediated change in postsynaptic GABA\textsubscript{A} receptor activity.

The origin of endogenous AngII is still under debate. An elegant study using transgenic mouse models expressing human renin and angiotensinogen has recently provided genetic evidence for *de novo* synthesis of AngII in the SFO as an essential contributor of water intake (34). This *de novo* synthesis of AngII combined with previous demonstration of neuronal
angiotensinogen and renin in the SFO (21, 42, 43) strongly suggests that endogenous AngII acts as a neurotransmitter originating from neurons in the SFO. Therefore, one possibility is that AngII is tonically released from synaptic terminals, a hypothesis supported by anatomical data showing the presence of the peptide in fibers originating in the SFO and terminating in the MnPO (25). Whether AngII has its own secretory pathway (packed into secretory granules) or is co-released with neurotransmitters (packed into vesicle-containing GABA or glutamate) has yet to be determined. It is, however, worth noting that the electrical stimulation of the fiber used here to evoked IPSCs was unlikely to be efficient enough to release secretory granules (trains of 10 IPSCs evoked at 0.2Hz). Indeed, it has been shown that the exocytosis of large dense core vesicles requires a Ca\(^{2+}\)-dependent priming step (35) or high order function of Ca\(^{2+}\) (2, 39), a condition which is probably not fulfilled with low frequency stimulation of the presynaptic fibers. In line with this, the modulatory effect of endogenous AngII reported in the paraventricular nucleus in vivo was evoked with pulse trains ranging from 100 to 200 Hz (4). Therefore, tonic release of AngII by axon terminal-containing secretory granules would require strong spontaneous activity of the presynaptic neurons. This scenario seems unlikely, however, because spontaneous synaptic events reflecting high level of spontaneous activity in the presynaptic neurons were not observed in the MnPO under our experimental conditions.

Alternatively, extracellular AngII might exert a constitutive action on MnPO neurons. This raises the possibility of local production and action of AngII within the MnPO. Interestingly, the cellular identification of two major components of the renin-angiotensin system in models of transgenic mice overexpressing human renin or angiotensinogen supports this possibility. In these animals, glia- and neuron-specific expression of renin has been detected in the MnPO, whereas angiotensinogen expression was restricted to neurons in this nucleus (31, 32). Here, angiotensinogen secreted by neurons would be cleaved by renin originating from neighboring neurons, or glial cells, to form extracellular AngII, which then would bind the AT\(_1\) receptors expressed by neurons in close proximity. The hypothesis of local production and release of AngII reconciles the action of endogenous AngII on eIPSCs and of exogenous AngII on the muscimol-activated GABA\(_A/Cl^-\) current. Indeed, focal application of AngII remained unefficient to potentiate eIPSCs, but the amplitude of the muscimol-activated GABA\(_A/Cl^-\) current. This observation suggests that under our recording conditions,
endogenous AngII acts at a restricted number of inhibitory synapses, namely those corresponding to the SFO inhibitory projection. However, when the postsynaptic AT1R population was globally activated with exogenous AngII, the peptide was able to mimic what was locally shown at individual synapses. Thus, an attractive hypothesis would suggest that unbalanced hydromineral conditions leading to enhanced production and release of AngII might expand the inhibitory action of AngII to additional synapses and strengthen the inhibition of the MnPO neurons responsive to the ambient Na⁺ level (see below).

Angiotensin II has dual actions in the MnPO, depending on the neuronal population

The identification of the neuronal population in which endogenous AngII tonically enhanced GABAergic input was based on their sensitivity to the ambient Na⁺ concentration (14). This finding differed, however, from previous reports describing a depolarizing effect of AngII on MnPO neurons via the activation of AT1R (3, 36, 41). Such AngII-induced depolarization was also observed during our recordings, but only in a neuronal population that was unresponsive to local application of hyponatriuric aCSF. Interestingly, the MnPO neurons displaying the AngII-induced depolarization were seldom recorded (7 out of 41 neurons, 17%). This percentage was similar to that reported in the other studies (16-25%) (36, 41), indicating that AngII might exert specific postsynaptic actions in the MnPO, depending on the neuronal subpopulations that express functional AT1 receptors.

The duality of AngII-induced responses observed in MnPO neurons might also shed light on neuroanatomical results obtained in the MnPO following Na⁺ depletion of body fluid compartments. Indeed, furosemide injection, a diuretic treatment known to activate AngII synthesis, has been shown to induce Fos or Fra expression in the ventral portion of the MnPO. Interestingly, double-labeled neurons (AT1A mRNA and Fos/Fra immunoreactivity) represented about 10-20% of the neuronal population expressing the AT1A mRNA receptor in these studies (6, 16). Despite a possible underestimation of Fos immunoreactive neurons, the percentage of double-labeled neurons was relatively similar to those displaying AngII-induced depolarization in the present and other studies. Among the various cellular mechanisms triggering Fos expression, an attractive hypothesis would be that the AngII-induced depolarization is a major determinant in the enhanced metabolic activity generated by Na⁺ deficit. Moreover, the absence of Fos immunoreactivity in a large population of AT1A receptor-
expressing neurons might be correlated to the AngII-induced facilitation of the GABAergic input, thereby reducing excitability and metabolic activation in these cells.

The opposite AngII-induced responses in two subpopulations of the MnPO neurons highlight the complex organization of the MnPO, probably reflecting the pivotal role of this nucleus in modulating the activity of the central network generating the neuroendocrine, autonomic and behavioral responses to a hydromineral challenge (9, 18, 30). The complex organization of the MnPO is clearly illustrated with in vivo extracellular single-unit recordings that identified distinct neuronal populations of the MnPO with monosynaptic connection to the PVN (MnPO-PVN neurons). Indeed elevated plasma AngII and hypertonicity were shown to increase cell discharges in distinct MnPO-PVN neurons (37). Interestingly, our in vitro results are in agreement with this observation. Indeed, MnPO-PVN neurons responding to systemic hypertonicity but not to AngII may correspond to the Na⁺-responsive neurons which inhibitory input is enhanced by AngII. Furthermore, the MnPO-PVN neurons responding to circulating AngII but not to hypertonicity may correspond to the Na⁺-unresponsive neurons that are depolarized by AngII. The in vivo study also reports a third subpopulation of MnPO-PVN neurons that did respond to both stimuli. It is possible that during our in vitro recordings, we were unable to record from these neurons for sampling reasons. The selection of the neurons was based on the operator criteria (shape of the cell body, appearance of the neuron under DIC illumination) and this might have introduced a selection of the neurons tested. Alternatively, the majority of the recorded neurons were localized in a region immediately adjacent to the anterior commissure. This criteria may therefore introduce a selection of the neurons dependent on the structural organization of the MnPO.

**Perspectives and significance**

The present study highlights an interesting functional aspect of central AngII, i.e. facilitation of the GABAergic inhibition via the recruitment of postsynaptic AT₁R. The underlying mechanism is an enhanced efficiency of the GABA_4 receptor activity and is thus, distinct from the modulation of GABA release (facilitation and attenuation) that occurs via the activation of presynaptic AT₁R (19, 22). In an integrative view of the physiology of the MnPO, the postsynaptic AT₁R-mediated facilitation of the inhibitory synaptic current by endogenous AngII may participate to the mechanisms underlying the neural basis of Na⁺ appetite. Indeed,
It has been reported that furosemide-induced Na⁺ depletion, a condition associated with enhanced production of AngII, was correlated with an increased proportion of septo-preoptic neurons which electrical activity was reduced by iontophoretic application of AngII (24). The inhibitory action of AngII on MnPO neuronal activity is well supported by our present data. Moreover, an attractive hypothesis is that, under Na⁺ deficit conditions, the enhanced production of local AngII in the MnPO would strengthen the inhibition of the subpopulation responsive to the change of ambient Na⁺ (14). The identification of this subpopulation in term of phenotype and projections remains an open question.

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**Figure 1:** Characteristics of the evoked IPSCs recorded in the neurons of the ventral MnPO. (A) Under the present experimental conditions (see methodology), electrical stimulation of the SFO or fiber track descending from the SFO evoked outward IPSCs in vMnPO neurons held at -60 mV that were abolished by 25 μM bicuculline (left traces). The outward direction of the IPSCs was correlated with hyperpolarized reversal potential of eIPSCs. (B) The presented time chart category graph indicates that the amplitude of eIPSCs remained stable during repeated low frequency stimulation (0.2Hz). Individual traces (a, b and c) illustrate an average of 10 consecutive eIPSCs sampled at three different time points after rupture of the patch membrane and stabilization of the eIPSC (outward direction).

**Figure 2:** GABAergic neurotransmission afferent to the vMnPO was tonically enhanced by angiotensin acting through the type 1 angiotensin receptor. (A) Time course of the reduction in eIPSC amplitude induced by bath application of 10 μM (Sar¹, Ile⁸)-AngII, a competitive AT₁ receptor antagonist. The amplitude of 10 consecutive IPSCs evoked at 0.2 Hz was averaged every 4 min. Typical averaged eIPSCs recorded in control and in the presence of (Sar¹, Ile⁸)-AngII were represented in a and b, respectively. (B) Bath application of 10 μM losartan, an non-competitive AT₁R antagonist, also reduced the amplitude of the eIPSCs. Typical averaged eIPSCs recorded in control and in the presence of losartan were illustrated in a and b, respectively. (C) Bath application of 10 μM PD 123319, an AT₂ receptor antagonist, was ineffective to modulate the amplitude of the eIPSCs, as illustrated with the time course chart and selected ePSCs in a and b. * indicates statistical significance with control eIPSCs (t₈ min., t₄ min., t₀ min.) and † indicates statistical significance with preceding averaged eIPSC.

**Figure 3:** Angiotensin does not change Cl⁻ homeostasis to modulate GABAergic neurotransmission. (A) Typical representation of individual eIPSCs recorded at different membrane potentials under control conditions (left traces) and in the presence of 10 μM (Sar¹, Ile⁸)-AngII (right traces). (B) Current-to-voltage relationship of the ePSCs recorded under control conditions (black circles) and in the presence of (Sar¹, Ile⁸)-AngII (open circles). This current-to-voltage
relationship indicates that (Sar$^1$, Ile$^8$)-AngII-induced inhibition of the eIPSCs was not correlated with a change in $E_{\text{IPSC}}$.

**Figure 4:** Angiotensin reduces eIPSC amplitude without changing release probability of GABA-containing vesicles.

The top traces illustrate representative average of 10 pairs of eIPSCs recorded under control conditions and after incubation of the slice with (Sar$^1$, Ile$^8$)-AngII (A1) or losartan (B1) for 12 min. The bar chart histogram illustrates the mean amplitude of the first and second eIPSC recorded under control conditions (white bars) and in the presence of (Sar$^1$, Ile$^8$)-AngII (A2) or losartan (B2). * indicates statistical difference (paired $t$-test, $P < 0.05$, $n = 5$). The bar chart histograms presented in A3 and B3 report the mean value of the paired-pulse ratio for all the neurons tested with (Sar$^1$, Ile$^8$)-AngII ($n = 5$) and losartan ($n = 4$), respectively. Stimulus parameters for the set of experiments depicted in A were $4.5 \pm 1.5$ mA; $127 \pm 37 \mu$s. Stimulus parameters for the set of experiments depicted in B were $15.7 \pm 5.5$ mA; $140 \pm 35 \mu$s.

**Figure 5:** Angiotensin II acts at a postsynaptic locus to modulate the amplitude of the $GABA_A$ response.

(A1) Transient application (30 sec.) of 1 µM muscimol triggered an outward $GABA_A/Cl^-$ current in a vMnPO neuron held at -60 mV. The amplitude of that current was potentiated in the presence of 1 µM Ang II. (A2) Note that two consecutive applications of muscimol elicited a $GABA_A/Cl^-$ current of similar amplitude. (B1) The bar chart histogram illustrates the AngII-mediated potentiation of the $GABA_A/Cl^-$ current. * indicates statistical difference (paired $t$-test, $P < 0.05$, $n = 6$). (B2) Current-to-voltage relationship of the muscimol-activated $GABA_A/Cl^-$ current ($I_{GABA_A}$) recorded under control conditions (black circles) and in the presence of AngII (open circles). Note that $I_{GABA_A}$ was obtained by subtracting the ramp current elicited before and at the peak of the muscimol-activated $GABA_A/Cl^-$ current (see truncated trace deflections in A1). (C1) The bar chart histogram illustrates the AngII-mediated potentiation of the $GABA_A/Cl^-$ current in neurons displaying inward $GABA_A/Cl^-$ current when held at -60 mV. * indicates statistical difference (paired $t$-test, $P < 0.05$, $n = 4$). (C2) Current-to-voltage relationship of $I_{GABA_A}$ obtained in neurons lacking a functional $K^+/Cl^-$ cotransport system.
**Figure 6:** Angiotensin differentially modulates the excitability of two neuronal subpopulations of the vMnPO.

(A) Typical example of a vMnPO neuron displaying a tonic facilitation of the synaptic GABA$_A$ response by endogenous AngII (upper trace). Note that bath application of 1 µM AngII had no effect on the holding potential of this cell (middle trace). Contrastingly, local application of a strict hyponatriuric aCSF (300 mOsm.l$^{-1}$, 100 mM NaCl; 1 min.) triggered a transient membrane hyperpolarization, identifying the cell as a specific Na$^+$-sensitive neuron (lower trace). (B) Typical example of a vMnPO neuron where bath application of 1µM AngII triggered a membrane depolarization (upper trace). That cell does not respond to transient application of hyponatriuric aCSF (lower trace). (C) Schematic representation of the two neuronal subpopulations of the vMnPO responding to AngII.
A

control

bicuculline

-60 mV

-60 mV

10 pA

20 ms

B

eIPSC (pA)

0  5  10  15  20  25

0  5  10  15  20  25

-60 mV

-60 mV

10 pA

25 ms

membrane potential (mV)
(Sar\(^1\)Ile\(^3\)) - Ang II

A

Losartan

B

PD 123319

C

- 60 mV

10 pA

25 ms

- 50 mV

25 pA

25 ms

- 60 mV

20 pA

25 ms
A

control  (sar\(^1\),ile\(^3\))-AngII

- 60 mV  - 60 mV
-70 mV  -70 mV
-80 mV  -80 mV
-90 mV  -90 mV
-100 mV -100 mV
-110 mV -110 mV

50 pA  50 ms

B

eIPSC (I/I\(_{max}\))

control  (sar\(^1\),ile\(^3\))-AngII

membrane potential (mV)
A

(Sar\(^1\), Ile\(^3\)) - Ang II

B

Ang II

C

GABAergic terminals

angiotensinergic terminals

Na\(^+\)-sensitive neuron

unidentified neuron

ventral MnPO