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

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Henry M, Grob M, Mouginot D. Endogenous angiotensin II facilitates GABAergic neurotransmission afferent to the Na⁺-responsive neurons of the rat median preoptic nucleus. Am J Physiol Regul Integr Comp Physiol 297: R783–R792, 2009.—The median preoptic nucleus (MnPO) is densely innervated by efferent projections from the subfornical organ (SFO) and, therefore, is an important relay for the peripheral chemosensory and humoral information (osmolality and serum levels ANG 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 ANG II modulates the strength of the SFO-derived GABAergic inputs to the MnPO. Our results showed that the amplitude of the inhibitory post synaptic currents (IPSCs) were progressively reduced by 44 ± 2.3% by (Sar1, Ile8)-ANG II, a competitive AT1 receptor (AT1R) antagonist. Similarly, losartan, a nonpeptidergic AT1R antagonist decreased the IPSC amplitude by 40.4 ± 5.6%. The facilitating effect of endogenous ANG II on the GABAergic input to the MnPO was not attributed to a change in GABA release probability and was mimicked by exogenous ANG II, which potentiated the amplitude of the muscimol-activated GABA, Cl− current by 53.1 ± 11.4%. These results demonstrate a postsynaptic locus of action of ANG II. Further analysis reveals that ANG II did not affect the reversal potential of the synaptic inhibitory response, thus privileging a cross talk between postsynaptic AT1 and GABA, receptors. Interestingly, facilitation of GABAergic neurotransmission by endogenous ANG II was specific to neurons responding to changes in the ambient Na⁺ level. This finding, combined with the ANG II-mediated depolarization of non-Na⁺-responsive neurons reveals the dual actions of ANG II to modulate the excitability of MnPO neurons.

hydromineral homeostasis; sodium homeostasis; neuropeptides; hypothalamus

THE MEDIAN PREOPTIC NUCLEUS (MnPO) is the midline structure of the lamina terminalis and a pivotal brain site for the hydromineral and cardiovascular homeostasis (for detailed reviews, see Refs. 18 and 30). Chemical lesions of the MnPO produce deficits in both osmotically- and angiotensin II (ANG 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 pressure responses elicited by sodium hyperosmolality or intracerebroventricular injection of ANG II (5, 17, 44).

In the context of a functional hypothalamic neuronal network, the MnPO is considered an integrator of chemosensory (osmolality) and humoral (ANG II) 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 cerebrospinal fluid (CSF) Na⁺ level, rather than CSF osmolality (14). Behavioral studies showed that ANG II 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 ANG II (38, 40) or following an increase in systemic ANG II (37). Finally, in vitro recordings revealed that ANG II increased spike frequency in MnPO neurons (41) via postsynaptic depolarization (3). All of these modulatory actions likely result from ANG II 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, ANG II has also been shown to alter the excitability of hypothalamic neurons by modulating their afferent synaptic neurotransmission. In the paraventricular nucleus (PVN), ANG II increases the excitability of spinally projecting PVN neurons by reducing their GABAergic inputs. This action is mediated by activation of the ANG type 1 receptor (AT1R) (22, 23). In contrast, ANG II 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 ANG II on brain regions involved in hydromineral and cardiovascular regulation, unraveling the action(s) of endogenous ANG 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 AT1R antagonists 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.

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Hypothalamic slices. Acute hypothalamic slices were prepared from male Wistar rats (4–5 wk old). The animals were at first anesthetized with a ketamine-xylasine solution (87.5 and 12.5 mg/kg ip, respectively) and decapitated. Brains were quickly removed from the skull and submerged in ice-cold (2°C) artificial CSF (aCSF) continuously bubbled with a gas mixture (55% O2-5% CO2) and containing (in mM): 2 KCl, 1 CaCl2, 3 MgCl2, 26 NaHCO3, 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 (model VT1000S; Leica, Nusloch, Germany) and then transferred to a submersion-type recording chamber (Warner Instruments, Hamden, CT) mounted on a Girbilar plate (Burleigh Instrument, 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-glucconate, 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 by using a heater.

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 by using the near-infrared-differential-interference contrast principle. Patch pipettes were made from borosilicate glass capillaries (model G75150T-4; Warner Instruments) with a resistance of ~4 to 5.5 MΩ. Pipettes were filled with a solution containing (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 osmolality to 298–300 mOsm/l with sorbitol. Recordings were performed with an EPC9 amplifier (Heka Electronics, 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 > 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 3 kHz, digitalized at 2 kHz, and stored on the computer hard drive for further analysis. Data analysis was performed using the Pulselit software (Heka Electronics).

Synaptic currents were evoked with a concentric bipolar tungsten electrode placed within the SFO or in the fiber track coursing in front of the fornix. To record pharmacologically isolated inhibited 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 GABA receptors, which have been reported to modulate IPSC amplitude at the MnPO inhibitory synapse (20). Contribution of HCO3− anions to the GABA 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 (EIPSC) was approaching the reversal potential of chloride ions (ECl−). Theoretical ECl−, 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 0.2 Hz. Direction of stable evoked IPSCs (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 fiber track linking the SFO to the MnPO was chosen 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 min after stabilization of the IPSC amplitude and three series of eIPSCs served as the baseline before drug application (t − 8 min, t − 4 min, t0 min; control). The change in eIPSC amplitude resulting from drug application was assessed on three to four series of eIPSCs (t4 min, t0 min, t12 min, and t16 min test) and expressed as a percentage of control eIPSCs.

A current-to-voltage relationship of IPSCs was built by varying the current elicited before the muscimol application. EGABAA was then determined with the intercept of the Δcurrent with the zero current line.

Drugs and application. Muscimol (Tocris Cookson, 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). ANG II, [Sar1, Ile5, Ile8]-ANG II, losartan-K (Sigma-Aldrich Canada, Oakville, ON, Canada), and PD 123,319 ditrifluoracetate (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 ANG II, [Sar1, Ile5, Ile8]-ANG II, and 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 ± SE. 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 (EIPSC; Fig. 1A). This is in agreement with our previous demonstration of a highly regulated Cl− homeostasis by a K+/Cl− cotransporter in ventral MnPO (vMnPO) neurons (15). As expected, the outward IPSC was abolished by bath application of bicuculline (25 μM), a selective antagonist for the GABA receptor subtype (Fig. 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 0.2 Hz. As illustrated in Fig. 1B, repetition of low-frequency stimulation of inhibitory input evoked outward IPSCs of stable amplitude over a period of at least 20 min. This result likely reveals that the efficiency of the Cl− gradient was maintained during low frequency stimulation enabling stable inhibition of vMnPO neurons.

Fast SFO-mediated inhibition of the MnPO is regulated by endogenous ANG II. The present study examined a possible regulatory action of endogenous ANG 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 min as control IPSCs. Four series of IPSCs were then recorded in the presence of a
specific peptide ANG type I receptor (AT₁R) antagonist, \((\text{Sar}^1, \text{Ile}^8)\)-ANG II (10 μM). Our data showed that the eIPSCs were progressively reduced in the presence of \((\text{Sar}^1, \text{Ile}^8)\)-ANG II compared with control (one-way ANOVA, \(F_{6,54} = 100, P < 0.0001, n = 10\), Fig. 2A). The reduction in eIPSC amplitude was stable after 12 min of drug incubation and was 44 ± 2.3% compared with control (Tukey’s test \(t_{12 \text{ min}}\) vs. \(t_{8 \text{ min}}\), \(t_{12 \text{ min}}\) vs. \(t_{4 \text{ min}}\), \(t_{12 \text{ min}}\) vs. \(t_0\), \(P < 0.001\), \(n = 10\)). Interestingly, bath application of 10 μM losartan, a noncompetitive AT₁R antagonist, reduced the amplitude of eIPSCs in the same range with an expected faster time course (one-way ANOVA, \(F_{6,24} = 4.4, P < 0.01, n = 5\), Fig. 2B). The reduction in eIPSC was maximal after 4 min of drug incubation and was 40.4 ± 5.6% compared with control (Tukey’s test \(t_{4 \text{ min}}\) vs. \(t_{8 \text{ min}}\), \(t_{4 \text{ min}}\) vs. \(t_{4 \text{ min}}\), \(t_{4 \text{ min}}\) vs. \(t_0\), \(P < 0.001\), \(n = 5\)). It has to be noted that recovery from \((\text{Sar}^1, \text{Ile}^8)\)-ANG II 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 three out of five neurons tested with losartan. In the two remaining cells, recovery was 62 and 80% compared with control after a washout period of 16 min. In contrast to what was observed with the AT₁R antagonists, bath application of a selective antagonist at the ANG type 2 receptor, the spinacine derivative PD 123,319 (10 μM), had no effect on the amplitude of eIPSCs (one-way ANOVA, \(F_{4,24} = 0.53, P = 0.71, n = 7\), Fig. 2C). These results demonstrated that the amplitude of the eIPSCs was specifically modulated by tonic activation of the AT₁R. The amplitude of the GABAA response was efficiently regulated by a functional K⁺/Cl⁻ cotransporter in this nucleus (15). It was thus possible that ANG II might control the strength of the GABAAergic synaptic events by regulating the activity of the K⁺/Cl⁻ cotransporter. This possibility is unlikely, however, since the action of \((\text{Sar}^1, \text{Ile}^8)\)-ANG II was not accompanied by a shift in the reversal potential of the eIPSCs. \(E_{\text{IPSC}}\) was \(-75.2 ± 6.5\) mV under control conditions and \(-75.9 ± 6.7\) mV in the presence of \((\text{Sar}^1, \text{Ile}^8)\)-ANG II (paired \(t\)-test, \(P = 0.8\); \(n = 5\); Fig. 3).

ANG II facilitates GABAA receptor-mediated responses via the activation of postsynaptic AT₁ receptors. The next series of experiments was designed to investigate the locus of action of...
ANG II. The presence of presynaptic AT1R 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 min incubation with (Sar1, Ile8)-ANG II. The amplitude of both the first and second synaptic events (eIPSC1, eIPSC2) was decreased by 36 \% and 33 \%, respectively (eIPSC1: paired t-test, \( P < 0.004 \); eIPSC2: paired t-test, \( P < 0.003 \); \( n = 5 \); Fig. 4, A1–A2). 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 (Sar1, Ile8)-ANG II; paired t-test, \( P = 0.422 \); \( n = 5 \); Fig. 4A3]. Similarly, steady-state application of losartan reduced the amplitude of two consecutive eIPSCs (150 ms) by 50.5 \% and 45.7 \%, respectively (eIPSCs1: paired t-test, \( P = 0.002 \); eIPSC2: paired t-test, \( P = 0.015 \); \( n = 4 \); Fig. 4, B1–B2). 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, \( P = 0.404 \); \( n = 4 \); Fig. 4B3). All these results make a presynaptic location of AT1R on GABAergic terminals unlikely, and to further validate the postsynaptic location of the AT1R, we investigated the effect of exogenous ANG II on the amplitude of the

Fig. 2. GABAergic neurotransmission afferent to the vMnPO was tonically enhanced by angiotensin acting through the ANG type 1 receptor (AT1R). A: time course of the reduction in eIPSC amplitude induced by bath application of 10 M (Sar1, Ile8)-ANG II, 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)-ANG II were represented in a and b, respectively. B: bath application of 10 M losartan, a noncompetitive AT1R antagonist, also reduced the amplitude of the eIPSCs. Typical averaged eIPSCs recorded in control and in the presence of losartan are illustrated in a and b, respectively. C: bath application of 10 M PD 123,319, an AT2 receptor antagonist, was ineffective to modulate the amplitude of the eIPSCs, as illustrated with the time course chart and selected eIPSCs in a and b.

*Statistical significance with control eIPSCs (t–8 min, f–4 min, t0 min); †Statistical significance with preceding averaged eIPSC.
The ANG II-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 to increase the driving force for Cl^- ions. At this membrane potential, bath application of 1 μM ANG II enhanced the amplitude of the GABA_A/Cl^- current by 86.5 ± 9.9% (paired t-test, P = 0.016; n = 4; Fig. 5C1). This facilitation of the GABA_A/Cl^- current was not accompanied by a change in the reversal potential of the current, as E_GABA_A was estimated at -58.3 ± 2 mV under control conditions and at -57 ± 3.7 mV in the presence of ANG II (paired t-test, P = 0.72, n = 4; Fig. 5C2). Taken together, the data reported above indicate that activation of postsynaptic AT_R modified the activity of GABA_A receptors expressed in the neurons of the vMnPO.

**ANG II has a dual postsynaptic action in the vMnPO neurons.** MnPO neurons have previously been shown to respond to exogenous ANG II by a sustained depolarization (3, 36). To reconcile these data with the present results, we introduced an identification criterion for neurons displaying ANG II-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)-ANG II-induced reduction in eIPSCs (n = 5) or the ANG II-induced facilitation of the muscimol-activated GABA_A/Cl^- current (n = 6) also responded to local application of hyponatremic aCSF (300 mOsm/l, 100 mM NaCl; 1 min) with a membrane hyperpolarization of 7 ± 0.9 mV (Fig. 6A, bottom trace). In these neurons, the ANG II-mediated effect on the GABA_A response was never combined with a change in the holding potential (Fig. 6A, middle trace). In a few cells tested for this Na^+-specific sensitivity, the hyponatremic 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 ANG II (1 μM; 3–4 min) triggered a sustained depolarization (11 ± 3.5 mV) as illustrated in Fig. 6B.

These results indicated that ANG II either facilitated the fast inhibitory transmission afferent to the Na^+-responsive neurons or increased the excitability of a nonidentified neuronal population in the vMnPO by depolarizing these neurons (Fig. 6C).

**DISCUSSION**

The present study revealed the tonic control of the inhibitory synaptic transmission afferent to the MnPO by ANG II. This peptide-mediated facilitation of the GABA_A response was based on a postsynaptic cellular mechanism involving cross talk between the AT_R and the GABA_A receptor. The present study shows that the ANG II-mediated modulation of the inhibitory neurotransmission was restricted to the neuronal population of the MnPO that specifically responded to variations in ambient Na^+. Interestingly, we also found that ANG II had a postsynaptic depolarizing action on MnPO neurons that were not sensitive to the variation of extracellular Na^+ ions,
indicating that ANG II could differentially modulate the excitability of two distinct neuronal subpopulations of the MnPO.

**Strength of the fast inhibitory response is modulated by endogenous ANG II.** Our data using specific AT₁R antagonists demonstrated that the amplitude of evoked IPSCs was under tonic control mediated by ANG II. This direct evidence for the control of inhibitory synaptic events was confirmed by the facilitating effect of exogenous ANG II on muscimol-activated GABA<sub>A</sub> currents. However, local application of ANG II rarely increased the IPSC amplitude (potentiation of eIPSCs was only observed in two neurons; M. Grob and M. Henry, personal communication), suggesting that the tonic release of endoge-

Fig. 4. ANG II reduces eIPSC amplitude without changing release probability of GABA-containing vesicles. Top traces: representative average of 10 pairs of eIPSCs recorded under control conditions and after incubation of the slice with (Sar<sup>1</sup>, Ile<sup>8</sup>)-ANG II (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 and in the presence of (Sar<sup>1</sup>, Ile<sup>8</sup>)-ANG II (A2) or losartan (B2). *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 (PPR) for all the neurons tested with (Sar<sup>1</sup>, Ile<sup>8</sup>)-ANG II (\(n = 5\)) and losartan (\(n = 4\)), respectively. Stimulus parameters for the set of experiments depicted in A were 4.5 ± 1.5 mA; 127 ± 37 s. Stimulus parameters for the set of experiments depicted in B were 15.7 ± 5.5 mA; 140 ± 35 μs.
Endogenous ANG II occluded the action of bath applied ANG II on eIPSCs. Endogenous ANG II enhanced the efficacy of inhibitory neurotransmission via the activation of postsynaptic AT1R. Since a majority of MnPO neurons express a functional Cl\(^{-}/H^+\) extrusion system (15), the mechanism of action of ANG II might be indirect, altering Cl\(^{-}\) homeostasis. Our demonstration that both the (Sar\(^{1}\), Ile\(^{8}\))-ANG II-induced reduction in IPSC amplitude and the ANG II-induced increase in the GABA\(_A/Cl^\text{-}\) current were not accompanied by a change in \(E_{\text{GABA}}\) was of great importance to rule out direct coupling between the AT1R and the K\(^{+}/Cl^\text{-}\) cotransport system, suggesting an ANG II-mediated change in postsynaptic GABA\(_A\) receptor activity.

The origin of endogenous ANG II 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 ANG II in the SFO as an essential contributor of water intake (34). This de novo synthesis of ANG II combined with previous demonstration of neuronal angiotensinogen and renin in the SFO (21, 42, 43) strongly suggests that endogenous ANG II acts as a neurotransmitter.

Fig. 5. ANG II acts at a postsynaptic locus to modulate the amplitude of the GABA\(_A\) response. A1: transient application (30 s) of 1 \(\mu\)M muscimol triggered an outward GABA\(_A/Cl^\text{-}\) current in a vMnPO neuron held at \(-60\) mV. The amplitude of that current was potentiated in the presence of 1 \(\mu\)M ANG II. (A2) Note that 2 consecutive applications of muscimol elicited a GABA\(_A/Cl^\text{-}\) current of similar amplitude. B1: The bar chart histogram illustrates the ANG II-mediated potentiation of the GABA\(_A/Cl^\text{-}\) current. *Statistical difference (paired \(t\)-test, \(P < 0.05\), \(n = 6\)). B2: current-to-voltage relationship of the muscimol-activated GABA\(_A/Cl^\text{-}\) current (\(I_{\text{GABA}}\)) recorded under control conditions (●) and in the presence of ANG II (○). Note that \(I_{\text{GABA}}\) was obtained by subtracting the ramp current elicited before and at the peak of the muscimol-activated GABA\(_A/Cl^\text{-}\) current (see truncated trace deflections in A1). C1: bar chart histogram illustrates the ANG II-mediated potentiation of the GABA\(_A/Cl^\text{-}\) current in neurons displaying inward GABA\(_A/Cl^\text{-}\) current when held at \(-60\) mV. *Statistical difference (paired \(t\)-test, \(P < 0.05\), \(n = 4\)). C2: current-to-voltage relationship of \(I_{\text{GABA}}\) obtained in neurons lacking a functional K\(^{+}/Cl^\text{-}\) cotransport system.
mitter originating from neurons in the SFO. Therefore, one possibility is that ANG II 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 ANG II has its own secretory pathway (packed into secretory granules) or is coreleased 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 0.2 Hz). 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 ANG II reported in the PVN in vivo was evoked with pulse trains ranging from 100 to 200 Hz (4). Therefore, tonic release of ANG II 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 a high level of spontaneous activity in the presynaptic neurons were not observed in the MnPO under our experimental conditions.

Fig. 6. ANG II differentially modulates the excitability of 2 neuronal subpopulations of the vMnPO. A: typical example of a vMnPO neuron displaying a tonic facilitation of the synaptic GABA\(_A\) response by endogenous ANG II (top trace). Note that bath application of 1 \(\mu\)M ANG II had no effect on the holding potential of this cell (middle trace). Contrastingly, local application of a strict hyponatriuric artificial cerebrospinal fluid (aCSF; 300 mOsm/l, 100 mM NaCl; 1 min) triggered a transient membrane hyperpolarization, identifying the cell as a specific Na\(^{+}\)-sensitive neuron (bottom trace). B: typical example of a vMnPO neuron where bath application of 1 \(\mu\)M ANG II triggered a membrane depolarization (top trace). That cell does not respond to transient application of hyponatriuric aCSF (bottom trace). C: schematic representation of the two neuronal subpopulations of the vMnPO responding to ANG II.
Alternatively, extracellular ANG II might exert a constitutive action on MnPO neurons. This raises the possibility of local production and action of ANG II 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 ANG II, which then would bind the AT1 receptors expressed by neurons in close proximity. The hypothesis of local production and release of ANG II on the muscimol-activated GABAergic input is attractive because it reconciles the action of endogenous ANG II on eIPSCs with the ability of extracellular ANG II 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 ANG II might expand the inhibitory action of ANG II to additional synapses and strengthen the inhibition of the MnPO neurons responsive to the ambient Na⁺ level (see below).

ANG II has dual actions in the MnPO, depending on the neuronal population. Identification of the neuronal population in which endogenous ANG II 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 ANG II on MnPO neurons via the activation of AT₁R (3, 36, 41). Such ANG II-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 ANG II-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 ANG II might exert specific postsynaptic actions in the MnPO, depending on the neuronal subpopulations that express functional AT₁ receptors.

The duality of ANG II-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 ANG II 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 ANG II-induced depolarization in the present and other studies. Among the various cellular mechanisms triggering Fos expression, an attractive hypothesis would be that the ANG II-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 ANG II-induced facilitation of the GABAergic input, thereby reducing excitability and metabolic activation in these cells.

The opposite ANG II-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 ANG II 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 ANG II may correspond to the Na⁺-responsive neurons, which inhibitory input is enhanced by ANG II. Furthermore, the MnPO-PVN neurons responding to circulating ANG II but not to hypertonicity may correspond to the Na⁺-unresponsive neurons that are depolarized by ANG II. 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 differential interference contrast 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 ANG II, i.e., facilitation of the GABAergic inhibition via the recruitment of postsynaptic AT₁R. The underlying mechanism is an enhanced efficiency of the GABA_A 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 ANG II 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 ANG II, was correlated with an increased proportion of septopreoptic neurons, which electrical activity was reduced by iontophoretic application of ANG II (24). The inhibitory action of ANG II 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 ANG II in the MnPO would strengthen the inhibition of the subpopulation responsive to the change of ambient Na⁺ (14). The identification of this subpopulation in terms of phenotype and projections remains an open question.
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