GABAergic Excitation of Vasopressin Neurons: A Possible Mechanism Underlying Sodium-Dependent Hypertension

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ABSTRACT

**Rationale:** Increased arginine-vasopressin (AVP) secretion is a key physiological response to hyperosmotic stress and may be part of the mechanism by which high-salt diets induce or exacerbate hypertension.

**Objective:** Employing deoxycorticosterone acetate (DOCA)-salt hypertension model rats, we sought to test the hypothesis that changes in GABA_A receptor-mediated inhibition in AVP-secreting magnocellular neurons contribute to the generation of Na^+^-dependent hypertension.

**Methods and Results:** In vitro gramicidin-perforated recordings in the paraventricular (PVN) and supraoptic nuclei (SON) revealed that the GABAergic inhibition in AVP-secreting neurons was converted into excitation in this model, due to the depolarization of GABA equilibrium potential (E_{GABA}). Meanwhile, in vivo extracellular recordings in the SON showed that the GABAergic baroreflexive inhibition of magnocellular neurons was transformed to excitation, so that baroreceptor activation may increase AVP release. The depolarizing E_{GABA} shift in AVP-secreting neurons occurred progressively over weeks of DOCA-salt treatment along with gradual increases in plasma AVP and blood pressure. Further, the shift was associated with changes in chloride transporter expression and partially reversed by bumetanide (Na^+^-K^+^-2Cl^- co-transporter inhibitor). Intracerebroventricular bumetanide administration during DOCA-salt treatment hindered the development of hypertension and rise in plasma AVP level. Muscimol (GABA_A agonist) microinjection into the SON in hypertensive rats increased blood pressure, which was prevented by prior intravenous V1a AVP antagonist injection.

**Conclusions:** We conclude that the inhibitory-to-excitatory switch of GABA_A receptor-mediated transmission in AVP neurons contributes to the generation of Na^+^-dependent hypertension by increasing AVP release. We speculate that normalizing the E_{GABA} may have some utility in treating Na^+^-dependent hypertension.

**Keywords:** Arginine-vasopressin (AVP), GABA, sodium, hypertension, NKCC1, supraoptic nucleus.

**Nonstandard Abbreviations and Acronyms:**
ACSF        artificial cerebrospinal fluid
AP5          DL-2-amino-5-phosphonopentanoic acid
AVP          arginine-vasopressin
DNQX         6,7-dinitroquinoxaline-2,3-dione
DOCA         deoxycorticosterone acetate
E_{GABA}     GABA equilibrium potential
EPSP/EPSC    excitatory postsynaptic potential/excitatory postsynaptic current
ICV          intracerebroventricular
IHC          immunohistochemical
IPSP/IPSC    inhibitory postsynaptic potential/inhibitory postsynaptic current
KCC2         K^+^-Cl^- co-transporter isotype 2
LJP          liquid junction potential
MNCs         magnocellular neurosecretory cells
NKCC1        Na^+^-K^+^-2Cl^- co-transporter isotype 1
NO           nitric oxide
OXY          oxytocin
PSP          postsynaptic potential
INTRODUCTION

It is well established that excess dietary salt is one of the most powerful risk factors for, and a major cause of, hypertension. One possible mechanism is that excess sodium (Na\textsuperscript{+}) increases the circulating levels of arginine-vasopressin (AVP), a neurohormone which can increase blood pressure not only through its well-known antidiuretic and vasoconstrictive actions but also through stimulating the arterial secretion of the potent vasoconstrictor endothelin. Consistent with this possibility, the plasma level of AVP is elevated in several animal models of Na\textsuperscript{+}-dependent hypertension, including the widely used “deoxycorticosterone acetate (DOCA)-salt” model. Further, Na\textsuperscript{+}-dependent hypertension does not develop in the Brattleboro rat, which genetically lacks AVP, and is suppressed by AVP antagonist.

AVP is synthesized in magnocellular neurosecretory cells (MNCs) in the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON), and it is released into the general circulation from the nerve terminals of MNCs located at the posterior pituitary. The release of AVP is regulated by synaptically-relayed signals to AVP-secreting MNCs from various sources including baro- and osmoreceptors. The major excitatory and inhibitory neurotransmitters involved in this synaptic transmission had been thought to be glutamate and γ-aminobutyric acid (GABA), respectively. Recent work from our laboratory indicated that GABA does not always function as an inhibitory neurotransmitter in this circuit. We demonstrated that, although GABA generally inhibits AVP neurons under resting conditions, it excites them under chronic hyperosmotic stress, a situation which demands increased AVP secretion. Further, we showed that an increase in intracellular chloride concentration ([Cl\textsuperscript{-}]) in the postsynaptic cell is responsible for the conversion of GABAergic inhibition to excitation. Our study provided evidence that the polarity of GABAA receptor-mediated responses of AVP neurons can reverse in response to physiological need through the change in transmembrane Cl\textsuperscript{-} gradient.

In the current study, rats were made hypertensive by a chronic administration of DOCA and exposure to 1% NaCl drinking water following uninephrectomy. We then sought to test the hypothesis that in this DOCA-salt model of hypertension, GABA excites AVP neurons to increase the release of AVP and consequently raise blood pressure. Here we present experimental results supporting this hypothesis. Our data indicate that altered Cl\textsuperscript{-} homeostasis resulting from the up-regulation of the Cl\textsuperscript{-} importer Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} co-transporter isotype 1 (NKCC1) and the down-regulation of the Cl\textsuperscript{-} extruder K\textsuperscript{+}-Cl\textsuperscript{-} co-transporter isotype 2 (KCC2) in AVP neurons make GABA excitatory in these cells. We discuss these findings with respect to the mechanisms responsible for the genesis of hypertension arising from excess salt intake.
METHODS

Animal.
Male Sprague-Dawley rats (250–400 g) from Orient Bio Co. (Sungnam, Korea) were used in the current study. They were housed in a temperature-controlled vivarium (22–24°C) with a 12:12 h light/dark cycle for at least a week before being used for experiments. The experimental procedures described below were approved by the Korea University College of Medicine Animal Research Policies Committee while conforming to the guidelines of Council of the Korean Physiological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All possible efforts were made to minimize the number of animals as well as suffering.

Hypertension models.
We used three different methods to induce hypertension in rats.
DOCA-salt hypertension model: Rats were anaesthetized with 2% isoflurane in oxygen. A lateral abdominal incision was used to access the left kidney for its resection. The incision site was sutured after the removal of the kidney. One day after surgery, uninephrectomized rats were given 1% NaCl in the drinking water for 4 weeks with subcutaneous injections of DOCA (25 mg in 0.4 ml of n,n-dimethyl formamide) every fourth day.
Nitric oxide (NO) inhibitor-induced hypertension model: Rats were made hypertensive by offering to drink water containing L-NAME (60-70 mg/100 ml) for 4 weeks.
Ethanol-induced hypertension model: In order to avoid a considerable loss of animals, the ethanol-treated group was submitted to a brief and gradual adaptation period during which rats received 5% ethanol in their drinking water in the first week, 10% in the second, and 20% ethanol in the third and fourth weeks of experimentation. The development of hypertension in the model rats was confirmed by measuring weekly their arterial blood pressures at zeitgeber time (ZT) 1:00-4:00 h (lights-on in the vivarium at ZT 0:00 h), with a non-invasive tail-cuff system (model CODA, Kent Scientific Co., Torrington, CT, USA). The animals were acclimated to the restraining device and the tail was heated (32°C) before the measurements were started. The average of 10 measurements was taken as the data point for each rat.

Hypothalamic slice preparation.
Brain slices were prepared as previously described. In brief, the animal was anesthetized with urethane (1.25 g/kg i.p.) and the brain was quickly excised from the skull and submerged in ice-cold artificial cerebrospinal fluid (ACSF) [(composition in mM) 124 NaCl, 1.3 MgSO₄, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 2.4 CaCl₂, and 10 glucose]. After being chilled for 1-2 min, the brain was trimmed to a block containing the hypothalami. With the use of a vibroslicer (Campden Instruments, UK), coronal slices (300–450 μm), containing the SON and/or the PVN, were cut from the tissue block in ice-cold ACSF. The slices were transferred to a gas interface recording chamber which was perfused with warm (34–35°C) aerated (95% O₂/5% CO₂) ACSF, at a rate of 0.5-1 ml/min, by a peristaltic pump-driven or gravity-fed bath-perfusion system. Warm (34–35°C) air humidified by 95% O₂/5% CO₂ gas mixture was continuously blown over the slices to further ensure adequate oxygenation of cells in the tissue. Drugs (see below) were applied to the brain slices by including them in the perfusion medium or focally with the “Y-tube” method.

Intracellular electrophysiological recording.
Current- or voltage-clamp recordings were obtained from neurons in the SON or PVN of hypothalamic slices equilibrated for 1-12 h in the recording chamber. The SON was identified as a translucent region right next to the optic chiasm, and the PVN as a triangular, translucent area between the fornix and the upper part of the third ventricle. Micropipettes (tip diameter, 1.5–2.0 μm; 3–6 MΩ) pulled from borosilicate tubings (P-97, Sutter Instrument Co., Novato, CA, USA) and filled with gramicidin (50 μg/ml)-containing solution [(composition in mM) 143 K-gluconate, 2 KCl, 10 HEPES, and 0.5 EGTA; pH 7.2–7.3] were used for recording in a perforated configuration. Our use of the low chloride (2 mM)
internal solution along with gramicidin provided a clear readout of when the patch was ruptured, resulting in a whole-cell recording configuration (i.e., GABAergic EPSPs/EPSCs were converted into IPSPs/IPSCs). Stable perforated-recording condition was usually achieved within 10–25 min after seal was formed. Those recordings having steady series resistances (range, 30–70 MΩ) and action potential amplitudes of ≥40 mV (measured from spike threshold) were the only ones included in the data pool; the reversal potentials of GABA<sub>A</sub> receptor-mediated responses were estimated only with the recordings that had a series resistance of <60 MΩ (typically 30–50 MΩ). The voltage errors resulting from the series resistance were compensated offline for voltage-clamp recordings and online for current-clamp recordings by using the bridge circuit. We corrected the liquid junction potential (LJP) prior to the experiments; we set the pipette potential to -9 mV just before the formation of patch configuration, knowing that the LJP was 15.8 mV (at 34.5 °C) while the perforated patch potential (Vpf) arising from the gramicidin perforation was -6.8 mV. We assumed that the change in resting membrane potential (RMP) detected when the recording mode was transformed from perforated to whole-cell configuration represented the Vpf. Neurons sampled in the SON and PVN, which responded to a depolarizing current pulse delivered at a holding potential between -70 mV and -80 mV with repetitive spikes having a delayed onset, were taken as MNCs 27. The signals from neurons amplified by Axoclamp-2B amplifier (bandwidth filter set at 10 kHz) were digitized and sampled at 50 μs intervals (Digidata1320, pClamp 8.0; Molecular Devices, Sunnyvale, CA, USA).

**Immunohistochemical (IHC) identification of recorded neurons.**
Please find the protocol in the online supplement.

**Measurement of plasma vasopressin and catecholamine levels.**
Please find the protocols in the online supplement.

**Western blot.**
Please find the protocol in the online supplement.

**In vivo extracellular single-unit recording and monitoring of blood pressure.**
Please find the protocol in the online supplement.

**Muscimol microinjection into the SON.**
Before the microinjection, rats were deeply anesthetized with urethane (1.25 g/kg i.p.), and catheterization was performed. One catheter (PE-50 tube) was inserted into the left carotid artery for blood pressure measurement and a second catheter was inserted into the left femoral vein for AVP antagonist injection. The catheterized rats were fixed in a stereotaxic instrument and kept warm (36–38°C) using a heating pad. Then, a 30-gauge injector was lowered into the SON (AP: 1.4 mm caudal to the bregma, ML: 1.8 mm lateral to the midline, DV: 9.3 mm below the skull surface). The GABA<sub>A</sub> receptor agonist muscimol was injected (1 and 10 nmol/100 nl) unilaterally over a period of 60 s by injection pump. These doses of muscimol were chosen based on earlier studies demonstrating that microinjection of a similar dose in the PVN effectively changed the blood pressure 28. Experimental procedures were initiated 1–1.5 h after anesthesia onset. Absence of somatic motor reflexes in response to tail pitching indicated deep anesthesia and analgesia. The baseline blood pressure was monitored for a period of at least 30 min before experimental procedures were performed. After microinjection experiment, “Direct Blue” dye (0.9% in saline) was injected (100 nl) into the SON. Brains were removed, sectioned (300 μm) and viewed through a light microscope to identify injection sites.

**Intracerebroventricular (ICV) infusion of bumetanide.**
The surgery to implant an osmotic mini-pump (infusion rate: 0.5 μl/h; Alzet, Model 2002; Cupertino, CA, USA) and an infusion cannula (Brain Infusion Kit 2, Alzet) targeting the right lateral ventricle (AP: 1.0 mm caudal to the bregma, ML: 1.6 mm from the midline, DV: 4 mm below the surface of the skull) was
performed on the 14\textsuperscript{th} day after the beginning of DOCA-salt treatment. Under anesthesia induced by an intraperitoneal injection of a combination of ketamine (43 mg/kg body weight) and xylazine (8.7 mg/kg body weight), the pump was positioned subcutaneously in the scapular region and attached to the cannula via polyethylene tubing. Prior to implantation, the pump had been filled with vehicle (ACSF, pH 7.4) or a solution containing bumetanide (500 μmol/l) and placed in warm (36°C) saline overnight for priming.

**Drugs.**

Please find the protocols in the online supplement.

**Statistical analysis.**

Numerical data are expressed as the mean ± SEM. Student’s \( t \)-test and Mann-Whitney \( U \)-test were used for the comparison of two independent data sets with and without normal distribution, respectively. Paired samples were compared with paired \( t \)-test. One-way analysis of variance (ANOVA) and pairwise comparison with Holm-Sidak method were performed to compare multiple independent data sets with normal distributions, while Kruskal-Wallis one-way ANOVA on ranks and pairwise comparison with Tukey test or Dunn’s method were performed to compare data sets without normal distributions. Comparison of multiple data sets collected with repeated measurement was performed with repeated measure of one-way ANOVA. Fisher Exact test was performed to see if there was a contingency between the two kinds of classification. \( P<0.05 \) was considered significant. When multiple comparisons were made with Fisher Exact test, the significance level was lowered by dividing the accepted \( P \) value criteria with the number of comparisons.

**RESULTS**

To determine if GABA\textsubscript{A} receptor-mediated inhibition is altered in AVP neurons of the DOCA-salt hypertension model rat, we compared the GABA\textsubscript{A} receptor-mediated postsynaptic potentials (PSPs) of putative AVP neurons recorded in the acute hypothalamic slices prepared from Sprague-Dawley rats treated with DOCA-salt for 4 weeks after uninephrectomy (\( n=17 \)) and untreated intact controls (\( n=15 \)). The GABAergic PSPs were isolated from glutamatergic ones by including AP5 (100 μM; NMDA receptor antagonist) and DNQX (20 μM; non-NMDA receptor antagonist) in the recording medium and monitored at various holding potentials with the use of gramicidin-perforated recording technique, which preserves the [Cl\textsuperscript{-}] of the recorded cell 29.

**Identification of AVP neurons.**

Many of the putative AVP neurons, sampled from the SON or the lateral magnocellular subnucleus of the PVN, exhibited the electrophysiological characteristics of AVP neurons 30, 31 including phasic firing patterns (Figure 1A) with little or no inward rectification at membrane potentials between -50 mV and -130 mV (Figure 1B). After the recordings, IHC analysis (Figure 1C) confirmed that the cells which exhibited the physiological signature of AVP neurons did indeed express AVP-neurophysin (21 out of 23 cells from 7 rats). In contrast, AVP-neurophysin was absent in most of the cells (8 out of 9 cells from 6 rats) which exhibited the physiological characteristics of oxytocin (OXY) neurons including the absence of phasic firing and strong inward rectification between -50 mV and -130 mV (Figure 1B). Therefore, for the remainder of this study, we used physiological properties to define MNCs as AVP-expressing neurons and focused our recordings on those cells that exhibited the physiological properties of AVP neurons. We could detect no physiological differences between the AVP neurons recorded in the SON and PVN regions and therefore pooled the resulting data for this study.
In control rats, GABA-mediated currents are predominately inhibitory in AVP neurons.

The GABAergic PSPs detected in AVP neurons (n=44) of the control rats (n=15) were predominantly inhibitory (41/44 cells) i.e., hyperpolarizing relative to action potential threshold, which was about -45 mV (Figure 2A, upper panel; Figure 2C). A few of the neurons (3/44 cells) did show an excitatory PSPs (example not shown). These PSPs were always mimicked by the focal application of GABA_A receptor agonist muscimol (10 μM, 10-20 ms, n=44; Figure 2A, upper panel) and blocked by bath-applied bicuculline (GABA_A receptor antagonist; 30 μM, n=15, example not shown), indicating that they were GABA_A receptor-mediated events. The reversal potential of these GABAergic responses (E_{GABA}), which was estimated with the currents elicited at various holding potentials by focal application of muscimol (10 μM, 10-20 ms) in the presence of tetrodotoxin (TTX, 0.5 μM) (Figure 2D), was -56 ± 1 mV on average (n=44 cells; range: -27 mV to -73 mV; Figure 2E). In many of the neurons having GABAergic IPSPs, the E_{GABA} was similar to or significantly more positive than the RMP (-61 ± 3 mV, n=44). Therefore, GABAergic IPSPs in these cells were either buried in the noise or detected as depolarizing events at RMP (middle trace of the upper panel of Figure 2A). Thus, our physiological data suggest that GABA functions typically as an inhibitory transmitter in AVP neurons of the normal control rat.

In DOCA-salt treated rats, GABA-mediated currents are predominately excitatory in AVP neurons.

In contrast, in the AVP neurons (n=54) of uninephrectomized rats treated with DOCA-salt (n=17), the GABAergic PSPs were almost always excitatory (52 of 54 cells; lower panel of Figure 2A and Figure 2C). These PSPs were always mimicked by muscimol (10 μM, 10-20 ms, n=54; Figure 2A, lower panel) and blocked by bicuculline (30 μM; n=15, Figure 2B), again indicating that they were GABA_A receptor-mediated events. The GABA_b receptor antagonist CGP 54626 hydrochloride (1 μM) had no significant effect on the PSPs (n=5, example not shown). The E_{GABA} of the AVP neurons of DOCA-salt treated rat averaged -35 ± 1 mV (range: -2.7 mV to -54 mV, n=54), which was significantly more positive than the action potential threshold and the E_{GABA} estimated for the neurons of control rats (P<0.001, Student’s t-test; Figures 2D and 2E). The RMP and input resistance of AVP neurons were not significantly different between control and DOCA-salt treated rats (Control: -61 ± 3 mV, n=44 vs. DOCA-salt: -64 ± 1 mV, n=54; Control: 356 ± 19 MΩ; n=44 vs. DOCA-salt: 318 ± 14 MΩ, n=51). These results indicate that the DOCA-salt treatment causes the E_{GABA} to depolarize beyond the action potential threshold in AVP neurons and therefore converts GABA_A receptor-mediated inhibition into excitation in these cells.

The depolarizing E_{GABA} shift and the resultant emergence of GABAergic excitation occur over several weeks.

Next, we examined the time course of the depolarizing E_{GABA} shift and the resultant emergence of GABAergic excitation in AVP neurons induced by DOCA-salt treatment. Prior work has shown that DOCA-salt treatment in the rat increases arterial pressure and the plasma levels of AVP gradually over several weeks 32. In the current study, we confirmed this (Figure 3A, upper panel; Figure 3B) and discovered that the progressive increases in arterial pressure and AVP concentration were paralleled by the gradual depolarizing shift of E_{GABA} (Figure 3A, lower panel) as well as an increase in the proportion of cells showing GABAergic excitation (Figure 3C). These findings indicate that the GABA-related changes in AVP neurons occur gradually over several weeks and may contribute to the development of hypertension by promoting AVP release.
GABAergic excitation is not found in all rat hypertension models.

The depolarizing \( E_{\text{GABA}} \) shift and the resulting GABAergic excitation detected in AVP neurons of the DOCA-salt model rat may be a general response to hypertension or a more specific physiological response to promote AVP release. To examine these possibilities, we investigated GABA\(_A\) receptor-mediated transmission in AVP neurons of rats made hypertensive by administering ethanol or NO inhibitor (Figure 4A, upper panel). Previous work has shown that the NO inhibitor-induced hypertension is independent of AVP secretion whereas the ethanol-induced hypertension requires enhanced AVP secretion \(^{23}\). We found that in the AVP neurons of the ethanol-induced hypertension model rats with increased plasma AVP levels (Figure 4B), the \( E_{\text{GABA}} \) was depolarized as compared to the control (Figure 4A, lower panel) and GABAergic PSPs were mostly excitatory (10 of 14 cells, Figure 4C). On the other hand, in the neurons of NO inhibitor-induced hypertension model rats with normal plasma AVP concentrations (Figure 4B), the \( E_{\text{GABA}} \) was not depolarized (Figure 4A, lower panel) and GABAergic PSPs were mostly inhibitory (18 of 19 cells, Figure 4C). These results indicate that the depolarizing \( E_{\text{GABA}} \) shift and the resulting GABAergic excitation in AVP neurons are not seen in every model of hypertension and may be found only under conditions that require enhanced AVP release.

GABAergic excitation promotes the release of AVP to increase blood pressure in the DOCA-salt hypertension model rat.

GABAergic excitation in the AVP neurons may contribute to increase AVP secretion in DOCA-salt model rats. To explore this possibility, we injected the GABA\(_A\) receptor agonist muscimol unilaterally into the SON of the rat under urethane anaesthesia (Figure 5A, lower panel) and measured the blood pressure. Muscimol produced no significant changes in blood pressure in the control rats, whereas it increased the blood pressure in the DOCA-salt hypertension model rats (Figures 5A and 5B), without altering the heart rate (Figure 5A, Online Figure IA) or the plasma levels of catecholamines (Online Figure IB). In comparison, the GABA\(_B\) receptor agonist baclofen injected into the SON (2.25 nmol in 100 nl ACSF) decreased the blood pressure in both the control and DOCA-salt model rats (Online Figure II). The muscimol-induced increase in blood pressure in the DOCA-salt hypertension model rats was blocked by a prior i.v. injection of the V1a AVP receptor antagonist (d(CH\(_2\))\(_5\))[Tyr(Me)\(_2\), Ala-NH\(_2\)]\(_9\)AVP; 100 \( \mu \)g/kg) (Figures 5C and 5D). Alone, his antagonist decreased the systolic blood pressure (SBP) significantly in a dose-dependent fashion in DOCA-salt model rats, while it had no effect in the control animals (Online Figure III). These results confirm the previous finding \(^{6,9}\) that AVP plays an important role in maintaining hypertension in DOCA-salt model rats. More importantly, they indicate that GABA\(_A\) receptor-mediated excitation in AVP neurons promotes AVP release to increase blood pressure in the DOCA-salt hypertension model rats.

The depolarized \( E_{\text{GABA}} \) converts baroreflexive inhibition into excitation in SON neurons of the DOCA-salt hypertension model rat, making increases in arterial pressure promote AVP release.

We assessed the impact of the depolarizing \( E_{\text{GABA}} \) shift on the reflexive inhibition of AVP neurons in the SON by arterial baroreceptor activation using extracellular single-unit recording technique in the anesthetized rat. It is well established that the baroreceptors in the aortic arch and carotid sinus buffer acute increases in arterial pressure by inhibiting AVP release from the posterior pituitary and decreasing the heart rate \(^{33}\). GABAergic afferents to AVP neurons mediate the reflexive inhibition of these cells by arterial baroreceptors \(^{15}\). In control rats (\( n=3 \)), we found that the activation of baroreceptors by an i.v. injection of the \( \alpha \)-adrenergic agonist phenylephrine (3–9 \( \mu \)g/kg body weight) resulted mostly in the inhibition of SON neurons (11/14 neurons; Figures 6A-6C, left panels). In contrast, in the DOCA-salt treated rats (\( n=4 \)), baroreceptor activation by phenylephrine mostly excited SON neurons (12/16 neurons; Figures 6A-6C, right panels). The proportions of cells showing inhibitory and excitatory responses to phenylephrine injections were significantly different between control and hypertensive rats (\( P=0.009, \)
Fisher’s exact test). To assess the functional significance of the conversion of the baroreflexive inhibition to excitation in SON neurons of the DOCA-salt model rats, we next examined the effect of an acute increase in arterial pressure on the plasma AVP concentration. As expected, the blood pressure increase led to a significant rise in the AVP concentration in the model rats (Figure 6D). Meanwhile, the increase in arterial pressure had no significant effect on the plasma AVP level in the control rats. These results suggest that the depolarization of $E_{\text{GABA}}$ in the DOCA-salt hypertension model rat impairs the GABA$_A$ receptor-mediated baroreflex in AVP neurons, such that an acute increase in arterial pressure promotes, rather than suppresses, AVP release.

**DOCA-salt treatment up-regulates NKCC1 and down-regulates KCC2 to induce the depolarizing shift of $E_{\text{GABA}}$ and the resultant emergence of GABAergic excitation.**

[Cl$^-$_i, the major determinant for $E_{\text{GABA}}$ and the polarity of GABA$_A$ receptor-mediated PSP, is regulated mainly by the Cl$^-$-importing transporter NKCC1 and Cl$^-$-extruding transporter KCC2 in mammalian central nervous system (CNS) neurons$^{34}$. To see if the levels of these transporters changed in AVP neurons of the DOCA-salt model rats, we examined expression levels of NKCC1 and KCC2 in the SON, PVN and hippocampus with Western blots. In the SON and PVN tissues, the level of NKCC1 was significantly higher and the level of KCC2 was significantly lower in the DOCA-salt model, than control, rats (Figures 7A and 7B). On the other hand, in the hippocampus, the levels of the Cl$^-$ transporters were not significantly different between the two groups (Online Figure IV A). Also, the levels of the NKCC1 and KCC2 in the SON and PVN of the rats treated with DOCA-salt for only 2 weeks did not differ from those of control rats (Online Figures IVB and IVC). This data suggests that the up-regulation of NKCC1 and the down-regulation of KCC2 in AVP neurons located in the osmoregulatory circuit contribute to the depolarizing $E_{\text{GABA}}$ shift and the resulting emergence of GABAergic excitation in the later stages of development of DOCA-salt hypertension.

To test this hypothesis more directly, we next examined the effects of the NKCC inhibitor bumetanide$^{35}$ and the KCC2 inhibitor VU0240551 on the $E_{\text{GABA}}$ and the GABAergic response profile. In AVP neurons ($n=5$) of the control rats ($n=2$), the bath application of bumetanide (10 μM) resulted in a small hyperpolarizing shift of the $E_{\text{GABA}}$ (Figure 7C, upper panel) while, in cells ($n=13$) of the DOCA-salt model rats ($n=8$), it caused the $E_{\text{GABA}}$ to hyperpolarize to a greater extent (Figure 7D, upper panel). The effect of bumetanide in these neurons was partially reversible, and it was accompanied by a significant change in GABAergic PSP profile; i.e., the ratio of cells showing EPSPs and IPSPs changed from 11:2 to 1:12, ($P \leq 0.001$, Fisher’s exact test). Meantime, bath-applied VU0240551 (75 μM) caused a significant depolarization of $E_{\text{GABA}}$ in AVP neurons ($n=10$) of the control rat ($n=3$) (Figure 7C, lower panel). In cells ($n=10$) of the DOCA-salt model rat ($n=3$), however, it had no significant effect on the $E_{\text{GABA}}$ (Figure 7D, lower panel). These effects of VU0240551 were not accompanied by any significant changes in GABAergic PSP profile. Thus, these results, coupled with the data from the Western blot experiments, indicate that the up-regulation of NKCC1 and the down-regulation of KCC2 are the molecular mechanisms underlying the depolarizing $E_{\text{GABA}}$ shift and the resultant emergence of GABAergic excitation in the AVP neurons of established DOCA-salt hypertension model rats.

NKCC1 plays an important role in the development of DOCA-salt hypertension.

If this hypothesis is correct, then pharmacologically blocking NKCC1 may hinder the development of hypertension in the DOCA-salt model by suppressing AVP release. To test this possibility, the NKCC blocker bumetanide was infused ICV using osmotic mini-pump into freely moving rats for a week between the 14th and 21st days of the DOCA-salt treatment. Infusion of bumetanide delayed the development of hypertension and lowered the plasma level of AVP (Figure 8). These results provide support for the idea that the up-regulation of NKCC1 in AVP neurons contributes to the development of hypertension in the DOCA-salt model rats by promoting the emergence of GABAergic excitation and the consequent increase in AVP release.
DISCUSSION

Previous studies suggested that hypertension can arise through impairments of inhibitory GABAergic transmission in the CNS regions involved in the regulation of blood pressure. Similarly, recent work found that the mechanism that underlies the development of spontaneous hypertension in a line of rats is the up-regulation of the Cl⁻ transporter NKCC1 which then impairs the GABA_A receptor-mediated inhibition of presynaptic neurons in the PVN. In the current study, we explored the possibility that, in the DOCA-salt hypertension model rat, GABAergic inhibition is converted into excitation in AVP neurons to increase the release of AVP and consequently blood pressure. We developed this hypothesis, in part, due to our earlier work which demonstrated that the polarity of GABA_A receptor-mediated transmission in AVP neurons can reverse in response to physiological need. AVP neurons in the PVN and SON are heavily innervated by GABAergic afferents, which seem to originate from the perinuclear zones in the adjacent hypothalamus and are stimulated by high-salt intake. In the present study, we found that in AVP neurons of DOCA-salt hypertension model rats GABAergic input evoked depolarizing, excitatory responses, and that the excitatory action of GABA increased blood pressure by promoting AVP release. GABA was also excitatory in a significant proportion of AVP neurons of the rat with ethanol-induced hypertension, a model dependent upon increased AVP secretion. In contrast, GABA remained inhibitory in AVP neurons of the rat made hypertensive by administering NO inhibitor. This treatment produced hypertension without increased AVP secretion. These findings suggest that the GABAergic excitation in AVP neurons is a general feature of hypertensions accompanied by increased AVP release, not an adaptive response to hypertension. More importantly, the findings suggest that one of the mechanisms through which high-salt intake, and perhaps excessive alcohol consumption, induce or aggravate hypertension is to bring about GABAergic excitation in AVP neurons and the resultant increase in AVP release.

The current study demonstrates that the depolarizing shift of E_GABA and consequent emergence of GABAergic excitation in AVP neurons occurred gradually from around the second week after the commencement of DOCA-salt treatment, while SBP and plasma AVP level began to increase from the very first week. Even after 4 weeks of treatment, the V1a AVP receptor antagonist reduced, but did not completely normalize, the elevated blood pressure. Furthermore, the administration of NKCC1 inhibitor during the DOCA-salt treatment blocked partially the development of hypertension and the associated rise in plasma AVP level. These findings indicate that the GABAergic excitation plays a crucial role in the development and maintenance of hypertension in this model but that other mechanisms (e.g., increases in plasma osmolality, renal sodium and water retention and sympathetic activity) must be involved.

Our results raise the possibility that the GABAergic excitation in AVP neurons contributes to the increase in blood pressure by altering the baroreflexive control of these cells. Baroreflex is responsible for the overall tone as well as the acute moment-to-moment regulation of blood pressure by modulating cardiac output and total peripheral resistance. Under resting conditions, baroreceptors respond to acute increases in arterial pressure by activating the vagal parasympathetic center. They also respond to the pressure increases by inhibiting the vasoconstrictive center of the medulla and suppressing the electrical activity of AVP neurons through the release of GABA. The resulting decreases in heart rate, cardiac contractility and AVP release from the posterior pituitary, along with vasodilation, cause blood pressure to decrease. Using the systemic administration of phenylephrine to interrogate the baroreflex, we discovered that arterial baroreceptor activation led to the excitation, rather than inhibition, of SON neurons in the DOCA-salt model rats. Moreover, we found that baroreceptor activation by phenylephrine raised the plasma levels of AVP in the DOCA-salt model, but not control, rats. These phenomena, which presumably resulted from the conversion of GABAergic inhibition to excitation, imply that the baroreflex in AVP neurons no longer functions to buffer blood pressure changes in these hypertensive rats but it rather contributes to the generation of hypertension by making increases in blood pressure promote AVP release.
The baroreflexive control of heart rate is significantly suppressed in the DOCA-salt hypertension model rats. In the current study, we found that muscimol (10 nmol) injection into the SON of the DOCA-salt hypertension model rat resulted in an increase in SBP by ~13 mmHg, with no significant change in heart rate. The absence of heart rate change might be related to the suppressed baroreflexive control of heart rate in the hypertension model rats. In support of this notion, our supplementary results (Online Figure V) demonstrate that an increase in SBP by 13 mmHg is too small a change to alter the heart rate significantly.

In our normotensive control rats, GABA was mostly inhibitory in AVP neurons. We examined GABA receptor-mediated transmission in AVP neurons identified with the electrophysiological characteristics and/or post-hoc IHC analysis. In most of the identified AVP neurons, the E$_{\text{GABA}}$ was negative to the action potential threshold (which was about -45 mV) and the GABA receptor agonist muscimol decreased the firing rate by causing hyperpolarization. These findings indicate that GABA is inhibitory in most AVP neurons as has been reported in most previous studies. One recent study reported that GABA receptor-mediated transmission produced excitatory responses in AVP neurons in the SON and PVN even under control conditions. At present, we cannot offer a satisfactory explanation for these differences but point out that these findings are the exception in the literature.

We found that the activation of GABA receptors in the SON by muscimol microinjection produced no significant changes in arterial pressure in the normotensive control rats. In addition, the GABA receptor-mediated baroreflex triggered by i.v. injected phenylephrine failed to alter plasma AVP concentration in the control animals. If GABA inhibits AVP neurons under normal conditions, activation of GABAergic system should lower blood pressure by suppressing AVP release. The reason for the lack of changes in arterial pressure and plasma AVP concentration after the activation of GABAergic system might be that the inhibitory effect of GABAergic transmission on the hormonal output of AVP neurons is already maximal in normal conditions. We speculate that the basal secretory activities of AVP neurons are low in normal conditions in such a way that AVP plays a negligible role in maintaining the arterial pressure. Consistent with this idea, the i.v. injected V1a receptor antagonist had no significant effect on the arterial pressure in the control, unlike DOCA-salt model, rat (Online Figure III).

The strength and polarity of GABA receptor-mediated responses of CNS neurons are determined predominantly by [Cl$^-$], which is regulated mainly by the Cl$^-$-importer NKCC1 and the Cl$^-$-extruder KCC2. We found evidence that the up-regulation of NKCC1 as well as the down-regulation of KCC2 is responsible for the depolarizing shift of E$_{\text{GABA}}$ and the resulting emergence of GABAergic excitation in AVP neurons. Direct stimulation by DOCA of the mineralocorticoid receptors in MNCs is unlikely to be the reason for the changes in the expression levels of the Cl$^-$ transporters because NKCC1 and KCC2 are up- and down-regulated, respectively, in MNCs of the SON and PVN after the removal of mineralocorticoid by bilateral adrenalectomy in the adult male rat (W.B. Kim, Ph.D., Y.-B. Kim, B.S., H. J. Chung, Ph.D. and Y.I. Kim, Ph.D., unpublished data, 2013). The up-regulation of NKCC1 in DOCA-salt treated rats might result from the central action of AVP and/or OXY released from the somata or dendrites of MNCs in response to osmotic stimuli presented by the DOCA-salt treatment. A recent study from our laboratory showed that a long-term ICV administration of selective oxytocin receptor antagonist partially blocked the NKCC1-dependent depolarizing E$_{\text{GABA}}$ shift and the resultant emergence of GABAergic excitation in the MNCs of rats subjected to chronic hyperosmotic stress. Meanwhile, the KCC2 down-regulation in DOCA-salt treated rats might arise from the action of brain-derived neurotrophic factor (BDNF) released locally from glia and/or neurons in the PVN and SON. Tropomyosin-receptor-kinase B (TrkB) receptors as well as BDNF mRNA and protein, are present in the SON and hyperosmotic stimulus increases the local release of BDNF in the SON. TrkB receptor-mediated action of BDNF from microglia was reported to down-regulate KCC2 in the lamina I of the spinal cord of the rat with neuropathic pain, enabling GABAergic excitation to occur in neurons located...
in this spinal region. Still the mechanisms that underlie the changes in expression of NKCC1 and KCC2 are not known and will require further investigation.

The present data raise the possibility that the Cl⁻ transporters (NKCC1 and KCC2) can be targeted for the treatment of hypertension. The finding that the NKCC inhibitor bumetanide retarded the development of hypertension in the DOCA-salt model rat provides some evidence in support of this hypothesis. With hypertension due to excess consumption of salt and alcohol being a growing problem throughout the world, there remains the need for novel treatment strategies.

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DISCLOSURES
None

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FIGURE LEGENDS

Figure 1: Identification of AVP-expressing MNCs recorded in hypothalamic slices. (A) Voltage trace illustrates phasic firing pattern that characterizes AVP neurons. (B) Graphs showing the current-voltage relations of putative AVP and OXY neurons. Inset (upper): current responses evoked by a series of hyperpolarizing voltage pulses (200-ms duration) applied at the holding potential of -50 mV with 10-mV increments. The voltage protocol is shown below the current responses. The red vertical line in each graph indicates the time point for current amplitude measurement. The ratio of amplitudes of the currents denoted with A1 and A2 gave rise to the rectification index (A1/A2), which was used as a criterion for classifying the MNCs as putative AVP and OXY neurons. Rectification index of >0.8 indicated putative AVP neuron, while rectification index of ≤0.8 OXY neuron. (C) Post-hoc IHC for AVP-neurophysin in MNCs injected with biocytin at the end of the recording. The top panels show that the cell classified as an AVP neuron based on its electrophysiological characteristics did indeed express AVP-neurophysin, whereas the lower panels show that a cell classified as an OXY neuron based on its physiology did not express AVP-neurophysin.

Figure 2: GABA<sub>A</sub> receptor-mediated PSPs in AVP neurons recorded in hypothalamic slices from control and DOCA-salt treated rats. (A) Spontaneous GABA<sub>A</sub> receptor-mediated PSPs recorded at various holding potentials in the cells of control and DOCA-salt model rats. ▲: focal muscimol application (10 μM, 10 ms). (B) Reversible, suppressive effect of bicuculline (30 μM; bath application) on GABAergic EPSPs (●) recorded from an AVP neuron of DOCA-salt treated rat. (C) Bar chart showing % of cells expressing inhibitory or excitatory GABAergic responses in control and DOCA-salt treated rats. (D) Illustration of how E<sub>GABA</sub> is estimated. Insets: Whole-cell currents elicited by focally applied muscimol (∆; 10 μM, 10 ms) at various holding potentials (V<sub>H</sub>) in the cells of control and DOCA-salt hypertension model rats. These current traces were obtained after blocking fast sodium currents and glutamatergic transmission with the cocktail of TTX (0.5 μM), AP5 (100 μM) and DNQX (20 μM). Peak amplitudes of the muscimol-elicited currents are plotted against V<sub>H</sub>. Linear regression was used to fit the data points. The intersections (↓, ↑) of the regression lines with the abscissa were taken as the reversal potentials of the muscimol-elicited responses (i.e., E<sub>GABA</sub>'s). (E) Bar chart shows E<sub>GABA</sub> of the cells recorded from control and DOCA-salt model rats. n: number of cells. In this and the rest of the figures, values are shown as means ± SEM. * indicates P < 0.05 and ** indicates P < 0.001, as compared to control.

Figure 3: DOCA-salt treatment leads to gradual increases in blood pressure, levels of AVP and GABA excitation. (A) Bar charts plot the weekly measurements of systolic blood pressure (SBP) and E<sub>GABA</sub> in the AVP neurons over the 4 weeks of treatment with DOCA-salt. (B) Bar charts plot the weekly measurements of plasma AVP during the 4 weeks of treatment. (C) Bar charts show the % of neurons exhibiting GABA-mediated inhibition or excitation during the 4 weeks of treatment. All four parameters (SBP, E<sub>GABA</sub>, AVP levels, and % cells showing GABA excitation) appear to gradually increase in response to the DOCA-salt treatment. n: number of rats (upper panel of A, B) or cells (lower panel of A, C). * indicates P < 0.05 and ** indicates P < 0.001, as compared to control.

Figure 4: GABA excitation in AVP neurons is also observed when hypertension is induced by treatment with ethanol. (A) Bar charts show the measurements of SBP and E<sub>GABA</sub> after treatment with an NO inhibitor (L-NAME) or ethanol. Both treatments increased blood pressure but only the ethanol-treated rats also showed a depolarized shift in E<sub>GABA</sub>. (B) Bar charts show the impact of the treatments on plasma AVP. (C) Bar charts show the impact of the treatments on the % of neurons exhibiting GABA-mediated inhibition or excitation. n: number of rats (upper panel of A, B) or cells (lower panel of A, C). * indicates P < 0.05 and ** indicates P < 0.001, as compared to control.

Figure 5: Microinjected muscimol into the SON of the DOCA-salt hypertension model rat increases blood pressure through a V1a receptor-dependent mechanism. (A, upper panel): Examples of
recording of blood pressure and heart rate obtained from the control and DOCA-salt model rats. Muscimol (M; 1 nmol in 100 nl) was injected into the SON and the impact on blood pressure and heart rate was measured. (A, lower panel): photomicrograph showing the muscimol injection sites (arrows). Note: muscimol injection experiment was often repeated in the same rat by injecting the agent into the SON on the opposite side. This was only to confirm the prior observation. OC: optic chiasm. (B) Graph summarizing the effects of two different doses (1 nmol and 10 nmol in 100 nl) of muscimol on SBP in the control and DOCA-salt treated rats. (C) Example of recording of blood pressure and heart rate obtained from a DOCA-salt model rat. V1a indicates the time of an i.v. injection of V1a AVP receptor antagonist (100 μg/kg) while M indicates the time of muscimol injection (10 nmol in 100 nl). (D) Graph summarizing the effects of intravenously injected V1a AVP receptor antagonist on the SBP increases produced by muscimol injection into the SON in the DOCA-salt model rats, as illustrated in (C). n: number of rats. * indicates P<0.05, as compared to the value at 0 min.

Figure 6: DOCA-salt treatment converts baroreflexive inhibition into excitation in SON neurons, making baroreceptor activation increase AVP release. (A) “Antidromic” stimulation was used to confirm that the SON neurons from which the recording were made projected to the posterior pituitary. Note the constant latency of antidromically evoked spikes (arrow heads) from the stimulation of the posterior pituitary (↑) (top and middle traces) and the lack of evoked spike when a spontaneous spike (*) arose a few milliseconds before the stimulation (bottom traces). Examples shown were recorded from control (left panel) and DOCA-salt hypertension model rats (right panel). The vertical dashed lines were placed on the peaks of antidromically evoked spikes for comparison of the latency. The stimulus artifacts were truncated. (B) Illustrates the responses of SON neurons in (A) to increases in arterial pressure produced by phenylephrine (P) injections. Note the dose-dependent inhibition (left panel, upper trace) and excitation (right panel, upper trace). (C) Summary of the effects of baroreceptor activation on the firing rates of SON neurons identified as projecting to the posterior pituitary in control (n=3) and DOCA-salt model rats (n=4). ☆: excitation, ●: inhibition. In control rats baroreceptors were activated by an i.v. injection of phenylephrine at a dose of 6 μg/kg body weight, while in the model rats they were activated with 3 μg/kg dose because these animals exhibited about twice as much pressor response to this drug; the mean increase in SBP was 47 ± 6 mmHg at 6 μg/kg dose in control and 51 ± 5 mmHg at 3 μg/kg dose in DOCA-salt model rats. (D) Illustrates the effects of baroreceptor activation on the plasma AVP concentrations of control and DOCA-salt model rats. The traces in the upper panel show the increases in arterial blood pressure induced by i.v. injections of phenylephrine in a control rat and a DOCA-salt model animal. The mean (±SEM) increase in SBP produced by phenylephrine (90 μg/kg body weight) in control rats (n=4) were 131 ± 14 mmHg, while the blood increase in DOCA-salt model rats (n=6) produced by phenylephrine (45 μg/kg) was 149 ± 9 mmHg. 0.5-ml blood samples for the measurements of AVP concentrations were taken at 20 min before and 2 min after the phenylephrine injection (▲) and replaced with the same amount of saline. * indicates P< 0.05.

Figure 7: DOCA-salt treatment increases levels of NKCC1 while down-regulating KCC2 expression in MNCs. (A, B) NKCC1, KCC2 and actin bands recognized by Western blot for the SON and PVN tissue samples obtained from the control and DOCA-salt hypertension model rats. Bar graphs showing the relative levels of NKCC1 and KCC2 in the SON and PVN of the control and DOCA-salt model rats. For each experiment, the values were normalized to the average value of the samples collected from the control rats. NKCC1 and KCC2 levels were normalized to actin to control for loading. Experiments were repeated three times for the SON, and five times for the PVN, tissues. (C, D) Graphs illustrate the impact of the NKCC1 inhibitor bumetanide (10 μM) and the KCC2 blocker VU2040551 (75 μM) on the E\textsubscript{GABA} in AVP neurons recorded in hypothalamic slices from the control and DOCA-salt hypertension model rats. The symbols connected by lines denote data from the same cells. In (A) and (B), * indicates P<0.05. In (C) and (D), * indicates P<0.05 and ** indicates P<0.001, as compared to the value before the drug treatment.
Figure 8: Administration of bumetanide delays the development of DOCA-salt hypertension and lowers plasma AVP. (A) Graphs showing the development of hypertension in DOCA-salt treated rats over the course of 21 days and the lack of hypertension in the control animals. The rats were infused ICV with bumetanide or vehicle for 1 week indicated with the shaded box. (B) Bar chart showing the effect of bumetanide infusion on the plasma concentration of AVP. The blood samples for AVP measurement were taken on the last day of bumetanide/vehicle infusion. n: number of rats. * indicates $P < 0.05$ and ** indicates $P < 0.001$. NS indicates no statistical difference.
Novelty and Significance

What Is Known?
- Excess dietary salt is a major risk factor for hypertension.
- Excess salt increases circulating levels of arginine-vasopressin (AVP).
- High AVP can increase blood pressure through a variety of mechanisms.

What New Information Does This Article Contribute?
- High-salt diet combined with mineralocorticoid treatment, which produces hypertension, converts the inhibitory effects of GABAergic input onto AVP secretory cells in the rat hypothalamus into excitatory ones.
- Increased AVP secretion may be due to an increase in intracellular chloride concentration driven largely by the chloride transporter NKCC1.
- Pharmacological inhibition of this transporter reduced AVP secretion and lowered blood pressure in this experimental model of hypertension.

It is well established that consumption of excess dietary salt is associated with hypertension. Yet, the mechanisms by which excess salt intake aggravates or produces hypertension have remained elusive. One possible mechanism is that excess sodium increases the circulating levels of AVP, as previous work has linked increased secretion of this neurohormone with raised blood pressure. This previous work raises the question of how a high-salt diet increases the secretion of AVP from hypothalamic neurons. In the present study, we found that under hypertensive conditions induced by a combination of high-salt diet and mineralocorticoid treatment, the normal GABA inhibition of AVP neurons is transformed into excitation, which results in an increase in AVP secretion. This increase in AVP secretion may be due to a shift in chloride homeostasis, which alters how these AVP cells respond to GABA. We speculate that increasing the GABA inhibition of AVP neurons by normalizing the chloride equilibrium potential may be useful in treating salt-dependent hypertension.
Figure 1

A

B

Putative AVP neuron

Putative OXY neuron

Rectification index = A1/A2

C

Biocytin  AVP-neurophysin  Merged

Putative AVP neuron

Putative OXY neuron
Figure 2
Figure 3

A

B

C

Figure 3

A

B

C
Figure 4

A

SBP (mmHg)

E_{\text{GABA}} (mV)

Control
NO inhibitor model
Ethanol model

(n=11) (n=9) (n=10)

B

Plasma AVP (pg/ml)

(n=9) (n=5) (n=4)

C

% Cell

IPSPs
EPSPs

Control NO inhibitor Ethanol

(n=44) (n=19) (n=14)

** *
Figure 5

A

Control

DOCA-salt

B

DOCA-salt

Control

C

D

DOCA-salt

Control

V1a-A

1 hour

M

M

1 hour

M

M

Δ SBP (mmHg)

Time (min)

Δ SBP (mmHg)

Time (min)
Figure 7

A

Control DOCA-salt

NKCC1
KCC2
β-actin

Relative level of NKCC1

Relative level of KCC2

SON

PVN

B

Control DOCA-salt


C

Bumetanide (-)
Bumetanide (+)
Wash

EGABA (mV)

VU0240551 (-)
VU0240551 (+)
Wash

EGABA (mV)

D

Bumetanide (-)
Bumetanide (+)
Wash

EGABA (mV)

VU0240551 (-)
VU0240551 (+)
Wash

EGABA (mV)
Figure 8

A

SBP (mmHg)

220
200
180
160
140
120
0 5 10 15 20 25
Day

DOCA (Vehicle), n=8
DOCA (Bumetanide), n=10
Control (Vehicle), n=5
Control (Bumetanide), n=5

ICV

** *

B

[Plasma AVP] (pg/ml)

200
150
100
50
0

DOCA-salt
Control

Vehicle
Bumetanide

** *

NS

(n=6) (n=7)
(n=5) (n=6)
GABAergic Excitation of Vasopressin Neurons: A Possible Mechanism Underlying Sodium-Dependent Hypertension
Yang In Kim, Young-Beom Kim, Yoon Kim, Woong Kim, Feng-Yan Shen, Seung Lee, Hyun Joo Chung, Jeong Kim, Hee C Han and Christopher S Colwell

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Supplemental Methods

Immunohistochemical (IHC) identification of recorded neurons. Some SON and PVN neurons recorded in slices from the control and DOCA-salt hypertension model rats were injected with biocytin (Sigma) for IHC identification. Biocytin (1.5 mg/ml) was included in the internal solution. Following perforated patch recordings, the membrane patch was ruptured to achieve the whole-cell configuration. Biocytin contained in the recording pipette was allowed to get into the cell for >10 min. On termination of the recording, the slice was fixed in 4% paraformaldehyde in 0.1 M PBS at a pH of 7.4 for 24 h, transferred to 30% sucrose-PBS, then cut into 25–35 μm sections. Fixed slices were incubated for 24 h at 4°C in a rabbit polyclonal antibody against AVP-neurophysin (1:200; Abcam). After the slices had been washed with PBS, a 1:200 dilution of DyLight 488-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch) was added with a 1:500 dilution of avidin-AMCA (7-amino-4-methylcoumarin-3-acetic acid; Vector Labs) to label biocytin for 1 h at room temperature. All antibodies and labeling reagents were dissolved in 0.1 M PBS containing 0.3% Triton X-100 and 2% normal goat serum. The sections were examined under a confocal fluorescence microscope for the presence of AVP-neurophysin immunoreactivity and biocytin labeling.

Measurement of plasma vasopressin levels. A total of 0.5-1.0 ml of blood was obtained through the femoral vein catheter or by decapitating the rat and placed into ice-cold tubes containing EDTA (1 mg/ml blood). Samples were placed on ice immediately. Plasma samples were separated by centrifugation at 1,600 × g for 15 min at 4 °C and stored at -80 °C until analyzed. Plasma vasopressin levels were determined in diluted samples (1:2) by using an Arg8-vasopressin enzyme immunoassay (ELISA) kit (Enzo Life Sciences, Plymouth Meeting, PA, USA). The intra- and inter-assay coefficients of variation for the assay for AVP were 8.2 % and 9.0%, respectively. The sensitivity was 0.25 pg/ml on average.

Measurement of plasma catecholamine levels. Blood samples were obtained through the femoral vein catheter and placed into ice-cold tubes containing EDTA (1 mg/ml blood). Samples were placed on ice immediately. Plasma samples were separated by centrifugation at 1,600 × g for 15 min at 4 °C and stored at -80 °C until analyzed. Plasma epinephrine and norepinephrine levels were quantified with a catecholamine ELISA kit (KA1877, Abnova, Taipei City, Taiwan). For competitive ELISA, epinephrine and norepinephrine were extracted with a cis-diol-specific affinity gel, acylated and derivatized enzymatically. The intra-assay coefficients of variation for the assay for epinephrine and norepinephrine were 10.2% and 10.6%, respectively. The sensitivity of epinephrine and norepinephrine were 10 pg/ml and 50 pg/ml, respectively.

Western blot. The SON, PVN and hippocampus were excised from brain slices prepared as above. Since the SON and PVN are very small structures, SON/PVN tissues from two rats were pooled to form a single sample. Each sample was lysed in triple-detergent lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenyl-methylsulfonyl fluoride). After the determination of protein concentration of total tissue lysate with Bradford assay, the tissue lysate was denatured with SDS-reducing buffer and heat (100°C, 5 min). The denatured lysate and prestained molecular size markers were resolved by 8% SDS-PAGE and transferred to nitrocellulose membrane. After being incubated in 5% skimmed milk/Tris-buffered saline for 1 h at room temperature, the membrane was reacted sequentially with primary antibodies [mouse monoclonal anti-NKCC1 (1:2000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), rabbit polyclonal anti-KCC2 (1:2000; Millipore), or rabbit polyclonal anti-β-actin (1:3000; Santa Cruz Biotechnology, CA, USA)] and
HRP-conjugated secondary antibodies (anti-mouse IgG or anti-rabbit IgG; 1:5000; Santa Cruz Biotechnology, CA, USA). The integrated optical density of bands was quantified using the imageJ software. Each sample was normalized to the content of β-actin, a constitutively expressed protein.

**In vivo extracellular single-unit recording and monitoring of blood pressure.** We obtained extracellular single-unit recordings from the SON of urethane-anesthetized rats (1.25 g/kg body weight, ip) placed in a stereotaxic instrument and kept warm (36–38°C) with a heating pad. Glass micropipettes (impedance: 10–15 MΩ) filled with 3.0 M-potassium acetate were used as the recording electrodes. The stereotaxic coordinate for the SON was as follows: AP: 1.4 ± 0.2 mm caudal to the bregma, ML: 1.8 ± 0.2 mm lateral to the midline, DV: 9.3 ± 0.2 mm below the skull surface. SON neurons were identified by antidromic activation following stimulation (pulse duration <0.5 ms, intensity <2 mA) of their axon terminals in the posterior pituitary. The signals from neurons amplified by DAM 80 AC differential amplifier (high-pass filter set at 300 Hz, low-pass filter set at 3 kHz, World Precision Instruments, New Haven, CT, USA) were digitized and sampled at 50 μs intervals (Digidata1320, pClamp 8.0). To activate baroreceptors during extracellular single-unit recording, we injected phenylephrine (3–90 μg/kg body weight) through a catheter inserted into the left carotid vein. The blood pressure was monitored through a catheter placed in the left carotid artery. A pressure transducer (BLPR2, World Precision Instruments, USA) and a pressure monitor (model BP-1, World Precision Instruments, USA) coupled to a computerized acquisition system (Digidata 1320, pClamp 8.0) were used for blood pressure recording. The moment-to-moment heart rate was calculated from the inter-pulse interval, using “Mini Analysis” program (Synaptosoft, Fort Lee, NJ).

**Drugs.** We purchased all the drugs and chemicals used in the current study from Sigma-Aldrich, Korea (Seoul, Korea), except muscimol (GABA_A receptor agonist; Ascent Scientific, Somerset, UK), CGP 54626 hydrochloride (GABA_A receptor antagonist, Tocris), VU0240551 (KCC2 inhibitor; Tocris), and d(CH2)5[Tyr(Me)2, Ala-NH2]9AVP (V1a AVP receptor antagonist; gift from Dr. M. Manning at the University of Toledo College of Medicine, Toledo, OH, USA). We prepared the solutions of muscimol, baclofen-HCl (GABA_B receptor agonist), bicuculline methiodide (GABA_A receptor antagonist), DL-2-amino-5-phosphonopentanoic acid (AP5, NMDA receptor antagonist) by dissolving these drugs in ACSF, the standard slice perfusion medium, and 6,7-dinitroquinoxaline-2,3-dione (DNQX, non-NMDA receptor antagonist), CGP 54626 hydrochloride and VU0240551 solutions by diluting their dimethyl sulfoxide-based stock solutions with ACSF (final concentrations of dimethyl sulfoxide: 0.001–0.1%). The working solutions of the NKCC inhibitor bumetanide (10 μM for electrophysiology and 500 μM for ICV infusion) were prepared by diluting the stock solution (25 mmol/l in 0.1 mol/l NaOH) with ACSF. The solutions of phenylephrine and V1a AVP receptor antagonist for intravenous (i.v.) injections were prepared by dissolving the agents in 0.9% NaCl solution.
Online Figure Legends

Online Figure I.  Muscimol injection into the SON does not increase sympathetic nerve activity.  
(A) Lack of effect on the heart rate of muscimol (1 or 10 nmol in 100 nl ACSF ) injected into the SON, 
with and without prior i.v. injection of the V1a AVP receptor antagonist (100 µg/kg body weight). The 
heart rate was measured immediately before and 16 min after the muscimol injection. In addition, it 
was measured at 4 and 6 min after 10 and 1 nmol muscimol injections, respectively. At these time 
points, the muscimol-induced increases in arterial pressure reached their peaks in DOCA-salt 
hypertension model rats (please see Figure 5B).  
(B) Lack of the effect of muscimol (10 nmol in 100 nl ACSF ) injected into the rat’s SON on the plasma levels of catecholamines.  
0.8-ml blood samples were taken at 40 min before and 4 min after the muscimol injection and replaced with the same 
amount of saline. The double arrowheads in the blood pressure record in the upper panel denote the 
increase in arterial blood pressure following the muscimol injection. Arrows: times of blood sampling.  
The numbers in parentheses in (A) and (B) denote the number of rats tested. NS indicates no 
statistical difference.

Online Figure II.  Effects on the blood pressure of the GABA_B agonist baclofen injected into 
the SON. Graph summarizing the effects on the SBP of baclofen (2.25 nmol in 100 nl ACSF) injected 
to the SON in the control and DOCA-salt model rats. n: number of rats tested. *: P< 0.05, **: P< 
0.001 compared to the value at 0 min.

Online Figure III.  Effect of V1a receptor antagonist on arterial pressure. Three different doses 
of the drug were i.v. injected at the time points indicated with arrowheads. The traces for arterial 
pressure in (A) were from 6 different rats.  
(B) Graph summarizing the effects of V1a receptor 
antagonist on the SBP in the control and DOCA-salt hypertension model rats. The numbers in 
parentheses denote the number of rats tested. *: P< 0.05, **: P< 0.001 compared to the value at 0 min.

Online Figure IV.  Lack of effects of DOCA-salt treatment on the levels of NKCC1 and KCC2 
in the hippocampus, SON and PVN of the rat. DOCA-salt was treated for 4 weeks (A) or 2 weeks 
(B,C) Experiments were repeated three times for the SON and hippocampal tissues and five times for 
the PVN tissues. NS indicates no statistical difference.

Online Figure V.  Effect of DOCA-salt treatment on the slope of baroreceptor reflex. Left panel, 
scatter plots showing the relationship between peak increases in SBP produced by i.v. injections of 
phenylephrine (3-90 µg/kg body weight) and the baroreceptor reflex-mediated increases in pulse 
interval in control (n=3) and DOCA-salt model rats (n=4). Linear regression lines were fitted through 
these points (Control: solid black line; Model: solid grey line). Right panel, bar charts compare the 
baroreceptor reflex slope (i.e., Δ pulse interval x Δ SBP’ ) between control (n=3) and DOCA-model 
rats (n=4). The graph in this panel was based on the slopes calculated with the separate sets of data 
from individual rats. *: P< 0.05.
Online Figure I

A

Control  DOCA-salt

Heart rate (pulses/min)

Before  Muscimol  After

(n=5)  (n=6)

Heart rate (pulses/min)

Before  Muscimol  After

(n=4)  (n=4)

Heart rate (pulses/min)

Before  Muscimol  After

(n=3)  (n=4)

With V1a antagonist

Heart rate (pulses/min)

Before  Muscimol  After

(n=3)  (n=4)

With V1a antagonist

Heart rate (pulses/min)

Before  Muscimol  After

(n=3)  (n=4)

B

Control  DOCA-salt

Muscimol

10 min

mmHg

Before

After

Norepinephrine (ng/ml)

Before  After

Control (n=5)  DOCA-salt (n=6)

Epinephrine (ng/ml)

Before  After

Control (n=5)  DOCA-salt (n=6)
Online Figure IV

A

Relative level of NKCC1

Control  DOCA-salt 4 w

Relative level of KCC2

Control  DOCA-salt 4 w

Hippocampus

B

Relative level of NKCC1

Control  DOCA-salt 2 w

Relative level of KCC2

Control  DOCA-salt 2 w

SON

C

Relative level of NKCC1

Control  DOCA-salt 2 w

Relative level of KCC2

Control  DOCA-salt 2 w

PVN

NS