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Blockade of ENaCs by Amiloride Induces c-Fos Activation of the Area Postrema

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Abstract

Epithelial sodium channels (ENaCs) are strongly expressed in the circumventricular organs (CVOs), and these structures may play an important role in sensing plasma sodium levels. Here, the potent ENaC blocker amiloride was injected intraperitoneally in rats and 2 hours later, the c-Fos activation pattern in the CVOs was studied. Amiloride elicited dose-related activation in the area postrema (AP) but only ~10% of the rats showed c-Fos activity in the organum vasculosum of the lamina terminalis (OVLT) and subfornical organ (SFO). Tyrosine hydroxylase-immunoreactive (catecholamine) AP neurons were activated, but tryptophan hydroxylase-immunoreactive (serotonin) neurons were unaffected. The AP projects to FoxP2-expressing neurons in the dorsolateral pons which include the pre-locus coeruleus nucleus and external lateral part of the parabrachial nucleus; both cell groups were c-Fos activated following systemic injections of amiloride. In contrast, another AP projection target - the aldosterone-sensitive neurons of the nucleus tractus solitarius which express the enzyme 11- β -hydroxysteroid dehydrogenase type 2 (HSD2) were not activated. As shown here, plasma concentrations of amiloride used in these experiments were near or below the IC₅₀ level for ENaCs. Amiloride did not induce changes in blood pressure, heart rate, or regional vascular resistance, so sensory feedback from the cardiovascular system was probably not a causal factor for the c-Fos activity

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DISCLOSURES

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seen in the CVOs. In summary, amiloride may have a dual effect on sodium homeostasis causing a loss of sodium via the kidney and inhibiting sodium appetite by activating the central satiety pathway arising from the AP.

1. INTRODUCTION

Epithelial sodium channels (ENaCs) facilitate movement of sodium across luminal epithelia such as found in the kidney and airways (Althaus, 2013; Alvarez de la Rosa et al., 2013; Kusche-Vihrog et al., 2013; Soundararajan et al., 2010; Warnock et al., 2014). Less is known about the ENaCs that are present in the brain (Giraldez et al., 2013). These nonvoltage-gated ion channels have been localized in five different types of brain cells: astrocytes, choroid plexus cells, ependymal cells, endothelial cells, and neurons (Amin et al., 2005; Miller and Loewy, 2013). ENaCs present in the choroid plexus regulate the $[Na^+]$ of the cerebrospinal fluid (Amin et al., 2009; Nakano et al., 2013; Wang et al., 2010). ENaCs expressed in brain endothelial cells function much like those found in peripheral endothelial cells (Kim et al., 2012; Kusche-Vihrog et al., 2013), serving to stiffen mechanically these cells and trigger the release of the nitric oxide which acts on its underlying smooth muscle to induce local vasodilation (Kusche-Vihrog et al., 2013). However, the role of ENaCs that are expressed in astrocytes (Miller and Loewy, 2013) and neurons (Amin et al., 2005; Miller et al., 2013; Teruyama et al., 2012) remains elusive.

ENaCs are densely concentrated in the neurons of the sensory circumventricular organs (CVOs) (Amin et al., 2005; Miller et al., 2013) which include the organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO) and area postrema (AP). Like all CVOs, these structures lack a blood-brain barrier and thus, their neurons and glial cells are continuously exposed to same chemical environment as found in the plasma. On the basis of this anatomical property, we hypothesized that ENaC-expressing CVO neurons may function as plasma sodium sensors since they become c-Fos activated following peripheral manipulations of plasma sodium levels (Miller and Loewy, 2014; Miller et al., 2013).

Earlier we reported that hypertonic saline intraperitoneal injections induced c-Fos activation of selective regions of the OVLT, SFO, and AP, while sodium deprivation elicited activation of the complimentary OVLT and SFO regions, but had no effect in the AP (Miller et al., 2013). Later studies established that serotonergic AP neurons were c-Fos activated by sodium repletion or hypertonic saline injections (Miller and Loewy, 2014). Some, but not all, of these neurons expressed ENaCs (Miller and Loewy, 2014). In addition, we identified a unique group of ENaC- γ expressing astrocytes that define the lateral border of the CVOs (Miller and Loewy, 2013), and presumably have the same vascular environment as the main part of the CVOs, functioning as an important glial-neuronal interface perhaps for monitoring the ionic content of the cerebrospinal fluid.

Here we tested whether ENaCs *per se* potentially play a functional role in the CVOs by injecting the highly selective ENaC-blocking drug – amiloride into the peritoneal cavity, and after two hours, examining whether this drug would have any effect on the CVOs. This experiment was begun as a pilot project, and since amiloride blocks ENaCs, we initially had assumed it would have no or a minimal effect on the CVOs. If this was established, our plan

was to use this drug in subsequent experiments to determine whether it would block c-Fos activation of the CVOs which occurs following hypertonic saline intraperitoneal injections (Miller et al., 2013). We reasoned that if amiloride blocked the c-Fos activation that occurs after hypertonic saline, these data would support the hypothesis that ENaCs localized in the CVOs may play a role in the brain sodium sensing system.

Instead, we were surprised to find that amiloride itself activated the AP in a dose-related manner. Moreover, it also caused activation of a group of synaptically-related neurons that lie in the dorsolateral pons; these neurons express the transcription factor Forkhead protein (FoxP2) and receive a direct input from the AP (Stein and Loewy, 2010). The activation pattern of both groups of neurons (AP and FoxP2 neurons) was correlated with changes in circulating and central levels of amiloride. To determine the possible mechanism of actions, we measured the effect of amiloride on blood pressure and vascular resistance in conscious rats, and found that this drug caused no changes in these cardiovascular parameters. While other possibilities could explain our findings, including the release of hormones from peripheral organs such as the kidney, we propose that the CNS effects observed here were due to the direct action of amiloride on AP neurons and offer a cellular mechanism to explain this phenomenon.

2. RESULTS

Our first goal was to determine whether systemic injections of amiloride would induce c-Fos activation of the CVOs, and as the study progressed, we found that the AP was the principal site where c-Fos activity was most consistently elicited. To refine these studies, we next measured the plasma and cerebrospinal fluid (CSF) levels of amiloride after intraperitoneal injections of graded doses of this drug. Once we established a “dose-response” relationship for amiloride and c-Fos activity in the AP, we analyzed the second-order AP projection targets and determined whether any of them were activated. Briefly, the FoxP2 neurons of the dorsolateral pons were activated by amiloride while the aldosterone-sensitive (HSD2) neurons of the NTS were not.

Figure 1 shows the total density of cells versus the density of ENaC positive cells in the AP. In a group of 3 rats, we found that ENaC positive cells represent 93% of the total AP cell population. There was no difference between the number of ENaC positive cells and the total number of AP cells.

Figure 2 illustrates the amiloride concentrations found in the plasma and CSF following intraperitoneal injections of this drug. Amiloride was highly concentrated in the plasma 15 to 30 minutes following the injections. Only two measurements of the amiloride levels in the CSF were available because the 0.1 mg/kg dose resulted in a concentration that was below the detectable limits of our method. The two higher doses of amiloride resulted in very low levels of amiloride in the CSF. This result is consistent with what has been described in earlier work pertaining to drug penetration into the CSF after peripheral administration (Pardridge, 2011). Table 1 summarizes these data.

Figure 3 demonstrates that a 2 mg/kg dose of amiloride had no effect on blood pressure, heart rate, mesenteric vascular resistance, or hindquarters vascular resistance as compared to the baseline measurements (n=7). Conscious rats had a resting arterial pressure of 113 ± 4 mmHg and a heart rate of 392 ± 8 beats per minute. Over a 2 hour period, amiloride elicited negligible responses in arterial pressure (-1 ± 1 mmHg) and heart rate (-10 ± 6 bpm/min) as compared to baseline levels. During each of the 15 minute intervals, any changes seen in blood pressure and heart rate were not statistically different from baseline values. In a group of four rats, vascular resistance was measured; hindquarters vascular resistance ($1 \pm 6\%$) and mesenteric vascular resistance ($10 \pm 4\%$) displayed very minor fluctuations. Dunnett's multiple comparison test failed to demonstrate that any of these four parameters were different from the baseline values. A one-way ANOVA also failed to show a difference for any of these data as compared to baseline values.

Figure 4 presents three photoimages of the three sensory CVOs showing the c-Fos activation pattern seen 2 hours following an intraperitoneal injection of amiloride (2 mg/kg). Figure 4A shows a slight amount of c-Fos labeling in the lateral zone of the OVLT, with an occasional c-Fos positive cell present in the dorsal cap of the OVLT. OVLT labeling was seen in only about 10% of the cases that received amiloride (2 mg/kg); lower doses produced no c-Fos labeling in this region. Figure 4B shows almost no c-Fos labeling in the SFO which was a typical finding in the majority of cases, although in ~10% of the amiloride-treated rats c-Fos labeling in the ventromedial core of the SFO was detected. This lack of labeling was likely not the result of a technical issue with the immunohistochemical staining procedure, since in the same tissue section, dense c-Fos labeling was consistently present in the paraventricular thalamic nucleus (Figure 4B). The most consistent finding was robust c-Fos labeling in the AP, as presented in Figure 4C, occurring in >90% of the cases. The medial NTS had very few c-Fos labeled neurons, and the zone lying immediately next to the AP had almost none.

Figure 5 illustrates a graded c-Fos response in the AP following various doses of amiloride. Figure 6A presents a bar graph showing an amiloride dose-related response pattern for the c-Fos activation in the AP. Even at the lowest dose of amiloride (0.1 mg/kg), the number of c-Fos labeled neurons in the AP was statistically different from the control rats at a $p < 0.01$ level. As described below, there was also a graded c-Fos response in the pre-locus coeruleus nucleus (pre-LC), but not in the parabrachial nucleus (Fig. 6).

Figure 7 reveals that ~20% of the c-Fos activated neurons in the AP contain tyrosine hydroxylase immunoreactivity while none of the tryptophan hydroxylase immunoreactive neurons were c-Fos positive. In addition, none of the 11- β -hydroxysteroid dehydrogenase type 2 (HSD2) immunoreactive neurons of the nucleus tractus solitarius were c-Fos activated.

Figure 8 shows that the dorsolateral pontine projection targets of the AP were also c-Fos activated. These include the FoxP2 neurons in the pre-LC and parabrachial nucleus - external lateral subdivision inner part (PBel-inner). Figure 6B shows a graded c-Fos activation response in the pre-LC, while Figure 6C illustrates that amiloride significantly increased the activation of the FoxP2 neurons of the PBel-inner.

3. DISCUSSION

3.1 Major Findings

The key findings of this report demonstrate that intraperitoneal injections of amiloride elicit c-Fos activation of the AP and of one of its key efferent projection targets which include the FoxP2 neurons of the pre-LC and PB-el inner. The degree of activation correlated with the plasma levels of amiloride. Not all AP projection targets were activated. For example, no c-Fos activity was seen in the HSD2 neurons of the NTS. The amiloride effect may have been relatively specific in terms of the neuronal phenotypes affected since the tyrosine hydroxylase (catecholamine) immunoreactive AP neurons were activated but the tryptophan hydroxylase (serotonin) neurons were not. Other AP neuronal phenotypes, such as cholecystokinin and GABAergic neurons (McKinley et al., 2003a), were not studied so we have only a limited view of the other types of AP neurons that might be responsive to amiloride.

Surprisingly, the OVLT and SFO were not c-Fos activated by amiloride except in ~10% of the cases. The reason for this differential response is difficult to explain unless higher plasma levels of amiloride are needed to induce c-Fos activation in the OVLT and SFO than those required in the AP. Consistent with this idea, we found in preliminary experiments in mice (2mg/kg) that c-Fos activation occurred in all three CVOs (unpublished data, Miller & Loewy). Thus, rats may have the ability to eliminate amiloride from the blood stream more efficiently than mice which may explain these species differences.

Previous work from our laboratory demonstrated that intraperitoneal injections of hypertonic saline and sodium repletion induced c-Fos activation in selective zones in the OVLT, SFO, and AP (Miller et al., 2013). Later we reported that serotonergic AP neurons were activated following the same procedures (Miller and Loewy, 2014). Some of these serotonergic neurons were ENaC immunoreactive (Miller and Loewy, 2014). In both of these studies, we hypothesized that the c-Fos responses were likely to be due to actions of plasma sodium effecting ENaC-expressing CVO neurons. Therefore, we were surprised to find that the ENaC blocker – amiloride also induced an increase c-Fos activity in the AP. Currently, we do not have an explanation for the c-Fos findings.

3.2 Methodological considerations

Amiloride potently and selectively blocks ENaCs at low concentrations, thus inhibits sodium transport across the apical epithelia of many different tissues including the airways, colon, endothelial cells, kidney, sweat glands, and salivary glands (Kleyman and Cragoe, 1988; Kleyman et al., 1999). This drug has been used clinically because of its selective ability to block sodium transport in the kidney, resulting in increased sodium excretion, while decreasing potassium excretion. As a result of these properties, amiloride has been classified as a potassium-sparing diuretic (Kleyman and Cragoe, 1988; Kleyman et al., 1999). Additional analogues have been synthesized, with some being more selective or more potent for blockade of ENaCs than amiloride (Kleyman and Cragoe, 1988), but amiloride is still regarded as the prototypical drug in this class of diuretics (Kleyman and Cragoe, 1988; Kleyman et al., 1999).

To our knowledge, this is the first study to demonstrate that amiloride has a CNS effect when delivered at a submicromolar concentration in the blood stream. The blood-brain barrier excludes most drugs from entering into the brain parenchyma, and here we show the most susceptible neurons are those lying in the CVOs, especially the AP. The CVOs lie outside the blood-brain barrier and thus, are immediately exposed to drugs carried in the plasma.

The c-Fos method is a powerful neuroanatomical tool for the identification of the excitatory cell groups in the central nervous system (Dragunow and Faull, 1989; Hoffman et al., 1993; Kovacs, 1998; Sagar et al., 1988). While c-Fos activity is a general indicator of neuronal excitation, a few exceptions have been noted (Hoffman et al., 1993). A major advantage of using this marker is the induction of this immediate-early gene transcription factor is rapid (1–2 hours), making the c-Fos method an extremely useful technique for functional studies of the brain.

3.3 Mechanism of amiloride actions

The c-Fos activity seen in the AP was most likely the result of amiloride acting directly on ENaC-expressing AP neurons. Other factors, such as an amiloride effect on the vagus nerve or its cell bodies, seem improbable because the number of c-Fos activated neurons in the NTS was consistently minimal. This suggests that vagal afferents, which project heavily to the NTS, were not excited by systemic application of amiloride. When c-Fos activity was present in the NTS, it did not match up with the NTS sites known to receive a strong input from the vagus nerve (Contreras et al., 1982; Norgren and Smith, 1988). For example, virtually no c-Fos activity was found in the rostral NTS – the so-called taste area that receives a dense input from the tongue (Hayakawa et al., 2001). This implies that the blockade of ENaCs on the salt taste receptors of the tongue (Chandrashekar et al., 2010) were surely not a factor that can explain the c-Fos labeling in the AP. A similar argument can be advanced regarding the ENaCs associated with the trigeminal nerve or its branches that innervate the teeth (Fricke et al., 2000; Hitomi et al., 2009). The amount of c-Fos labeling that occurred in the spinal trigeminal nucleus in amiloride-treated rats never exceeded the number of activated cells observed in the control animals. Overall, there is no neuroanatomical evidence in our material that activation of the sensory components of the Vth, VIIth, IXth, and Xth cranial nerves occurred following peripheral administration of amiloride.

Short-term treatment with amiloride did not alter blood pressure, heart rate, or vascular resistance in the gastrointestinal tract or in skeletal musculature. Conceivably, other vascular beds such as those supplying the cerebral, cutaneous, pulmonary, renal, and coronary circulations may have been affected by amiloride. None of our experiments address this possibility.

ENaCs may be still involved in other types of cardiovascular functions since they are expressed in the terminal sensory endings of the baroreceptor nerves (Drummond et al., 1998). Since other types of ion channels besides ENaCs might be involved in the mechanical transduction of baroreceptor terminals (Ditting et al., 2003), the role of ENaCs in the baroreceptor reflex needs further clarification.

Amiloride is a diuretic and the effects identified here could have been a secondary effect resulting from acute fluid volume and/or sodium loss. Because we did not measure these two parameters, we are uncertain whether changes in these two physiological conditions affected the CVOs. As stated above, it seems unlikely that renal afferents, which project into the spinal cord via the dorsal root system, were a factor responsible for the c-Fos activity found here because ascending spinal projection systems do not innervate the AP. Thus, no reported multisynaptic pathway arising from the spinal cord could account for these findings. Likewise, volume receptors associated with heart were not likely to be involved since only minimal c-Fos labeling was present in the NTS, but we cannot rule out whether cardiac hormones, such as atrial natriuretic peptide, was involved (Hindmarch et al., 2011).

Finally, since the renin is released from the kidney following amiloride treatment (Kurtz et al., 1991), there is a possibility that this hormone played a role in inducing c-Fos activity in the CVOs. Additional evidence suggests that the renin-angiotensin system is operative in the CVOs (McKinley et al., 2003a; McKinley et al., 2003b) including the AP (Hindmarch et al., 2011), so this possibility needs further analysis.

3.4 Amiloride concentration in the plasma and CSF

The plasma protein binding of amiloride is 40% (Spahn et al., 1987). Thus, only 60% of the drug remains unbound in the plasma and free to bind to the neurons and/or glial cells in the CVOs. The plasma concentrations of amiloride after different doses and time points are presented in Figure 2. During the first 0.5 hour of the 2.0 mg/kg experiments, the concentration of amiloride was 0.43 μM which approximates the IC_{50} value of amiloride for ENaCs and is 0.35 μM (Kleyman and Cragoe, 1988). For the 0.5 mg/kg dose, the amiloride concentration was 0.20 μM which is less than the IC_{50} value, and for the 0.1 mg/kg dose, it was 0.03 μM . All three doses elicited c-Fos activation of the AP and the FoxP2 neurons in the pre-locus coeruleus and parabrachial nuclei.

At low concentrations ($< 1 \mu\text{M}$), amiloride preferentially blocks ENaCs. However, at higher concentrations, it also inhibits other ion transport systems, such as the Na^+/H^+ exchanger, $\text{Na}^+/\text{Ca}^{++}$ exchanger, Na^+ pump, Ca^{++} pump, or acid-sensing ion channels- (ASICs) (Abrams and Osborn, 2008; Kellenberger and Schild, 2002). As noted above, the IC_{50} of amiloride for ENaCs is 0.35 μM (Kleyman and Cragoe, 1988) while the IC_{50} for the Na^+/H^+ exchanger is 84 μM , and the IC_{50} for the $\text{Na}^+/\text{Ca}^{++}$ exchanger is 1100 μM , and the IC_{50} for homomeric ASICs is 10 μM (Kellenberger and Schild, 2002; Kleyman and Cragoe, 1988).

Amiloride does not exclusively bind to ENaCs, and its affinity for the ASIC2a channel has an IC_{50} of 0.15 μM (Kellenberger and Schild, 2002). This suggests that ASIC2a channels were potentially involved in mediating the c-Fos responses seen here. In this context, however, it should be noted that c-Fos responses were elicited with amiloride doses as low as 0.1 mg/kg which resulted in a plasma concentration of 0.03 μM . If ASIC2a channels were involved, future experiments might be able to resolve this with the use of a venom peptide derived from black mamba snake which selectively blocks ASIC1a and ASIC2a channels (Diochot et al., 2012).

3.5 Potential Mechanisms of Activation of ENaC-Expressing AP Neurons

Three possibilities could explain how amiloride might have induced c-Fos activation in the AP: 1) direct effect on the AP neurons; 2) indirect effect involving activation of sensory nerves that project directly or indirectly to the AP; and 3) indirect effect involving the release of hormones from target organs such as the kidney or gastrointestinal tract, and then, these hormones subsequently target the AP neurons.

The AP receives a relatively minuscule input from the vagus nerve, and its projection is restricted mainly to its lateral portions (Contreras et al., 1982; Norgren and Smith, 1988; Shapiro and Miselis, 1985). This restricted input does not correlate with the widespread c-Fos activation seen in the AP following amiloride treatment so it seems highly improbable that the c-Fos activation seen in the AP was the result of vagal activation. Other sensory systems conceivably could be responsible, such as the dorsal root afferent system which has been reported to express ENaCs (Drummond et al., 2000). This hypothesis has little support because there is no evidence that the spinal cord projects to the AP (Craig, 1995; Gamboa-Esteves et al., 2001; Potts et al., 2002).

Theoretically, amiloride could induce the release of hormones from various target tissues, such as the kidney, gastrointestinal tract, or even the pituitary gland, and these agents, in turn, could produce an effect in the AP. This possibility, to our knowledge, has not yet been examined.

After ruling out these alternative explanations, we suggest that the most likely explanation for the present observations is amiloride acts directly on the ENaC-expressing AP neurons. Direct proof of this hypothesis, however, requires based further proof, such as data obtained from single-cell electrophysiological studies.

How would amiloride blockade of the ENaCs in AP neurons lead to increased level of excitability? Blockade of ENaCs can lead to cell hyperpolarization (Chavez et al., 2013), which can result in either increasing or decreasing cell excitability. For a neuron resting at a relatively depolarized membrane potential where voltage-dependent sodium and/or calcium channels were maintained in a stable inactivated state, hyperpolarization would remove their inactivation, permitting them to participate in action potential activity. A remaining element needed to initiate endogenous action potential activity could be supplied by a hyperpolarization-activated inward current such as I_H (Maccaferri and McBain, 1996) to provide excitatory drive to stimulate phasic and tonic firing. Such effects are common, as has been demonstrated by endogenous pacemaker activity of area postrema neurons (Funahashi et al., 2003) so a direct action of amiloride resulting in an increase in the activity of AP neurons is plausible.

3.5 Conclusion

Amiloride induces c-Fos activation of both the AP neurons and their connectionally-related FoxP2 neurons of the dorsolateral pons (Stein and Loewy, 2010). The AP projection to the parabrachial/pre-locus coeruleus regions represents the 2nd order components of this pathway. While the AP has been implicated in a variety of different functions (Hindmarch et al., 2011), one of them may be to detect plasma sodium levels (Miller et al., 2013). Since the

AP is linked multisynaptically to the ventral tegmental area and nucleus accumbens (Miller and Loewy, 2014; Shekhtman et al., 2007; Shin et al., 2011) which are two brain regions implicated in reward/pleasure functions, the amiloride-activated AP pathway described here may function in sodium satiety.

4. EXPERIMENTAL PROCEDURES

4.1 Animals

All the experimental procedures that were used in this study were approved by the Institutional Animal Care and Use Committees at St. Louis University Medical School and Washington University School of Medicine and followed NIH guidelines. The experiments were performed on male and female Sprague-Dawley rats (Charles Rivers, Wilmington, MA) that had been maintained in a heat controlled room maintained at 23° C with a 12h/12h light-dark schedule. The rats were allowed free access to standard rat chow (PicoLab rodent diet #20, containing 0.33% sodium; Lab-Diet, Richmond, IN) and tap water. At the end of each experiment, the rats were anesthetized with an intraperitoneal (i.p.) injection of 8 % chloral hydrate (1 ml/100 g body weight) or sodium pentobarbital (50 mg/kg), and perfused through the heart with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.4). The brains were removed and placed in fixative for 1–3 days before histochemical processing.

4.2 Cell Counts of the ENaC-Expressing Neurons in the AP

For a description of the *in situ* hybridization method used for visualization of the ENaC-expressing neurons in rat brain - see (Miller et al., 2013). These sections were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) which is a histochemical stain that binds to A-T rich regions in DNA. For cell counts, DAPI was used to visualize each cell in the AP to get a total cell count, and subsequently, the *in situ* hybridization stained cells were counted to identify ENaC positive cell bodies. The sections were sampled at 200 µm intervals starting with the rostral section at the bregma –14.04 mm level. Cell counts and areal measurements of each AP section were made using the MetaMorph program (Molecular Devices, Sunnyvale, CA). Cell densities for each rat ($n = 3$) were averaged, and the final value was expressed as a density measurement, namely, the number of cells per unit area. These mean values were evaluated by the two-tailed Student's *t*-test in GraphPad Prism software (San Diego, CA). A *p*-value of <0.05 was chosen as signifying a level of statistical significance.

4.3 Drug Injections

Amiloride hydrochloride (Cat # A7410; Sigma, St. Louis, MO) was dissolved in distilled water, and injected i.p. into conscious rats; the doses used were 0.1, 0.5, and 2.0 mg/kg. All injections were delivered in a volume less than 1.0 ml. Control rats received i.p. injections with 1 ml of distilled water. The experiments were performed between 7:30 – 8:30 AM, and 2 hours later, the rats were anesthetized and killed as described above. Three experiments were performed on three groups of rats. The first group was used for determination of amiloride levels in the plasma and cerebrospinal fluid (CSF), a second group was prepared for the evaluation of the effect of amiloride on the cardiovascular system, and the third group was used for c-Fos immunohistochemistry.

4.4 Plasma and CSF Amiloride Measurements

A total of 116 adult rats were injected i.p. with amiloride (0.1, 0.5, and 2.0 mg/kg) dissolved in 1 ml distilled water or 1 ml of distilled water. After 15, 30, 45, or 120 min post-injection, the rats were anesthetized with 8% chloral hydrate, the chest was opened, and blood samples were obtained by cardiac puncture. The blood was centrifuged at 5000 RPM for 5 min, and the plasma (1.5 ml) separated and stored at -80°C . Thus, plasma samples based on 8 rats for each amiloride dose and post-injection period were available. Similarly, control samples from 5–7 rats were collected for each of the four post-injection time periods (15, 30, 45, and 120 min).

In order to obtain the CSF samples after amiloride treatment, rats were anesthetized and placed in a stereotaxic apparatus. A dorsal midline incision was made on the neck. The neck muscles were reflected, and the atlanto-occipital membrane exposed. Using a 100 μM micropipette, CSF samples were collected from the cisterna magna (25 μl) under direct microscope observation; the samples were frozen and stored at -80°C . CSF samples were taken at 30 and 120 min after intraperitoneal injections of amiloride of 0.1, 0.5, and 2.0 mg/kg doses. The amount of amiloride in the CSF for 0.1 mg/kg dose was below the detectable limit for the assay.

The CSF and plasma samples were assayed by liquid chromatography/electrospray tandem mass spectrometry (Song et al., 2007). A TSQ Quantum-Ultra triple-quadrupole mass spectrometer was used. The internal standard curves for amiloride in rat plasma versus amiloride in water demonstrated that the assay detected amiloride in the nanogram range.

4.5 Cardiovascular Measurements following Intraperitoneal Injections of Amiloride

The cardiovascular effects of amiloride were tested in awake Sprague-Dawley rats ($n=7$) that had been implanted with femoral arterial and venous catheters and with pulsed Doppler flow probes on the superior mesenteric artery and the abdominal aorta caudal to the renal and mesenteric arteries. After a recovery period of 3–7 days, rats were tested in their home cages.

Arterial pressure and heart rate were determined using a strain gauge and Grass Instruments Model 7 polygraph (Natus Neurology Inc, Warwick, RI) with a tachometer for heart rate determination. Mesenteric and hind limb blood flows were measured using miniaturized pulsed Doppler flow probes (Iowa Doppler Products, Iowa City, IA) implanted on the superior mesenteric artery and the abdominal aorta as previously described (Knuepfer and Branch, 1992; Knuepfer et al., 1989). Flows were obtained using a directional pulsed Doppler flow meter (Bioengineering Department, University of Iowa, Iowa City, IA). All data were collected at 1000 Hz/channel using a data acquisition device and WINDAQ software from DATAQ Instruments (Akron, OH). Control data were collected during a 30–60 min period, and then, the rats were briefly handled to administer amiloride (2 mg/kg, i.p.) and returned to their home cage. Hemodynamic parameters were recorded for 10–30 second intervals during the next two hours at 15 min intervals. Since no trends in any of the parameters were observed, the data were averaged to determine over the two hour period to determine whether amiloride produced any effects on cardiovascular parameters.

4.6 Cobalt-DAB Immunohistochemistry

Amiloride (0.1, 0.5, and 2 mg/kg) dissolved in distilled water or distilled water (control) i.p. injections were made in awake animals (n=25). The rats were returned to their home cage, and 2 h later, they were anesthetized with 8% chloral hydrate (i.p.) and perfused. The brains were removed and post-fixed for 1–3 days prior to histological processing. Serial sections through the OVLT, SFO, and AP regions were cut in the transverse plane on a freezing microtome at 50 μ M. Serial sections of all three CVOs per each rat were processed in the same staining batch. The sections reacted overnight at room temperature on a rotary shaker in a primary antibody solution of rabbit anti c-Fos (1:10K; cat. #PC38; Calbiochem, EMD Chemicals, San Diego, CA). Brain sections from groups of two to four rats were reacted during a single histological staining session. Brain samples from both amiloride- and vehicle-treated animals were reacted at the same time.

The primary antibody solution contained 0.3% Triton-X (Sigma, St. Louis, MO) and 5% donkey serum in 0.1M sodium phosphate (pH = 7.4). The sections were washed in potassium phosphate buffered saline (KPBS; 0.01M, pH = 7.4), transferred to a biotinylated donkey anti-rabbit (1:250; Jackson ImmunoResearch, West Grove, PA) solution made in 5% donkey serum (as above), washed in KPBS, treated for 1 hour in the avidin-biotin complex (ABC, Vectastain kit, Vector Labs, Burlingame, CA) solution, washed in KPBS, and colorized in a cobalt-diaminobenzidine (Co-DAB) solution (D-0426, Sigma). A single Co-DAB tablet was dissolved in 15 ml of distilled water containing one tablet of urea hydrogen peroxide. The sections were reacted for 15 min in this activated solution, washed three times in KPBS, mounted on gelatinized slides, and air dried. The slides were passed through ascending alcohols, transferred to xylene, and coverslipped with DPX mountant (VWR International, Poole, UK).

4.7 Double Indirect Immunohistochemistry for c-Fos and FoxP2

A double indirect immunohistochemical method described previously was used (Miller et al., 2011). All histochemical reactions were performed at room temperature. Pyrex spot plates (Thermo Fisher #7220-85, Waltham, MA) containing the tissues sections of the pons were agitated continuously on a rotary shaker. Sequential blocking steps containing avidin, formaldehyde, and biotin were applied following each primary antibody application (Miller et al., 2011). First, the sections were reacted in an avidin solution (100 μ g/ml) made in 0.1 M sodium phosphate buffer (pH = 7.4) for 15 min, rinsed in 0.01 M potassium phosphate buffer with 5% donkey serum (KPBS), placed in a biotin solution (20 μ g/ml) for 15 min, and washed in KPBS. Then, they were transferred to a rabbit c-Fos antibody solution (1:10K) for 16 hours and the sections were reacted with biotinylated donkey anti-rabbit antibody solution (1:250, Jackson ImmunoResearch, West Grove, PA) for 2.5 hours, washed in KPBS, and then placed in ABC complex (Vectastain kit PK-4000; Vector Laboratories, Burlingame, CA) for 1 hour. The sections were transferred to Cy3-streptavidin solution (1:250; Jackson) for 3 hours, washed in KPBS, and then transferred to sheep anti-FoxP2 antibody solution (1:5K; cat. #AF5647; R&D Systems, Minneapolis, MN) for 16 hours. The sequential blocking procedure was applied (Miller et al., 2011), and then they sections were transferred in biotinylated donkey anti-sheep solution (1:250, Jackson) for 2.5 hours, and reacted in the ABC complex. Next, the sections were placed in a Cy2-streptavidin solution

(1:250; Jackson) for 3 hours, washed in KPBS, mounted on gelatinized slides, air-dried, and coverslipped with a non-fade glycerol mountant containing 0.1% *n*-propyl gallate. The Cy2- or Cy3-streptavidin linked dyes did not bleed through the microscopic filters when tissues were reacted as a single immunohistochemical procedure.

4.8 Cell counts of the Area Postrema

Using brightfield optics, the AP sections (12–18 sections; average = 15 per rat) were photoimaged using the 10× lens of a standard microscope. These images were montaged in Adobe Photoshop (Adobe, San Jose, CA), and the contrast of the Co-DAB images was increased. Each c-Fos positive cell found in both sides of the AP was counted using MetaMorph software. The data were entered into a Microsoft Excel spreadsheet and tested by the one-way ANOVA using GraphPad Prism software (San Diego, CA, USA). A p-value of less than 0.05 was chosen as signifying statistical significance for this study.

4.9 Cell Counts of the Parabrachial and Pre-locus Coeruleus Regions

Photoimages of serial sections through the parabrachial and pre-locus coeruleus regions were made with a Nikon Eclipse E800 fluorescence microscope using a Q Imaging CCD camera and PM Capture Pro software (Surrey, BC Canada V3S 6K3). The images were montaged, and adjusted for brightness, contrast, and pseudocolor using Adobe Photoshop. Cell counts were made in selected dorsolateral pontine regions using MetaMorph software (Molecular Devices, Sunnyvale, CA) in a similar manner as in earlier report from our laboratory (Geerling and Loewy, 2007). The number of neurons that co-contained c-Fos and FoxP2 were determined. Montages were first constructed, and then various templates outlining a particular region of interest was placed over the site and the area within this region used for the cell counts. The template used for the analysis of the PBel-inner = 350 × 200 μm rectangle taken at the bregma –9.00 level (average of right and left sides); this rectangle was positioned over the ventrolateral part of the superior cerebellar peduncle. For the pre-locus coeruleus (pre-LC), a circle with a diameter of 500 μm was positioned over the montage including the mesencephalic tract of the trigeminal nerve and the lateral edge of the periventricular gray matter. Six to eight serial sections containing the PBel-inner were used; average = 7 sections per case, while 9–12 serial sections containing the pre-LC were used; average = 10 sections per case. Data from both sides of the brain were pooled.

All comparisons shown here were tested by a one-way ANOVA in GraphPad Prism software (San Diego, CA, USA). A p-value of less than 0.05 was chosen as signifying statistical significance for this study. All group data were presented as mean ± SEM.

4.10 Double Indirect Immunohistochemistry: c-Fos and HSD2, c-Fos and Tyrosine hydroxylase, and c-Fos and Tryptophan Hydroxylase

The presence of c-Fos activity in specific neuronal phenotypes in the caudal medulla was studied in 11 rats that had been injected intraperitoneally with amiloride and killed 2 hours later. A double indirect immunohistochemical method was used for all of paired staining combinations. In the first group of rats (n=4), c-Fos immunostaining was performed as described above on serial sections through the medulla and this was followed by immunostaining with a sheep antibody directed against 11-β-hydroxysteroid dehydrogenase

type 2 (HSD2; 1:40K; product # 1296; Chemicon International, Temecula, CA). A second group of rats (n=4) were processed for c-Fos and tyrosine hydroxylase (MAB318; Chemicon; 1:1000). A third group of rats (n=3) that had received 10 mg/kg of amiloride i.p. were processed for both c-Fos and tryptophan hydroxylase (synthetic enzyme producing 5-hydroxytryptamine, 5-HT = serotonin) immunoreactivity. A monoclonal antibody to Tryp-OH (MAB5278, Chemicon) was used at a dilution of 1:2K. This group of rats served as the pilot experiment of this project; the dose used in these three rats was higher than those used in the rest of the study.

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Literature Cited

- Abrams JM, Osborn JW. A role for benzamil-sensitive proteins of the central nervous system in the pathogenesis of salt-dependent hypertension. *Clin Exp Pharmacol Physiol*. 2008; 35:687–694. [PubMed: 18387084]
- Althaus M. ENaC inhibitors and airway re-hydration in cystic fibrosis: state of the art. *Curr Mol Pharmacol*. 2013; 6:3–12. [PubMed: 23547930]
- Alvarez de la Rosa D, Navarro-Gonzalez JF, Giraldez T. ENaC modulators and renal disease. *Curr Mol Pharmacol*. 2013; 6:35–43. [PubMed: 23547933]
- Amin MS, Reza E, Wang H, Leenen FH. Sodium transport in the choroid plexus and salt-sensitive hypertension. *Hypertension*. 2009; 54:860–867. [PubMed: 19635991]
- Amin MS, Wang HW, Reza E, Whitman SC, Tuana BS, Leenen FH. Distribution of epithelial sodium channels and mineralocorticoid receptors in cardiovascular regulatory centers in rat brain. *Am J Physiol Regul Integr Comp Physiol*. 2005; 289:R1787–R1797. [PubMed: 16141309]
- Chandrashekar J, Kuhn C, Oka Y, Yarmolinsky DA, Hummler E, Ryba NJ, Zuker CS. The cells and peripheral representation of sodium taste in mice. *Nature*. 2010; 464:297–301. [PubMed: 20107438]
- Chavez JC, de la Vega-Beltran JL, Escoffier J, Visconti PE, Trevino CL, Darszon A, Salkoff L, Santi CM. Ion permeabilities in mouse sperm reveal an external trigger for SLO3-dependent hyperpolarization. *PLoS One*. 2013; 8:e60578. [PubMed: 23577126]
- Contreras RJ, Beckstead RM, Norgren R. The central projections of the trigeminal, facial, glossopharyngeal and vagus nerves: an autoradiographic study in the rat. *J Auton Nerv Syst*. 1982; 6:303–322. [PubMed: 7169500]
- Craig AD. Distribution of brainstem projections from spinal lamina I neurons in the cat and the monkey. *J Comp Neurol*. 1995; 361:225–248. [PubMed: 8543660]
- Diochot S, Baron A, Salinas M, Douguet D, Scarzello S, Dabert-Gay AS, Debayle D, Friend V, Alloui A, Lazdunski M, Lingueglia E. Black mamba venom peptides target acid-sensing ion channels to abolish pain. *Nature*. 2012; 490:552–555. [PubMed: 23034652]
- Ditting T, Linz P, Hilgers KF, Jung O, Geiger H, Veelken R. Putative role of epithelial sodium channels (ENaC) in the afferent limb of cardio renal reflexes in rats. *Basic Res Cardiol*. 2003; 98:388–400. [PubMed: 14556084]
- Dragunow M, Faull R. The use of c-fos as a metabolic marker in neuronal pathway tracing. *J Neurosci Methods*. 1989; 29:261–265. [PubMed: 2507830]
- Drummond HA, Abboud FM, Welsh MJ. Localization of beta and gamma subunits of ENaC in sensory nerve endings in the rat foot pad. *Brain Res*. 2000; 884:1–12. [PubMed: 11082481]
- Drummond HA, Price MP, Welsh MJ, Abboud FM. A molecular component of the arterial baroreceptor mechanotransducer. *Neuron*. 1998; 21:1435–1441. [PubMed: 9883735]

- Fricke B, Lints R, Stewart G, Drummond H, Dodt G, Driscoll M, von Düring M. Epithelial Na⁺ channels and stomatin are expressed in rat trigeminal mechanosensory neurons. *Cell Tissue Res.* 2000; 299:327–334. [PubMed: 10772247]
- Funahashi M, Mitoh Y, Kohjitani A, Matsuo R. Role of the hyperpolarization-activated cation current (I_h) in pacemaker activity in area postrema neurons of rat brain slices. *J Physiol.* 2003; 552:135–148. [PubMed: 12897173]
- Gamboa-Esteves FO, Tavares I, Almeida A, Batten TF, McWilliam PN, Lima D. Projection sites of superficial and deep spinal dorsal horn cells in the nucleus tractus solitarii of the rat. *Brain Res.* 2001; 921:195–205. [PubMed: 11720726]
- Geerling JC, Loewy AD. Sodium deprivation and salt intake activate separate neuronal subpopulations in the nucleus of the solitary tract and the parabrachial complex. *J Comp Neurol.* 2007; 504:379–403. [PubMed: 17663450]
- Giraldez T, Dominguez J, Alvarez de la Rosa D. ENaC in the brain--future perspectives and pharmacological implications. *Curr Mol Pharmacol.* 2013; 6:44–49. [PubMed: 23547934]
- Hayakawa T, Takanaga A, Maeda S, Seki M, Yajima Y. Subnuclear distribution of afferents from the oral, pharyngeal and laryngeal regions in the nucleus tractus solitarii of the rat: a study using transganglionic transport of cholera toxin. *Neurosci Res.* 2001; 39:221–232. [PubMed: 11223468]
- Hindmarch CC, Fry M, Smith PM, Yao ST, Hazell GG, Lolait SJ, Paton JF, Ferguson AV, Murphy D. The transcriptome of the medullary area postrema: the thirsty rat, the hungry rat and the hypertensive rat. *Exp Physiol.* 2011; 96:495–504. [PubMed: 21317217]
- Hitomi Y, Suzuki A, Kawano Y, Nozawa-Inoue K, Inoue M, Maeda T. Immunohistochemical detection of ENaCβ in the terminal Schwann cells associated with the periodontal Ruffini endings of the rat incisor. *Biomed Res.* 2009; 30:113–119. [PubMed: 19420735]
- Hoffman GE, Smith MS, Verbalis JG. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. *Front Neuroendocrinol.* 1993; 14:173–213. [PubMed: 8349003]
- Kellenberger S, Schild L. Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure. *Physiol Rev.* 2002; 82:735–767. [PubMed: 12087134]
- Kim EC, Ahn DS, Yeon SI, Lim M, Lee YH. Epithelial Na⁺ channel proteins are mechanotransducers of myogenic constriction in rat posterior cerebral arteries. *Exp Physiol.* 2012; 97:544–555. [PubMed: 22090066]
- Kleyman TR, Cragoe EJ Jr. Amiloride and its analogs as tools in the study of ion transport. *J Membr Biol.* 1988; 105:1–21. [PubMed: 2852254]
- Kleyman TR, Sheng S, Kosari F, Kieber-Emmons T. Mechanism of action of amiloride: a molecular prospective. *Semin Nephrol.* 1999; 19:524–532. [PubMed: 10598540]
- Knuepfer MM, Branch CA. Cardiovascular responses to cocaine are initially mediated by the central nervous system in rats. *J Pharmacol Exp Ther.* 1992; 263:734–741. [PubMed: 1432699]
- Knuepfer MM, Han SP, Trapani AJ, Fok KF, Westfall TC. Regional hemodynamic and baroreflex effects of endothelin in rats. *Am J Physiol.* 1989; 257:H918–H926. [PubMed: 2675635]
- Kovacs KJ. c-Fos as a transcription factor: a stressful (re)view from a functional map. *Neurochem Int.* 1998; 33:287–297. [PubMed: 9840219]
- Kurtz A, Della Bruna R, Scholz H, Baier W. Amiloride enhances the secretion but not the synthesis of renin in renal juxtaglomerular cells. *Pflugers Arch.* 1991; 419:32–37. [PubMed: 1658729]
- Kusche-Vihrog K, Jeggle P, Oberleithner H. The role of ENaC in vascular endothelium. *Pflugers Arch.* 2013; 466:851–859. [PubMed: 24046153]
- Maccaferri G, McBain CJ. The hyperpolarization-activated current (I_h) and its contribution to pacemaker activity in rat CA1 hippocampal stratum oriens-alveus interneurons. *J Physiol.* 1996; 497(Pt 1):119–130. [PubMed: 8951716]
- McKinley MJ, McAllen RM, Davern P, Giles ME, Penschow J, Sunn N, Uschakov A, Oldfield BJ. The sensory circumventricular organs of the mammalian brain. *Adv Anat Embryol Cell Biol.* 2003a; 172:1–122.
- McKinley MJ, Albiston AL, Allen AM, Mathai ML, May CN, McAllen RM, Oldfield BJ, Mendelsohn FA, Chai SY. The brain renin-angiotensin system: location and physiological roles. *Int J Biochem Cell Biol.* 2003b; 35:901–918. [PubMed: 12676175]

- Miller RL, Loewy AD. ENaC gamma-expressing astrocytes in the circumventricular organs, white matter, and ventral medullary surface: sites for Na⁺ regulation by glial cells. *J Chem Neuroanat.* 2013; 53:72–80. [PubMed: 24145067]
- Miller RL, Loewy AD. 5-HT neurons of the area postrema become c-Fos-activated after increases in plasma sodium levels and transmit interoceptive information to the nucleus accumbens. *Am J Physiol Regul Integr Comp Physiol.* 2014; 306:R663–R673. [PubMed: 24598462]
- Miller RL, Stein MK, Loewy AD. Serotonergic inputs to FoxP2 neurons of the pre-locus coeruleus and parabrachial nuclei that project to the ventral tegmental area. *Neuroscience.* 2011; 193:229–240. [PubMed: 21784133]
- Miller RL, Wang MH, Gray PA, Salkoff LB, Loewy AD. ENaC-expressing neurons in the sensory circumventricular organs become c-Fos activated following systemic sodium changes. *Am J Physiol Regul Integr Comp Physiol.* 2013; 305:R1141–R1152. [PubMed: 24049115]
- Nakano M, Hirooka Y, Matsukawa R, Ito K, Sunagawa K. Mineralocorticoid receptors/epithelial Na(+) channels in the choroid plexus are involved in hypertensive mechanisms in stroke-prone spontaneously hypertensive rats. *Hypertens Res.* 2013; 36:277–284. [PubMed: 23096235]
- Norgren R, Smith GP. Central distribution of subdiaphragmatic vagal branches in the rat. *J Comp Neurol.* 1988; 273:207–223. [PubMed: 3417902]
- Pardridge WM. Drug transport in brain via the cerebrospinal fluid. *Fluids Barriers CNS.* 2011; 8:7. [PubMed: 21349155]
- Potts JT, Lee SM, Anguelov PI. Tracing of projection neurons from the cervical dorsal horn to the medulla with the anterograde tracer biotinylated dextran amine. *Auton Neurosci.* 2002; 98:64–69. [PubMed: 12144043]
- Sagar SM, Sharp FR, Curran T. Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science.* 1988; 240:1328–1331. [PubMed: 3131879]
- Shapiro RE, Miselis RR. The central organization of the vagus nerve innervating the stomach of the rat. *J Comp Neurol.* 1985; 238:473–488. [PubMed: 3840183]
- Shekhtman E, Geerling JC, Loewy AD. Aldosterone-sensitive neurons of the nucleus of the solitary tract: multisynaptic pathway to the nucleus accumbens. *J Comp Neurol.* 2007; 501:274–289. [PubMed: 17226797]
- Shin JW, Geerling JC, Stein MK, Miller RL, Loewy AD. FoxP2 brainstem neurons project to sodium appetite regulatory sites. *J Chem Neuroanat.* 2011; 42:1–23. [PubMed: 21605659]
- Song M, Hang T, Zhao H, Wang L, Ge P, Ma P. Simultaneous determination of amiloride and hydrochlorothiazide in human plasma by liquid chromatography/tandem mass spectrometry with positive/negative ion-switching electrospray ionisation. *Rapid Commun Mass Spectrom.* 2007; 21:3427–3434. [PubMed: 17902196]
- Soundararajan R, Pearce D, Hughey RP, Kleyman TR. Role of epithelial sodium channels and their regulators in hypertension. *J Biol Chem.* 2010; 285:30363–30369. [PubMed: 20624922]
- Spahn H, Reuter K, Mutschler E, Gerok W, Knauf H. Pharmacokinetics of amiloride in renal and hepatic disease. *Eur J Clin Pharmacol.* 1987; 33:493–498. [PubMed: 3428342]
- Stein MK, Loewy AD. Area postrema projects to FoxP2 neurons of the pre-locus coeruleus and parabrachial nuclei: brainstem sites implicated in sodium appetite regulation. *Brain Res.* 2010; 1359:116–127. [PubMed: 20816675]
- Teruyama R, Sakuraba M, Wilson LL, Wandrey NE, Armstrong WE. Epithelial Na(+) sodium channels in magnocellular cells of the rat supraoptic and paraventricular nuclei. *Am J Physiol Endocrinol Metab.* 2012; 302:E273–E285. [PubMed: 22045317]
- Wang HW, Amin MS, El-Shahat E, Huang BS, Tuana BS, Leenen FH. Effects of central sodium on epithelial sodium channels in rat brain. *Am J Physiol Regul Integr Comp Physiol.* 2010; 299:R222–R233. [PubMed: 20427723]
- Warnock DG, Kusche-Vihrog K, Tarjus A, Sheng S, Oberleithner H, Kleyman TR, Jaisser F. Blood pressure and amiloride-sensitive sodium channels in vascular and renal cells. *Nat Rev Nephrol.* 2014; 10:146–157. [PubMed: 24419567]

HIGHLIGHTS

- ENaCs are highly expressed in the sensory circumventricular organs
- The ENaC blocker amiloride elicited c-Fos activation in the area postrema (AP)
- AP projection targets in dorsolateral pons were c-Fos activated by amiloride
- FoxP2 neurons in the parabrachial nucleus and pre-locus coeruleus were activated

Fig. 1. Bar graphs demonstrating that the majority (93%) of AP cells express ENaCs. NS, not statistically significant.

Fig. 2.

A: Plasma concentrations of amiloride following 0.1, 0.5, and 2.0 mg/kg doses were plotted at 15 min intervals for a 2 hour period. **B:** CSF concentration of amiloride following 0.5 or 2.0 mg/kg at two time intervals: 30 and 120 minutes are shown.

Cardiovascular effects of Amloride (2 mg/kg)

Fig. 3.

Graphs showing the cardiovascular effects of 2 mg/kg on blood pressure (BP), heart rate (HR), superior mesenteric resistance (MsR), and hindquarter resistance (HqR) in awake rats. Abbreviation: bpm, beats per minute. None of these measurements were statistically different from the baseline values indicating the amloride did not elicit cardiovascular changes.

Fig. 4.

Sections through the circumventricular organs from cobalt-intensified c-Fos immunohistochemical preparations showing the effects of a single dose of amiloride (2 mg/kg) after 2 hours. **A:** The organum vasculosum of the lamina terminalis (OVLT) expressed c-Fos activation in its lateral zone in ~10% of cases. **B:** In this experiment, the subfornical organ (SFO) expressed only a few c-Fos activated cells following amiloride treatment, but in ~10% of the cases the ventromedial core of the SFO was activated. In contrast, the nearby paraventricular thalamic nucleus (PVT) consistently expressed strong c-

Fos activity. **C:** The area postrema (AP) expressed dense c-Fos activity following amiloride treatment in nearly all experiments. However, the medial nucleus tractus solitarius (med NTS) showed scant numbers of c-Fos activated neurons. Abbreviation: CC, central canal.

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Fig. 5. c-Fos activity in the AP following intraperitoneal injections of water or graded doses of amiloride. **A:** Control injections of distilled water produced almost no c-Fos labeling in the AP. **B:** Intraperitoneal injection of 0.1 mg/kg amiloride produced significantly more c-Fos labeling in the AP than the control rats. **C:** Intraperitoneal injection of 0.5 mg/kg amiloride resulted in stronger c-Fos activity than the 0.1 mg/kg dose. **D:** Intraperitoneal injections of 2.0 mg/kg amiloride elicited strong c-Fos labeling in the AP. Very little c-Fos activity was found in the nucleus tractus solitarius (med NTS) in any of these experiments. Abbreviation: t, solitary tract.

Fig. 6.

A: Cell counts of the c-Fos activated neurons in the AP following different amiloride doses. Note that even at the lowest dose (0.1 mg/kg), the number of c-Fos activated neurons in the AP was statistically different than the control (water) experiments. **B:** Cell counts of the c-Fos activated FoxP2 neurons in the pre-locus coeruleus nucleus showed a graded response. Only the 2 mg/kg dose was statistically different from the control (water) experiments. **C:** Cell counts of the c-Fos activated FoxP2 neurons localized in the parabrachial external lateral –inner division (PBel-inner).

Fig. 7.

A: Graph showing the c-Fos activation of tyrosine hydroxylase (TH = catecholamine) AP neurons following amiloride treatment. These data were compared to rats receiving intraperitoneal injections of distilled water. ~20% of the c-Fos activated neurons in the AP were TH immunoreactive. The graph to the right shows the complete number of c-Fos activated neurons found in the AP, and its y-axis is different than that for the two graphs on the left side. **B:** Photoimages in the AP showing c-Fos (red), TH (green), and double-labeled (red + green) immunoreactive neurons.

Fig. 8. c-Fos activated FoxP2 neurons in the dorsolateral pons 2 hours after amiloride treatment. **A:** Transverse section through the dorsolateral pons showing c-Fos (red), FoxP2 (green) and c-Fos + FoxP2 (yellow) neurons in the parabrachial nucleus. **A':** Enlargement of the PBel-inner showing double-labeled neurons (arrows). **B:** Dorsolateral pons showing the pre-locus coeruleus nucleus (pre-LC) 2 hours after amiloride treatment. **B':** Neurons in the external lateral parabrachial nucleus- inner division (PBel-inner) were co-labeled with antibodies directed against c-Fos and FoxP2 (arrows). Abbreviations: MeV, mesencephalic nucleus of the trigeminal nerve; mtV, tract of the mesencephalic trigeminal nerve; PBdl, dorsal lateral parabrachial nucleus; PBm, medial parabrachial nucleus; scp, superior cerebellar peduncle.

TABLE 1

Amloride Levels in CSF and Plasma

Intraperitoneal injections of 0.1, 0.5, or 2.0 mg/kg amloride were given to adult rats, and after various time intervals, CSF and plasma samples were collected. Measurements given in the Table are expressed as ng/ml. The values are presented as mean±standard error of mean (SEM).

0.1 mg/kg						
CSF			Plasma			
Minutes	Mean	SEM	Minutes	Mean	SEM	
30	ND	ND	15	12.15	2.19	
120	ND	ND	30	8.48	1.23	
			45	4.21	0.47	
			120	1.60	0.30	

0.5 mg/kg						
CSF			Plasma			
Minutes	Mean	SEM	Minutes	Mean	SEM	
30	1.90	0.50	15	32.95	6.42	
120	0.59	0.21	30	54.40	4.71	
			45	16.46	3.19	
			120	12.32	0.47	

2 mg/kg						
CSF			Plasma			
Minutes	Mean	SEM	Minutes	Mean	SEM	
30	17.04	3.36	15	193.83	31.11	
120	6.94	1.32	30	191.36	13.35	
			45	107.28	6.54	
			120	71.25	6.67	

ND = None Detected