Research report

Aldosterone infusion into the 4th ventricle produces sodium appetite with baroreflex attenuation independent of renal or blood pressure changes

S. Gasparini^{a,b}, M.R. Melo^a, G.M.F. Andrade-Franzé^a, J.C. Geerling^b, J.V. Menani^a, E. Colombari^{a,*}

^a Department of Physiology and Pathology, School of Dentistry, São Paulo State University, UNESP, Araraquara, SP, Brazil

^b Department of Neurology, University of Iowa Carver College of Medicine, Iowa City, IA, USA

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ABSTRACT

Aldosterone infusion into the 4th ventricle (4th V), upstream the nucleus of the solitary tract (NTS), produces strong 0.3 M NaCl intake. In the present study, we investigated whether aldosterone infusion into the 4th V activates HSD2 neurons, changes renal excretion, or alters blood pressure and cardiovascular reflexes. Chronic infusion of aldosterone (100 ng/h) into the 4th V increased daily 0.3 M NaCl intake (up to 44 ± 10 , vs. vehicle: 5.6 ± 3.4 ml/24 h) and also c-Fos expression in HSD2 neurons in the NTS and in non-HSD2 neurons in the NTS. Natriuresis, diuresis and positive sodium balance were present in rats that ingested 0.3 M NaCl, however, renal excretion was not modified by 4th V aldosterone in rats that had no access to NaCl. 4th V aldosterone also reduced baroreflex sensitivity (-2.8 ± 0.5 , vs. vehicle: -5.1 ± 0.9 bpm/mmHg) in animals that had sodium available, without changing blood pressure. The results suggest that sodium intake induced by aldosterone infused into the 4th V is associated with activation of NTS neurons, among them the HSD2 neurons. Aldosterone infused into the 4th V in association with sodium intake also impairs baroreflex sensitivity, without changing arterial pressure.

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1. Introduction

The renin-angiotensin-aldosterone system (RAAS) is an important hormonal mechanism activated in response to water and/or sodium deficiency in the body (Fitzsimons, 1998; Johnson and Thunhorst, 2007). Angiotensin II (ANG II) has numerous physiological functions, increasing blood pressure, sodium excretion, water and sodium intake, vasopressin secretion and sympathetic activity (Daniels et al., 2009; Fitzsimons, 1998; Liu et al., 2014; Xue et al., 2011). In addition to ANG II, the mineralocorticoid aldosterone plays an important role in the control of sodium in the body, acting on mineralocorticoid receptors (MR) located peripherally in the kidney and in the colon to regulate the (re)absorption of sodium and secretion of potassium, as well as centrally, inducing sodium appetite (Booth et al., 2002; Evans et al., 2016; Formenti et al., 2013; Geerling et al., 2006a; Geerling and Loewy, 2007; Sakai et al., 1996). Both aldosterone and glucocorticoids bind to MR, so selectivity for aldosterone depends on the presence of the enzyme

11 β hydroxysteroid dehydrogenase type 2 (HSD2), which catalyzes the conversion of endogenous glucocorticoid, cortisol (in humans) and corticosterone (in rodents), into biologically inactive analogs (Naray-Fejes-Toth and Fejes-Toth, 1996; Odermatt et al., 1999; Odermatt et al., 2001). Therefore, the presence of the HSD2 enzyme allows only aldosterone to have access to MR. Aldosterone sensitive neurons (those containing the HSD2 enzyme and MR) are present in the nucleus of the solitary tract (NTS) at the back of the 4th V (Geerling et al., 2006b). The HSD2 neurons from the NTS project to important areas involved in the control of sodium intake, including the lateral parabrachial nucleus (LPBN) and the bed nucleus of the stria terminalis (Geerling and Loewy, 2006b; Geerling and Loewy, 2006c). These HSD2 neurons are activated under various conditions that stimulate sodium intake (Geerling and Loewy, 2006a; Geerling and Loewy, 2007), and activating or ablating them stimulates or reduces sodium consumption (Jarvie and Palmiter, 2017; Resch et al., 2017), suggesting that they play an important role in sensing the need for sodium and generating sodium appetite.

In addition to the neuroanatomical studies, functional studies have shown that chronic infusion of low dose of aldosterone into the 4th ventricle (4th V) induces strong daily NaCl intake in rats, without changing food and water intake or body weight

* Corresponding author at: Department of Physiology and Pathology, School of Dentistry, Sao Paulo State University, UNESP, Rua Humaitá, 1680, 14801-903 Araraquara, SP, Brazil.

E-mail address: eduardo.colombari@unesp.br (E. Colombari).

(Formenti et al., 2013). This effect is blocked by an MR antagonist (Formenti et al., 2013) and the knockdown of MR receptors in the NTS reduces sodium appetite induced by aldosterone infusion into the 4th V, suggesting that aldosterone acting on hindbrain MR induces sodium intake (Koneru et al., 2014). Further, systemic inhibition of HSD2 in rats, which exposes MR in aldosterone target cells to glucocorticoids, increases sodium appetite despite also increasing sodium retention (Cooney and Fitzsimons, 1996) and the brain-specific deletion of *Hsd11b2* in mice produces sodium intake, baroreflex attenuation and hypertension (Evans et al., 2016). Spironolactone, an antagonist of MR, reduced sodium intake in these *Hsd11b2* brain-deleted mice, suggesting important role of MR for this effect (Evans et al., 2016). Although the deletion of *Hsd11b2* increases sodium intake, it is not clear if aldosterone infusion into the 4th V affects the activity of HSD2 neurons in the NTS or non-HSD2 neurons.

Besides the control of sodium in the body, aldosterone may also affect arterial pressure. The administration of aldosterone into the lateral ventricle increases arterial pressure, a response suggested to depend on increased sympathetic activity and abolished by the administration of MR antagonist (Gomez-Sanchez et al., 1990; van den Berg et al., 1989). The subcutaneous (s.c.) infusion of aldosterone also causes hypertension that was reduced by silencing NADPH oxidase isoform NOX2 and NOX4 in the hypothalamic paraventricular nucleus (PVN) (Xue et al., 2012). Moreover, MR and HSD2 in the PVN are suggested to affect sympathetic activity (Zhang et al., 2006). However, no study investigated if aldosterone acting in the hindbrain changes arterial pressure, particularly at the dose that it produces sodium appetite. Studies showing that the MR antagonist spironolactone increased baroreceptor reflex sensitivity in a renovascular hypertension model or that injection of the MR antagonist eplerenone into the NTS increased baroreflex sensitivity in salt-loaded rats suggest that endogenous aldosterone or corticosterone may be involved in the control of baroreflex function (Lincevicius et al., 2015; Masuda et al., 2010).

The deletion of *Hsd11b2* in mice increases sodium intake, without changing sodium excretion, suggesting no correlation between sodium intake and sodium excretion (Evans et al., 2016). In spite of this, chronic infusion of aldosterone in the region of the subcommissural organ (SCO), near the aqueduct, induces natriuresis (Dundore et al., 1984) which opens the possibility that sodium intake induced by aldosterone infused into the 4th V is secondary to sodium appetite produced by an increase in sodium excretion. Thus, it seems important to investigate the renal excretion in rats treated with chronic infusion of aldosterone into the 4th V with or without access to hypertonic NaCl to ingest.

Therefore, in the present study, we investigated if sodium appetite produced by aldosterone infused into the 4th V is influenced by changes in renal excretion and if it influences the activity of HSD2 neurons in the NTS. In addition, arterial pressure, cardiovascular reflexes and plasma renin activity (PRA) were also tested in rats treated with aldosterone into the 4th V.

2. Results

2.1. Expression of c-Fos in HSD2 neurons and in other NTS neurons in animals treated with aldosterone infusion into the 4th V

The infusion of aldosterone into the 4th V induced c-Fos expression in HSD2 neurons of the intermediate NTS (3 ± 1 , vs. vehicle: 0 ± 0 cells/section, $p < 0.05$) and rostral NTS (7 ± 2 , vs. vehicle: 0 ± 0 cells/section, $p < 0.05$) (Figs. 1A–D and 2). Although c-Fos activation in HSD2 neurons of the caudal NTS was not statistically different between the groups, double labeling was present in two

animals (2 ± 2 , vs. vehicle: 0 ± 0 cells/section, $p > 0.05$). In addition, c-Fos expression also increased in non-HSD2 neurons in the caudal NTS in animals treated with aldosterone infusion into the 4th V (aldosterone, $n = 5$: 22 ± 6 vs. vehicle, $n = 4$: 5 ± 3 cells/section, $p < 0.05$) (Fig. 2).

In the rats used for immunohistochemistry, chronic infusion of aldosterone (100 ng/h) into the 4th V during 2 h daily access increased daily 0.3 M NaCl intake (10.3 ± 3 , vs. vehicle: 2.3 ± 1 ml/2 h) [$F(1, 7) = 71.64$; $p < 0.05$], without significantly changing daily water intake (1.7 ± 0.7 , vs. vehicle: 0.6 ± 0.4 ml/2 h) [$F(1, 7) = 1.41$; $p > 0.05$].

Fig. 1E shows the tract of the cannula directed to the 4th V. In the present study, the injections into the 4th V were performed upstream of the NTS, where HSD2 neurons are located.

2.2. Daily fluid ingestion and renal excretion in rats infused with aldosterone into 4th V

Daily fluid ingestion and renal excretion were analyzed in rats with and without access to 0.3 M NaCl *ad libitum*.

In rats with access to 0.3 M NaCl, chronic infusion of aldosterone (100 ng/h) into the 4th V strongly increased daily 0.3 M NaCl intake (aldosterone, $n = 8$: up to 44 ± 10 , vs. vehicle, $n = 7$: 5.6 ± 3.4 ml/24 h) [$F(1, 117) = 42.42$; $p < 0.05$], urinary sodium excretion (up to 8.17 ± 2.2 , vs. vehicle: 2.2 ± 1.6 mEq/24 h) [$F(1, 117) = 12.81$; $p < 0.05$], urinary volume (up to 29.1 ± 6.8 , vs. vehicle infusion: 6.4 ± 1.3 ml/24 h) [$F(1, 117) = 59.25$; $p < 0.05$] and kaliuresis (up to 7.2 ± 1.8 mEq/24 h, vs. vehicle: 2.4 ± 0.2 to 2.7 ± 0.4 mEq/24 h) [$F(1, 117) = 19.98$; $p < 0.05$]. Aldosterone-infused rats showed a variable, slight reduction in water intake some days, but across the treatment period this was not significantly different from the control group [$F(1, 117) = 0.03$; $p > 0.05$] (Fig. 3A–D and Table 3). Aldosterone infusion also produced a positive sodium balance (up to 6.4 ± 2.9 on the 6th day of infusion, vs. vehicle: 0.4 ± 2.2 mEq/24 h) [$F(1, 117) = 12.81$; $p < 0.05$] (Fig. 3E). As a consequence of the increases in 0.3 M NaCl intake, the total fluid intake also increased in rats infused with aldosterone into the 4th V (up to 71.4 ± 4.6 ml/24 h, vs. vehicle infusion: 41 ± 3.7 ml/24 h) [$F(1, 117) = 29.43$; $p < 0.05$] (Fig. 3F).

In rats that had no access to ingest 0.3 M NaCl, chronic infusion of aldosterone (100 ng/h) into the 4th V did not modify daily water intake (aldosterone, $n = 8$: 40 ± 7 ml/24 h, vs. vehicle, $n = 6$: 39.7 ± 6.4 ml/24 h) [$F(1, 108) = 0.42$; $p > 0.05$], urinary sodium excretion (up to 0.9 ± 0.1 mEq/24 h, vs. vehicle infusion: 0.7 ± 0.1 mEq/24 h) [$F(1, 108) = 2.02$; $p > 0.05$] or urinary volume (up to 11.6 ± 2.4 , vs. vehicle infusion: 9.3 ± 1.2 ml/24 h) [$F(1, 108) = 0.33$; $p < 0.05$] (Fig. 4A–C). The infusion of aldosterone into the 4th V did reduce potassium excretion (up to 2.5 ± 0.3 , vs. vehicle infusion: 4.4 ± 0.8 mEq/24 h) [$F(1, 108) = 15.10$; $p < 0.05$] (Table 1).

2.3. Arterial pressure and heart rate in rats infused with aldosterone into the 4th V

In rats with access to water and 0.3 M NaCl, chronic infusion of aldosterone did not modify mean arterial pressure recorded by telemetry in the light (aldosterone, $n = 13$: 110 ± 10 , vs. vehicle, $n = 7$: 108 ± 2 mmHg) [$F(1, 270) = 3.47$; $p > 0.05$] or dark period (108 ± 7 , vs. vehicle: 103 ± 7 mmHg) [$F(1, 270) = 1.56$; $p > 0.05$] (Fig. 5A and B). HR was also not different between groups in the light (333 ± 17 , vs. vehicle: 357 ± 16 bpm) [$F(1, 270) = 3.47$; $p > 0.05$] or dark period (381 ± 8 , vs. vehicle: 390 ± 12 bpm) [$F(1, 270) = 1.56$; $p > 0.05$] (Table 2). The infusion of aldosterone (100 ng/h) into the 4th V in the same rats strongly increased daily 0.3 M NaCl intake (up to 43 ± 13 , vs. vehicle: 5.1 ± 1.6 ml/24 h) [$F(1, 271) = 83.68$; $p < 0.05$] (Table 3) and decreased water intake on

Fig. 1. Photomicrographs of rostral NTS (*rNTS*) and intermediate NTS (*iNTS*) of animals treated with (A and C) vehicle or (B and D) aldosterone infusion (100 ng/h) into the 4th V for 6 days. In A–D, green color represents HSD2 expression and red c-Fos expression, both at 20× magnification. (E) Photomicrograph of a coronal section of a rat brain showing the injection site into the 4th V (arrow).

Fig. 2. Quantitative c-Fos and HSD2 expression in the caudal NTS (cNTS), intermediate NTS (iNTS) and rostral NTS (rNTS) in animals treated with vehicle (VEH) or aldosterone (ALDO) infusion (100 ng/h) into the 4th V for 6 days. Data expressed as means $\pm$ SEM.

the 2nd, 5th, 8th and 12nd day of treatment [$F(1, 271) = 25.14$; $p < 0.05$] (Table 3).

2.4. Cardiovascular reflexes in rats infused with aldosterone into the 4th V

Cardiovascular reflexes were tested in rats with and without access to 0.3 M NaCl *ad libitum*.

In rats that had access to 0.3 M NaCl to ingest, on the 7th day of aldosterone infusion into the 4th V, baroreflex reduction in HR was blunted in response to intravenous phenylephrine (aldosterone, $n = 5$: slope: -0.72 ± 0.25 , vs. vehicle, $n = 4$: -5.1 ± 0.9 bpm/mmHg, $R = 0$) (Fig. 6A), but there was no difference with nitroprusside (slope: -1.93 ± 0.52 , vs. vehicle: -1.15 ± 0.9 bpm/mmHg, $R = 0.246$) (Fig. 6B). The bradycardic response to chemoreflex activation with intravenous potassium cyanide was also reduced (-30 ± 12 , vs. vehicle: -101 ± 15 bpm) [$F(1, 7) = 9.12$; $p < 0.05$], without changing the pressor response (Fig. 6C, D). However, infusion of aldosterone did not change the cardiopulmonary reflex tested with intravenous injection of the 5HT₃ agonist phenylbiguanide (Fig. 6C, D) in the same group of animals. The infusion of aldosterone into the 4th V in these rats again did not modify MAP (107 ± 4 , vs. vehicle: 99 ± 4 mmHg) [$F(1, 7) = 3.19$; $p > 0.05$] or HR (395 ± 16 , vs. vehicle: 355 ± 20 bpm) [$F(1, 7) = 2.19$; $p > 0.05$].

Fig. 3. Daily (A) 0.3 M NaCl intake, (B) urinary Na⁺ excretion, (C) urinary volume, (D) water intake, (E) Na⁺ balance and (F) total fluid intake in rats treated with chronic infusion of aldosterone (100 ng/h) or vehicle into the 4th V (days 1–6). Baseline represents the period before starting the infusion (days –2 to 0). Data expressed as means $\pm$ SEM.

Fig. 4. Daily (A) water intake, (B) urinary Na⁺ excretion, (C) urinary volume in rats that had no access to NaCl to ingest in rats treated with chronic infusion of aldosterone (100 ng/h) or vehicle into the 4th V (days 1–6). Baseline represents the period before starting the infusion (days –2 to 0). Data expressed as means $\pm$ SEM.

In rats that had no access to 0.3 M NaCl, aldosterone infusion into the 4th V did not alter baroreflex responses to phenylephrine (aldosterone, $n = 6$: slope: -1.33 ± 0.30 , vs. vehicle, $n = 4$: -1.47 ± 0.16 bpm/mmHg, $R = 0.241$) (Fig. 7A) or sodium nitroprusside (slope: -2.90 ± 0.55 , vs. vehicle: -5.11 ± 1.29 bpm/mmHg, $R = 0.285$) (Fig. 7B). Bradycardic responses to chemoreflex with KCN activation were again attenuated (-46 ± 13 , vs. vehicle: -111 ± 4

bpm) [F (1, 8) = 14.02; $p < 0.05$], without significantly changing the pressor response (Fig. 7C, D), whereas the hypotensive response to phenylbiguanide increased (Fig. 7C, D) [F (1, 8) = 5.67; $p < 0.05$]. The infusion of aldosterone into the 4th V in these rats again did not modify MAP (111 ± 2 , vs. vehicle: 111 ± 2 mmHg) [F (1, 8) = 0.04; $p > 0.05$] or HR (350 ± 12 , vs. vehicle: 389 ± 23 bpm) [F (1, 8) = 2.63; $p > 0.05$].

Table 1

Renal K⁺ excretion in rats that had no access to NaCl to ingest treated with chronic infusion of aldosterone (100 ng/h) or vehicle into the 4th V during 6 days (days 1–6). Baseline represents the period before starting the infusion (days –2 to 0).

	–2	–1	0	1	2	3	4	5	6 Days
<i>K⁺ excretion (mEq) – 0.3 M NaCl access</i>									
Vehicle (n = 7)	2.5 ± 0.2	2.7 ± 0.6	2.5 ± 0.1	3.0 ± 0.5	3.1 ± 0.9	3.3 ± 0.7	2.4 ± 1.1	3.3 ± 0.6	2.6 ± 0.4
Aldosterone (n = 8)	2.5 ± 0.6	3.06 ± 0.6	3.0 ± 3.6	3.5 ± 0.7	6.6 ± 1.5*	6.7 ± 1.1*	7.2 ± 1.8*	6.6 ± 1.9*	7.2 ± 2.1*
<i>K⁺ excretion (mEq) – no 0.3 M NaCl access</i>									
Vehicle (n = 8)	3.3 ± 0.6	3.0 ± 0.7	2.6 ± 0.7	3.4 ± 0.8	3.6 ± 0.7	2.5 ± 0.4	2.5 ± 0.6	3.3 ± 0.7	1.0 ± 4.2
Aldosterone (n = 6)	2.9 ± 0.3	2.4 ± 0.4	2.4 ± 0.4	2.0 ± 2.6*	2.1 ± 0.3*	2.7 ± 0.6	2.3 ± 0.3	2.0 ± 0.4*	2.5 ± 0.2*

Data expressed by means ± SEM. * Different from vehicle ($p < 0.05$). Aldosterone (100 ng/h). Vehicle (1% ethanol-saline). Baseline, intake before starting the infusion (–2 to 0 days); 4th V infusion of aldosterone or vehicle (0–6 days). n = number of animals. Data expressed as means ± SEM.

Fig. 5. Mean arterial pressure (MAP) in (A) the light and (B) dark period in rats treated with chronic infusion of aldosterone (100 ng/h) or vehicle into the 4th V. Baseline represents the period before starting the infusion (days –2 to 0). Data expressed as means ± SEM.

2.5. PRA in rats treated with aldosterone infusion into the 4th V

The PRA was measured on the 7th day of aldosterone or saline infusion into the 4th V and it was not different between groups. All PRA values were less than 0.2 mg/ml/h.

3. Discussion

The present results confirm that chronic aldosterone infusion into the 4th V strongly increased daily 0.3 M NaCl intake, as previously demonstrated by [Formenti et al. \(2013\)](#), and produced a small, inconsistent decrease in water intake. Aldosterone infusion also induced c-Fos activation in HSD2 neurons of the intermediate NTS and rostral NTS and in non-HSD2 neurons of the caudal NTS. Elevated NaCl intake due to 4th V aldosterone infusion increased renal excretion, but rats without access to NaCl did not exhibit this effect, indicating that aldosterone's effect on HSD2 neurons or non-HSD2 neurons do not directly influence renal excretion. Aldosterone infusion into the 4th V also impaired baroreflex sensitivity in animals that had access to sodium. The bradycardic response to chemoreflex activation was reduced by aldosterone, with or without saline access, without changing baseline MAP or HR. These results suggest that HSD2 neurons of the NTS are part of the mechanisms activated by aldosterone acting in the hindbrain to stimulate sodium appetite. In addition, aldosterone in the hindbrain reduces the sensitivity of the baroreflex when combined with sodium ingestion.

Neuroanatomical studies initially showed that HSD2 neurons in the NTS were activated in mineralocorticoid (DOCA) treated rats and deactivated by hypertonic NaCl ingestion, suggesting that these neurons might play a role in sodium appetite ([Geerling and Loewy, 2006a](#)). Behavioral studies showed that chronic infusion of aldosterone into the 4th V strongly increases daily sodium intake, and that sodium intake can be reduced by MR blockade or by reducing MR expression in the NTS ([Formenti et al., 2013; Konecny et al., 2014](#)). More recently, two groups found that chemo-genetic activation of HSD2 neurons drives mice to consume large volumes of concentrated 0.5 M NaCl or eat salty chow ([Jarvie and Palmiter, 2017; Resch et al., 2017](#)). The present results show that the HSD2 neurons located in the NTS are activated by the same dose of aldosterone that induces sodium appetite when infused into the 4th V, which expands the results of previous functional and neuroanatomical studies suggesting a correlation between the activity of HSD2 neurons in the NTS and sodium appetite caused by aldosterone acting in the hindbrain. Although we know that HSD2 neurons regulate sodium appetite ([Jarvie and Palmiter, 2017; Resch et al., 2017](#)), our observation that non-HSD2 neurons also showed c-Fos expression raises the possibility that other neurons participate as well. Aldosterone could affect these neurons via G protein-coupled estrogen receptor (GPER) ([Funder, 2011; Qiao et al., 2016](#)). Alternatively, non-HSD2 neurons could be activated synaptically by HSD2 neurons. This hypothesis could be tested in ex vivo brainstem slices ([Resch et al., 2017](#)) by selectively stimulating HSD2 neurons while recording non-HSD2 neurons in the caudal NTS.

The infusion of aldosterone into the 4th V produced strong natriuresis and diuresis in rats that had access to 0.3 M NaCl to ingest, yet it produced no change in urinary volume or sodium

Table 2

Heart rate (HR) in the light or dark period in rats treated with chronic infusion of aldosterone (100 ng/h) or vehicle into the 4th V (days 1–14). Baseline represents the period before starting the infusion (days –2 to 0).

	–2	–1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14 Days
<i>HR (bpm) in the light period</i>																	
Vehicle	345 ± 15	324 ± 10	335 ± 6	331 ± 6	345 ± 8	329 ± 8	331 ± 9	340 ± 5	352 ± 19	336 ± 10	324 ± 12	324 ± 12	316 ± 7	325 ± 7	328 ± 10	344 ± 12	353 ± 14
Aldosterone	336 ± 11	338 ± 9	342 ± 9	328 ± 6	333 ± 7	327 ± 10	362 ± 10	334 ± 9	325 ± 8	324 ± 5	324 ± 8	340 ± 8	337 ± 9	341 ± 13	330 ± 7	323 ± 6	343 ± 10
<i>HR (bpm) in the dark period</i>																	
Vehicle	392 ± 8	355 ± 18	393 ± 9	360 ± 9	377 ± 5	370 ± 8	377 ± 15	389 ± 8	411 ± 16	387 ± 7	375 ± 9	378 ± 8	380 ± 7	387 ± 6	383 ± 5	391 ± 3	390 ± 12
Aldosterone	382 ± 12	372 ± 9	388 ± 8	363 ± 8	362 ± 7	365 ± 8	392 ± 10	352 ± 19	379 ± 8	375 ± 7	386 ± 16	376 ± 8	379 ± 8	367 ± 8	391 ± 9	384 ± 10	381 ± 8

Data expressed by means ± SEM. Aldosterone (100 ng/h, n = 7–13). Vehicle (1% ethanol-saline, n = 7). Baseline, intake before starting the infusion (–2 to 0 days); 4th V infusion of aldosterone or vehicle (0–14 days).

Table 3

Daily 0.3 M NaCl and water intake produced by chronic infusion of aldosterone into the 4th V over a 14 day period.

	–2	–1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14 Days
<i>0.3 M NaCl intake (ml)</i>																	
Vehicle	8 ± 4	11 ± 5	5 ± 1	4 ± 1	8 ± 2	9 ± 2	9 ± 2	10 ± 3	5 ± 1	10 ± 3	9 ± 2	8 ± 4	11 ± 5	5 ± 1	6 ± 2	7 ± 2	5 ± 1
Aldosterone	9 ± 1	11 ± 2	12 ± 2	15 ± 3*	20 ± 2*	28 ± 4*	29 ± 7*	31 ± 7*	35 ± 6*	29 ± 8*	30 ± 9*	31 ± 9*	35 ± 9*	35 ± 11*	37 ± 11*	39 ± 13*	43 ± 14
<i>Water intake (ml)</i>																	
Vehicle	37 ± 4	37 ± 3	47 ± 4	23 ± 7	45 ± 3	42 ± 6	39 ± 4	45 ± 4	38 ± 3	36 ± 4	36 ± 6	34 ± 5	37 ± 5	36 ± 7	46 ± 3	40 ± 6	41 ± 5
Aldosterone	36 ± 4	41 ± 3	39 ± 4	28 ± 4	32 ± 2*	35 ± 3	29 ± 3	27 ± 3*	38 ± 4	32 ± 5	22 ± 4*	21 ± 5	29 ± 7	28 ± 6	25 ± 4*	28 ± 6	29 ± 4

Data expressed by means ± SEM. * Different from vehicle ($p < 0.05$). Aldosterone (100 ng/h, n = 7–13). Vehicle (1% ethanol-saline, n = 7). Baseline, intake before starting the infusion (–2 to 0 days); 4th V infusion of aldosterone or vehicle (0–14 days).

Fig. 6. (A, B) Baroreflex sensitivity, (C) changes in mean arterial pressure (Δ MAP) and (D) changes in heart rate (Δ HR) produced by i.v. injection of potassium cyanide (40 μ g/0.1 ml per rat) or phenylbiguanide (50 μ g/kg) in rats that received aldosterone (100 ng/h) or vehicle into the 4th V during 6 days with access to 0.3 M NaCl. Data expressed as means ± SEM.

excretion if NaCl solution was not available to ingest, suggesting that aldosterone into the 4th V produces no direct effect on renal sodium or water excretion. These results suggest that infusing aldosterone into the 4th V is primarily natrioregic, whereas

natriuresis and diuresis occur secondarily to excess ingestion of 0.3 M NaCl produced by the infusion of aldosterone. We also evaluated potassium excretion, which was higher in aldosterone treated animals that had access to 0.3 M NaCl, possibly due an increase

Fig. 7. (A, B) Baroreflex sensitivity, (C) changes in mean arterial pressure (Δ MAP) and (D) changes in heart rate (Δ HR) produced by iv injection of potassium cyanide (40 μ g/0.1 ml per rat) or phenylbiguanide (50 μ g/kg) in rats that had no access to NaCl to ingest treated with chronic infusion of aldosterone (100 ng/h) or vehicle into the 4th V during 6 days. Data expressed as means $\pm$ SEM.

in urinary flow (Welling, 2013). However, in animals without access to sodium, kaliuresis varied across time in both groups, which we cannot explain, but could have to do with subtle changes in potassium handling not captured in the present dataset.

In spite of the strong ingestion of hypertonic NaCl induced by infusing aldosterone into the 4th V, water intake did not change or was variably reduced when compared to control group, confirming that aldosterone specifically stimulates sodium intake. Chronic infusion of aldosterone with 0.3 M NaCl ingestion also produced a tendency of positive sodium balance that was different from vehicle on the 4th and 6th day of infusion, suggesting that urinary excretion may not completely compensate for the increased sodium intake.

Besides increasing sodium appetite, aldosterone infusion into the 4th V modified reduced baroreflex sensitivity when the animals had sodium hypertonic NaCl solution available. However, it is difficult to know exactly whether their increased sodium intake *per se* or the combined action of with aldosterone infusion into the 4th V was responsible for the change in baroreflex sensitivity. Sodium intake produces changes in baroreflex sensitivity in humans (Creager et al., 1991). In addition, Nakamura and colleagues (Nakamura et al., 1988) demonstrated that DOCA-salt loaded rats have the central component of baroreflex impaired. Moreover, in DOCA-salt hypertensive rats, chorthalidone treatment, which reduces plasma sodium, normalizes baroreceptor responses (Bissoli et al., 2000).

Although we showed that sodium intake is important to change baroreflex sensitivity in 4th V aldosterone-treated animals, others showed that peripherally administered aldosterone has an independent effect. Infusion of aldosterone in isolated carotid sinus

acutely or subcutaneous infusion chronically reduced baroreceptor activity in dogs, a response prevented by previous infusion of the MR receptor antagonist spironolactone (Wang et al., 1992; Wang, 1994). In healthy, normotensive volunteers, acute i.v. infusion of aldosterone also impaired the baroreflex, with marked decrease in the bradycardic response to phenylephrine (Yee and Struthers, 1998). It is difficult to know if the change in baroreflex produced by aldosterone is due to the activity of HSD2 or non-HSD2 neurons. In the present study, aldosterone was infused close to the NTS, an area that receives baroreceptor afferents and where HSD2 neurons are located (Geerling et al., 2006b; Guyenet, 2006; Roland et al., 1995). It is possible that aldosterone acting on HSD2 neurons in the NTS indirectly affects the activity of other NTS neurons that process baroreceptor signals, via local synaptic connection in the NTS.

Aldosterone infused chronically into the lateral ventricle increased MAP in rats and dogs, independent of sodium retention (Gomez-Sanchez et al., 1990; Peysner et al., 1990), whereas here aldosterone infused into the 4th V produced no change in MAP and HR, despite of a tendency to increase sodium retention. Our data complement the recent finding that HSD2 neuron chemogenetic activation does not change blood pressure or heart rate (Resch et al., 2017). On the other hand, Evans and colleagues (Evans et al., 2016) showed an increase in blood pressure in mice with Hsd11b2 expression eliminated from the brain when these mice were given saline to drink. Besides the possibility of a species difference between mice and rats, a potentially important distinction is the breeding strategy in that study. Evans et al. crossed Nestin-Cre transgenic mice with their floxed Hsd11b2 strain, which should eliminate the developmental expression of Hsd11b2

in many brain regions outside the NTS (Naray-Fejes-Toth and Fejes-Toth, 2007; Robson et al., 1998) impeding neurogenesis by exposing cells to glucocorticoids (Holmes et al., 2006a; Noguchi et al., 2008). Developmental elimination of Hsd11b2 reduces adult cerebellar size (Holmes et al., 2006b), but the potential cardiovascular consequences for central autonomic neurons are unknown. Although in the hindbrain it appears that MR and HSD2 neurons may not modulate blood pressure, Gomes Sanchez (Gomez-Sanchez, 1986), infused aldosterone into the lateral ventricle at 5 ng/h in nephrotomized rats with only saline available to drink and was found an increase in systolic blood pressure when measured by tail-cuff. If the kidney was not removed, a higher dose (45 ng/h) was necessary to increase blood pressure (Gomez-Sanchez, 1988).

Separately, Van den Berg et al. (1994) demonstrated a depressor effect of injecting the MR antagonist RU28318 in normotensive, spontaneous hypertensive, and DOCA-salt hypertensive rats. This depressor effect did not occur in free-moving rats, and required warming and restraint-stress conditions mimicking tail-cuff measurement of BP (Van den Berg et al., 1994). Rahmouni showed that this depressor effect of RU28318 depends on renal innervation (Rahmouni et al., 1999; Rahmouni et al., 2002). MR activation in the AV3V region of the hypothalamus was proposed to explain the increase of arterial pressure by aldosterone infused into the lateral ventricles (Gomez-Sanchez, 1986), but the cell types, locations, and mechanisms remain unclear.

In the present work we infused a low dose of aldosterone (100 ng/h) and we exclude the action of aldosterone on other brain structures based on the fact that infusion of aldosterone into the lateral ventricle at the same concentration infused into the 4th V produces no sodium intake (Formenti et al., 2013). We also exclude the likelihood of a peripheral action of our 4th V aldosterone infusions, based on a prior dose-response study on peripheral administration of aldosterone (Garwitz and Jones, 1982). Subcutaneous infusion required at least $2.5\times$ more aldosterone (250 ng/h) to produce a statistically significant rise in the plasma concentration of aldosterone, and at least $5\times$ more (500–1000 ng/h) to produce a statistically significant increase in saline ingestion, relative to the dose we infused into the 4th V (100 ng/h). Some portion of our 4th V aldosterone infusion probably did escape into the periphery, but based on these dose-response data the quantity would not be sufficient to change sodium appetite or even plasma aldosterone significantly, even if we had infused it peripherally.

In conclusion, the present results suggest that aldosterone activates HSD2 neurons in the NTS to produce a natriorexigenic effect, which is the cause, not a consequence of changes in renal excretion. In addition, the reduction in baroreflex sensitivity produced by aldosterone and sodium intake is not associated with changes in baseline blood pressure or heart rate. Future investigations are necessary to provide information on whether HSD2 neurons are involved in other cardiovascular and behavioral functions.

4. Experimental procedure

4.1. Animals

Male Holtzman rats weighing 280–320 g were used housed individually in stainless steel cages in a room with controlled temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 10\%$). Lights were on from 7:00 am to 7:00 pm. Biobase rat chow (Águas Frias, SC, Brazil) and tap water were available *ad libitum*. Rats also had access to 0.3 M NaCl *ad libitum* or for 2 h/day depending on the protocol. The experimental protocols used in the present study were approved by the Ethical Committee for Animal Care and Use from the Dentistry School of Araraquara - UNESP.

4.2. Brain surgery

The animals were anesthetized with ketamine (80 mg/kg) and xylazine (7 mg/kg) intraperitoneally (*i.p.*) and placed in a Kopf stereotaxic instrument. The skull was positioned with bregma and lambda at the same horizontal level. An intracerebral 23-gauge stainless steel guide cannula was implanted in the 4th V according to the following coordinates: 12.9 mm caudal to bregma, in the midline and 4.8 mm below the surface of the skull. The tips of the cannulas were positioned at a point 2 mm above the 4th V. The cannulas were fixed to the cranium using dental acrylic resin and jeweler screws. Post-surgery, animals received an intramuscular injection of penicillin (30,000 IU) and a subcutaneous (*s.c.*) injection of the analgesic Ketoflex (ketoprofen 1%, 0.03 ml/rat).

After the surgery, rats were allowed to recover for one week before starting water and NaCl intake tests or arterial pressure recordings.

4.3. Intracerebroventricular infusions

The infusions were performed using osmotic mini-pumps (model 2001, Durect Corp. Cupertino, CA) filled with aldosterone (100 ng/ μl , dissolved in 1% ethanol in 0.9% NaCl) or vehicle. The osmotic mini-pumps were incubated for at least 24 h in saline at room temperature prior to the implantation in the scapular region under 1% halothane anesthesia. For the infusions into the 4th V, the pumps were connected with polyethylene tubing to a 30-gauge stainless steel injector cannula (2 mm longer than the guide cannula).

4.4. Water and 0.3 M NaCl intake and urine analyses

Rats chronically infused with aldosterone (100 ng/ μl /h) or vehicle were housed in metabolic cages with water and food *ad libitum*. Two groups also had access to 0.3 M NaCl solution *ad libitum*. Water and 0.3 M NaCl were provided from burettes with 1 ml divisions that were fitted with metal drinking spouts. Urine samples were collected in 0.1 ml graduated polypropylene tubes, and urinary volume was measured. Sodium and potassium urinary concentration was measured by an ion-specific electrode (Nova Biomedical Analyser, model Nova 1) and the amount of sodium and potassium in the urine was determined by the product of urine volume times the concentration of each ion in the urine. Water and 0.3 M NaCl intake and urine samples were measured on a 24-h basis for nine days.

4.5. Baroreflex, chemoreflex and cardiopulmonary reflex tests

Six days after inserting mini-pumps, animals were anesthetized with ketamine (80 mg/kg of body weight *i.p.*) and xylazine (7 mg/kg of body weight *i.p.*), and polyethylene tubing (PE-10 connected to a PE-50) was inserted into the abdominal aorta through the femoral artery on the day before the experiments. Polyethylene tubing was also inserted into the femoral vein for drug administration. These catheters were tunneled subcutaneously and exposed on the back of the rat to allow access in awake rats. To record pulsatile arterial pressure, mean arterial pressure (MAP) and heart rate (HR), the arterial catheter was connected to a Stathan Gold (P23 Db) pressure transducer connected with an ETH-200 amplifier (CB Sciences, Dover, NH) and to a PowerLab data acquisition system (ADInstruments, Dunedin, New Zealand).

We evaluated baroreflex sensitivity with *i.v.* phenylephrine (40 $\mu\text{g/kg}$) and sodium nitroprusside (30 $\mu\text{g/kg}$). We analyzed one second mean HR values in response to 10 mmHg incremental or decremental changes in MAP, starting at 0 mmHg up to a maximal change of 25 or -25 mmHg. The values were plotted and we used

GraphPad to perform a linear regression for each animal. The regression coefficient (slope) was used to analyze baroreflex sensitivity as the increase or decrease of HR across changes in mean arterial pressure (MAP).

To test chemoreflex and cardiopulmonary reflex, potassium cyanide (KCN, 40 µg/0.1 ml/rat) and phenylbiguanide (50 µg/kg) were administered i.v., respectively. The interval between i.v. injections was 5 min.

4.6. Chronic arterial pressure and heart rate recordings

A telemetry method was used to record arterial pressure and heart rate chronically. Using aseptic techniques, a laparotomy was performed and the catheter of the pressure telemetry transmitter (Model TA11PAC40, Data Sciences International, New Brighton, MN) was inserted into the descending abdominal aorta, distal to the kidneys according to the manufacturer's instructions. The catheter was fixed in the aorta with cyanoacrylate adhesive and the transmitter was secured to the abdominal wall with sutures. Mean daily 24-h blood pressure (BP) and heart rate data were derived from the average BP measured by bursts of 10 s every 5 min using the software (Dataquest 4.0) provided by the manufacturer.

4.7. Plasma renin activity

Trunk blood was collected after decapitation for determination of plasma renin activity (PRA). Samples for PRA were collected in tubes pre-refrigerated at 0 °C containing sodium EDTA (2 mg/ml of blood) to obtain plasma. All samples were centrifuged (3000 rpm for 20 min at 4 °C) then stored at –20 °C.

One hundred microliters of the plasma sample were mixed with 200 µl of 0.2 M Tris–HCl buffer pH 7.4, 20 µl of 48 mM of 8-OH-quinoleine and 10 µl of 161 mM phenylmethylsulfonyl fluoride and was incubated at 37 °C for 3 h. Blanks were prepared by incubating plasma samples in the same conditions at 4 °C. After incubation, the amount of angiotensin I generated from angiotensinogen was determined by radioimmunoassay, utilizing rabbit antibody for angiotensin I from the Maia Biodata Kit for renin activity (Biochem Imunosystem).

4.8. Immunohistochemistry

Animals were deeply anesthetized with sodium thiopental (70 mg/kg body weight i.p.), transcardially perfused with 0.9% saline followed by 4% paraformaldehyde and brains were prepared as detailed before (Melo et al., 2015). Four sets of axial sections (40 µm) of the brainstem and forebrain were sectioned on a cryostat (Leica, CM1850, Wetzlar, Hesse, Germany). One of the sets of the brain sections was pre-incubated for 15 min in a blocking solution composed of 10% (vol/vol) normal horse serum (NHS, Sigma, St Louis, MO, USA) and 0.3% (vol/vol) Triton X-100 (Sigma) in 0.1 M PBS followed by rinses in PBS (3 × 10 min). Subsequently, sections were incubated with a sheep anti-HSD2 primary antibody (1:15,000, Millipore) and rabbit anti-c-Fos (1:1,500, Santa Cruz Biotechnology), in PBS containing 1% (vol/vol) NHS and 0.3% Triton-X-100 for 48 h at 4 °C. Sections were rinsed in PBS between the steps. After this, a 1-h incubation in biotinylated horse anti-goat IgG (1:500, Vector Laboratories, Burlingame) was followed by 1-h incubation in Streptavidin Alexa Fluor 488 conjugate and donkey anti-rabbit Alexa Fluor 594 (both 1:500; Life Technologies). Then, the sections were mounted onto slides in 0.5% gelatin and allowed to air-dry for 10–15 min before being cover slipped using antifade fluorescent mounting solution (Fluoromount, Sigma-Aldrich, St. Louis, MO, USA). The sections were examined in a

fluorescence microscope (Leica DM5500 B, Wetzlar, Hessen, Germany) with the appropriated filters.

Fos-IR nuclei and the presence of HSD2 neurons were counted in one section of each area previously defined, in each case the section that appeared to have the greatest number of Fos-positive nuclei. Neuroanatomical sites were identified according to Paxinos and Watson's Atlas (Paxinos and Watson, 1997).

For assessing c-Fos in HSD2 neurons in the nucleus of the solitary tract (NTS), 20 µm z-stacks (containing 20 images) were collected through c-Fos and HSD2 stained sections at 20x magnification and counted manually on a computer using Leica software (Application Suite Advanced Fluorescence, LAS X).

4.9. Histology

At the end of the experiments, all animals not used for immunofluorescence received through a single injection of 2% Evans blue solution (2 µl) into the 4th V. They were then deeply anesthetized with sodium thiopental (60 mg/kg of body weight) and perfused transcardially with 10% formalin. The brains were removed and fixed in 10% formalin for at least 2 days. Following post fixation, the brains were frozen, cut in 60 µm sections, Giemsa-stained and analyzed by light microscopy to confirm the central sites of the injections. Forty-four animals did not have Evans blue in the 4th V or their acrylic cement detached during the experiment, and these animals were excluded from further analyses.

4.10. Statistical analysis

The results were analyzed by two-way ANOVA with treatments and times as factors or one-way ANOVA. In all the cases, ANOVA was followed by Newman-Keuls test. For c-Fos data, a *t*-test was used to analyze differences between groups. Differences were considered significant at *p* < 0.05.

4.11. Experimental protocols

4.11.1. Quantification of c-Fos and HSD2 expression in animals treated with aldosterone or vehicle infusion into the 4th V

Rats with cannulas implanted into the 4th V were used. They had free access to water and food, whereas 0.3 M NaCl was offered during 2 h/day at 2:00 pm in the course of 8 days. The infusions of vehicle or aldosterone (100 ng/µl/h) into the 4th V started 3 days after the access to 0.3 M NaCl.

On the sixth day after the beginning of the infusion, at 2:00 pm, animals were deeply anesthetized with sodium thiopental (70 mg/kg body weight ip) and had the brains removed and the number of c-Fos and HSD2 expression was quantified in different sites of the brain by immunohistochemistry procedures. On the day of the perfusion the animals had no access to 0.3 M NaCl.

4.11.2. Daily water and 0.3 M NaCl intake, renal excretion and cardiovascular reflexes in rats treated with aldosterone infused into the 4th V

Rats maintained in metabolic cages with access to water, 0.3 M NaCl and food *ad libitum* received chronic infusion of aldosterone (100 ng/µl/h) or vehicle into the 4th V. Daily ingestion of water and 0.3 M NaCl was recorded continuously for a 10 days period (control period of 3 days before starting the infusion of aldosterone or vehicle, 7 days of continuous infusion of aldosterone or vehicle). Urine volume was measured and urine samples to measure the concentration of sodium and potassium were collected daily.

At the end of the 6th day of aldosterone or vehicle infusion into the 4th V, catheters were inserted into the femoral artery and vein to record MAP and HR and for drug administration, respectively.

MAP and HR were recorded 24 h after the surgery and the reflexes were tested with i.v. injections of phenylephrine (5 µg/kg body weight), sodium nitroprusside (30 µg/kg body weight), KCN (40 µg/0.1 ml/rat) and phenylbiguanide (50 µg/kg body weight). The injection time did not last more than 30 s and interval between two i.v. injections was 5 min.

The same protocol described above was repeated in another two groups of animals chronically infused with aldosterone (100 ng/µl/h) or vehicle into the 4th V, except that these animals had no access to 0.3 M NaCl to ingest.

4.11.3. Chronic telemetry arterial pressure and heart rate in rats treated with aldosterone into the 4th V

Rats implanted with telemetry probes were maintained in polypropylene boxes with access to water, 0.3 M NaCl and food *ad libitum* and received chronic infusion of aldosterone (100 ng/µl/h) or vehicle into the 4th V. Daily ingestion of water and 0.3 M NaCl, MAP and HR were recorded continuously for a period of 17 days (control period of 3 days before starting the infusion of aldosterone or vehicle, 14 days of continuous infusion of aldosterone or vehicle).

4.11.4. PRA in rats treated with aldosterone infusion into the 4th V

Rats with cannulas implanted into the 4th V had free access to water, food and 0.3 M NaCl. Daily ingestion of water and 0.3 M NaCl was recorded continuously for 10 days (control period of 3 days before starting the infusion of aldosterone or vehicle, 7 days of continuous infusion of aldosterone or vehicle). On the 7th day of the infusion, the animals were decapitated and had blood collected for the measurement of PRA.

5. Author contributions

Conception and format of the paper was made by SG and MRS. Acquisition, analysis, or interpretation of data was made by SG, MRS, GMFAF, JCG, JVM and EC. SG drafted the article and MRM, GMFAF, JCG, JVM and EC revised it critically for important intellectual content. All authors approved the final version of the manuscript, agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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