MINERALOCORTICOID RECEPTOR IN THE NUCLEUS OF THE SOLITARY TRACT STIMULATES SALINE INTAKE DURING 4TH VENTRICULAR INFUSIONS OF ALDOSTERONE

Running Title: NTS and saline intake

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Abstract

The purpose of this study was to determine if neurons within the NTS that express the mineralocorticoid receptor (MR) play a role in aldosterone stimulation of salt intake. Adult WKY rats received microinjections into the NTS of a short-hairpin RNA for the MR, to site-specifically reduce levels of the MR by RNA interference (shRNA; n=9), or scrambled RNA as a control (scRNA; n=8). After injection of the viral construct, aldosterone-filled osmotic mini-pumps were implanted subcutaneously and connected to a cannula extending into the 4th ventricle to infuse aldosterone at a rate of 25ng/h. Prior to and after surgeries, rats had ad libitum access to normal sodium (0.26%) rat chow and two graduated drinking bottles filled with either distilled water or 0.3M NaCl. Prior to surgeries, basal saline intake was 1.6ml ± 0.6ml in the scRNA group and 1.56ml ± 0.6ml in the shRNA group. 24 days post-surgery, saline intake was elevated to a greater extent in the scRNA group (5.9ml± 1.07ml) than in the shRNA group (2.41ml ± 0.6ml). Post-mortem immunohistochemistry revealed a significant reduction in the number of NTS neurons exhibiting immunoreactivity for MR in shRNA-injected rats (23 ± 1 cells/section) vs. scRNA-injected rats (33 ± 2 cells/section; p=.008). shRNA did not alter the level of HSD2 protein in the NTS as judged by the number of HSD2 immunoreactive neurons. These results suggest that 4th ventricular infusions of aldosterone stimulate saline intake and that this stimulation is at least in part mediated by hindbrain NTS neurons that express MR.

Key Words: Mineralocorticoid receptor, NTS, HSD2 neurons, sodium intake
**Introduction**

Sodium plays a very important role in maintaining electrolyte homeostasis (27). Sodium ingestion is often essential to restore lost body fluids. Excess sodium intake can lead to elevation in blood pressure and metabolic syndrome (3, 18). Many health organizations across the world suggest that hypertensive patients reduce their salt intake as a first line of defense in their anti-hypertensive therapy. Increased salt intake is also associated with oxidative stress (20). Furthermore, studies also show increased salt preference in heart failure patients, suggesting another link between salt intake and cardiovascular disease (9, 22).

The renin-angiotensin-aldosterone and vasopressin systems are important mechanisms by which the body maintains fluid and ion balance (12, 27). Aldosterone is released in response to falling concentrations of circulating sodium, as well as increased circulating concentrations of potassium and angiotensin II (7, 19). After being released, aldosterone binds the mineralocorticoid receptor (MR) to promote $\text{Na}^+$ and simultaneous water absorption and potassium excretion (6). This role of aldosterone in maintaining sodium balance was discovered long ago (23, 24), and in fact it was shown in the 1960’s that high doses of aldosterone can increase sodium intake even in the absence of sodium deficiency (30).

The MR has equal affinity for mineralocorticoids such as aldosterone as it does for glucocorticoids such as cortisol and corticosterone (4, 13). 11-beta-hydroxysteroid dehydrogenase type II (HSD2) is an enzyme that catalyzes the conversion of active cortisol to inert cortisone (1). This conversion plays an important role in MR-expressing cells by preventing binding of cortisol (a glucocorticoid) to the MR and allowing aldosterone to bind in its place.
Thus, co-expression of MR and HSD2 are the two key characteristics that define whether a cell is selective for aldosterone over corticosterone.

Despite numerous ongoing studies examining the mechanisms by which MR and aldosterone regulate renal function, the central mechanisms whereby aldosterone stimulates Na+ intake still remain unclear. Identifying the underlying neural circuitry responsible for modulating salt intake will aid in developing treatments that could specifically target key points in the regulatory cascades involved and prevent the deleterious cardiovascular effects of excess salt intake.

Previous studies have shown that MR is expressed in many CNS sites (5), but it is the presence of HSD2 that makes cells selective for aldosterone. Roland et al. in 1995, demonstrated that HSD2 mRNA is expressed in neurons within the nucleus tractus solitarius (NTS) (25). Adding to this, Geerling et al. in 2006 discovered a group of cells within the NTS which were immunoreactive for both MR and HSD2. These “HSD2 neurons” are situated in the NTS extending ventrally beneath the area postrema—an area with a diminished blood-brain barrier that allows easier influx of circulating aldosterone (14, 15).

It has been previously suggested that mechanisms driving the inhibition of salt appetite originate from the NTS. Right atrial stretch leads to activation of atrial mechanoreceptors and reduced salt intake, and NTS lesions increase salt intake (8, 21). Other studies have shown activation of HSD2 neurons in the NTS during sodium depletion, evidenced by increased immunoreactivity for c-fos—an early gene product of neuronal activation—suggesting a stimulatory role for these neurons during periods of sodium depletion (15). While these findings suggest that activation of NTS HSD2 neurons stimulates salt intake, a definitive role has yet to be established. An alternative hypothesis is that NTS HSD2 neurons function as part of a negative feedback circuit
that inhibits or limits sodium intake. Therefore, the goal of this study was to determine if NTS HSD2 neurons play a role in the mediation/modulation of salt intake.

**Materials and Methods**

**General:** Rats (Wistar-Kyoto WKY, Charles River Laboratories, Inc., Wilmington, MA) were allowed to acclimatize for at least one week after arrival in the animal facility maintained at 23°C with 12-hour light: 12-hour dark cycle (12L:12D, on at 7 AM, off at 7 PM), before any surgical procedure was performed. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Texas Health Science Center.

**AAV microinjections:** The adeno-associated viral vector with shRNA (short hairpin RNA) used to knock-down the MR (the construct sequence is AAV1/2-U6 Rat.Nr3c3/MR.shRNA terminator- CAG-EGFP-WPRE-BGH-polyA) and the scRNA (scrambled RNA, which acts as control) are commercially available (Catalog number: GD1009-RV; GeneDetect, New Zealand; previously cited by Xue et al.) (31). The U6 promoter drives expression of the shRNA and the CAG promoter drives expression of enhanced green fluorescent protein (EGFP). The CAG promoter consists of chicken β-actin promoter hybridized with the cytomegalovirus (CMV) immediate early enhancer sequence and is highly efficient in neurons. The Woodchuck post-transcriptional regulatory element (WPRE) and the presence of a bovine growth hormone (BGH) polyadenylation sequence ensure high transcription following transduction. Microinjections were performed in an aseptic environment as described previously (10, 29) to inject shRNA and scRNA into the NTS of WKY rats under 2% isoflurane anesthesia. To cover the rostral-caudal
extent of NTS HSD2 neurons, three injections of the viral construct (100 nl; > 1 x 10^{12} genomic particles/ml) were injected 0.5 mm below the surface at calamus scriptorius and bilaterally at 0.5 mm rostral and 0.5 mm lateral to calamus. The NTS regions affected by these injection parameters have been shown to contain the highest number of HSD2 immunoreactive neurons (5, 14, 15). Importantly, although the AAV construct freely transfects any neurons in the regions encompassed by injections and drives GFP synthesis, the shRNA only acts to knock down MR in neurons that synthesize MR.

**Osmotic mini-pumps:** Immediately after injection of the AAV constructs and while still under isoflurane anesthesia, osmotic mini-pumps (Alzet model 2004, 0.25μl/h) were implanted into a small pocket under the skin at the base of the neck. The pumps were connected to the free end of a 23 gauge cannula that had been extended into in the 4th ventricle using stereotaxic coordinates then cemented into place with dental acrylic and jeweler’s screws. Prior to implantation, pumps were filled with the MR receptor agonist aldosterone (Sigma, St.Louis, MO), dissolved in artificial cerebrospinal fluid (final concentration 100μg/ml). Infusions of aldosterone into the 4th ventricle occurred at a rate of 0.25μl/hr (25ng/hr).

**Measurement of saline intake:** Rats had *ad libitum* access to normal sodium (0.26%) rat chow. Two graduated glass bottles, one containing deionized water and one containing 0.3 M NaCl, were provided in the front of each cage and placed on opposite sides to prevent mixing of solutions. The position of the tubes was alternated every 24 h to control for placement preference and intake was measured to the nearest milliliter at noon every day.

**Immunohistochemistry:** To verify that the shRNA reduced MR levels in the NTS, rats were anesthetized with Inactin (100mg/kg IP, Sigma, St. Louis, MO) and transcardially perfused with
Brains were removed and stored in 4% paraformaldehyde at 4°C for 1-2 hours, then transferred into 30% sucrose solution. Each hindbrain was sectioned (40 μm thick) coronally into 3 sets and stored at -20°C in a cryoprotectant solution until processed for immunohistochemistry. Different sets of serial sections were processed either for the MR (Primary antibody: rMR-1-18 1D5, provided by Dr. Elise Gomez-Sanchez; 1:500. Secondary antibody: biotinylated anti mouse, Jackson immunoresearch laboratories Inc, PA; 1:1000.) or HSD2 (Primary antibody: Chemicon/Millipore, Billerica, MA. 1:20,000 and secondary antibody: biotinylated anti sheep, Jackson immunoresearch laboratories Inc, PA; 1:100). Tissue sections were incubated in primary antibody for 48 hrs at 4°C after a series of washes with phosphate buffered saline (PBS), then for 2 hrs at room temperature with secondary antibody. At this point, sections were reacted with an avidin-peroxidase conjugate (Vectastain ABC kit, PK-4000; Vector labs, Burlingame, CA, USA) and PBS containing 0.04% 3, 3’–diaminobenzidine hydrochloride and 0.04% nickel ammonium sulfate for 10-11 min. The tissues were then mounted on gelatin coated slides and cover slipped with Permount mounting medium (Fisher Scientific, NJ)

**Imaging and cell counts:** An Olympus microscope (BX41) equipped with epifluorescence and Olympus DP70 digital camera and DP Manager Software (version 2.2.1., Olympus, Tokyo, Japan) was used to capture images of the MR and HSD2 immunostaining. ImageJ software (v 1.44, NIH, Bethesda, MD, USA) was used to count the number of MR or HSD2 positive cells from each rat and expressed as an average number per section.

**Statistical analysis:** All data are presented as mean ± SE. Differences between the group injected with shRNA and the one injected with scRNA over several days were determined by 2-way analysis of variance (ANOVA) with repeated measures on time. A Holm-Sidak *post hoc* test
was performed to reveal any significant difference among mean values. Student t-test was conducted to compare the mean number of MR immunoreactive and HSD2 immunoreactive cells in the scRNA and shRNA groups. P < 0.05 was considered statistically significant.

**Results**

Prior to injections of the viral constructs into the NTS and implantation of 4th ventricular osmotic mini-pumps, the weights of shRNA-injected rats (n=9, 275±8 g) and scRNA-injected rats (n=8, 273±6 g) were not significantly different. Prior to sacrifice (28 days later) shRNA-injected rats still weighed approximately the same (323±6 g) as scRNA-injected rats (320±7 g) suggesting that the intake of sodium via the chow was not different between the 2 groups. Figure 1 illustrates the mean saline intake values of shRNA-injected and scRNA-injected rats during the period of the study. Days 1-4 represent saline intake preceding NTS injections and implantation of the aldosterone-filled osmotic mini-pumps. Basal saline intake was low in both groups prior to surgery (1.6ml ± 0.6ml in the scRNA group and 1.56ml ± 0.6ml in the shRNA group). On the first post-surgical day (day 5) saline intake began to increase in both groups indicating that the 4th ventricular infusion of aldosterone was stimulating saline intake. Saline intake remained elevated for the duration of the study in the scRNA-injected rats. However, the aldosterone stimulated saline appetite began to fall one week post-surgery in the shRNA–injected rats and remained lower than saline intake in the scRNA –injected rats for the duration of the study. Furthermore, this increased saline intake in scRNA compared to shRNA was sustained 24 days post-surgery (5.9ml± 1.07ml in the scRNA group and 2.41ml ± 0.6ml in the shRNA group).
Two-way ANOVA revealed significant difference in saline intake both as a function of group (shRNA vs. scRNA, p<.001) and as a function of time (p<.001). Post-hoc analysis (Holm-Sidak) revealed that in scRNA-injected rats, there was a significant increase in saline intake following implantation of the osmotic mini-pump compared to control (day 4), except on days 5,6,7,10,11,18,19 & 27 (p<.05). In shRNA injected rats, there was no significant increase in saline appetite compared to control on any day following implantation of the osmotic mini-pump. Water intake was not different between the groups.

Figure 2A depicts the robust difference in mean saline intake between scRNA-injected rats vs. those injected with shRNA during the period from 14-21 days after microinjections and osmotic minipump implantation. Figure 2B shows that the mean water intake of scRNA- and shRNA-injected groups during this same period after the microinjections and osmotic minipump implantation was not significantly different. Figure 2C illustrates the absence of any difference in total fluid intake between shRNA- and scRNA-injected groups during this time.

Immunohistochemistry was performed to visualize the reduction in apparent amount of MR within the NTS following shRNA injection (Figure 3). GFP expression and immunofluorescence (Figure 3A) marked a successful transfection of NTS neurons with the viral constructs, and MR immunoreactivity was predominantly nuclear (Figure 3C and 3D). The number of cells showing immunoreactivity for MR was greater in scRNA-injected brains vs. shRNA-injected brains (33 ± 2 cells/section and 23 ± 1 cells/section, respectively; p=.008). This significantly decreased number of MR immunoreactive neurons in shRNA-injected brains demonstrates the efficacy of the virus in successfully knocking down the MR.
Since HSD2 and MR are co-localized within the NTS (HSD2 neurons) (14, 15), we counted neurons demonstrating immunoreactivity for HSD2 in both the scRNA- and shRNA-injected groups (Figure 3E and 3F), and observed no difference between groups. This suggests that the observed reduction in MR immunoreactivity following injections of the shRNA constructs was not due to neurotoxicity. Figure 3B shows the comparison of NTS neurons per section exhibiting MR immunoreactivity (left bar graph; p<0.05) and HSD2-immunoreactivity (right bar graph) in rats injected with scRNA and rats injected with shRNA.

**Discussion:**

Stimulation of salt intake is understood to originate primarily from the structures of the hypothalamus and forebrain, whereas inhibition of salt intake is proposed to arise from a pathway between the NTS and parabrachial nucleus, which can be activated by right atrial stretch (8). The findings by Geerling and Loewy describing NTS neurons that contain both MR and HSD2 in 2006 (14, 15) generated a great deal of interest in how these neurons may play a role in the stimulation of salt intake (14). However this evidence is correlative and not demonstrative. They demonstrated that reductions in dietary sodium induced the expression of c-fos in NTS HSD2 neurons, while re-establishing access to dietary sodium reduced c-fos expression in NTS HSD2 neurons to basal levels (15). These changes in activity are consistent with a role for HSD2 neurons in the stimulation of salt intake during periods of sodium depletion. However, such responses are also consistent with a negative feedback system that serves to suppress sodium intake during periods when salt intake is increased.
To test the hypothesis that NTS neurons play a role in mediating stimulated saline intake we utilized shRNA to reduce the amount of MR in these cells, then stimulated saline intake by infusing aldosterone at the rate of 25ng/hr into the 4th ventricle. RNA-interference using viral-mediated shRNA delivery can reduce protein levels in a region of interest. shRNA integrates into the cell’s nucleic acid and silences genes in a sequence specific manner at the mRNA level by forming a RNA-induced silencing complex (RISC) after being cleaved into small interference RNA (siRNA) by ribonuclease III enzyme. The mechanism of shRNA-mediated gene silencing has been discussed in detail in a previous publication (28). Unlike, knockout models which are limited by developmental defects and lack of regional specificity, shRNA delivery through AAV can result in in vivo reduction of a selective gene in a precise region. Many groups, including ours, have successfully demonstrated the advantage and specificity of shRNA (2, 17, 26, 32).

Since cerebrospinal fluid flows rostral to caudal within the central ventricular system, the effects of 4th ventricular infusion should be restricted to the hindbrain. Our data indicate that increased saline intake during 4th ventricular infusions of aldosterone can be attributed to activation of the MR within the NTS, which lie near enough to the 4th ventricle for adequate diffusion of the drug, and also in an area which has an incomplete blood brain barrier (16).

Based upon the functional anatomical studies of Geerling and Loewy cited previously, we feel the neurons most likely to mediate the increased saline intake stimulated by 4th ventricular infusions of aldosterone are the NTS HSD2 neurons. However, it is important to consider that while the majority of NTS neurons that contain MR also contain HSD2, not all MR-containing NTS neurons contain HSD2. While non-HSD2 neurons would not be likely to respond to physiological levels of aldosterone due to occupancy of the MR by corticosterone, we cannot exclude the possibility that they may participate in the responses to 4th ventricular infusions of aldosterone.

A recent study by Formenti et al. support our results—they showed that 4th ventricular infusions of aldosterone increased the saline intake in Wistar Hanover rats in a dose-dependent fashion (11). The increase in the saline intake they observed after aldosterone infusion in their study is
quite higher (~40ml in response to infusion of aldosterone at the rate of 25ng/hr) than what we observed in the scrambled group (~6ml in response to infusion of aldosterone at the rate of 25ng/hr). They also reported decreased water intake in aldosterone-treated (10ng/hr) and vehicle-treated groups on 6th day after the minipump implantation, which was absent in our current study following a higher dose of aldosterone. One potential explanation for this discrepancy in water intake could be the difference in animal strain used. Also, Formenti et al. used a higher rate of infusion (2μl/hr) with low concentration of aldosterone (5μg/ml), while we used a higher concentration of aldosterone (100μg/ml) at a low rate of infusion (0.25μl/hr). Despite their higher rate of infusion, the amount of aldosterone accessible to the target neurons was higher in our study (0.25 ug/hr) than in the study of Formenti et al. (.01ug/hr). Therefore, at this time there is not an obvious explanation for the difference between the two studies.

The initial increase in saline intake observed following aldosterone infusion is similar between both experimental groups in our study. During the period of time when the shRNA is reported to have begun exerting its effects (i.e. 14-21 days post-injection), saline intake of the shRNA-injected group dropped significantly compared to the scRNA-injected group. Water intake did not differ between the two groups 14-21 days post-op, but the saline intake of shRNA-injected group was reduced. This demonstrates that the shRNA-mediated MR knockdown altered saline intake independently. Reducing NTS MR expression by this method did not exert any apparent effect on basal saline intake, however we noted that basal saline intake is very low in WKY (and in the Sprague-Dawley, unpublished observations, 2012).

MR knock-down in the NTS did not appear to modulate food intake, as weight gain during the protocol was not different in shRNA-injected rats vs. scRNA- injected rats. The expression of GFP in the NTS offered assurance that successful transfection was achieved in the desired
region. shRNA knock-down reduced the number of cells with MR immunoreactivity by 27%, but did not alter the number of cells with HSD2 immunoreactivity. Since MR and HSD2 are co-localized in most NTS HSD2 neurons, this suggests that the viral constructs had no obvious neurotoxic effects.

**Conclusion**

Reductions in the expression of MR within the rostral-caudal NTS do not appear to alter basal saline intake. However, infusion of aldosterone into the 4th ventricle evokes a prompt and dramatic increase in saline appetite that is suppressed by shRNA knock-down of MR. These viral constructs do not appear to exhibit any neurotoxicity, as the shRNA does not alter the number of HSD2 immunoreactive neurons. Since HSD2 and MR are co-localized in the NTS, these results indicate a reduction in MR level that is acting to reduce the increased saline appetite stimulated by 4th ventricular infusions of aldosterone.

**Perspectives**

Infusion of aldosterone into the 4th ventricle evoked a prompt and significant increase in saline appetite. Injection of a viral construct to reduce the expression of MR within the NTS attenuated the increase in saline appetite stimulated by 4th ventricular infusions of aldosterone. The constructs did not appear to have any toxicity as the shRNA did not alter the number of HSD2 immunoreactive neurons and HSD2 and MR are co-localized in the NTS. These results suggest that HSD2 neurons in the NTS are responsible for increased saline intake following stimulation by aldosterone.
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Conflicts of Interest/Disclosures

None
References:


Figure Legends

Figure 1: Saline intake before and after injections of viral constructs into the NTS

Days 1-4 represent saline intake prior to NTS injections of viral vectors and implantation of aldosterone-filled osmotic mini-pumps. Following implantation of the osmotic mini-pump there was a significant increase in saline intake in the scRNA group (n=8) compared to control while in shRNA group there is no significant change in saline intake. Comparing scRNA to shRNA injected rats, the saline intake of scRNA injected groups was significantly more than shRNA injected group after surgeries. Asterisks indicate the days on which the saline intake between the two groups was significantly different. (p<0.05)

Figure 2: Saline and water intake comparison between scRNA and shRNA injected groups

14 days after mini-pump implants and virus injections

A. Mean values of saline intake of scRNA and shRNA injected groups after mini-pump implantation. The shRNA injected group has significantly lower saline intake.

B. Mean values of water intake of scRNA and shRNA injected groups after mini-pump implantation. There was no significant difference between the two groups.

C. Total fluid intake of scRNA and shRNA injected groups after mini-pump implantation. There was no significant difference between the two groups.

Values are expressed as mean ± Standard error. Asterisk indicate p<0.05.
Figure 3: Expression of GFP (A), MR (C and D) and HSD2 (E and F) immunoreactivity in NTS of respective groups.

A. Expression of GFP in the NTS of a scRNA injected rat, indicating successful transfection of neurons with the viral construct.

B. Mean number of MR immunoreactive (left bar graph) and HSD2-immunoreactive (right bar graph) NTS neurons per section in scRNA and shRNA injected rats.

C. Section showing pseudo yellow colored MR immunoreactive neurons in the NTS of a scRNA injected rat. The majority of MR immunoreactivity is nuclear.

D. Section showing pseudo yellow colored MR immunoreactive neurons in the NTS of a shRNA injected rat. Number of MR immunoreactive neurons were less when compared to image in C.

E. Section showing pseudo red colored HSD2 immunoreactive neurons in the NTS of a scRNA injected rat. They lie in same region as neurons demonstrating MR immunoreactivity.

F. Section showing pseudo red colored HSD2 immunoreactive neurons in the NTS of a shRNA injected rat

c = central canal; scale bars = 100 µm. Asterisk denotes p < 0.05.
Figure 1
Figure 2

A

Saline intake (ml)

scRNA
shRNA

*  

B

Water intake (ml)

C

Total fluid intake (ml)

Figure 2