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The purpose of this study was to determine if neurons within the NTS that possess the mineralocorticoid receptor (MR) and the enzyme 11-β-hydroxy steroid dehydrogenase type II (HSD2) play a role in aldosterone stimulation of salt intake.

Adult WKY rats received microinjections of a short hairpin RNA for the MR or a scrambled RNA into the NTS and aldosterone-filled osmotic mini-pumps were implanted subcutaneously and connected to tubing within the 4th ventricle to infuse aldosterone at a rate of 25ng/h. Aldosterone infusion stimulated salt intake and MR knock down successfully reduced the aldosterone stimulated salt intake. Post-mortem immunohistochemistry revealed a significant reduction in the number of NTS neurons exhibiting immunoreactivity for the MR

Spontaneously hypertensive rats have a greater salt intake than WKY, therefore another study determined the correlation between the activity of HSD2 neurons, indicated by expression of c-fos and FosB, and increased salt intake in SHR. Very little evidence for co-localization of HSD2 and c-fos or FosB was found using immunohistochemistry and Western blotting.

The results indicate that HSD2 neurons in NTS can mediate the increased salt intake induced by aldosterone in the 4th ventricle. Increased activation of HSD2 neurons does not appear to account for the elevated salt intake observed in SHR.

ROLE OF MINERALOCORTICOID RECEPTOR OF THE NUCLEUS TRACTIS SOLITARIUS ON DRIVING SALT INTAKE

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THE ROLE OF MINERALOCORTICOID RECEPTOR OF THE NUCLEUS TRACTUS SOLITARIUS IN DRIVING SALT INTAKE.

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CHAPTER – I

THE OVERALL VIEW

Introduction:

The human body has several mechanisms to maintain fluid balance. Sodium plays a very important role in maintaining body's homeostasis (37, 50). Sodium ingestion is often essential to restore lost body fluids. High sodium intake can cause elevations in blood pressure and can also cause metabolic syndrome (4, 30, 37). Many health organizations across the world suggest hypertensive patients reduce their salt intake as part of therapy. Increased salt intake is also known to cause oxidative stress (33). Studies also show increased salt preference in heart failure patients (15, 44).

The renin-angiotensin-aldosterone and vasopressin systems are important systems in the body that maintains the fluid and electrolyte balance (34, 37). Aldosterone is released in response to low sodium levels, increased angiotensin II or increased potassium levels (13, 32). Aldosterone acts by binding to the Mineralocorticoid Receptor (MR) (12) which promotes Na^+ and simultaneous water absorption in the body (10). The MR is a nuclear receptor i.e. once bound by ligand they migrate into the nucleus of the cell and initiate the transcription of the responsible genes. The role of aldosterone in sodium balance was discovered long ago (11). It was shown back in 1960's that higher doses of aldosterone and other adrenal mineralocorticoids increase sodium intake even in the absence of sodium deficiency (53).

MR and HSD2

The marginal brain penetration of aldosterone through blood brain barrier (BBB) is low (42-43). Because of this the vast majority of cells in the brain are not sufficiently exposed to aldosterone. Also, the MR has equal affinity for aldosterone and for glucocorticoids cortisol (human) and corticosterone (rat) (2, 5, 49). They circulate at 1,000-fold higher (micromolar) concentrations compared to aldosterone. An important enzyme that plays a role in the binding of aldosterone to the MR is 11-beta-hydroxysteroid dehydrogenase type II (HSD2). This enzyme catalyzes the conversion of active cortisol and corticosterone to inert cortisone and 11-dehyroxycorticosterone respectively (1). This conversion helps in preventing binding of cortisol/corticosterone (glucocorticoid) to the MR. Thus HSD2 is a key to identify MR-expressing cells that are aldosterone sensitive (Figure1).

Neural Circuitry

Central mechanisms that regulate Na+ intake still remain unclear. Identifying the neural circuitry that is modulating salt intake will help in developing therapies that specifically target these sites in the brain. This would help in regulating salt intake which would in turn help in preventing the various disorders caused by increased salt intake.

Studies showed that MR is expressed in many CNS sites. However, the presence of HSD2 makes neurons aldosterone sensitive. Ronald et.al in 1995 showed that HSD2 is expressed in nucleus tractus solitarius (NTS), subcommissural organ and the ventromedial nucleus of the hypothalamus (8, 48). Geerling et.al in 2006 found a group of cells in the NTS which are immunoreactive for MR and HSD2 (21-22) (Figure 2). These neurons, termed HSD2 neurons, are situated in the NTS extending ventrally beneath the area postrema. This is an area with leaky Blood Brain Barrier (6, 25) and hence is accessible to circulating aldosterone.

Salt Intake in Hypertension

Spontaneously hypertensive rat (SHR) was obtained from a Wistar rat colony in Japan in early 1960's, by mating a hypertensive male Wistar rat to a female Wistar rat with blood pressure slightly higher than normal (41). Almost 100% of rats in the F_3 generation were spontaneously hypertensive. SHR is considered to be the genetic model of essential hypertension (46).In SHR the degree of hypertension depends upon salt intake. Evidence show that there is involvement of central nervous system in the salt sensitive hypertension (7). Several studies showed that SHR have high salt intake under normal conditions (17-18). Increased salt intake can cause an increase in hypertension in SHR (31). But still the reason behind the exaggerated salt intake in these animals remains unclear. In 2005, Geerling et al, observed an increased number of aldosterone sensitive neurons (i.e. neurons with HSD2 and MR) in the NTS region of Dahl salt sensitive rats (23), which is also a model of salt sensitive hypertension.

In the present study where the role of MR in stimulated salt intake was studied, SHR was used as the hypertensive model with exaggerated salt intake and WKY as the normotensive model.

Rationale for this study

There are studies which show that mechanisms that inhibit salt appetite can originate from the NTS such as, right atrial stretch and activation of atrial mechanoreceptors reduce salt intake while NTS lesions increase salt intake (14, 40). In 1997, Houpt et al observed c-fos-like immunoreactive neurons in NTS after salt intake in sodium deprived rats (29). The activity of NTS neurons (as seen by expression of c-fos) was found to be returning to normal when access to salt was restored after sodium depletion (35). Further studies by Geerling et al also showed that HSD2 neurons in the NTS are activated during sodium depletion (20). This was revealed by

the increased immunoreactivity for the immediate early gene product c-fos in the HSD2 neurons during the periods of sodium depletion. These findings suggest but do not prove, that activation of NTS HSD2 neurons stimulates salt intake, but their definitive role has yet to be established. It could just as easily be proposed that NTS HSD2 neurons function as negative feedback circuit that inhibits or limits sodium intake. Therefore the goal of present studies is to determine if NTS HSD2 neurons play a role in the mediation/modulation of salt intake.

Global Hypothesis

Aldosterone sensitive neurons in the Nucleus Tractus Solitarius, contribute to stimulated salt intake.

Specific Aims

1. To determine the effect of MR knock down on aldosterone stimulated salt intake inWKY
2. To determine whether the increased salt intake in SHR correlates with increased activity of HSD2 neurons.
3. To determine if MR mediates the increased salt intake in SHR.

Figures and Figure Legends

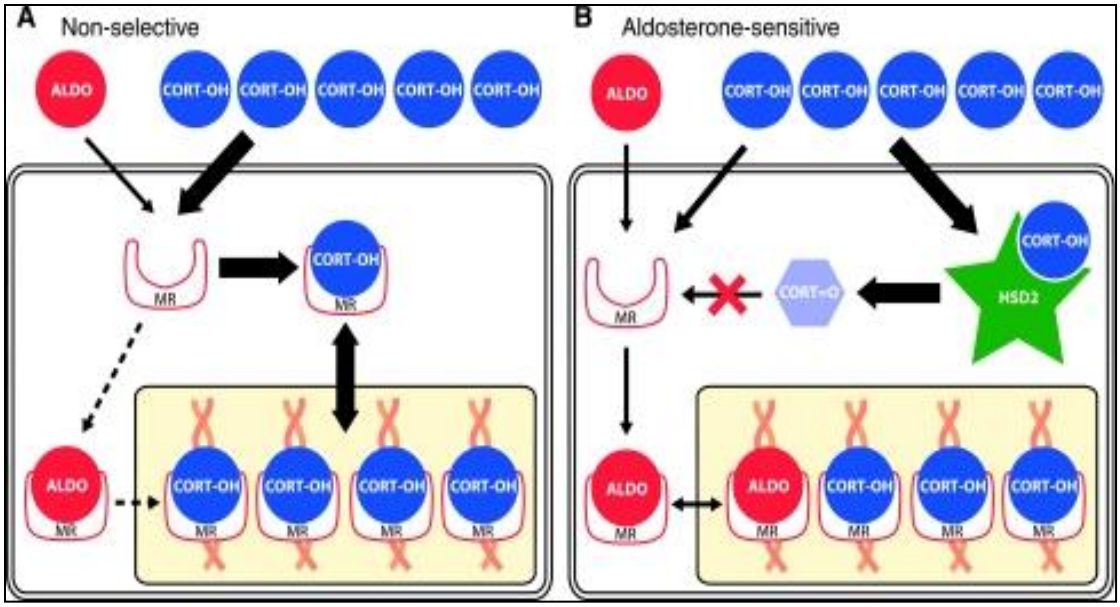

Figure 1: Mechanism of aldosterone selectivity in MR

A) Cortocosterones (CORT) circulates 1,000 fold higher concentrations than the aldosterone (ALDO). In most MR expressing cells, the basal occupation of this receptor is by glucocorticoids. (22)

B) In contrast cells with HSD2 can inactivate the CORT and increase the MR selectivity for Aldosterone. (22)

Figure 2 (21)**:**

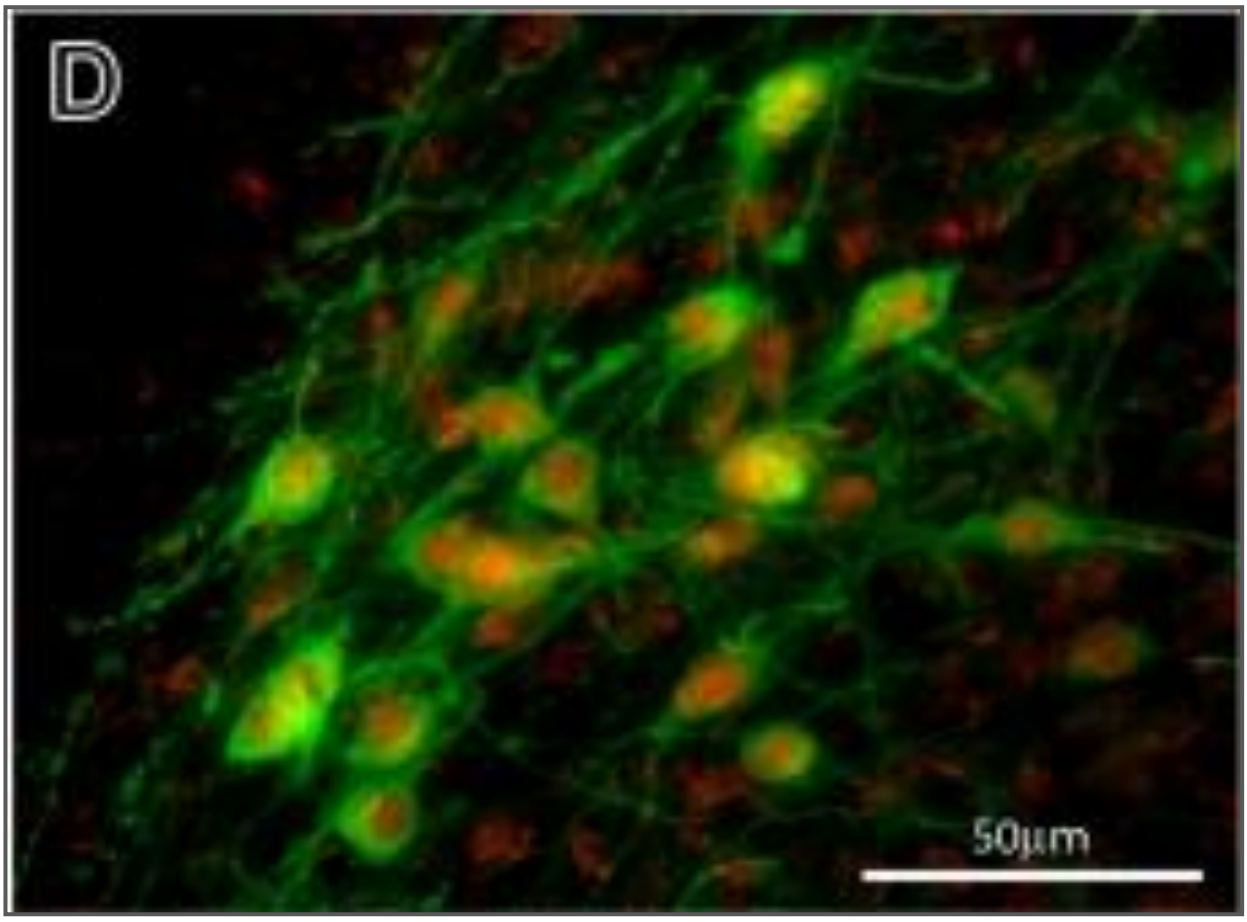

Figure 2: MR and HSD2 colocalization in NTS

Aldosterone sensitive cells in the NTS showing immune reactivity for both MR (Red) and also HSD2 (green) (21)

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CHAPTER – II

EFFECT OF MR KNOCK DOWN ON ALDOSTERONE STIMULATED SALT INTAKE IN WKY RATS

Introduction:

WKY and Sprague dawley rats have a low baseline salt intake (unpublished data). Aldosterone infusion into 4th ventricle can stimulate salt intake (19). The nucleus tractus solitarius (NTS) contains a group of neurons which express both the MR and HSD2 and have been hypothesized to be a site within the brain where aldosterone acts to stimulate salt intake.

To test the hypothesis that MR mediates the aldosterone stimulated salt intake, we injected short hairpin RNA (shRNA) and scrambled RNA (scRNA) for the MR into the NTS and then measured salt intake in response to 4th ventricular infusions of aldosterone. The shRNA is supposed to knockdown the MR protein, while the scRNA acts as a control.

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 12hour light: 12hour dark cycle, before any surgical procedures were 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: Adeno Associated Viral vector with shRNA was used to knock-down the MR (the construct sequence is AAV1/2-U6 Rat.Nr3c3/MR.shRNAterminator- CAG-EGFP-WPRE-BGH-polyA) and also the scRNA. Both are commercially available (Gene Detect, New Zealand; previously cited by Xue et al (54)). 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. A standard microinjection approach as described previously (16, 52) was used to inject shRNA and scRNA into the NTS of the WKY under 2% isoflurane anesthesia and aseptic conditions. To cover the rostral-caudal extent of NTS HSD2 neurons, three injections of 100 nl of the viral construct (> 1 x 10^{12} genomic particles/ml) were injected 0.5 mm below the surface at calamus and bilaterally at 0.5mm rostral and 0.5mm lateral to calamus. These NTS regions contain the highest number of NTS HSD2 immunoreactive neurons (22) . The AAV construct enters neurons in the regions of the injection and drives GFP synthesis; however the shRNA can only inhibit MR synthesis in those 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, 0.25 ml/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 23 gauge tubing placed in the 4th ventricle using stereotaxic coordinates then cemented into place with dental acrylic and jeweler's screws. Prior to implantation, the pumps were filled with aldosterone (Sigma, St.Louis, MO), the MR receptor agonist, dissolved in artificial cerebrospinal

fluid (final concentration 100µg/ml). Infusions of aldosterone into the 4th ventricle occurred at a rate of 25 ng/h (or 0.25ml/hr).

Measurement of salt intake: Rats had ad libitum access to normal sodium (0.26%) rat chow. Two calibrated 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. Intake was measured to the nearest milliliter at noon every day.

Immunohistochemistry: To verify if the shRNA reduced MR levels in the NTS, rats were anesthetized with Inactin (100mg/kg ip, Sigma, St. Louis, MO.) and transcardially perfused with 4% paraformaldehyde. Post fixation of brains for 1-2 hours was done, before cryoprotecting at 4°C in 30% sucrose solution. The hindbrains were sectioned (40 µm) coronally into 3 sets and stored at -20°C in cryoprotectant until processed for immunohistochemistry. Different sets of serial sections were processed either for the MR, using a primary antibody (Primary antibody: MR-18f, provided by Dr. Elise Gomez-Sanchez; 1:500 and 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). The tissues were incubated in primary antibody for 48hrs at 4^{o}C after a series of washes with PBS. After incubating for 2hrs at room temperature with secondary antibody, the 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 media (Fisher Scientific, NJ)

Imaging and Cell counts: Olympus microscope (BX41) equipped with epifluorescence and Olympus DP70 digital camera with DP manager software (version 2.2.1; Olympus, Tokyo, Japan) was used to image the MR or 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 were presented as mean ± SE. Differences between groups (shRNA and scRNA) and between days were determined by 2-way ANOVA with repeated measures. Holm-Sidak post hoc test was used to identify significant difference among mean values. Student t-test was conducted to determine the differences between the scRNA and shRNA group MR immunoreactive and HSD2 immunoreactive cell count means values. $P < 0.05$ 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. At the time of sacrifice (28 days later) shRNA-injected rats weighed 323±6 g and scRNA-injected rats weighed 320±7 g which were not significantly different.

Figure 1 illustrates the mean salt intake values of shRNA-injected and scRNA-injected rats during the period of the study. Days 1-4 are measures of salt intake preceding the NTS injections and implantation of the aldosterone-filled osmotic mini-pumps. Basal levels of salt intake were low (1.6ml ± 0.6ml in the scRNA group and 1.56ml ± 0.6ml in the shRNA group) in both groups prior to surgery. On the first post-surgical day (day 5) salt intake began to increase in both groups indicating that the infusion of aldosterone was stimulating salt intake. Salt intake

remained elevated for the duration of the study in the scRNA-injected rats. However, a week post-surgery the aldosterone stimulated salt intake began to fall in the shRNA–injected rats and remained lower than salt intake in the scRNA injected rats for the duration of the study. Salt intake was significantly greater in scRNA compared to shRNA (24 days post-surgery salt intake was 5.9ml± 1.07ml in the scRNA group and 2.41ml ± 0.6ml in the shRNA group).

Two-way ANOVA indicated a significant source of variation as a function of group (shRNA vs. scRNA, $p<.001$) and as a function of day ($p<.001$). Post-hoc analysis (Holm-Sidak) revealed that in scRNA injected rats, there was a significant increase in salt intake following implantation of the osmotic mini-pump compared to control (day 4) except on days 5,6,7,10,11,18,19 & 27. In shRNA injected rats, following implantation of the osmotic mini-pump there was no significant increase in salt appetite compared to control on any day.

Water intake was not significantly different between the groups. Figure 2A shows the mean values of water intake in shRNA and scRNA for 14-21 days after surgery.. Figure 2B depicts the mean values of salt intake of shRNA and scRNA injected groups 14-21 days after surgeries, which were significantly different. Also the total fluid intake (Figure 2C) was not different between the two groups 14 – 21 days after microinjections. Therefore it is only the amount of saline intake that altered between the two groups.

Immunohistochemistry was performed to verify that the shRNA injections reduced the apparent amount of MR in the NTS (Figure 3). GFP expression (Figure 3A) shows successful transfection of NTS neurons with the viral constructs. MR immunoreactivity was predominantly nuclear (Figure 3B). The number of cells showing immunoreactivity for MR was significantly different between shRNA and scRNA injected groups (scRNA 33 ± 2 cells/section; shRNA 23 ± 1

cells/section; p=.008). Significantly fewer MR immunoreactive neurons in shRNA injected group indicate that the virus successfully reduced the MR.

Since within the NTS, HSD2 and MR are co-localized (HSD2 neurons) (20-21), neurons demonstrating immunoreactivity for HSD2 in both shRNA and scRNA injected groups (Figure 3C) were counted. The number of HSD2 neurons was not significantly different among the two groups. This suggests that the observed reduction in immunoreactivity following injections of the constructs was not due to a toxic effect on the neurons. Figure 3D shows the quantification of the number of NTS neurons per section exhibiting MR immunoreactivity (left bar graph) and HSD2-immunoreactivity (right bar graph) in rats injected with scRNA and rats injected with shRNA.

Discussion

Stimulated salt intake has been considered to result primarily from the structures located in the hypothalamus and forebrain with inhibition of salt intake arising from a pathway between the NTS and the parabrachial nucleus which can be activated by right atrial stretch (14). The finding by Geerling and Loewy of NTS neurons that contain both the MR and the enzyme HSD2 in 2006 (20-21), generated a great deal of interest. These studies of Geerling and Loewy suggest that NTS HSD2 neurons may play a role in the stimulation of salt intake (20). However this evidence is correlative and not demonstrative. For example, reductions in dietary sodium induce the expression of the immediate early gene c-fos in NTS neurons and re-establishing access to dietary sodium reduces c-fos expression in NTS HSD2 neurons to basal levels (21). These responses are consistent with a role for NTS 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 prevent excessive sodium intake during periods when salt intake is increased.

To test the hypothesis that NTS HSD2 neurons play a role in mediating stimulated salt intake we utilized shRNA to reduce the amount of MR in NTS HSD2 neurons. The animals were either injected with shRNA or scRNA. The group injected with scRNA served as the control for the shRNA injected group. The results demonstrate that salt intake is significantly stimulated by infusion of aldosterone at the rate of 25ng/hr into the 4th ventricle. Since the flow of cerebrospinal fluid is rostral to caudal within the central ventricular system, 4th ventricular infusions should produce effects restricted to actions at hindbrain sites. In the present study, the increase in salt intake during 4th ventricular infusions of aldosterone can be attributed due to activation of the MR within the NTS, which are present close to the 4^{th} ventricle and also in a area which has an incomplete blood brain barrier (22).

The observations from a recent study by Formenti et al support our results(19). They showed that 4^{th} ventricular infusions of aldosterone increased the salt intake in Wistar Hanover rats in a dose dependant fashion. The amount of increase in the salt intake after aldosterone infusion in their study is quite higher (~40ml in response to infusion of aldosterone at the rate of 10ng/hr) than we observed in the scrambled group (~6ml in response to infusion of aldosterone at the rate of 25ng/hr). They also observed a significant decrease in water intake in aldosterone (10ng/hr) and vehicle treated groups on 6^{th} day after the minipump implantation. But in the present study, there was no significant decrease in water intake in response to aldosterone stimulation of salt intake (25ng/hr). This can be because of the strain variation in the experimental model. Also, they 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). It is possible, because of their higher rate of infusion the amount of aldosterone accessible to the target neurons is high compared to our study.

The initial increase in salt intake in response to aldosterone infusion is similar between the two groups. As the shRNA begins to exert its effect, the salt intake of the shRNA group dropped significantly compared to the scRNA group. Water intake did not differ between the two groups, during 14-21 days after the surgery but the salt intake of shRNA group was reduced. This further proves that the MR knockdown altered the salt intake alone. Our study design cannot determine the effect of reducing NTS MR levels on basal salt intake. However basal salt intake is very low in WKY (and in the Sprague-Dawley, unpublished observations, 2012).

MR in the NTS does not appear to modulate food intake as weight gain during the protocol was not different in shRNA-injected rats compared to scRNA- injected rats. The expression of GFP in the NTS, showed a successful transfection in the desired region. shRNA 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 toxic effects; that is NTS HSD2 neurons were not killed but rather had reduced expression of MR.

Conclusion

Reductions in the level of MR within the NTS do not appear to alter basal levels of salt intake, although under control conditions basal salt intake is very low in WKY (and in Sprague-Dawley rats, unpublished observations). Infusion of aldosterone into the 4^{th} ventricle evokes a prompt and dramatic increase in salt appetite. The constructs do not appear to have any toxicity 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. Reductions in the level of MR within the NTS reduce the increased salt appetite stimulated by 4^{th} ventricular infusions of aldosterone.

Figures & Figure Legends

Figure 1:

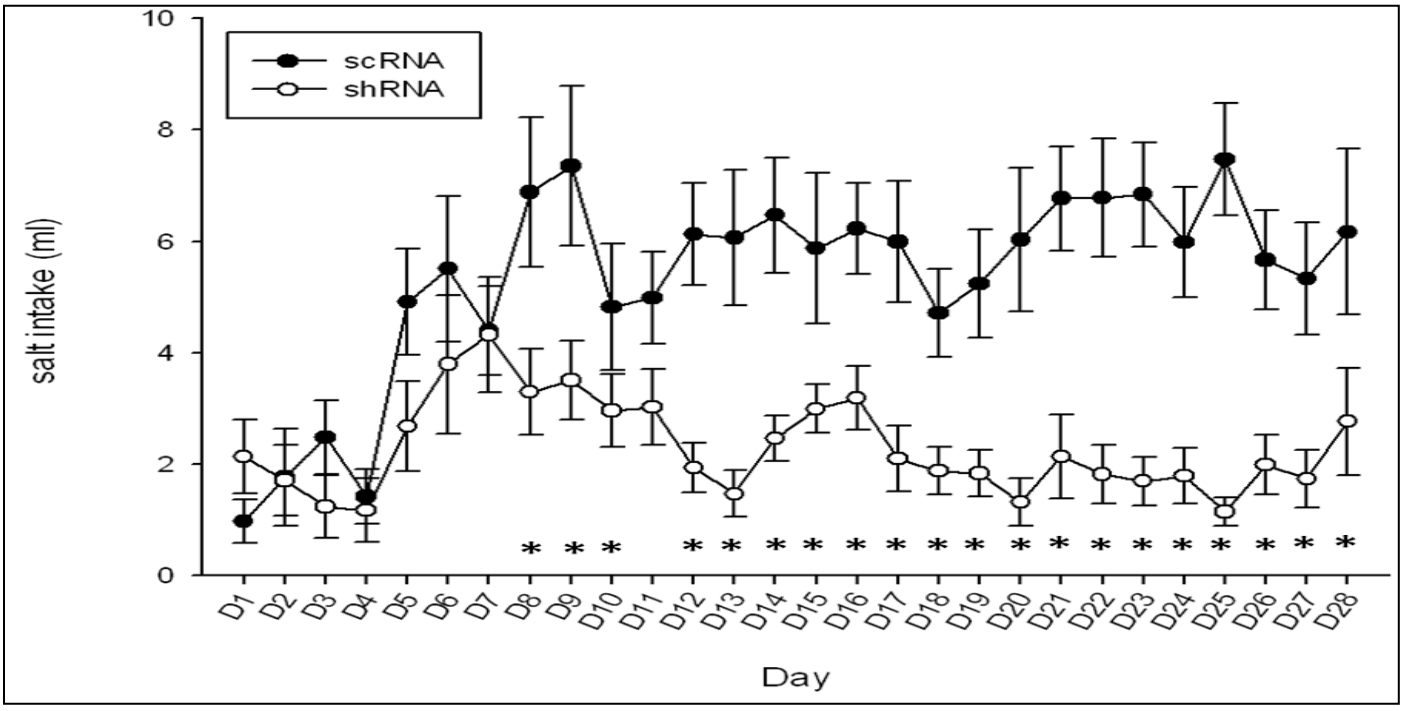

Figure 1: Salt intake before and after injections of viral constructs into the NTS

Days 1-4 represent salt 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 salt intake in the scRNA group (n=8) compared to control on all days except days 1,3,6,6,15,21 while in shRNA group there is no significant change in salt intake. Comparing scRNA to shRNA injected rats, the salt intake of scRNA injected groups was significantly more than shRNA injected group after surgeries. Asterisks indicate the days on which the salt intake between the two groups was significantly different. ($p<0.05$)

Figure 2:

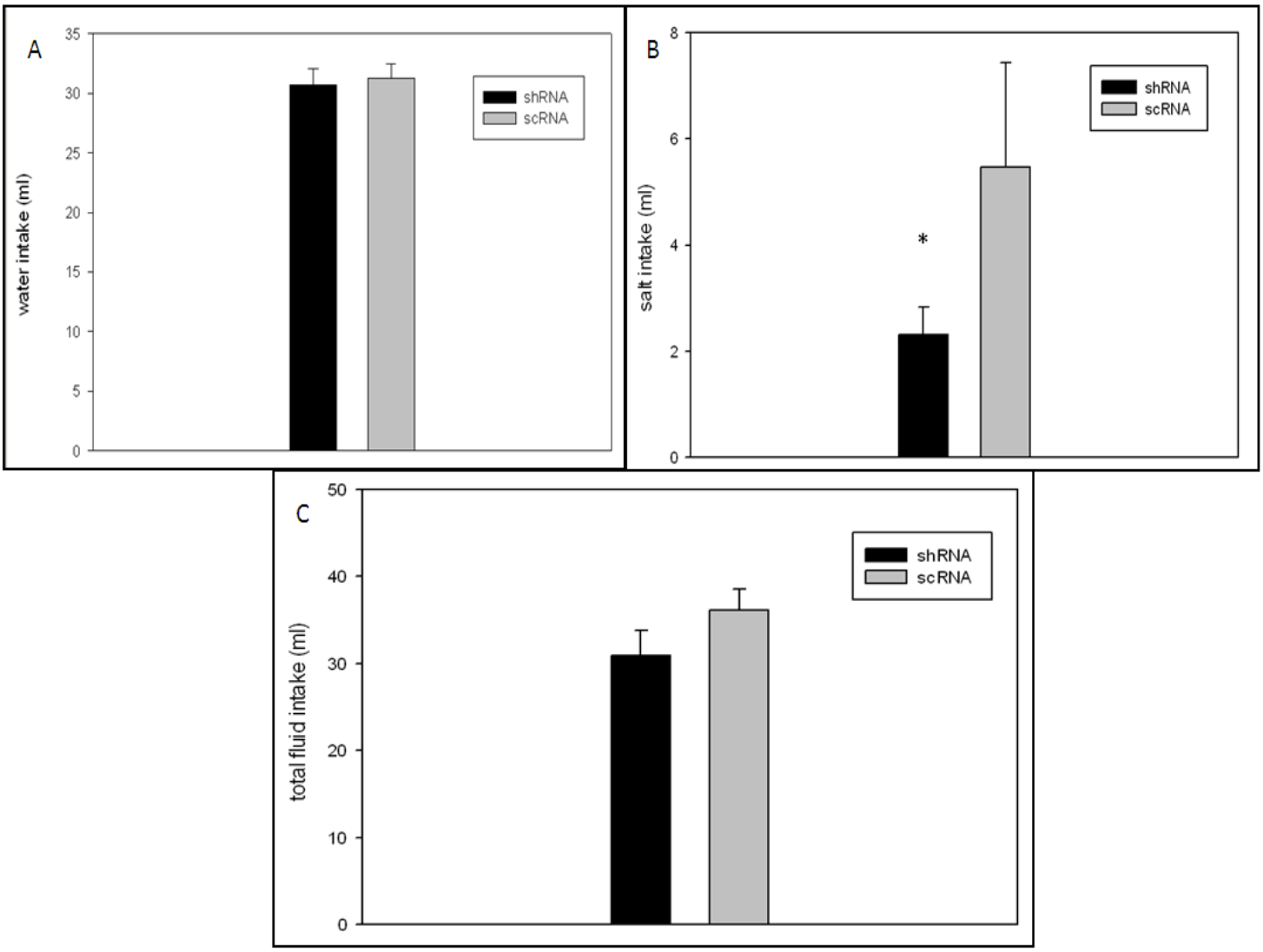

Figure 2: salt and water intake comparison between shRNA and scRNA injected groups after mini-pump implants

A. Mean values of water intake (per day) of shRNA and scRNA injected groups from 14-21 days after microinjections. The scRNA injected group has significantly more of salt intake.

B. Mean values of salt intake (per day) of shRNA and scRNA injected groups 14-21 days after mini-pump implantation. There was no significant difference between the two groups.

C. Mean values of total fluid intake (per day) of shRNA and scRNA injected groups 14 – 21 days after microinjections. The total fluid intake was not significantly different between the groups.

Values are expressed as mean ± Standard error. Asterisk indicate $p<0.05$.

Figure 3:

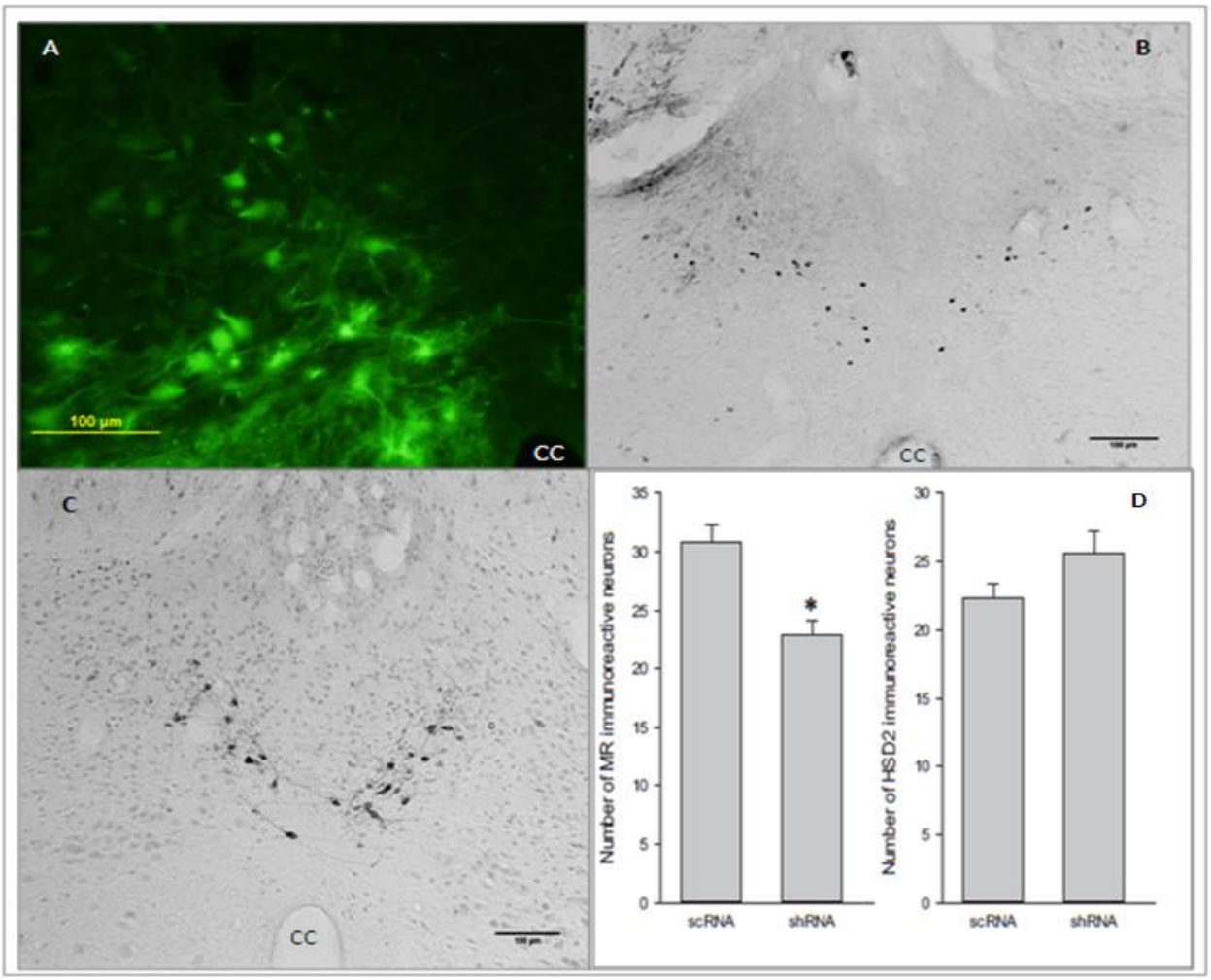

Figure 3: Expression of GFP (A), MR (B) and HSD2 (C) immunoreactivity.

A. Expression of GFP in the NTS indicating successful transfection of neurons with the viral construct.

B. Section showing MR immunoreactive neurons in the NTS. The majority of MR immunoreactivity is nuclear.

C. Section showing HSD2 immunoreactive neurons in the NTS. They lie in same region as neurons demonstrating MR immunoreactivity.

D. Mean number of MR immunoreactive (left bar graph) and HSD2-immunoreactive (right bar graph) NTS neurons per section in scRNA and shRNA injected rats. Sections taken from rat injected with scRNA for the MR. cc = central canal; scale bars = 100 µm. Asterisk denotes $p < 0.05$.

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CHAPTER III

To Determine Whether the Increased Salt Intake in SHR Correlates With Change in the Activity of HSD2 Neurons

Introduction

The spontaneously hypertensive rat (SHR) is considered to be a genetic model of essential hypertension (46). SHR was obtained from a Wistar rat colony in Japan in early 1960's, when investigators mated a hypertensive male Wistar rat to a female Wistar rat with blood pressure slightly higher than normal(41). SHR has also evolved as a model of salt sensitive hypertension. Several studies have shown that SHR have a higher salt intake than WKY under normal conditions (17-18).

Neurons in the NTS were activated during sodium depletion (29, 35). In 2006 Geerling et al showed that the NTS HSD2 neurons had increased immunoreactivity for the immediate early gene product c-fos in the HSD2 neurons during the periods of sodium depletion. To date the mechanism behind the exaggerated salt intake in SHRs remains unclear.

C-fos and FosB belong to the Fos family of proteins. Fos family proteins form active AP-1 (activator protein -1) transcription factors by heterodimerizing with Jun family proteins (c-Jun, JunB, or JunD). These transcription factors bind to AP-1 sites (consensus sequence: TGAC/GTCA) present in the promoters of certain genes to regulate their transcription. C-fos is an immediate early gene that is used as a marker of neuronal activity under acute stress (27-28)

while FosB is an indicator of activation of neurons in response to chronic stress. Chronic stimulation or stress causes expression of FosB which have a longer time course and stable expression (26, 36, 38-39).

The present study was designed to see if there is a correlation between HSD2 neuronal activity (as indicated by expression of either c-fos or FosB) and the enhanced salt intake in the SHRs.

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 12hour light: 12hour dark cycle before any surgical procedures were performed. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Texas Health Science Center.

Measurement of Salt Intake:

Two groups of animals, WKY (n=5) and SHR (n=5) were housed in cages and had ad libitum access to normal sodium (0.26%) rat chow. Two calibrated 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. Intake was measured to the nearest milliliter at noon every day. Percent saline intake was calculated as volume of saline intake divided by total fluid intake.

$$\% \text{ Saline Intake} = [\text{NaCl intake} / (\text{NaCl} + \text{water intake})] \times 100.$$

Salt intake was measured for 2 weeks.

Immunohistochemistry: To see if the increased salt intake in SHR correlates with the activity of the HSD2 neurons, we performed immunohistochemical studies for HSD2 and c-fos/FosB in the NTS sections of the hind brain of these animals. Rats were anesthetized with Inactin (100mg/kg ip, Sigma, St. Louis, MO.) and transcardially perfused with 4% paraformaldehyde. Post fixation of brains for 1-2 hours was done, before cryoprotecting at 4°C in 30% sucrose solution. The hindbrains were sectioned (40 µm) coronally into 3 sets and stored at -20°C in cryoprotectant until processed for immunohistochemistry. Same set of sections from all the animals were processed for double immunolabelling of either HSD2 and c-Fos or HSD2 and FosB.

For the double immunolabelling of HSD2 (Chemicon/Millipore, Billerica, MA, 1:20,000) and c-fos (For c-fos - Rabbit anti-c-Fos Ab5; Calbiochem, San Diego, CA, 1:30,000), hindbrain sections (40µm) were first incubated in primary antibody cocktail for 48hrs at 4^{o}C. After series of washes with PBS, the sections were incubated in secondary antibody for HSD2 (Cy3 conjugated anti sheep, Jackson immunoresearch laboratories Inc, PA; 1:800) for 2hrs at room temperature. After washing with PBS, the tissues were then incubated for 2 hrs in secondary antibody for c-fos (Cy2 conjugated anti rabbit Jackson immunoresearch laboratories Inc, PA; 1:100). After a series of washes with PBS, the tissues were then mounted on gelatin coated slides and coverslipped with VECTASHIELD® Mounting Media (Vector Laboratories, Burlingame, CA).

For the double immunolabelling of HSD2 and FosB, the tissues were washed with PBS and then incubated in 1% H2O2 for 2 hrs at room temperature. The tissues were then incubated in a primary antibody cocktail against HSD2 (Chemicon/Millipore, Billerica, MA, 1:40,000) and FosB (1:5000, FosB goat sc-48-G, Santa Cruz biotechnology, CA, USA) for 48hrs at 4^{o}C. After

series of washes with PBS, the sections were incubated in secondary antibody for FosB (Biotinylated anti goat, Jackson immunoresearch laboratories Inc, PA; 1:100) for 2hrs at room temperature. After washing with PBS, the 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. Then the tissues were then incubated for 2 hrs in secondary antibody for HSD2 (Cy3 conjugated anti rabbit Jackson immunoresearch laboratories Inc, PA; 1:800). After a series of washes with PBS, the tissues were then mounted on gelatin coated slides and coverslipped with permount mounting media (Fisher Scientific, NJ)

Imaging and Cell counts: Olympus microscope (BX41) equipped with epifluorescence and Olympus DP70 digital camera with DP manager software (version 2.2.1; Olympus, Tokyo, Japan) was used to image the HSD2 and c-fos/ HSD2 and FosB immunostaining. ImageJ software (v 1.44, NIH, Bethesda, MD, USA) was used to count the number of immunopositive cells from each rat.

Western Blots: Western blot analysis was carried out to determine if there is a difference in the amount of HSD2 in SHR and WKY groups. Salt intake was measured as mentioned above for two groups of animals, WKY (n=5) and SHR (n=5). The animals were then sacrificed and their hindbrains were snapfreezed in ice-cold Isopentane. NTS punches were collected from each rat and stores at -80^{o}C. Protein was extracted from the NTS punches by treating the tissue with a mixture of lysis buffer and sigma inhibitor. The solution was then homogenized and centrifuged at 14000 RPM at 4^{o}C for 25 min. The supernatant was collected and the pellet was discarded.

The protein concentration of each sample was determined using the Bradford Assay. Various concentrations (ranging from 0µg/ml to 2.5µg/ml) of diluted albumin standards (BSA) were

used. The samples and the standards were filled in duplicates in a 96 well plate. To each well, 250µl of Bradford reagent (Sigma, St. Louis, MO) was added and the protein concentration was determined based on the color intensity on a spectrophotometer (xMark™ microplate spectrophotometer, Bio-Rad, Hercules, CA) and the plate reader software (Microplate Manager® 6 software). The protein concentration was normalized with the BSA standards. The sample was then denatured by heating at 95^{o}C on a water bath for 5 minutes.

SDS page was run using 12% gel loaded with different samples from rats. Blue marker (All Blue, BioRad, Hercules, CA) was also loaded into the gel to locate the protein of interest. After running the gel, the protein in the gel was transferred on to a nitrocellulose membrane (BioRad, Hercules, CA). After blocking in TBS-Tween 5% non-fat milk, the membranes were incubated over night with primary antibody against HSD2 (Chemicon/Millipore, Billerica, MA, 1:1500). After rinse, the membrane was incubated in secondary antibody (1:5000, Anti sheep secondary antibody, Sigma, st.louis, MO) for 45 minutes.

The membrane was then incubated in stripping buffer at room temperature for 30 min. After blocking in TBS-Tween 5% non-fat milk, the membranes were incubated over night with primary antibody against GAPDH (1:2000, Anti GAPDH, clone 6C5/MAB374, Chemicon (Millipore), Temecula, CA). After rinse, the membrane was incubated in secondary antibody (Anti mouse secondary antibody (1:5000, Sigma, St.Louis, MO) for 45 minutes. The detection system used was enhanced chemiluminescence (ECL reagents, Amersham). The detection signal was developed digitally using software from Syngene (G:Box, GeneSnap; Syngene). Densitometry of the bands of interest was performed by Image J (v 1.44, NIH, Bethesda, MD, USA), and GAPDH was used in normalizing each sample.

Statistical Analysis:

All data were presented as mean ± SE. Differences in salt intake between groups (SHR and WKY) was determined by 2-way ANOVA with repeated measures. Student t-test was conducted to determine the differences between the WKY and SHR group HSD2 and c-fos/FosB cell count means values. $P < 0.05$ was considered statistically significant.

Results:

Salt intake of WKY and SHR was measured for 13 days. The salt intake of SHR was significantly higher than the WKY on the days (Figure 1). Figure 2 shows the quantification of the salt intake of the two groups.

Double immunolabelling for HSD2 and c-fos was performed. Figure 3 shows the immuno reactivity for HSD2 (red) and c-fos which is a neuronal activity marker (green) in both WKY and SHR. In Figure 3A and 3C, the HSD2 immunoreactive cells in WKY and SHR were shown. Figures 3B and 3D show the c-fos immunoreactive cells in WKY and SHR. These were small number c-fos immunoreactive cells in the NTS. c-fos positive cells do not colocalize with HSD2 positive cells.

Double immunolabelling of HSD2 and FosB was done to see if the HSD2 neuronal activity can be understood using this chronic neuronal activity marker. Figure 4A and 4C shows the HSD2 positive cells in WKY and SHR. The cell count of HSD2 positive neurons revealed significantly fewer HSD2 positive cells in the SHR group (27±3 cells/section in WKY and 14±1 cells/section in SHR group; Figure 5). FosB immunoreactive cells in the NTS did not colocalize with the HSD2 positive cells. Figures 4B and 4D show the FosB immunoreactive neurons in WKY and SHR.

To further confirm about the difference in the levels of HSD2 in both groups, Western blot analysis was performed. Figure 6 shows the western blots for HSD2 in WKY and SHR. GAPDH was used as the housekeeping gene. Figure 6A shows the bands for HSD2 (top) and GAPDH (bottom) in WKY and 6B shows the HSD2 (top) and GAPDH (bottom) in SHR group. Bands were analyzed using densitometry and the band density is normalized against that of GAPDH. In figure 6C, the quantification of the normalized band densities (mean) of HSD2 in both groups can be seen. This shows that there is no significant difference in the protein levels of HSD2 between the two groups.

Discussion:

NTS HSD2 neurons are activated during salt depletion (20). This study showed that the neurons expressed c-fos, which is a neuronal activity marker, when the animals were deprived of salt. The c-fos expression in the HSD2 neurons was reduced once the salt was restored.

SHR were known to have an exaggerated salt intake under normal conditions (17-18). In this study the hypothesis that the exaggerated salt intake in SHRs correlate with the activity of HSD2 neurons was tested. For this purpose, double immunolabelling was done for HSD2 and c-fos in WKY and SHR groups. The labeling of c-fos in the NTS was sparse and it did not colocalize with HSD2. As a second try, FosB which is a chronic activity marker was chosen to test for the neuronal activity. The double immunolabelling of HSD2 and FosB also did not show any colocalization. Therefore the acute and chronic activity markers used to test the hypothesis did not show any colocalization with HSD2. Hence it appears that the activity of HSD2 neurons does not correlate to the exaggerated salt intake of SHR.

But counting the cells that are immunoreactive for HSD2 in both groups, showed that SHRs have significantly low number of HSD2 positive cells in SHR compared to WKY. HSD2 is the

enzyme that makes MR specific for Aldosterone. If HSD2 is present in less number, it is possible that the MR is being activated by both Aldosterone and cortisol/corticosterone. This might cause more activation of MR and there by more salt intake. However, counting the MR and HSD2 colocalized cells would help in even better understanding of these results. But unfortunately, we did not perform a double immunolabelling technique in this set of animals.

Western blot analysis was done on NTS punches to compare the HSD2 protein levels in the NTS of both the groups. In contrast to what was observed with cell counts before, this did not show a significant difference in the levels of HSD2 between the two groups. It can be possible that there is some amount of HSD2 synthesized and detectable by western blot, but because of its low amount of expression, it was not detected by immunohistochemistry.

Conclusion:

The salt intake of SHR is significantly greater than WKY. There was no correlation between activity of HSD2 neurons and the elevated salt intake during hypertensive conditions as observed by the expression of c-fos/FosB. The number of cells positive for HSD2 was significantly less in SHR compared to the WKY group.

Figures & Figure Legends:

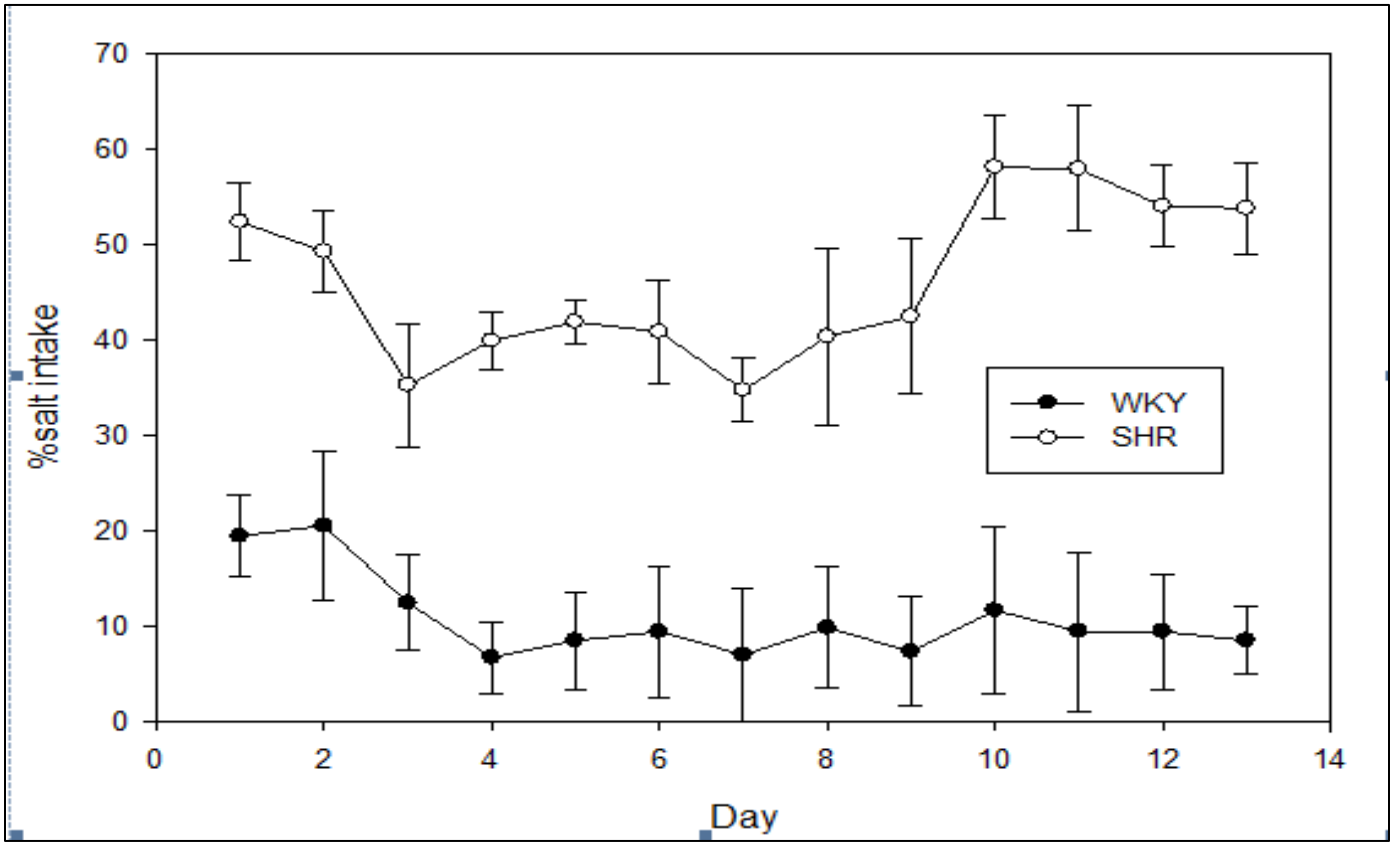

Figure 1: Salt intake of WKY and SHR

Salt intake of WKY (n=5) and SHR (n=5) groups over a period of 2 weeks. SHR have significantly higher salt intake at base level compared to WKY. All values are expressed as mean± standard error.

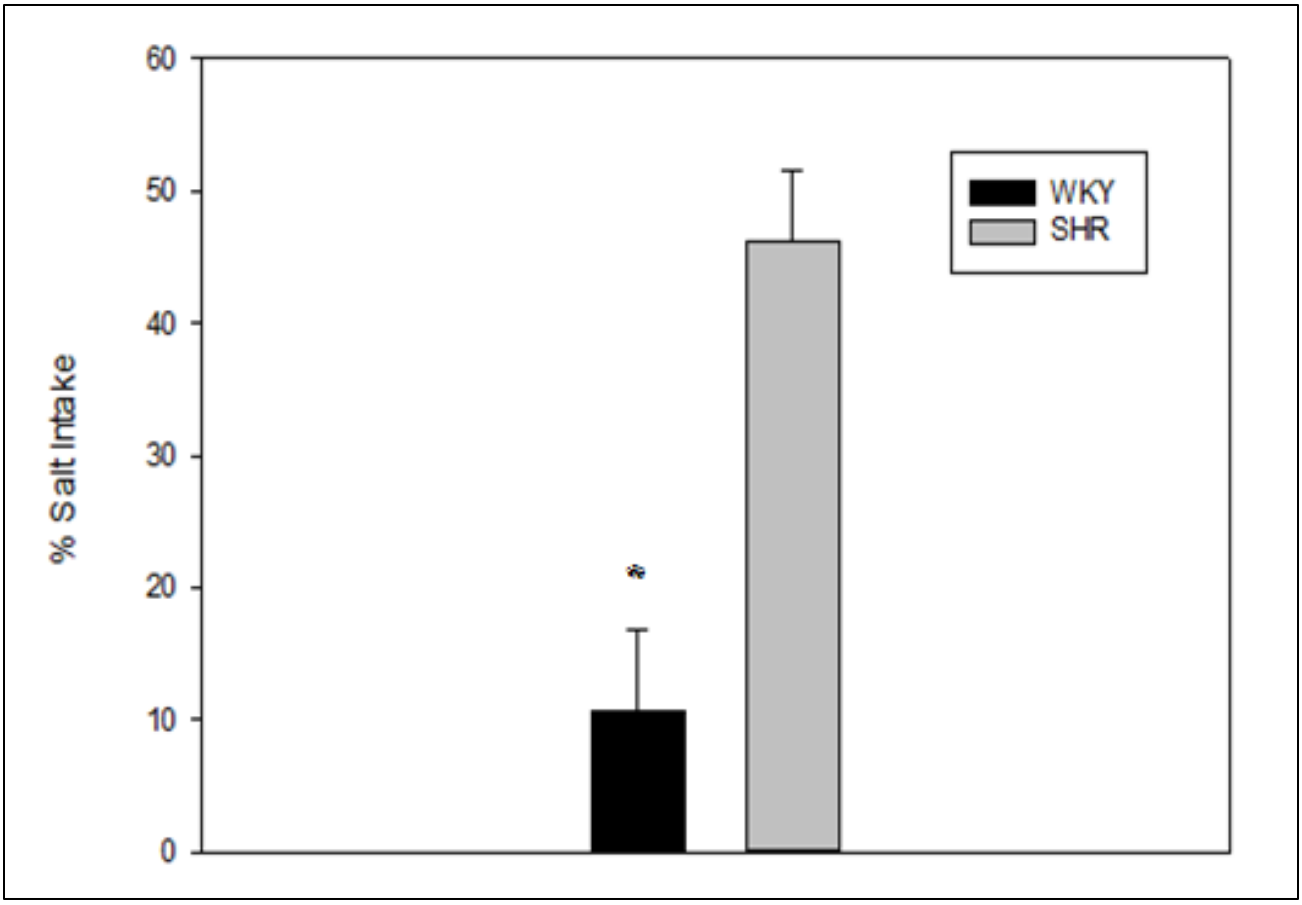

Figure 2: Comparison of Salt intake between WKY and SHR

Bar graph showing difference in salt intake between WKY and SHR groups. SHR has significantly high salt intake than WKY. A t-test revealed a p value <0.001. All the values were expressed as mean± standard error.

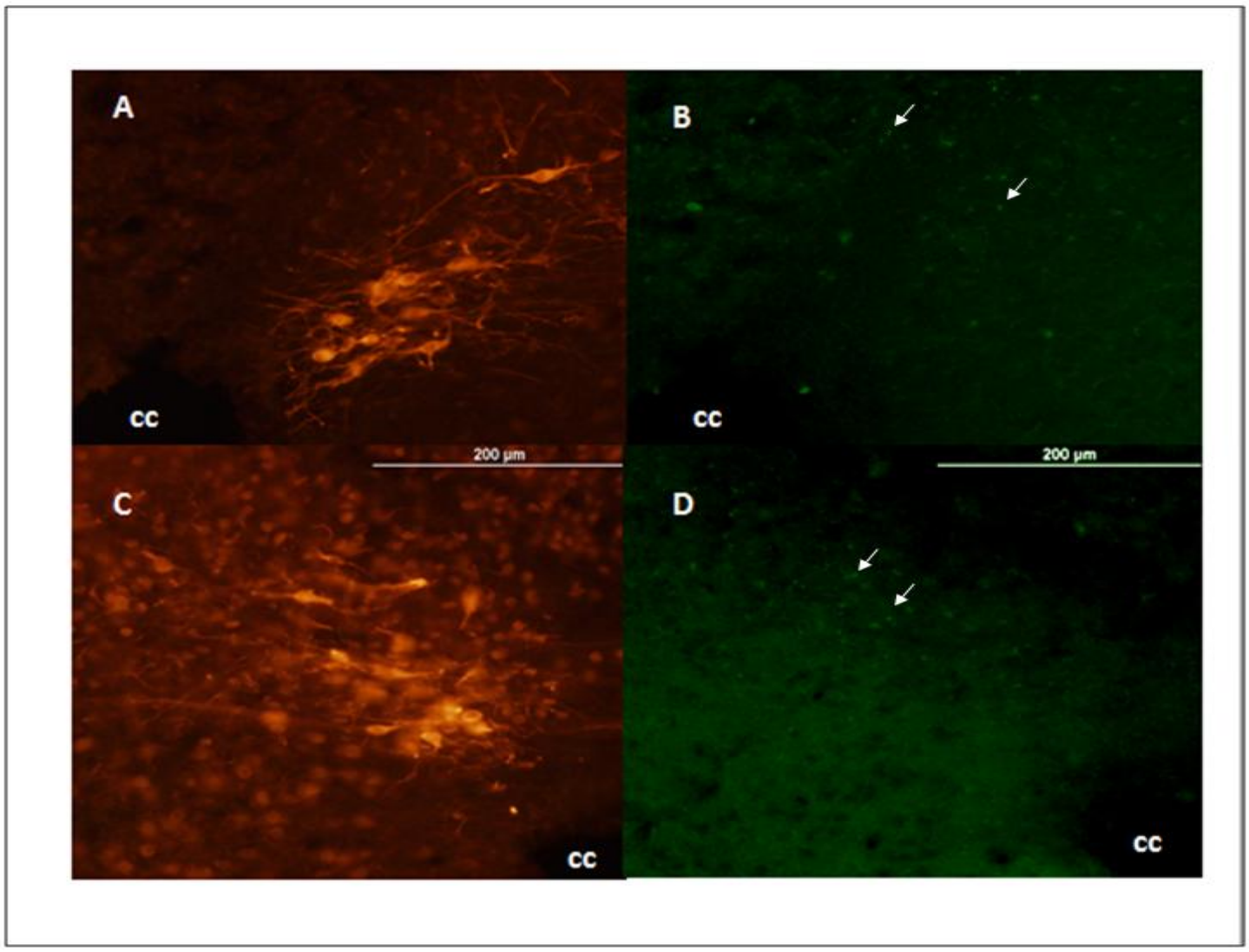

Figure 3: HSD2 and c-fos double immune staining

Double immunolabelling for HSD2 and c-fos.

A) HSD2 positive cells in WKY.

B) C-fos positive cells in WKY.

C) HSD2 positive cells in SHR.

D) c-fos positie cells in SHR. In both the groups, c-fos staining was seen in NTS region but the HSD2 and c-fos positive cells did not colocalize. Arrows point to c – fos or FosB immunoreactive cells

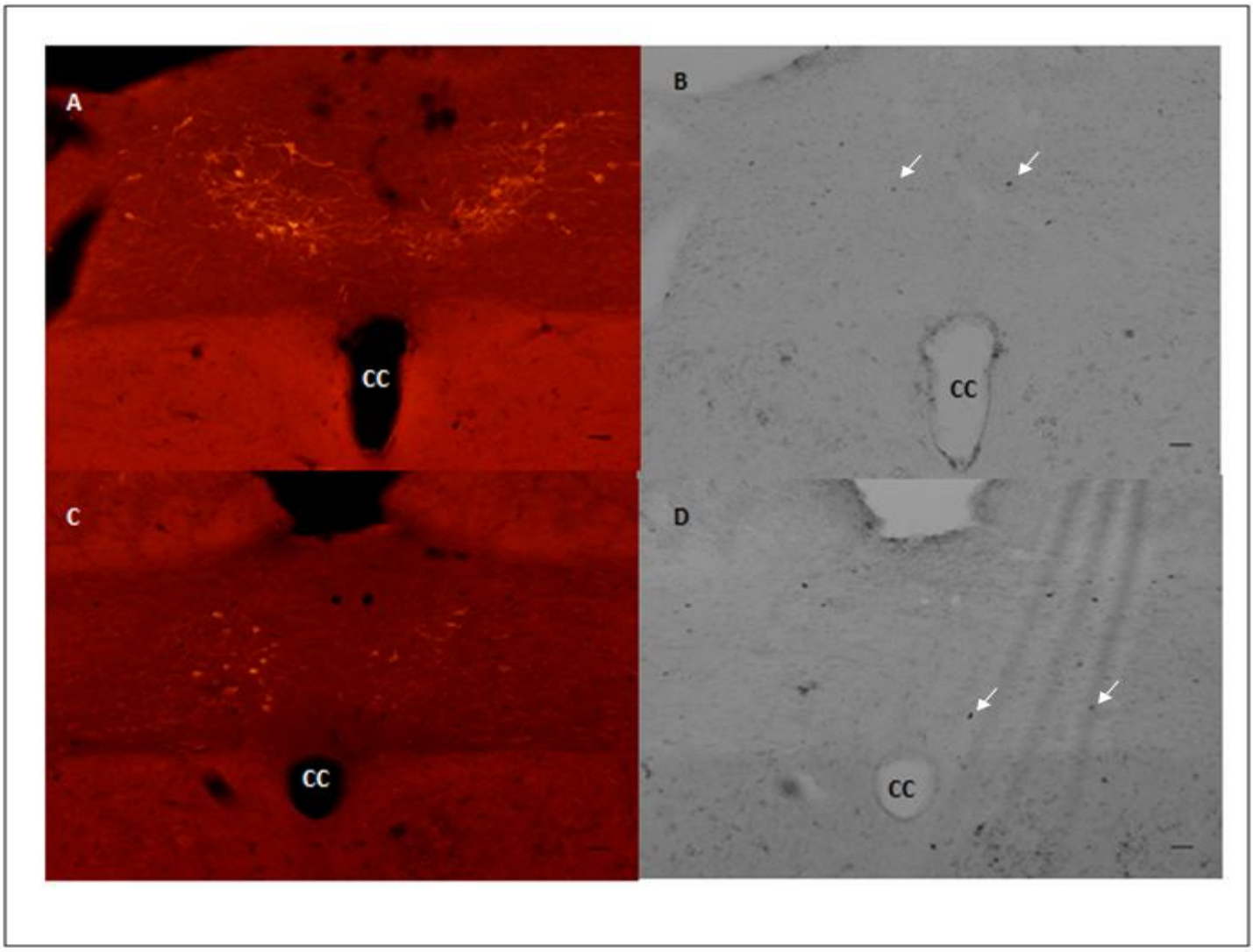

Figure 4: HSD2 and FosB double immune staining

A) HSD2 positive cells in WKY. B) FosB positive cells in WKY. C) HSD2 positive cells in SHR. D) FosB positive cells in SHR. In both the groups, c-fos staining was seen in NTS region but the HSD2 and c-fos positive cells did not colocalize. Arrows point to FosB immunoreactive neurons

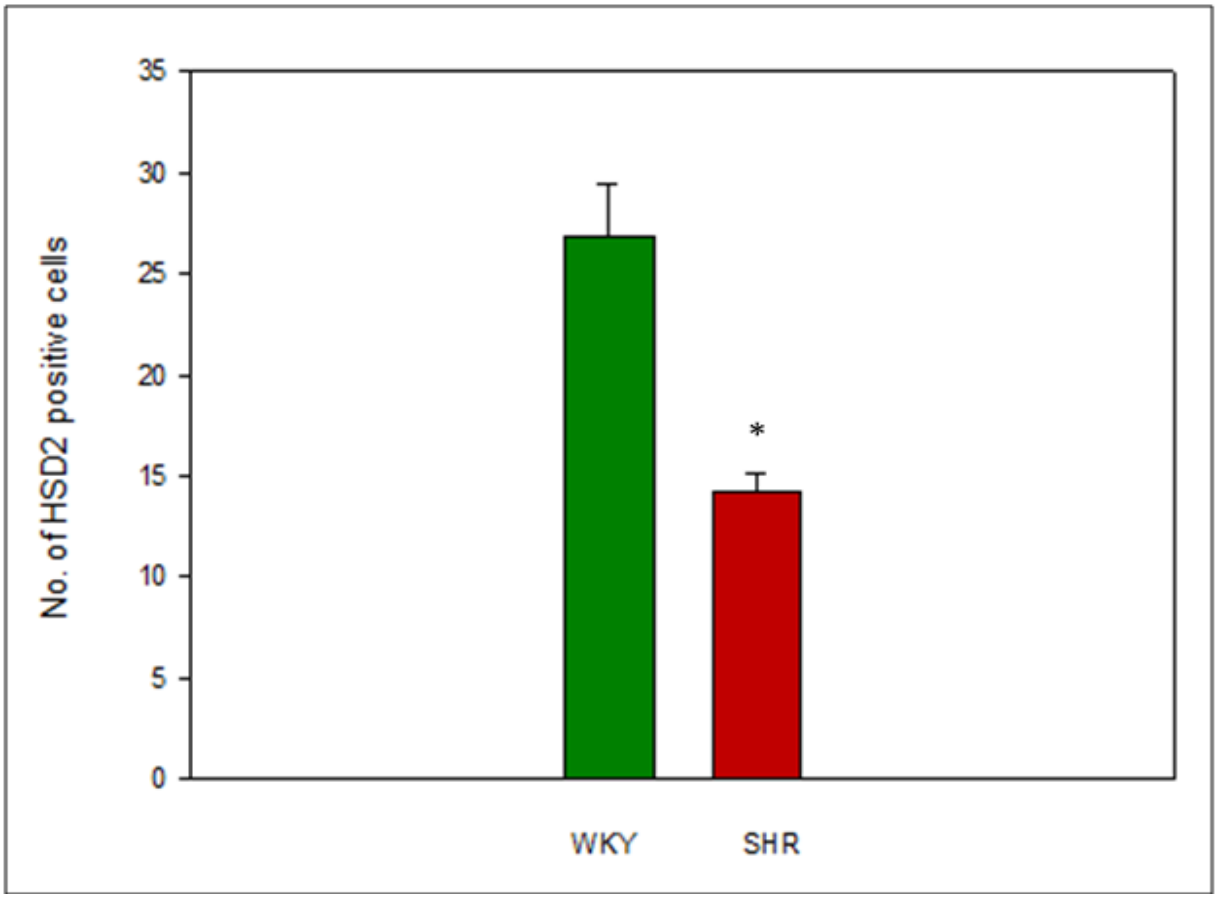

Figure 5: HSD2 immunoreactive cell count

HSD2 immunopositive cell count in both WKY and SHR groups. WKY has significantly more HSD2 positive cells compared to WKY. Values are expresses as mean± standard error. $P<0.05$

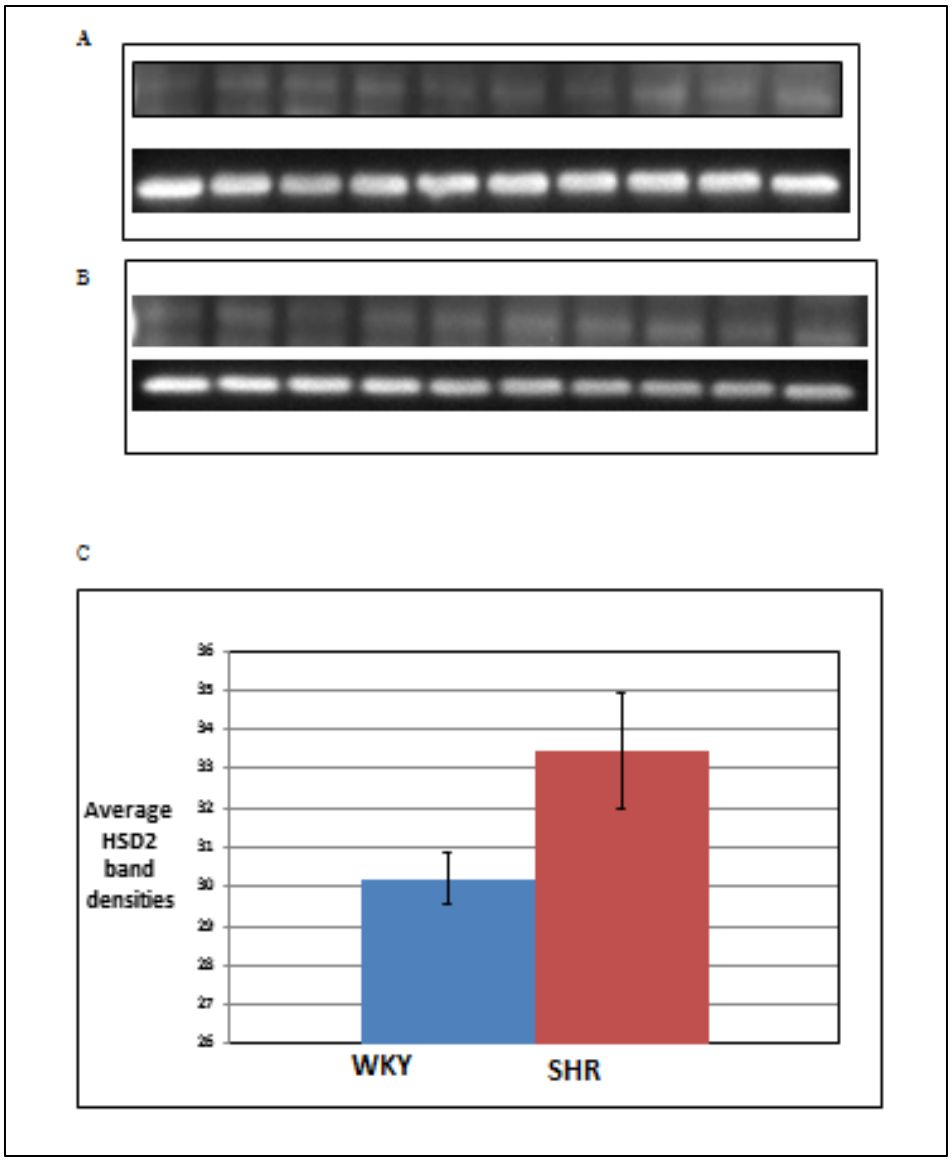

Figure 6: Western Blot analysis

A): Bands of HSD2 (top) and GAPDH (bottom) in WKY group. B) Bands of HSD2 (top) and GAPDH (bottom) in SHR. C) Comparison of the levels of HSD2 in WKY and SHR from the western blot data. The densities of the HSD2 bands are normalized with those of GAPDH in both of the WKY and SHR groups. The HSD2 levels were not different between two groups P>0.05

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Chapter IV

To Determine If MR Is Mediating the Increased Salt Intake in Hypertensive Conditions

Introduction:

The spontaneously hypertensive rat (SHR) was obtained from a Wistar rat colony in Japan in the early 1960's, when investigators mated a hypertensive male Wistar rat to a female Wistar rat with blood pressure slightly higher than normal (41). SHR has evolved as a model of essential hypertension (46). Many studies also used it as a model of salt sensitive hypertension. Several studies showed that SHR have high salt intake under normal conditions (17-18). There are studies which show a direct correlation between salt intake and hypertension in SHR (31, 47). Central nervous system is involved in the salt sensitive hypertension (7), but the reason behind the exaggerated salt intake in SHR under normal conditions remains unclear. Experimental evidence show there is an increased expression of MR in the brain of SHR (45). In 2005, Geerling et al, observed an increased number of aldosterone sensitive neurons (i.e. neurons with HSD2 and MR) in the NTS region of Dahl salt sensitive rats (23), which is also a model of salt sensitive hypertension. Data from our lab (Figure 5, Chapter 3) showed decrease in the number of aldosterone sensitive neurons in SHR in the NTS. In this study we tested the hypothesis that MR in the NTS mediated the exaggerated salt intake in the SHR.

Materials and Methods:

General: Rats (Spontaneously Hypertensive rats, SHR, 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 12hour light: 12hour dark cycle before any surgical procedures were 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: Adeno Associated Viral vector with shRNA (short hairpin RNA) was used to knock-down the MR (the construct sequence is AAV1/2-U6 Rat.Nr3c3/MR.shRNAterminator- CAG-EGFP-WPRE-BGH-polyA) and also the scRNA (scrambled RNA, which acts as control) is commercially available (Gene Detect, New Zealand). 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. A standard microinjection approach as described previously (16, 52) was used to inject shRNA and scRNA into the NTS of the WKY under 2% isoflurane anesthesia and aseptic conditions. To cover the rostral-caudal extent of NTS HSD2 neurons, three injections of 100 nl of the viral construct (> 1 x 10^{12} genomic particles/ml) were injected 0.5 mm below the surface at calamus and bilaterally at 0.5mm rostral and 0.5mm lateral to calamus. These NTS regions contain the highest number of NTS HSD2 immunoreactive neurons(22). The AAV construct enters neurons in the regions of the injection and drives GFP synthesis; however the shRNA can only inhibit MR synthesis in those neurons that synthesize MR.

Measurement of salt intake: Rats had ad libitum access to normal sodium (0.26%) rat chow. Two calibrated 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. Intake was measured to the nearest milliliter at noon every day.

Immunohistochemistry: To verify if the shRNA reduced MR levels in the NTS, rats were anesthetized with Inactin (100mg/kg ip, Sigma, St. Louis, MO.) and transcardially perfused with 4% paraformaldehyde. Post fixation of brains for 1-2 hours was done, before cryoprotecting at 4°C in 30% sucrose solution. The hindbrains were sectioned (40 μm) coronally into 3 sets and stored at -20°C in cryoprotectant until processed for immunohistochemistry. Different sets of serial sections were processed either for the MR and HSD2, Tissues were first incubated in a primary antibody for MR (Primary antibody: MR-18f, provided by Dr. Elise Gomez-Sanchez; 1:500) for 48 hrs at 4^{o}C. After a series of washes with PBS, tissues were incubated in secondary antibody (biotinylated anti mouse, Jackson immunoresearch laboratories Inc, PA; 1:1000) for 2 hrs at room temperature. The sections were then 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 and washed with PBS. After blocking with PBS diluents, tissues were incubated in primary antibody for HSD2 (Chemicon/Millipore, Billerica, MA. 1:40,000) for 48hrs at 4^{o}C. After a series of washes with PBS, tissues were incubated in secondary antibody (Cy3 conjugated anti sheep, Jackson immunoresearch laboratories Inc, PA; 1:800). After incubating for 2hrs at room temperature with secondary antibody, tissues were washed with PBS and were then mounted on gelatin coated slides and cover slipped with per mount mounting media (Fisher Scientific, NJ)

Imaging and Cell counts: Olympus microscope (BX41) equipped with epifluorescence and Olympus DP70 digital camera with DP manager software (version 2.2.1; Olympus, Tokyo,

Japan) was used to image the MR or 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 were presented as mean ± SE. Differences between groups (shRNA and scRNA) and between days were determined by 2-way ANOVA with repeated measures. Student t-test was conducted to determine the differences between the scRNA and shRNA group MR immunoreactive and HSD2 immunoreactive cell count means values. $P < 0.05$ 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=6, 308.16±2.63 g) and scRNA-injected rats (n=6, 306±2.4 g) were not significantly different. Prior to sacrifice (6 weeks later) shRNA-injected rats weighed 359±4 g and scRNA-injected rats weighed 354±6 g which were not significantly different.

Figure 1 illustrates the mean salt intake values of shRNA-injected and scRNA-injected rats during the period of the study. The first five days are measures of salt intake preceding the NTS injections. Basal levels of salt intake were 11.7ml ± 3.3ml in the scRNA group and 21.9ml ± 3.4ml in the shRNA group prior to surgery, which were significantly different. The salt intake in scRNA group was 16.3ml ± 3.7ml, while that of shRNA group was 25ml ± 4.6ml after the microinjections. A two way repeated measures ANOVA showed significant difference in salt intake between the two groups before and after NTS microinjections. The shRNA group also did not show any significant difference in the salt intake before and after the microinjections. The

water intake of scRNA group is significantly low compared to shRNA group (Figure 2) 14 – 44 days after microinjectstions

Immunohistochemistry was performed to verify if the shRNA injections reduced the apparent amount of MR in the NTS (Figure 3). GFP expression (Figure 3, A) shows successful transfection of NTS neurons with the viral constructs in the scRNA group. In Figure 3B, the viral transfection in the NTS was not as good as in scRNA as the GFP expression is concentrated in the DMV region more than the NTS. MR immunoreactivity was predominantly nuclear (Figure 4 A&C). The number of cells showing immunoreactivity for MR was less in shRNA group, but this was not significantly different between shRNA and scRNA injected groups (scRNA 15±2 cells/section; shRNA 8 ± 3 cells/section; p=0.07). The number of HSD2 immunoreactive cells was also not significantly different in both the groups (scRNA-16±1cells/section and shRNA-16±2cells/section). Figure 4B, D shows HSD2 immunopositive cells in both groups. Figure 5 shows the bar graphs depicting the number of MR (right) and HSD2 (left) immunoreactive neurons in the scRNA and shRNA groups.

Discussion:

The finding by Geerling and Loewy of NTS neurons that contain both the MR and the enzyme HSD2 in 2006(21-22), generated a great deal of interest and suggested that NTS HSD2 neurons may play a role in the stimulation of salt intake. Studies described in Chapter 2 also indicate that the MR in the NTS plays a role in mediation of aldosterone stimulated salt intake. The goal of the experiments in this chapter was to test the role of MR in the mediation of exaggerated salt intake in the spontaneously hypertensive rats.

Several studies have shown that salt intake affects the hypertension in SHRs (9, 31) and that the SHRs have an elevated salt intake under normal conditions (17-18). But the mechanisms behind

the elevated salt intake are unknown. Our first study (chapter 2) on determining the role of MR in the NTS on stimulated salt intake helped us confirm the role of MR in driving the stimulated salt intake in response to 4th ventricular infusions of aldosterone in WKY rats.

In the present study we tried to test the role of MR in SHR's exaggerated salt intake. The results demonstrated that there is no significant difference in salt intake between the two groups. The MR cell count in the shRNA group was less compared to the scRNA group, but the difference was not statistically significant. There can be several reasons for these results. In our first study using MR knockdown in WKY, the knockdown just inhibited the stimulated salt intake in response to 4th ventricular aldosterone infusion in the shRNA group. It did not affect the base level of salt intake. In WKY a 30% of knock down in the MR cells did not affect the basal salt intake. In case of SHR, the base line salt intake was very high compared to the WKY, almost double the aldosterone stimulated salt intake in WKY. Also based on the GFP transfection in the shRNA group, it can be possible that either the virulence of the AAV construct was lost or the site of injection is not precisely into the NTS. This experiment is being repeated on another group of animals. Another aspect can be the amount of MR knocked down is low the SHR group compared to the WKY group (Chapter 2). This also might have been the reason why no decrease in salt intake was seen. As the basal salt intake in SHR was very high, a larger reduction in MR may be required to observe an affect.

Conclusion:

The shRNA for MR did not affect the elevated salt intake in the SHR. The number of MR immunoreactive neurons in NTS was not significantly different from the scrambled group. Therefore it appears that the viral vector did not reduce the levels of MR sufficiently to adequately test the hypothesis of this study.

Figures & Figure Legends:

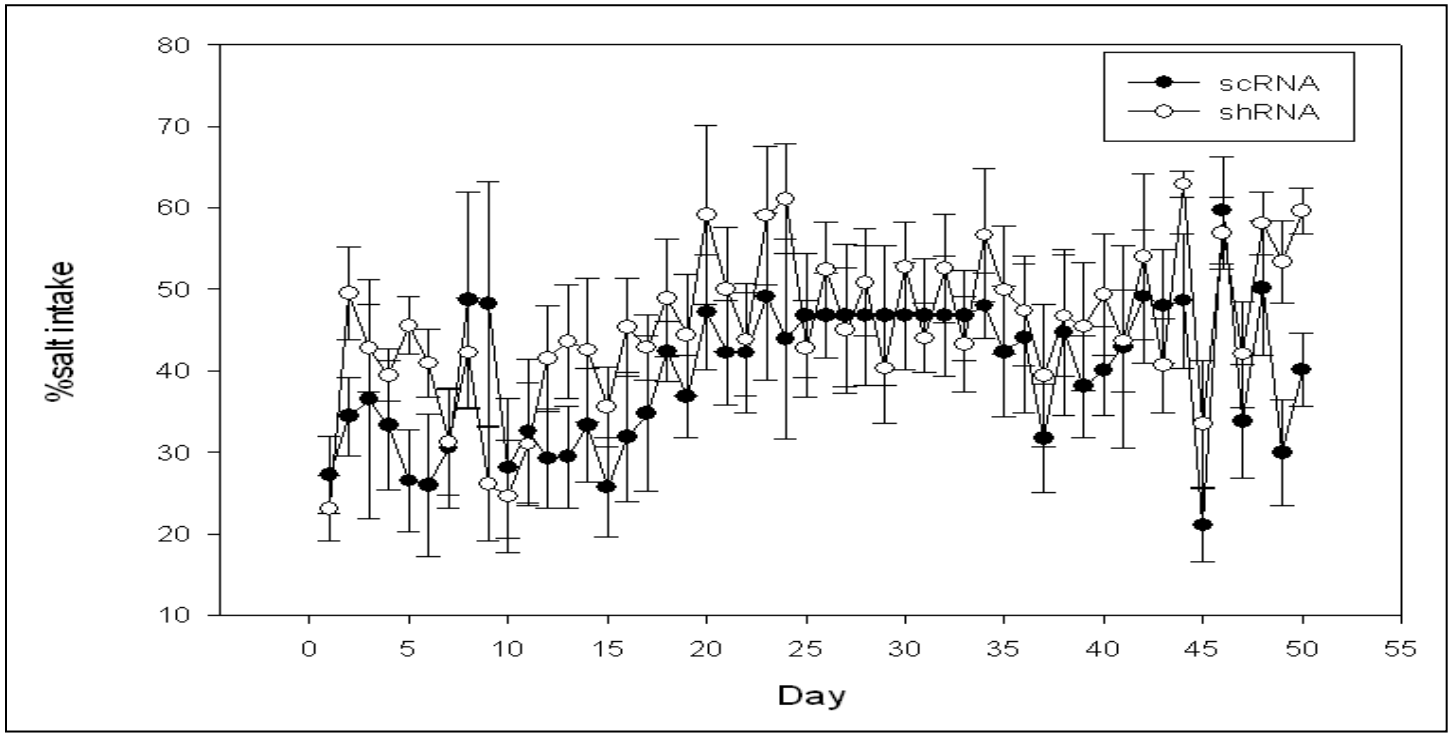

Figure 1: Salt intake of scRNA and shRNA groups before and after microinjections

Days 1-5 represent salt intake prior to NTS injections of viral vectors. Salt intake was measured for about 6 weeks after the microinjections. For both the groups, the salt intake before microinjections was very high. Comparing scRNA to shRNA injected rats, the salt intake of both groups were not significantly different. There was no significant difference between the salt intake before and after microinjections with the groups. ($p>0.05$)

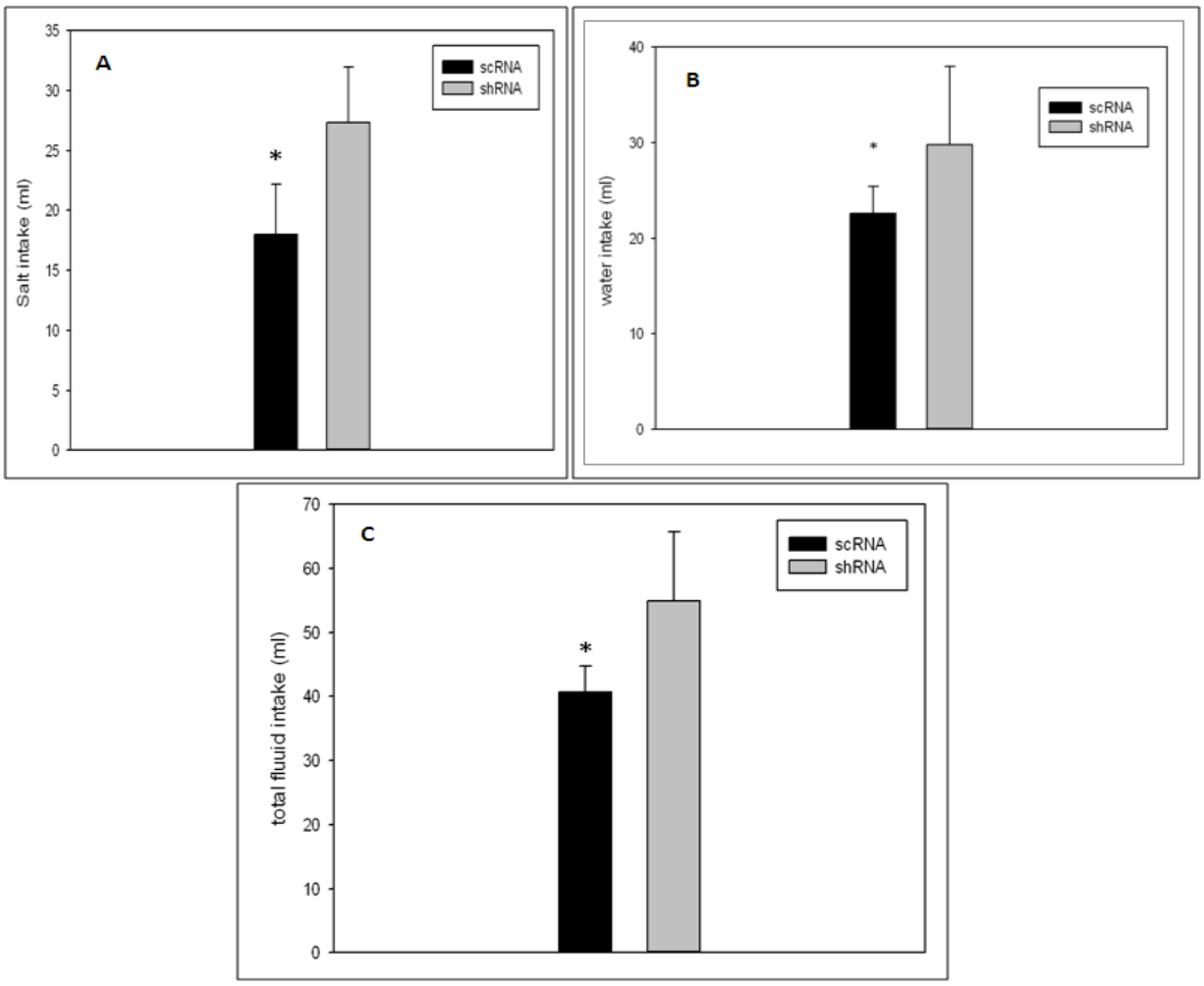

Figure 2: Salt, Water and total fluid intake comparison between scRNA and shRNA groups 14-44 days after microinjections: The scRNA group has significantly low salt intake (A) water intake (B) and fluid intake (C) compared to shRNA group. Values expressed as mean of water intake per day over the period of 14-44 days after injections.

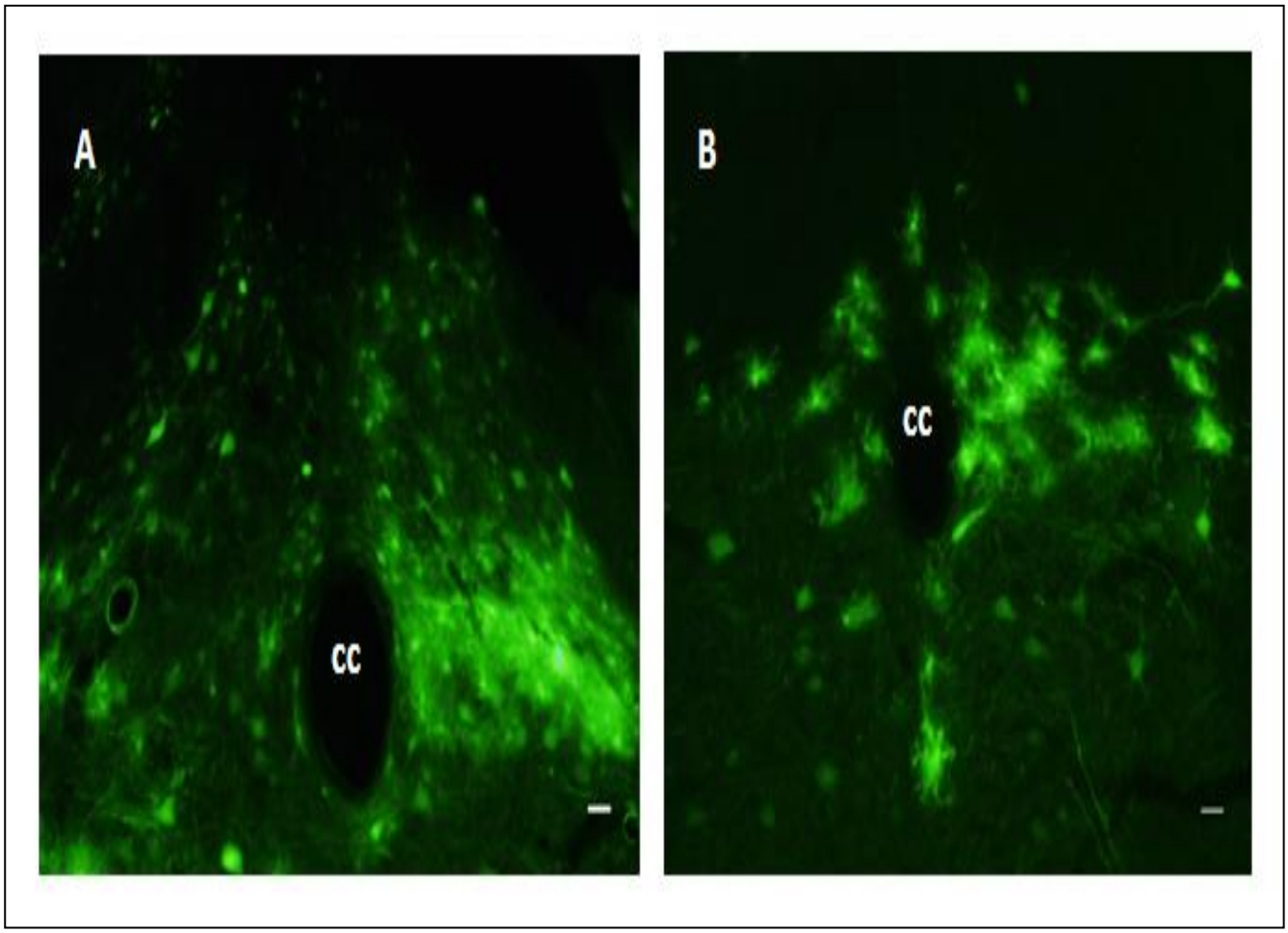

Figure 2: Expression of GFP in scRNA and shRNA groups

A. GFP transfection in NTS. A section from scRNA group.

B. A section form shRNA group, showing GFP transfection in NTS.

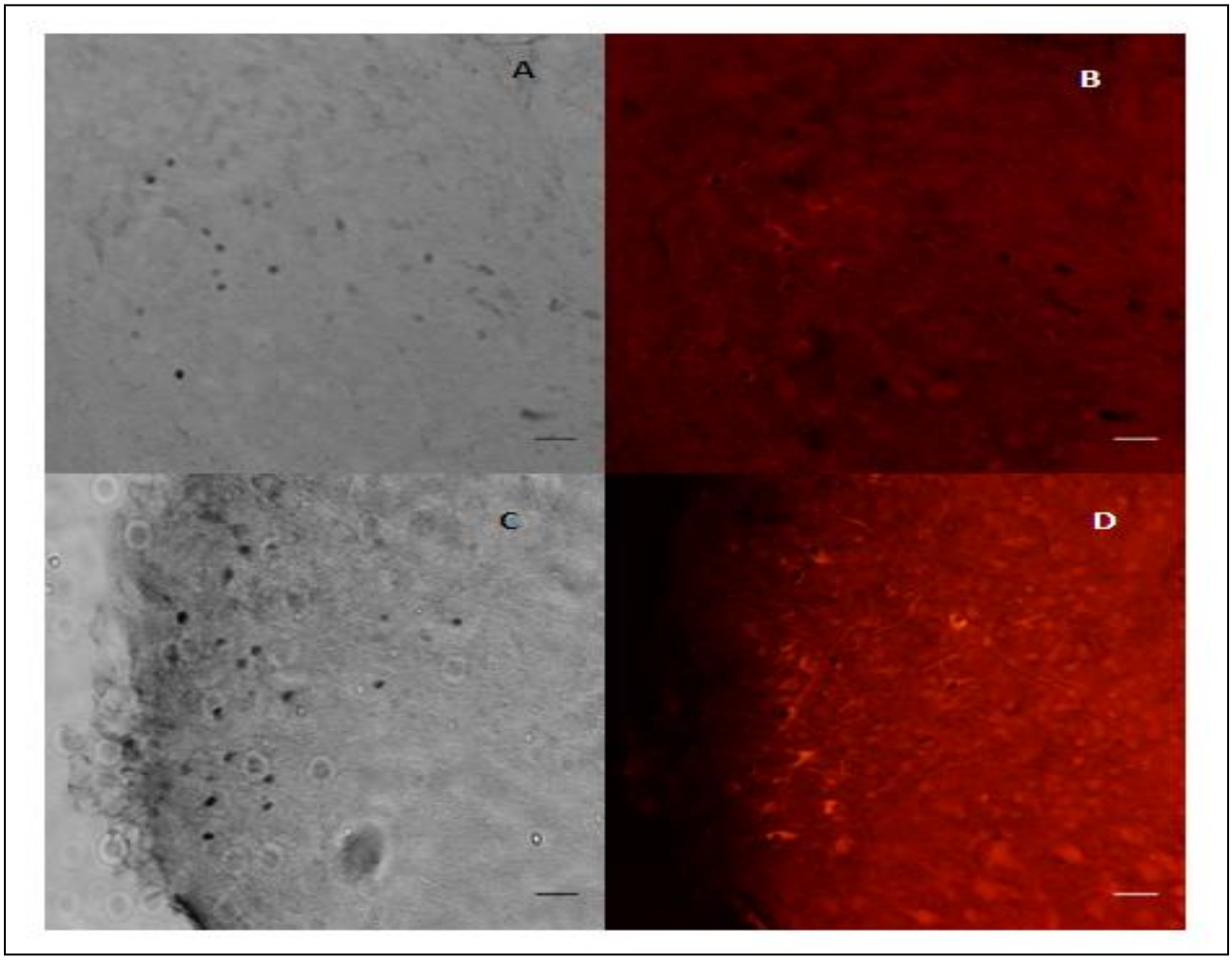

Figure 4: Expression of MR and HSD2 immunopositive cells in scRNA and shRNA groups

A. MR immunopositive cells in scRNA group

B. Cells showing HSD2 immunoractivity. A section from scRNA group

C. MR immunopositive cells in shRNA group

D. Cells showing HSD2 immunoractivity. A section from shRNA group

Cell counts revealed that there is no significant difference between the number of MR/HSD2 immunopositive cells among the two groups ($p>0.05$)

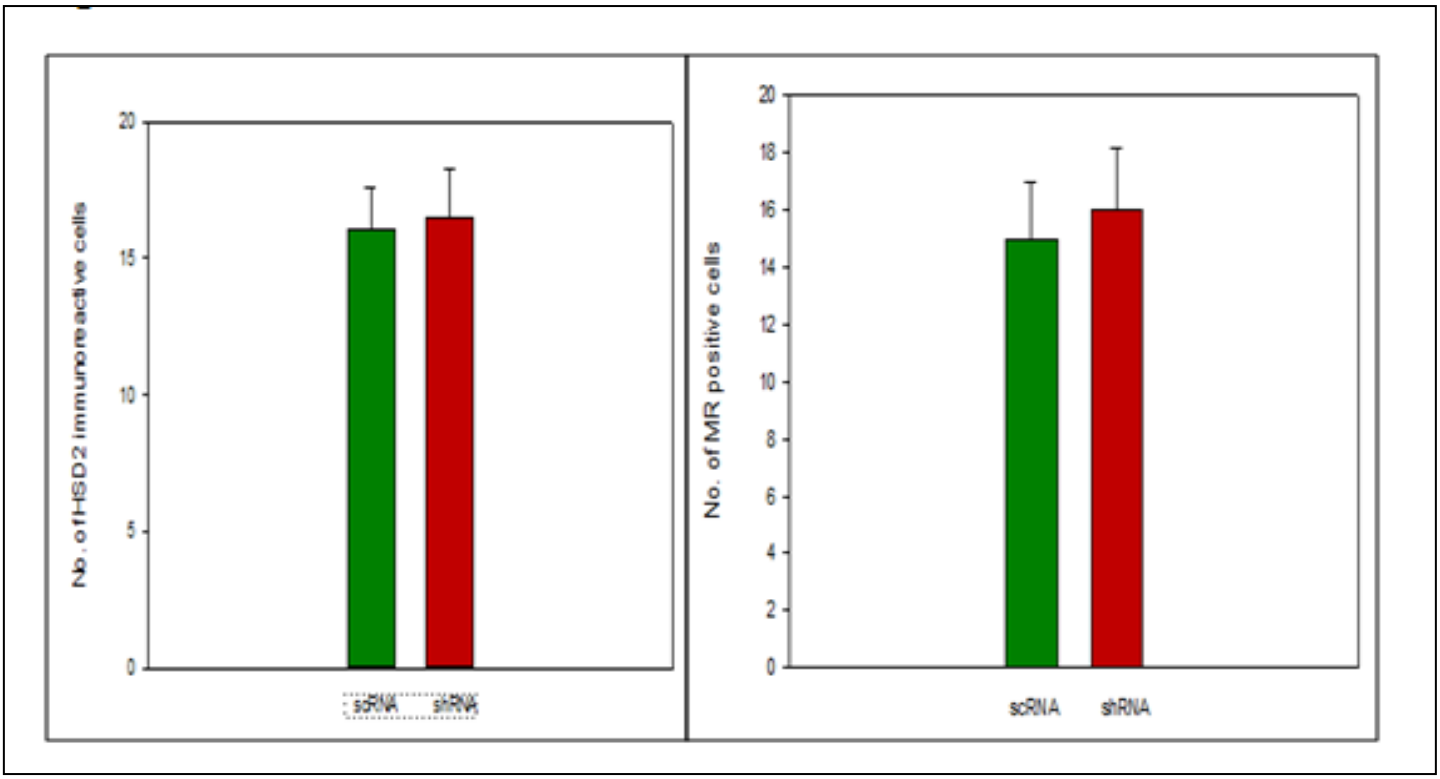

Figure 5: Bar graphs depicting the number of MR (left) and HSD2 (right) immunoreactive neurons in the scRNA and shRNA groups.

The MR and HSD2 cells counts were not significantly different among the two groups. Values expressed as mean ± standard error. ($p>0.05$)

APPENDIX

EFFECT OF MR KNOCKDOWN ON THE EXXAGGARATED SALT APPETITE IN SHR – REPEATED STUDY

The study described in chapter IV was repeated in another group of SHRs. The animals were assigned to two groups. One group received microinjections of scrambled RNA (scRNA; n=5) and the other group received microinjections of short hairpin RNA (shRNA; n=6) into the NTS using standard microinjection procedure described in chapters II and IV. The salt intake of the animals was measured for 4weeks after the injections. At the time of thesis submission, postmortem immunohistochemistry studies have not been completed.

Results:

Figure 1 shows the comparison between scRNA and shRNA group's salt intake. Days 1-4 are control days. Day 5 is the day after microinjections. The salt intake of the shRNA group was not affected by the shRNA for MR injected into the NTS. Figure 2A shows the salt intake comparison between both groups from 14-30 days after injections. The scRNA group has a significantly low salt intake compared to the shRNA group. Figure 2B shows the comparison between water intake of both the groups 14-30 days after injections while Figure 2C shows that the total fluid intake did not differ between the two groups. scRNA group had significantly more water intake compared to shRNA group. Analysis of brain tissues from these animals is currently being done

Discussion: There was a baseline difference in salt intake between scRNA and the shRNA groups, with shRNA gourp having a higher salt intake compared to scRNA group prior and after microinjections. Injection of shRNA did not seem to alter the salt intake in these animals.

Whether or not there is a significant knockdown in the MR levels in shRNA group compared to scRNA will determine what conclusion can be drawn on the role of MR in modulating exaggerated salt intake in SHRs. If we see a significant decrease in the number of MR immunoreactive neurons in the shRNA group then, the knock down of MR in NTS does not contribute to the exaggerated salt intake in SHRs. Presumably other mechanisms are responsible.

One such potential mechanism could be angiotensin. ICV infusion of angiotensin II (Ang II) could increase salt appetite in rats (3). Quantitative autoradiography shows that there is increased number of Ang II receptors in several parts of the brain in SHRs including NTS (24). SHRs are have an increased brain rennin angiotensin system (RAS) with increased salt load (34). SHRs are known to have an increased sodium retention compared to WKY (51). Increased RAS in brain leading increased sodium retention might be a mechanism behind the exaggerated salt intake in SHR and is an interesting aspect to examine in future.

Figures and Figure Legends:

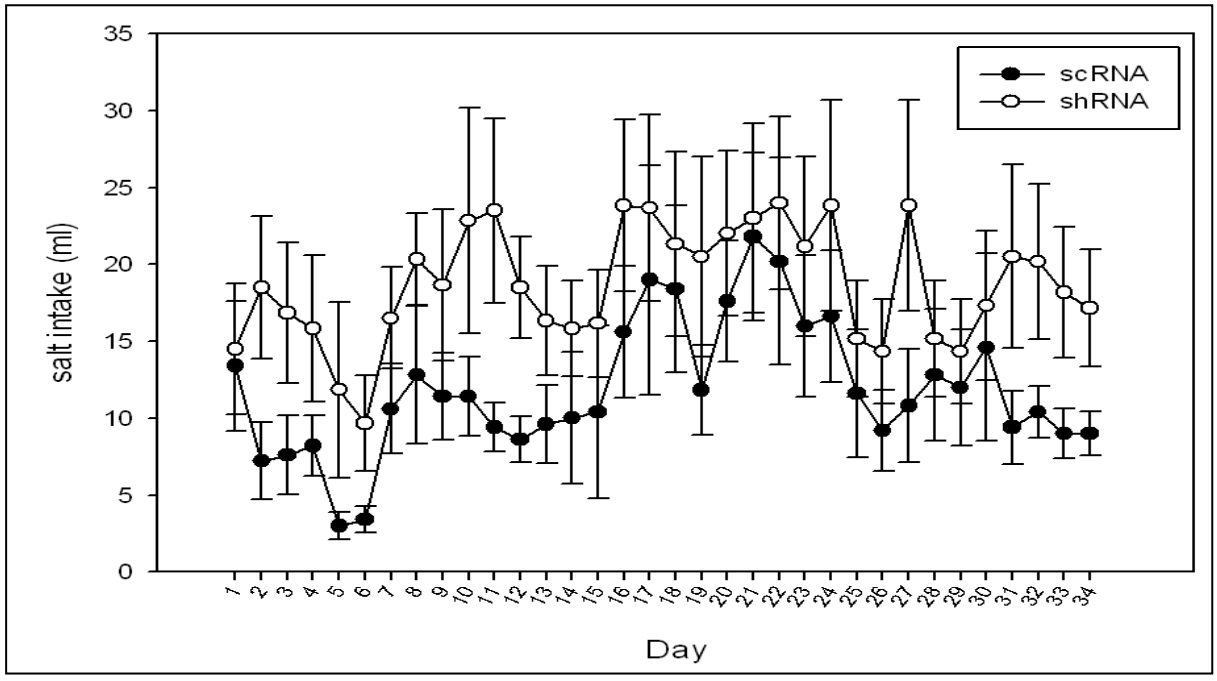

Figure 1: **Comparison of salt intake between scRNA and shRNA injected groups:** Days 1-4 are the control days (i.e. before microinjections). Both groups had a significant increase in salt intake after microinjections compared to the control days (day 4). Also after the microinjections, the salt intake of shRNA injected group is significantly more compared to scRNA injected group. All values are presented as average salt intake per day ± standard error

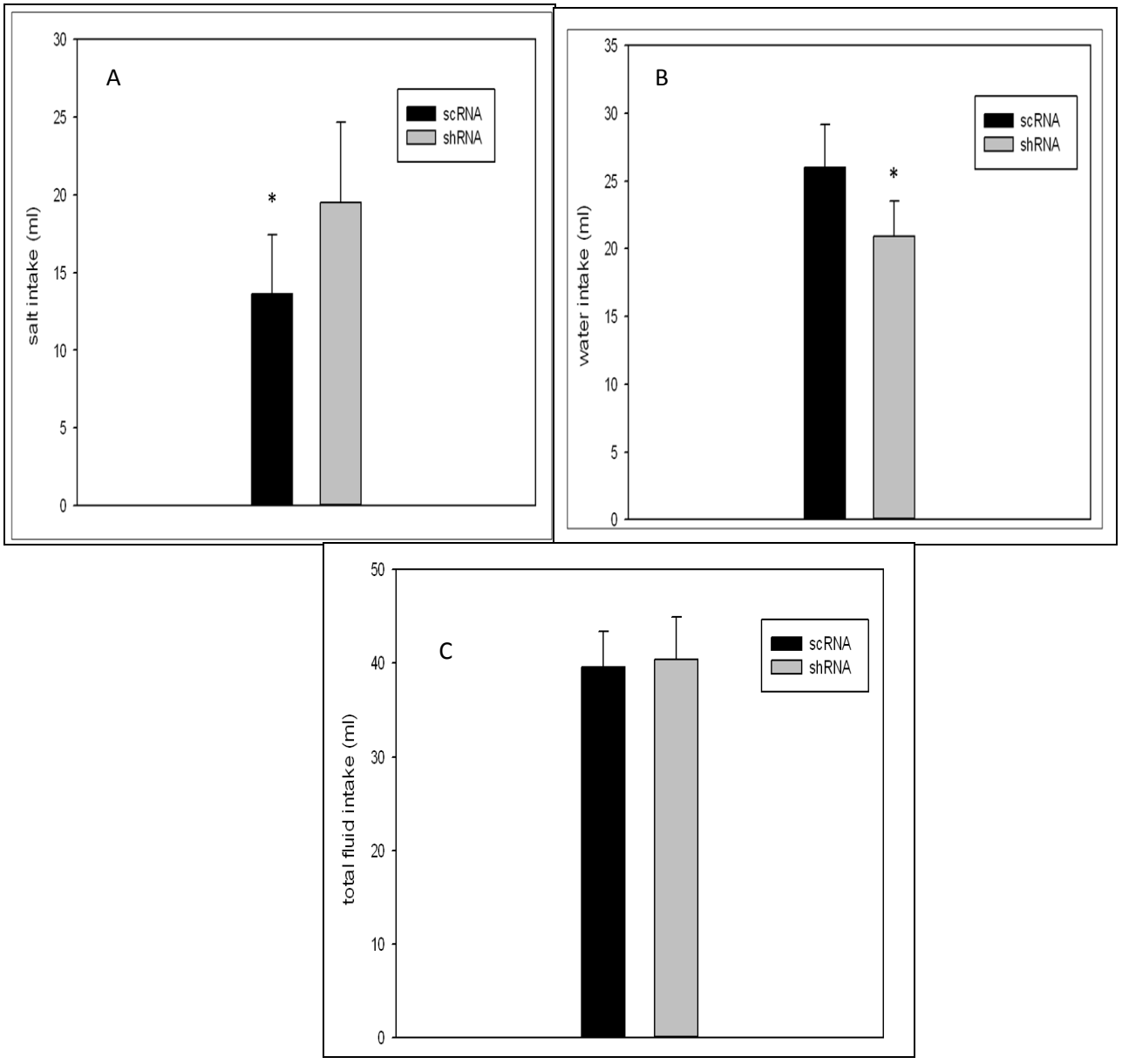

Figure 2 - Comparison of salt, water and total fluid intake between scRNA and shRNA groups, 14-30 after injections: The scRNA group has significantly low salt intake (A) compared to the shRNA group. Water intake (B) is significantly more inscrna compared to shrna group but the total fluid intake (C) between the groups is not different.

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