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ABSTRACT

Mechanisms of Prenatal High-Salt "Fetal Programming" Resulting in Stress Hyperresponsiveness in the Adult Female Offspring in the Sprague Dawley Rat

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Female offspring of Sprague-Dawley rats fed a high-salt diet (HS) during pregnancy show an enhancement of mean arterial pressure (MAP) and heart rate (HR) response to acute stress in adulthood compared to offspring whose mothers were fed a normal-salt diet (NS) [1]. In the present study, we first examined the expression of soluble epoxide hydrolase (SEH) protein in brain tissue. Whole brains were collected and SEH gene (EPHX2) mRNA and SEH protein expression were analyzed using RT-PCR and Western blot, respectively. mRNA levels were relatively decreased in high-salt rats (1.0 ± 0.32 NS vs 0.39 ± 0.07 HS, n=6). However, the relative expression of SEH protein was significantly increased in HS rats (0.97 ± 0.06 NS vs. 1.72 ± 0.32 HS, n=10). SEH is an enzyme that inactivates epoxyeicosatrienoic acids (EETs), which can increase the level of oxygen free radical production and potentially produce an increase in blood pressure. Tempol, a free radical scavenger, was administered intracerebroventricularly to HS (n=12) and NS (n=11) offspring to determine if the stress-induces cardiovascular hyperresponsiveness could be reversed. We were unable to conclusively show that this was the case. Hence, the expression of SEH protein in the brains of HS offspring was increased, but a role, if any, for this change in explaining the exaggerated response to acute stress remains elusive. Second, the expression of the glucocorticoid receptor (GR) gene was investigated. We focused on the methylation patterns of the exon 1_7 GR promoter and 17 CpG dinucleotide sites that include the NGFI-A transcription factor binding site. Female rats (HS n=8, NS n=8) were sacrificed and brains were immediately extracted. Tissue from the pituitary, hypothalamus, and hippocampus was removed and DNA was extracted from each of these areas. CT conversion was performed on the DNA samples followed by cloning and sequencing. Methylation patterns between HS and NS in the pituitary, hypothalamus, and hippocampus did not vary. RT-PCR and Western blot were performed to investigate differences in the levels of GR transcription and/or translation. There were no significant differences found. However, the trends found may suggest different levels of GR mRNA and protein between HS and NS female rats. DNA methylation may play a role in the regulation of GR in prenatal high-salt female offspring. Additional studies will be needed to pinpoint the mechanisms responsible for the exaggerated cardiovascular response to acute stress in HS offspring.

Keywords: soluble epoxide hydrolase, glucocorticoid receptor, hypertension, methylation

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INTRODUCTION, REVIEW OF LITERATURE

A study done by the National Health and Nutrition Examination Survey (NHANES) from 2005 to 2008 found that 33.5% of adults 20 years and older in the U.S. have hypertension. This amounts to about 76,400,000 US adults that suffer from this condition. Out of those that are aware of their condition, only 48% have it under control[2].

It is a well known fact that hypertension is a risk factor for cardiovascular disease. Hypertension predisposes people to atherosclerosis and serious heart conditions, such as myocardial infarction and atrial fibrillation[3]. It is estimated that 14% of cardiovascular deaths worldwide are rooted from high blood pressure[4]. Hypertension is not only a risk factor for cardiovascular disease, but can target brain damage and impair cognitive function. Nearly half of all strokes can be attributed to hypertension[5]. Hypertension is the most important risk factor for small vessel disease which is predisposing to lacunar infarction, leucoaraiosis, white matter changes, and intracerebral hemorrhages[3]. There is also evidence that supports an association between hypertension and the development of cognitive disorders and dementia, including Alzheimer's disease[6].

Unfortunately, the prevalence of hypertension is growing. A study that looked at high blood pressure trends in the US from 1963 to 2002 found that since the late 1980s, the frequency of high blood pressure in children and adolescents is on the rise[7]. With an estimated cost of $43.5 billion directly and indirectly due to high blood pressure[2] and it being the most common primary diagnosis for office visits in the US[5], the increasing incidence of hypertension will be a challenge that medical professionals and the health care system will have to face.

One of the challenges of treating hypertension is that the origin or mechanism is not known. In fact, 95% of all cases of hypertension are due to essential hypertension, where the

cause is unknown. However, we do know that there are a number of risk factors that can contribute to or cause hypertension. These include obesity, insulin resistance, high alcohol intake, high salt intake (in salt-sensitive patients), aging, a sedentary lifestyle, stress, low potassium intake, and low calcium intake. There are also genetic factors (family history) that can influence a person's risk of hypertension[8].

Several animal models have been developed to investigate fetal programming, the idea that environmental factors can affect the health of the embryo or fetus later in life, and the underlying mechanisms responsible for hypertension. Most of these models cause low birth weight in offspring, which later develop hypertension as adults. Among these is the protein undernutrition model that limits maternal protein consumption during pregnancy and results in hypertension in the adult offspring[9]. Another model uses ligation of the uterine arteries to decrease perfusion of the placenta, resulting in small offspring who later develop hypertension [10]. A third model uses an injection of dexamethasone into animals near parturition. This once again causes the offspring to be small and hypertensive later in adulthood[11].

In our lab, we use a modified perinatal high salt model to induce hypertension in offspring rats. This model, initially developed by Contreras et al. [1] produces a higher mean arterial pressure in offspring of female Sprague-Dawley rats fed chow consisting of 3% (high) NaCl. Their model consists of feeding a high-salt diet before and during pregnancy, during lactation, and to the offspring until postnatal day 10, when their diet is switched to normal-salt (NS) chow. Blood pressure is then measured in a stress-free and freely moving environment. Our lab has modified this model, by extending the HS diet to the offspring until postnatal day 30. We also use a higher diet concentration of NaCl (8%) in order to produce a larger blood pressure difference from the NS subjects. Using this model, hypertension is present in the offspring as

early as week 4. Increase in sympathetic outflow and AT_{1a} receptor expression may be contributing factors[12].

Although the model successfully produced hypertension in the offspring, it was not limited to the conditions of a fetal programming model because high salt was given during lactation and after weaning. Therefore, another study was designed to provide evidence that this high-salt model can affect the fetal environment and produce hypertension in the offspring. This model involved giving a high-salt diet one week before conception and throughout pregnancy. No significant differences in basal HR and MAP were found between HS and NS groups in either male or female offspring. This suggests that a HS diet during the prenatal period is not sufficient to program hypertension in adult offspring. However, the HS female offspring did show an enhanced pressor and tachycardic response to acute stress from restraint and an increase in relative expression of corticotrophin-releasing hormone (CRH) mRNA in the paraventricular nucleus of the hypothalamus during basal and stressed conditions. In contrast, CRH mRNA did not differ in the adult male offspring. This suggests that the effects of fetal programming are sex specific in this model[13]. We subsequently determined that the stress-induced cardiovascular hyperresponsiveness was mediated primarily by an exaggerated activation of the sympathetic nervous system and hypothesized that the increased expression of CRH in the brain mediated the effect[14].

To further explore the possible underlying mechanisms that may have caused the female offspring to experience this hypertensive response to the acute stress, our lab used microarray analysis to look at differences in brain gene expression between the HS and NS groups. Among other genes, there was a significant 1.65 fold decrease in the expression of the epoxide hydrolase 2 gene (EPHX2), which codes for the protein, soluble epoxide hydrolase (SEH)[15].

SEH is an enzyme that regulates the levels of epoxyeicosatrienoic acids (EETs) by converting them into dihydroxyeicosatrienoic acids (DHETs) which are inactive. EETs are known to have an effect on the regulation and vascular tone of pulmonary, renal, and cardiac function[14]. EETs are cytochrome P_{450} metabolites of arachidonic acid and are produced in the endothelium. They hyperpolarize vascular smooth muscle, open Ca^{2+}-activated K^{+} channels, and relax arteries[16]. The vasodilatory effect caused by EETs becomes less potent after their metabolism by SEH [17, 18]. The importance of the SEH enzyme in regulation of cardiovascular function is apparent in models of hypertensive rats where the expression of SEH is increased [19, 20]. In fact, inhibition of SEH by pharmacological drugs lowered blood pressure and protected renal vascular function in angiotensin hypertensive rats[21]. Furthermore, complete knockout of the SEH gene decreased the systolic blood pressure of the EPHX2-null mice compared to wild-type mice in both the presence and absence of dietary salt loading[22]. This evidence points to the cardiovascular benefits of disrupting SEH expression in the peripheral circulation.

However, there are some studies investigating the effects of SEH in the brain that do not support the view that high levels of SEH expression lead to hypertension[23, 24]. One particular study by Sellers [25] found that inhibition of SEH in the brain caused an increase in blood pressure as opposed to observing lowered blood pressure when peripheral levels of SEH were significantly decreased. This led them to propose that EETs are involved with an increase in blood pressure through depression of the baroreceptor reflex and central neural pathways that regulate the set-point of arterial pressure. Lower levels of SEH cause an increase in EETs activity, which leads to increased reactive oxygen species (ROS) that results in high blood

pressure. ROS has been shown to contribute to the central nervous system mechanisms underlying hypertension in stroke-prone spontaneously hypertensive rats [26].

If EPHX2 mRNA levels are indeed decreased in the brain of the high-salt offspring in our model, then the expression of the SEH enzyme in the brain may also be decreased and contribute to high ROS levels that result in a greater hypertensive response when undergoing acute stress. To test this hypothesis in our model, we will first verify that EPHX2 mRNA expression is decreased in the brain of HS offspring using RT-PCR. Then we will determine the expression of SEH protein by performing Western blot. The distribution of SEH protein in the brain will be determined using immunohistochemistry. To investigate the role of ROS in acute hypertension under stress, we will inject tempol (free radical scavenger) into the rat brain (intracerebroventricularly), expecting to see a reversal in the stress-induced cardiovascular hyperresponsiveness of high-salt offspring.

Exposure to prenatal nutritional stress, prenatal corticosterone administration, and prenatal stressors from the environment such as cold, noise, crowding, and restraint, result in an exaggerated stress response in offspring[27-31]. A maternal high-salt or low-salt diet is a stress on the mother and also a stress to the developing offspring *in utero* [32]. The consequences of both high-salt and low-salt diets include enlarged adrenal glands and hyper responsiveness of ACTH and corticosterone when stressed by restraint. This points to an increased activation of the hypothalamic-pituitary-adrenal (HPA) axis in the HS offspring compared to the NS offspring[32]. The HPA axis develops throughout the prenatal and early postnatal periods [33], possibly making its development more sensitive to environmental stresses.

After blastocyst implantation, the bulk of the genome is demethylated and certain genes that are not needed for differentiation and development of specific cells are methylated. Since

this process of demethylation and methylation occurs in utero and in early postnatal development, it is a likely mechanism for fetal programming[34].

Out of the four bases that make up DNA, cytosine is the only one that is either methylated or unmethylated on the fifth carbon position of the pyrimidine ring. Cytosines are generally methylated when a guanine immediately follows[35]. Regions of genomic DNA where these CpG dinucleotides are found in relatively high concentrations are referred to as CpG islands. DNA methylation is generally found throughout the genome and is globally methylated with the exception of the CpG island regions, which are usually unmethylated. In contrast, CpGs found outside of the islands are typically methylated. CpG islands have an abundant GC-base composition compared to the rest of the genomic DNA, which relatively has a higher percentage of AT-bases[36]. CpG islands are usually around 500-1,000 base pairs and are normally found in the promoter region or exon 1 of genes. It is estimated that there are about 30,000 CpG islands in the human genome[35]. In fact, 56% of human genes have CpG islands in their promoters[36].

The methylation status of these CpG islands is significant because it can affect gene expression. When there is methylation, methyl-binding domain (MBD) proteins recognize the methylated region and recruit factors for chromatin condensation and gene inactivation. Therefore, hypermethylation of a gene's transcription region could be similar to a gene deletion or mutation. However, methylation of some islands plays a vital role in health. Global hypomethylation of CpGs not part of CpG islands has been linked to genomic instability[35]. Although relatively rare, methylation can directly prevent transcription factors from binding to target genes in vivo. DNA methylation can also increase gene expression by preventing the binding of repressor proteins[37]. Whatever the mechanisms, changes in normal methylation

patterns are shown to have dire consequences and influence diseases, such as cancer, atherosclerosis, schizophrenia, endometriosis, Alzheimer's disease[38], and fragile X syndrome[35].

The mechanism that determines which areas of the genome are methylated or not are still not understood. However, DNA methylation is catalyzed by DNA methyltransferase 1 (Dnmt1), which methylates the hemi-methylated CpG dinucleotides on the nascent strand of DNA after the DNA is replicated. This enzyme maintains the methylation patterns in proliferating cells. DNA methyltransferase 3a and 3b initiate de novo methylation in vivo and create new methylation patterns during development[37].

Maternal care early in the postnatal period can cause epigenetic modifications (eg. CpG methylation) that affect the expression of the glucocorticoid receptor (GR) in the hippocampus. These studies looked at the methylation patterns on CpG sites located on the non-coding exon 1 region of the hippocampal GR gene, particularly the binding site for nerve growth factor-inducible protein A (NGFI-A, which is a transcription factor)[39, 40]. DNA methylation in this region prevents NGFI-A from binding to the exon 1 promoter, resulting in decreased expression of GR[41].

GR expression in the brain, notably in the hippocampus, plays an important role in the HPA axis by regulating release of corticotrophin-releasing factor (CRF) through negative feedback[42]. Glucocorticoid feedback helps facilitate an animal's ability to adapt and recover from stress by terminating the stress-induced activation to the HPA axis[43].

Fetal programming models in rats have also shown effects on the HPA axis due to decreased expression of GR in the brain. Fetal glucocorticoid exposure or prenatal stress decreases hippocampal GR expression. This lessens the effect of the negative feedback system

and results in an increase of basal CRH, ACTH, and glucocorticoid levels [44-46]. The increase in CRH has been linked to an increase in the sympathetic nervous response to acute stress[47]. This includes a role for CRH in mediating increased expression of tyrosine hydroxylase in neurons of the locus coeruleus (main relay nucleus for noradrenergic pathways) and increased release of adrenal epinephrine in response to restraint. Furthermore, enhanced glucocorticoid levels in adults lead to hypertension and dampen the HPA response to stress[42]. Meaney et al. [39] have shown that rats with less methylation in the exon 1_7 promoter region around the NFGI-A binding site are able to respond better to restraint stress than the group of rats that were hypermethylated, especially on the 5' CpG of the NFGI-A binding site.

We hypothesize that our prenatal high-salt treatment causes epigenetic programming of the GR gene in areas of the brain that are involved in the stress response. Specifically, the increased CRH mRNA expression that we have found in the hypothalamus of HS offspring may be due to loss of negative feedback because of down regulation of GR in the hippocampus, hypothalamus, and/or pituitary, areas that are considered primary feedback sites in stress-induced HPA axis activation[48]. To investigate this, we will determine the methylation patterns of the exon 1_7 region of the GR promoter in these brain areas of HS and NS female offspring. We will also determine the expression of GR mRNA and protein in these brain areas. We expect to find the HS group to be hypermethylated, resulting in lower levels of GR in the hippocampus, hypothalamus, and/or pituitary. This could explain the increase in CRH expression and hypertensive response to acute stress.

EXPERIMENTAL OBJECTIVES

In the present study, our experimental objectives were:

1) To determine if a prenatal high-salt diet significantly decreases the level of SEH mRNA and protein expression in the whole brain of Sprague-Dawley rats.
2) To determine what areas of the brain express SEH protein.
3) To determine if increased brain oxygen free radicals contribute to the exaggerated cardiovascular response to acute stress in HS offspring.
4) To determine if a prenatal high-salt diet causes changes to methylation patterns in the exon 1_7 transcription region of the GR gene and, therefore, changes in expression of the GR mRNA and protein.

MATERIALS AND METHODS

Prenatal HS and NS protocol

Female Sprague Dawley rats received 8% NaCl diet (Harlan Teklad, Madison, WI) as the HS treatment. For the NS control treatment, a diet of 0.7% NaCl was provided. Breeder males were given standard lab chow (0.29% Na^+, 0.49% Cl^-). Female rats were allowed to adjust to the high salt diet, while caged, 1 week before breeding. At this point, one breeder male was placed in the cage with each female. Pregnancy was confirmed by the presence of a mucus plug in the bottom of the cage. The male was removed from the cage and the pregnant female continued to eat the HS or NS diet throughout gestation. After giving birth, litters were culled to 10 offspring (approximately equal male and female) and the experimental diet was discontinued. The dams were fed standard lab chow while lactating. The pups were weaned on PD 21 and given standard lab chow to eat. Therefore, the only exposure of offspring to the HS diet was during the *in utero* period.

RNA isolation

One to two naïve female offspring were randomly selected from each litter for analysis. Animals were sacrificed by decapitation at 12-13 weeks of age and brains were removed. For determination of SEH mRNA, whole brains were immediately frozen in liquid nitrogen. For determination of GR mRNA, hypothalamic and hippocampal blocks of tissue were first dissected and then frozen. Pituitaries (anterior and posterior) were also frozen.

Total RNA was extracted by homogenization in TRIzol (Invitrogen) reagent according to the manufacturer's instructions. Chloroform was added to the homogenate and the mixture was shaken and centrifuged. The aqueous phase was transferred to a fresh tube where the RNA was

precipitated in isopropyl alcohol, centrifuged, and washed in 75% ethanol. The resultant RNA was redissolved into water and the concentration was measured using spectrophotometry.

RT-PCR

RNA (1 µg) was reverse transcribed to cDNA using 100 U of Superscript II (Invitrogen, Carlsbad, CA) in the presence of .5 µl of dNTP mix (10.0 mM each), 1.0 µl of random decamers (250 µM, Ambion), 1 µl of DTT (0.1 M), 20 U of RNAse inhibitor (Invitrogen), and 1 µl of 10X buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$). Each tube had a final volume of 10 µl and was incubated at 42^{o}C for 60 minutes and then cooled immediately to 4^{o}C. For SEH, PCR was performed on the cDNA using 1 µl of the RT reaction product combined with 1 µM of the SEH primer pairs (Invitrogen) or 18S primer pairs + competimers (Ambion, Austin, TX), 0.313 µl of the dNTP mix, 2.5 µl of a 10X buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1.4 mM $MgCl_2$, and 0.2 µl of JumpStart *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO). Each tube had a final volume of 25 µl and was subjected to the following optimized temperature profile for amplification, 94^{o}C (20 sec), 60^{o}C (30 sec), 72^{o}C (45 sec) repeated 35 times followed by a final elongation period (72^{o}C) for 10 minutes. The SEH primer pairs (272 bp fragment) used were, sense:
5'-GCTTTGAGAGAACTGGAGAAAGTCA and antisense:

5'-AAGATGAGTCTCCATAGCCTTTCA. The 18S RNA pairs amplified a 324 bp fragment (324 bp) that was used as an internal control.

For investigating GR mRNA expression, the RT protocol was the same as outlined above and the PCR protocol only differed in that an anneal temperature of 61^{o}C was used. The primer

pairs (271 bp) were, sense: 5'-ACCCTACGCCCCTTTCCT and antisense: 5'-CTCCTGACCTCTCCTTCCAC.

Relative RT-PCR data were analyzed as follows. PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide. The optical density (O.D.) of the SEH band or GR band was determined using FluorChem SP (Alpha Innotech). This O.D. value was then standardized by dividing it by the O.D. value of its corresponding 18S band. This O.D. ratio was then normalized to an average of 1.0 for NSF animals and the O.D. ratio of the HSF rats was adjusted accordingly. Each RT-PCR protocol was repeated at least twice and the results for each analysis were averaged. Differences in these averaged values (NSF versus HSF) were determined using the Student t test. For all analyses, a $p < 0.05$ was considered to be significant.

Western blot

The SEH and GR protein expression was assessed by Western blot. Brain tissue from each rat was homogenized in a Tris-EDTA buffer. The proteins (10 μg) were separated by SDS-PAGE (9% gel) and then electrotransfered to supported nitrocellulose (1 h at 100 V). The nitrocellulose was washed in blocking buffer (non-fat dry milk 5%) for 1 hr. In order to detect SEH protein bands, the nitrocellulose was incubated with primary antibody (1:200 dilution) specific to the soluble epoxide hydrolase protein (H-215, Santa Cruz Bioltechnology, Santa Cruz, CA) for 45 minutes. In order to detect GR bands, primary antibody specific to the glucocorticoid receptor protein (M-20, Santa Cruz Biotechnology) with a dilution of 1:100 was used for 45 minutes. The same secondary antibody (anti-rabbit IgG-HRP, Santa Cruz Biotechnology) was used for the SEH samples at a dilution of 1:7500 and for the GR samples at a dilution of 1:000 for 30 minutes. Blotting buffer was used to wash the nitrocellulose paper three times for five

minutes each in between the primary and secondary antibody, and before adding the TBS (see below). The membrane was then washed for five minutes in TBS and then for one minute in luminol reagent (Santa Cruz Biotechnology). The nitrocellulose was then blotted and placed in a transparent plastic holder. This was exposed to X-ray film for 30 minutes. The O.D. of each band was determined and normalized as above. The experiment was performed at least twice and the average was used to calculate the final values. The Student t test was used to determine differences.

Immunohistochemistry

HS and NS female adult offspring (12-13 wks) were anesthetized with a mixture of ketamine and acepromazine (140 mg/kg/1.4 mg/kg, i.m.) and an incision was made from the middle abdomen through the rib cage, gaining access to the heart. A thin needle and syringe was inserted into the left ventricle of the heart and an incision was made in the right atrium. 100 ml of physiologic saline was injected into the left ventricle in order to remove the blood from the rat's system. When all the blood was drained, 100 ml of 4% buffered formalin was then injected through the same system. The formalin caused the tissue to be fixed and firm, thus preserving all the original proteins and structures of the rat's brain. The brain was then removed and stored in the formalin solution at 4°C for at least 48 hours. 40 µm sections were cut through the hypothalamus and brainstem using a freezing microtome. Endogenous peroxidase activity was inhibited by incubating the tissue (free floating) in 0.1 M phosphate buffer (PO_4) containing methanol (10% v/v) and hydrogen peroxide (3%). The sections were then washed in 0.1 M PO_4 until all the foam was gone. The tissue was then blocked with 10% normal goat serum in 0.1 PO_4 containing 3% Triton X-100 for 20-30 minutes. Following washing, the sections were then

incubated with SEH antibody overnight at 4°C. Two different primary polyclonal anti-rabbit antibodies were used. The first was H-215 (Santa Cruz Biotechnology) and was directed to the C-terminal end of the protein. The second was Y-13 (Santa Cruz) and was directed toward "internal" amino acids. The next day, a commercially available ABC kit (anti-rabbit, Vector Laboratories, Burlingame, CA) was used to visualize the SEH immunoreactivity. 3,3'-diamino-benzidine was used to produce a brown reaction product. Thus, the brown staining on the tissue indicated the presence of SEH proteins in the tissue. The sections of tissue were then transferred onto glass slides, cover slipped, and visualized using a light microscope with digital camera. Digital pictures of the areas of interest were taken at different magnifications to localize the immunoreaction. In the case of the Y-13 antibody, a blocking peptide was commercially available. Pre-incubation of the antibody with the blocking peptide (at 5-fold excess) prevented any immunostaining.

This study was done to identify the location of SEH in the brain and was not used to look for difference in intensity of immunostaining in NS versus HS rats.

Effect of icv Tempol on response to stress

Adult female offspring (12-13 wks) were anesthetized with ketamine/acepromazine (140 mg/kg/1.4 mg/kg, *im*) and the abdominal aorta was exposed through a ventral midline incision. Blood pressure probes (TA11PA-C40, DSI, Arden Hills, MN) were inserted into the aorta distal to the renal arteries and cemented in place with Vetbond (3M, St. Paul, MN) and secured with a cellulose (0.5 cm^2) patch. The incision was closed using 3-0 silk for the abdomen and surgical staples for the skin. The rats were then placed in a stereotaxic instrument (Kopf, Tujunga, CA) and an icv infusion cannula connected to a pre-filled osmotic minipump (Alzet, Cupertino, CA)

was inserted into the right cerebroventricle and cemented to the skull using cyano acrylate. The osmotic minipump contained either sterile physiologic saline (HS, n=11 and NS, n=11 rats) or tempol (HS rats, n=12) dissolved in sterile saline (0.5 g/ml). The pump delivered solution at a rate of 0.5 □l per hour. Each rat was put in a plastic cage placed on top of radio receiving units (RPC-1, DSI, 1 per rat). Two weeks after surgery, mean arterial pressure and heart rate were sampled for 30-seconds every 5 minutes before, during, and for 1 hour after they were placed into Plexiglas restraining tubes (5 cm x 7 cm x 14 cm) for 1 hour. Data were digitized and stored on a hard drive using Dataquest ART software (DSI, St. Paul, MN). For each rat, pre-stress values for MAP and HR were averaged (2-10 values) to give a single baseline number. The change in MAP and HR from the initial was then determined for each 5-min sample during the 60 minutes of stress and the 60 minutes of recovery. Two-way ANOVA with repeated measures (time) was used to compare the effects in the three groups (NS + saline, HS + saline, HS + tempol) across the 120 minutes.

Sodium bisulfite mapping

One to two naïve female offspring (NS, n=4; HS, n=4) were selected from each litter for analysis. Animals sacrificed by decapitation at 12-13 weeks of age and tissue from the pituitary, hypothalamus, and hippocampus was collected and stored in liquid nitrogen.

Genomic DNA was extracted and isolated from the tissues by using the DNeasy blood & tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Homogenization was performed using a glass homogenizer and tissue was briefly grinded in ATL buffer. RNase A (Qiagen) was used to give a RNA-free product. DNA concentrations were determined by spectrophotometry and DNA quality was determined by gel electrophoresis in 1% agarose.

Bisulfite modification of the extracted DNA was performed using the Epitect bisulfite kit (Qiagen) according the protocol given. 200-500 ng of extracted DNA was used for the reaction.

The methylation status of the exon 1_7 glucocorticoid receptor promoter region (GenBank accession number AJ271870) was assessed by sequencing the bisulfite modified DNA. The bisulfite-treated DNA was subjected to two rounds of PCR using hemi-nested primers with the same reverse primer being used in both rounds.

Round 1: Forward primer:

1639-YGTTTTTTTTTTTAGGTTTTT-1659

Round 1: Reverse primer:

1909-CRAATTTCTTTAATTTCTCTTCTCC-1933

Round 2: Forward primer:

1737-GCCCCTCTGCTAGTGTGACAC-1757

Round 2: Reverse primer:

1909-CRAATTTCTTTAATTTCTCTTCTCC-1933

PCR amplification was performed in a reaction mixture containing 7 µl of bisulfite modified DNA, 2.5 µl of 10 × PCR buffer, 0.313 µl 10 mM dNTP's, 0.5 µl of each primer (50mM), and 0.2 µl Jumpstart TAQ polymerase (Sigma-Aldrich) and made up to 25 µl with H_20. The thermocycler protocol for each round of PCR involved an initial denaturing cycle (4 min, 95 °C), 5 cycles of denaturation (30 seconds, 95 °C), annealing (90 seconds, 56.7 °C), and extension (120 seconds, 72 °C), followed by 30 cycles of denaturation (30 seconds, 95 °C), annealing (90 seconds, 56.7 °C), and extension (90 seconds, 72 °C), followed by a final extension cycle (4 min, 72 °C) terminating at 4 °C.

The second round PCR product (197bp) was then run on a 2% agarose gel to confirm the product size. The product band was then cut out of the gel and the QIAquick gel extraction kit (Qiagen) was used to purify the DNA sample from any agarose. The DNA was then amplified with one round of PCR as described above using the round 2 primers. A sample of this product was run on a 2% agarose gel to confirm the product had been amplified. The amplified DNA was then cleaned with the QIAquick PCR Purification Kit (Qiagen). The final DNA product, containing the exon 1_7 promoter region of interest was then subcloned and transformed using chemically competent *E. coli* and TOP10 competent cells (TOPO TA Cloning Kit for Sequencing, Invitrogen) according to the manufacturer's instructions. 200 µl and 20 µl from each transformation was spread on kanamycin agar plates and colonies were allowed to grow overnight at 37 °C. The next day, ten colonies were randomly chosen from each plate. Pipette tips were used to transfer the colonies to PCR tubes. PCR was performed to amplify the ligated exon 1_7 GR promoter DNA fragment using M13 Reverse and M13 Forward (-20) primers, their priming sites being located in the plasmid. The thermocycler protocol for each round involved an initial denaturing cycle (3 min, 94 °C), 25 cycles of denaturation (45 seconds, 94 °C), annealing (30 seconds, 50 °C), and extension (1 min, 72 °C), followed by a final extension cycle (1 min, 72 °C) terminating at 4 °C. To purify the product from dNTP's and primers, 5 µl of the PCR product was mixed with an EXO I/SAP (USB) mixture containing 1.1 µl of SAP 10X RX buffer, 1 µl SAP (1 un/ µl, 0.1 µl EXO I (10 un/ µl), and 2.8 µl of ddH_2O for a total of 10 µl. The samples were run on a PCR thermocycler for (15 min 37 °C), activating the EXO I/SAP enzymes, and (15 min, 80 °C), inactivating the enzymes, terminating at 4 °C. The EXO I/SAP treated samples were then directly sequenced, using the M13 Reverse primer sequence provided

in the TOPO TA cloning kit, per animal per brain area at the BYU DNA Sequencing Center (Provo, Utah).

RESULTS

SEH mRNA measurements

RNA was extracted from the whole brains of adult (12-13 weeks) female rats. RT-PCR was performed and showed that the HS offspring (n=6) had significantly decreased levels of epx2 mRNA compared to the expression levels in NS (n=6) rats (fig.1).

SEH protein measurements

In order to verify that lower levels of mRNA led to a lower expression of the SEH protein, we measured the SEH protein expressed in the whole brain of female rats using Western blot. We were surprised to find that there was a significant increase in SEH protein in the brains of the HS group (n=10) compared to the NS group (n=10) (fig. 2).

SEH immunohistochemistry

Brains were sectioned (40µm) and immunohistochemistry specific to the SEH protein was performed. Sections were incubated for 24-48 hr with two different 1° antibodies (H-215 or Y-13). Immunoreactivity was detected primarily in the area of the arcuate nucleus of the hypothalamus (ARC), the subfornical organ (SFO), and the area postrema (AP). H-215 staining was diffuse, while the Y-13 staining was more localized into various punctate shapes. In the SFO and AP, H-215 staining was located within each structure, while Y-13 staining surrounded each structure (fig. 3). There was no staining detected in the hippocampus (not shown).

Icv Tempol

As expected, 1 hour of restraint produced a significant increase in HR and MAP in all groups (fig. 4). This effect gradually declined over the hour period and then a second increase in

both parameters was evoked when the rats were removed from the restraining tubes. Two-way ANOVA showed that there was no difference in response between the HS rats that received icv saline and those that received icv tempol. That is, the hyperresponsiveness observed in prior experiments was reversed by the free radical scavenger. However, it is impossible to tell if this reversal was due to the scavenging of free radicals because the expected exaggerated response in HS rats treated with icv saline compared to NS rats treated with icv saline was also absent. It appears that something related to the implanting of a chronic icv cannula system may be sufficient to interfere with the expected hyperresponsiveness.

GR mRNA measurements

Glucocorticoid receptor mRNA levels were measured in the pituitary, hypothalamus, and hippocampus by performing RT-PCR. Although there appeared to be several trends, none of the mRNA expression levels were significantly changed ($p > 0.05$) by the HS treatment (fig. 5). GR mRNA expression in the pituitary and hippocampus tended to be increased in the HS animals while expression in the hypothalamus tended to be lower.

GR protein

Glucocorticoid receptor protein levels in the hypothalamus and hippocampus of HS and NS adult female rats were analyzed through western blot. There were no significant differences between HS and NS in the expression of the GR protein, though both brain areas showed a tendency for GR protein to be higher in the HS animals (fig. 6). We were unable to extract sufficient protein from the pituitary to perform the Western blot analysis.

GR methylation

The methylation patterns for the exon 1_7 promoter region that contains the NFGI-A transcription binding site (sequence shown in fig. 7) was investigated by sequencing up to 10 subclones of bisulfite treated DNA for each animal (HS n=4 and NS n=4) and from each site (pituitary, hypothalamus, and hippocampus). The pituitary sequencing resulted in 7-9 readable sequences for each animal (HS n=3, NS n=3) (see Table 1). Overall, NS pituitary samples had 7 CpG dinucleotides sites that were 4% methylated, with no two animals having the same site methylated. The HS group only showed one site that was 4% methylated. The hypothalamus sequencing resulted in 9-10 readable sequences for each animal (HS n=3, NS n=3) (see Table 2). Overall, NS hypothalamus samples had 6 sites with 3-10% methylation. The HS group also had 6 sites with 3-17% methylation. The hippocampus samples had 2-8 readable sequences for each animal (HS n=4, NS n=3) and there was little to no methylation (see Table 3). Thus, there were no significant patterns or differences in methylation between HS and NS adult offspring female rats.

To further analyze the methylation data, the total number of sites methylated was determined for each rat and each brain site. This value was divided by the total number of possible CpG sites sequenced per animal to give an overall percentage of methylation in the exon 1_7 promoter region. For each brain site, the difference in percentage of methylation was compared between NS and HS rats using the Rank Sum Test (fig. 8). Overall methylation of the exon 1_7 promoter region was low; ranging from 5% to 1%. Though not significant, there was a trend for the overall methylation to be decreased in the DNA extracted from the pituitary of HS rats.

DISCUSSION

SEH mechanism

We were successful in showing that a prenatal high-salt diet leads to a significant change of expression in the SEH protein compared to a prenatal normal-salt diet. However, our results were counter to our hypothesis that the HS group would have less expression of the protein. Instead, SEH protein levels in the HS were significantly higher than the NS, despite having lower levels of epx2 mRNA. This argues against the proposed mechanism that a decrease in the SEH enzyme causes a significant increase in EETS, resulting in the increased production of ROS that downregulates or inhibits the effects of the baroreceptor reflex, thus increasing blood pressure[25]. Our data, instead, support the hypothesis that increases in SEH enzyme levels may diminish the effects of EETs by degradation. A decrease of SEH in the brain is protective against ischemic stroke by a vascular mechanism linked to the activity of EETs[49]. Thus it would seem that areas of the brain with high levels of SEH may be less efficient in receiving blood than normal and thus have impaired function. The areas of the brain where SEH was found in the present study, the arcuate nucleus[50], subfornical organ[51] and area postrema, are known to be involved in cardiovascular regulation[52]. It is therefore possible that the decreased amount of EETs caused by the upregulated SEH protein impairs the increase of blood flow to these areas, thus causing them to be less efficient in their function of cardiovascular regulation and stress response, resulting in a hyperresponsive pressor and hypertension when stressed.

It is not clear why epx2 mRNA levels are lower and SEH protein expression is higher in the HS group. Microarray analysis showed a 1.65 fold decrease in epx2 mRNA[15], but the Western blot we performed shows that SEH protein is more abundant. The mechanism for this

discrepancy is unknown, but it could involve differences in degradation rates of the mRNA versus the protein or negative feedback regulation of transcription.

A limitation of the RT-PCR and Western blot data is that the HS animals were selected for brain collection without first verifying that stress hyperresponsiveness was present. We felt confident in doing this because the exaggerated response to stress in HS rats has been repeated multiple times in our lab. Obviously, each individual rats shows different levels of hyperresponsiveness, but our sample size (n=10 in the case of the Western blot data) should have ensured a good representation of the range of response.

The experiment using icv infusion of tempol did eliminate the exaggerated increase in MAP and HR in HS offspring. However, since the HS rats that received icv saline did not exhibit the expected hyperresponsiveness compared to the NS that received icv saline, we cannot be sure that the absence of effect in the tempol treated rats was due to scavenging of free radicals. We speculate that the added stress associated with chronic implantation of an icv cannula may have already affected the HS animals in such a way that they could not exhibit the typical cardiovascular hyperresponsiveness with acute stress. The rationale for using icv tempol was derived from our expectation that SEH protein would be down-regulated in the brains of HS offspring. Since this did not occur, the idea of increased free radical production due to increased EETs seems less compelling.

At this point, though we have shown that SEH mRNA and protein are changed in the brains of female rats born to mothers who consumed high salt during pregnancy, we have not been able to show a role for this change in explaining the exaggerated cardiovascular response to acute stress.

GR mechanism

The results from our experiments concerning the glucocorticoid receptor suggests that it, too, may not play a role in the enhanced stress response of HS female rats. In the study done by Weaver et al.[39], high pup licking and grooming caused hypomethylation of the exon 1_7 promoter region in hippocampus DNA while low pup licking and grooming caused a hypermethylation of the region, especially the site 16, the 5' CpG dinucleotide of the NFGI-A transcription binding region. Both groups of rats were highly methylated at site 17, the 3' CpG dinucleotide of the NFGI-A binding region. Therefore, we expected our group of rats to have significant methylation at that site. There was no methylation detected at site 16 or 17 of hippocampus DNA in either HS or NS rats. However, in the pituitary and hypothalamus, a very small number of clones did have methylation at those sites, but they did not differ between HS and NS rats. Long-Evans hooded rats were used in Weaver's experiments while we used Sprague-Dawley rats. Another study[53] looked at this region to see if early maternal separation of Sprague-Dawley rats altered the methylation status of young pups. They too saw no change between their two experimental groups and no methylation of the NGFI-A binding site. This may indicate that DNA methylation status may be strain-specific along with condition-specific.

It is recognized that only a limited number of animals were used in the present study. Both GR mRNA and protein expression showed some non-significant trends when comparing NS to HS brain regions. It is possible that with additional animals some of these trends, if maintained, may become significant. The data showing methylation in the exon I_7 promoter region of the GR gene represents a new direction for the lab. Future studies examining epigenetic changes in gene expression are likely to provide an added dimension to the output of the lab.

CONCLUSIONS

The first experimental objective in the present study was to determine if a prenatal maternal high-salt diet in Sprague-Dawley rats affects the expression levels of soluble epoxide hydrolase in their female offspring as compared to normal-salt diet offspring. There is an increase in the expression of the SEH protein in HS offspring compared to NS offspring. This raises the possibility that high SEH levels may be influential in the acute hyper-responsiveness seen when the HS offspring experience acute stress during restraint. Finding specific immunoreactivity of SEH primarily in the arcuate nucleus (ARC) of the hypothalamus, the subfornical organ (SFO), and the area postrema (AP), areas of the brain that are known to regulate cardiovascular function, indicate that the upregulation of SEH may play a role in the enhanced response to stress in the HS offspring.

The second experimental objective was to investigate if expression of the glucocorticoid receptor had a role in the enhanced stress response HS female offspring experienced. There were no differences in methylation patterns between HS and NS female offspring of the exon 1_7 promoter region that contained the NFGI-A transcription binding site. No differences of GR protein and mRNA expression levels were seen between the HS and NS groups. This suggests that glucocorticoid receptor levels are not affected by the prenatal maternal high-salt diet model and is not a mechanism for the hyperresponsive pressor and tachycardic response to acute restraint. However, sample sizes were small and the trends found may suggest that additional samples could possibly produce significant differences in GR mRNA and Protein levels.

FUTURE RESEARCH

The assumption that SEH is a mechanism that contributes to the hyper-responsive pressor of the females of high-salt offspring seems valid due to our data showing a significant difference in SEH proteins found in cardiovascular regulating areas of the brain.

Further research could be done to reinforce this idea. It would be useful to investigate the level of EETs between HS and NS rats and to investigate their location in the brain compared to that of SEH in the brain to see if they coincide with our SEH data. The amount of influence SEH levels have on the stress response of rats could be evaluated by giving HS rats a SEH inhibitor and putting them through stress restraint to see if they respond with a lesser hypertensive response or even go back to NS levels.

The investigation of the mechanism behind the sex-specific response experienced by the HS female offspring could also be further researched. Perhaps testosterone plays a direct or indirect part in blood vessel dilation or constriction. Studies have experimented with castration and how testosterone levels influence prenatally programmed hypertension in male rats[54]. This same experiment could be used to study the male offspring in the high-salt model to see if they would have a similar response as females. SEH levels between males and females could also be studied.

Methylation of the SEH promoter region represses the expression of SEH and demethylation of the site increases expression[55]. Perhaps the prenatal high-salt diet causes an epigenetic change to the methylation patterns of the offspring in the SEH promoter. Other factors that play a role in the HPA-axis stress response, such as CRH, ACTH, and glucocorticoids could also be looked into. It would be interesting to explore the epigenetic

changes that our model may cause in offspring by performing a global methylation analysis with a DNA microarray chip.

Angiotensin II up-regulates SEH through a transcriptional mechanism, which results in decreasing the concentration of EETs[56]. Therefore, in our high-salt model we could also investigate the relationship between angiotensin II and SEH. Furthermore, we could not only observe brain levels but examine expression of these proteins in the kidneys.

The SEH RT-PCR and western blot experiments in the present study looked at whole brain levels of mRNA and protein. It could be possible that some areas of the brain express different levels of the protein and mRNA, as suggested by our immunohistochemistry results. Instead of looking at whole brains, specific areas, such as the arcuate nucleus, subfornical organ, and area postrema can be cut out and analyzed. This would give a more accurate depiction of the protein expression in those areas.

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TABLES

Table 1. Methylation of 17 CpG sites in the exon I_7 promoter region of the GR gene of DNA extracted from the pituitary of normal salt female (NSF) and high salt female (HSF) offspring.

		CpG Site																
Animal	Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
NSF1	9	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	1
NSF2	9	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
NSF3	8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
HSF1	9	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
HSF2	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HSF3	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2. Methylation of 17 CpG sites in the exon I_7 promoter region of the GR gene of DNA extracted from the hypothalamus of normal salt female (NSF) and high salt female (HSF) offspring.

		CpG Site																
Animal	Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
NSF2	10	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
NSF3	10	1	0	1	0	0	0	0	0	2	0	0	0	1	0	0	0	0
NSF4	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
HSF1	9	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
HSF2	9	1	2	0	0	3	0	0	0	1	0	0	0	0	0	0	0	0
HSF3	10	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3. Methylation of 17 CpG sites in the exon I_7 promoter region of the GR gene of DNA extracted from the hippocampus of normal salt female (NSF) and high salt female (HSF) offspring.

		CpG Site																
Animal	Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
NSF2	3	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
NSF3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NSF4	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HSF1	5	2	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0
HSF2	4	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
HSF3	8	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HSF4	6	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0

FIGURE LEGENDS

Figure 1: RT-PCR of epx2 mRNA extracted from whole brains of adult normal salt (NS) and high salt (HS) female rats (12-13 wks). * $p < 0.05$

Figure 2: Western blot of SEH protein extracted from whole brains of NS and HS adult female rats. * $p < 0.05$

Figure 3: Immunohistochemical staining of SEH in the arcuate nucleus (ARC), subfornical organ (SFO), and area postrema (AP). Brains were fix-perfused with buffered formalin and sectioned (40 μm) with a freezing microtome. Two different primary antibodies (1^o Ab) were used.

Figure 4: Effect of icv tempol or saline on the change in mean arterial pressure (MAP) and heart rate (HR) during restraint in normal salt females (NSF) or high salt females (HSF).

Figure 5: RT-PCR of GR mRNA extracted from the pituitary, hypothalamus, and hippocampus of NS and HS adult female offspring

Figure 6: Western blot of GR proteins extracted from the hypothalamus and hippocampus of NS and HS adult female offspring.

Figure 7: The sequence of the exon 1_7 promoter region of interest (GenBank accession number AJ271870). CpG islands are numbered 1-17. The NFGI-A binding site that contains site 16 and 17 is highlighted. The hemi-nested primers used in Round 1 and 2 of the bisulfite treated DNA PCR amplification are underlined.

Figure 8: Percentage methylation of the entire GR exon 1_7 promoter in (A) pituitary, (B) hypothalamus, and (C) hippocampus.

FIGURES

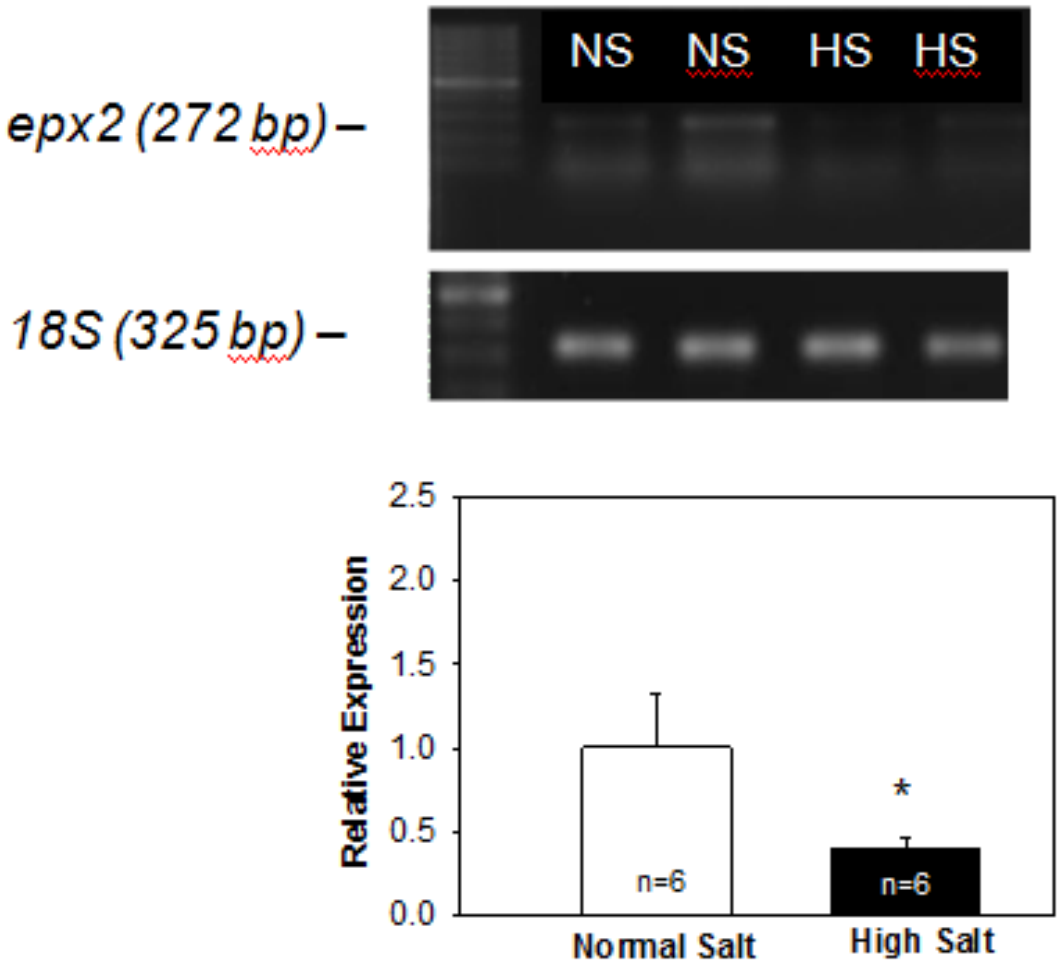
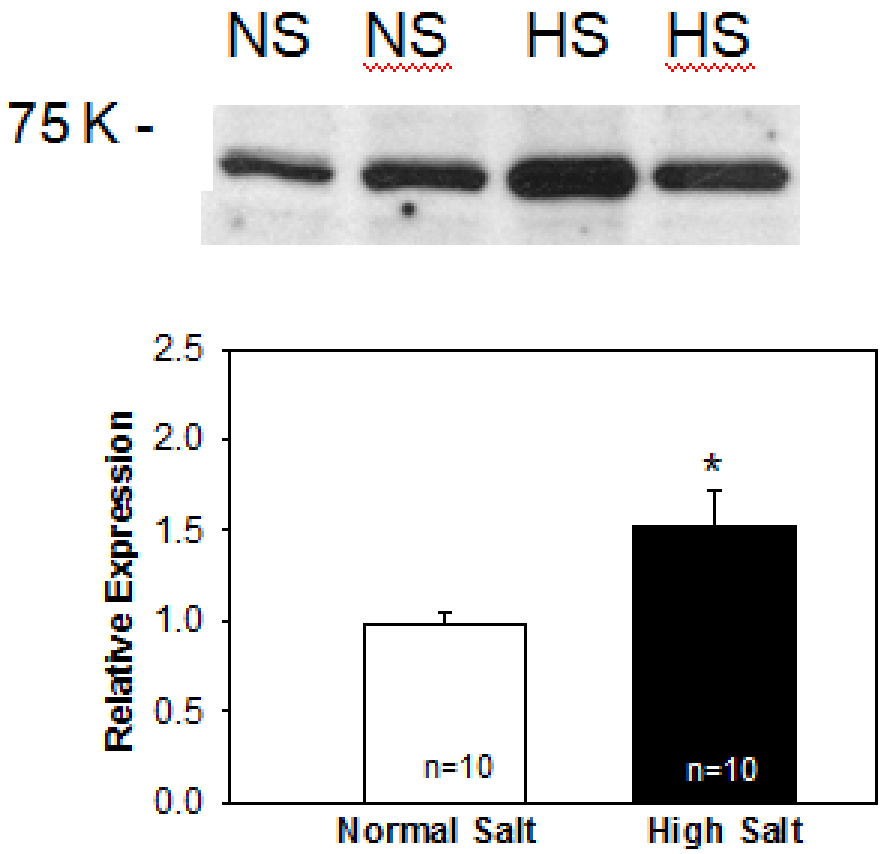


Figure 1


NS
NS
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HS
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Relative Expression
2.5
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1.0
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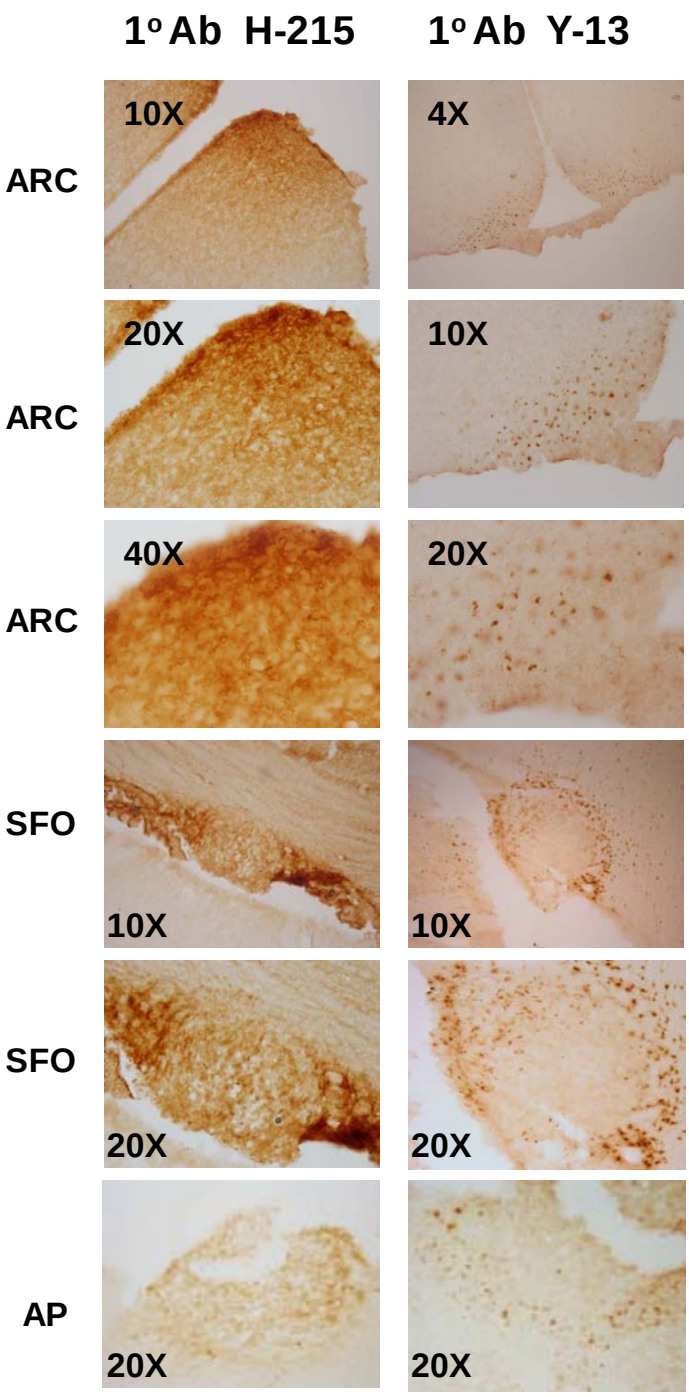
n=10
n=10
Normal Salt
High Salt

Figure 2


1° Ab H-215
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AP
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20X

Figure 3

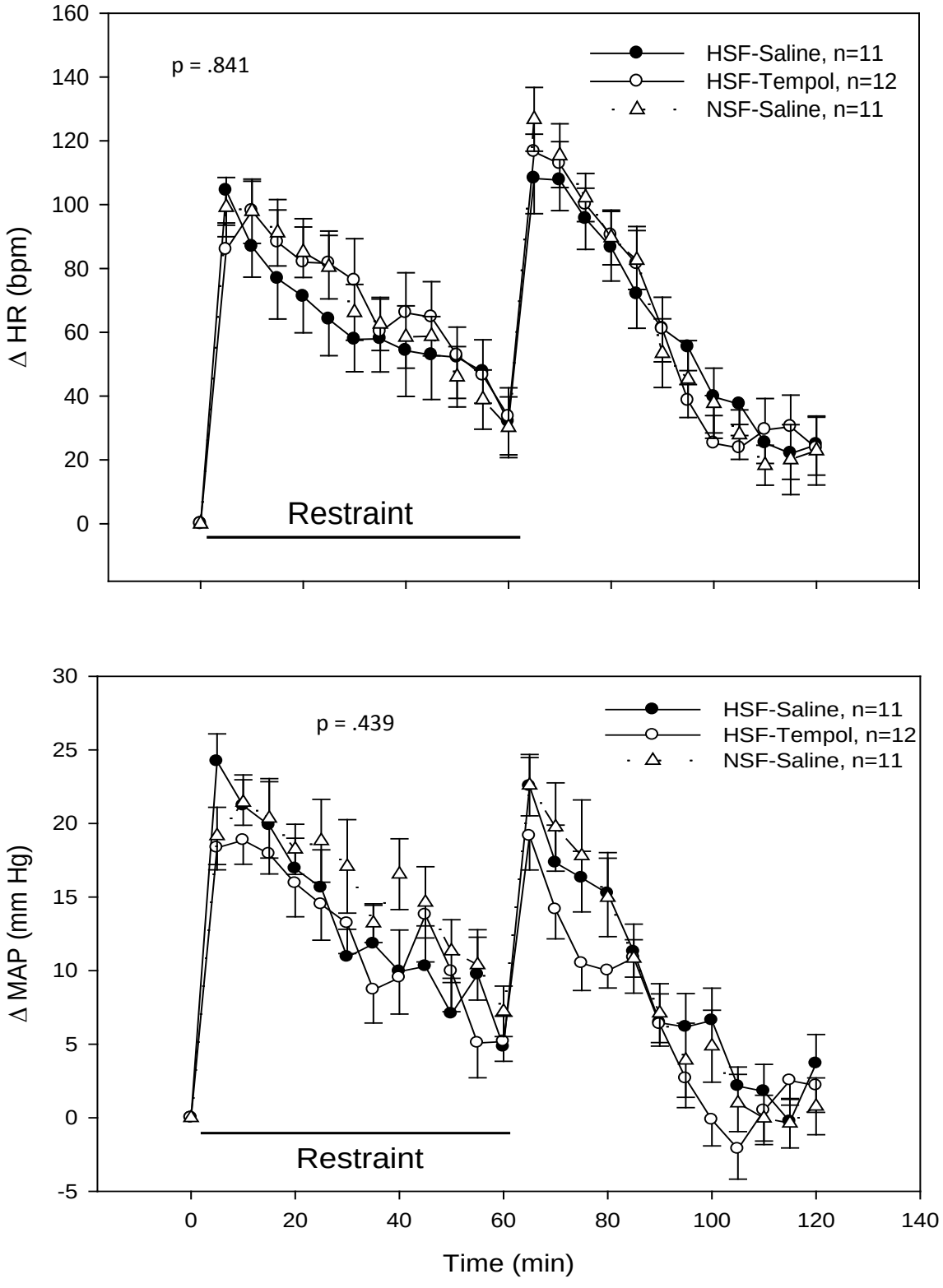
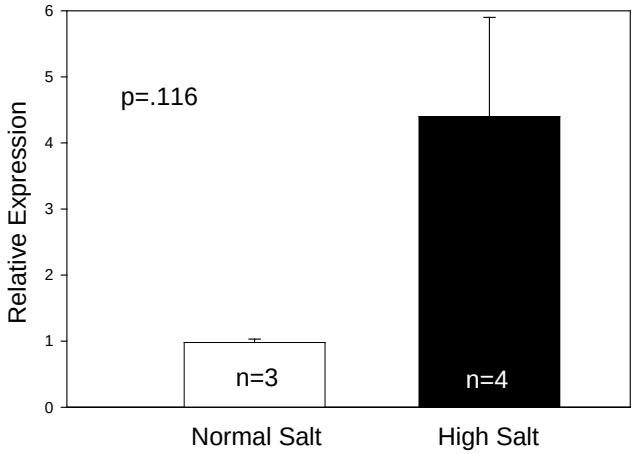
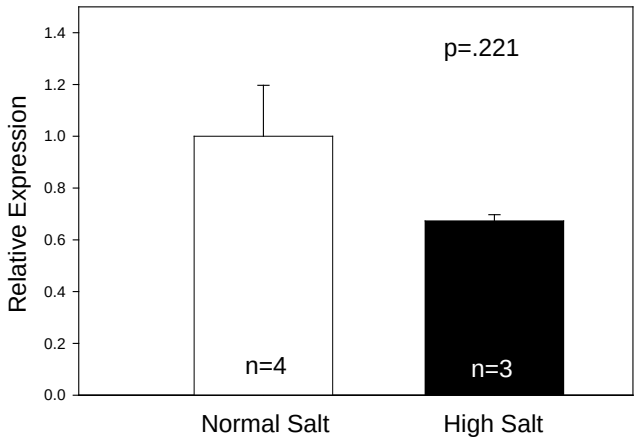

p = .841
HSF-Saline, n=11
HSF-Tempol, n=12
NSF-Saline, n=11
Δ HR (bpm)
Restraint
p = .439
HSF-Saline, n=11
HSF-Tempol, n=12
NSF-Saline, n=11
Δ MAP (mm Hg)
Restraint
Time (min)

Figure 4

Pituitary GR mRNA Expression



Hypothalamus GR mRNA Expression



Hippocampus GR mRNA Expression

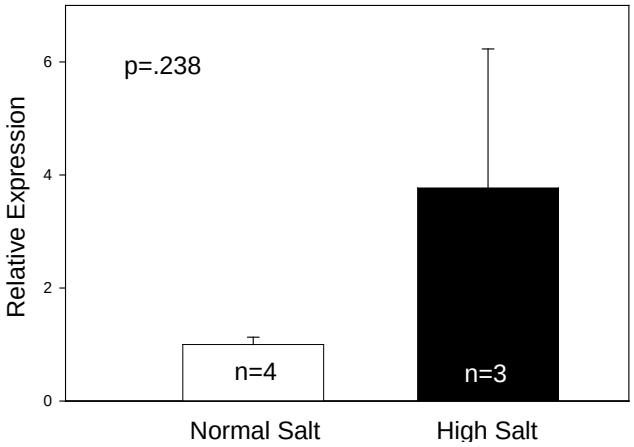
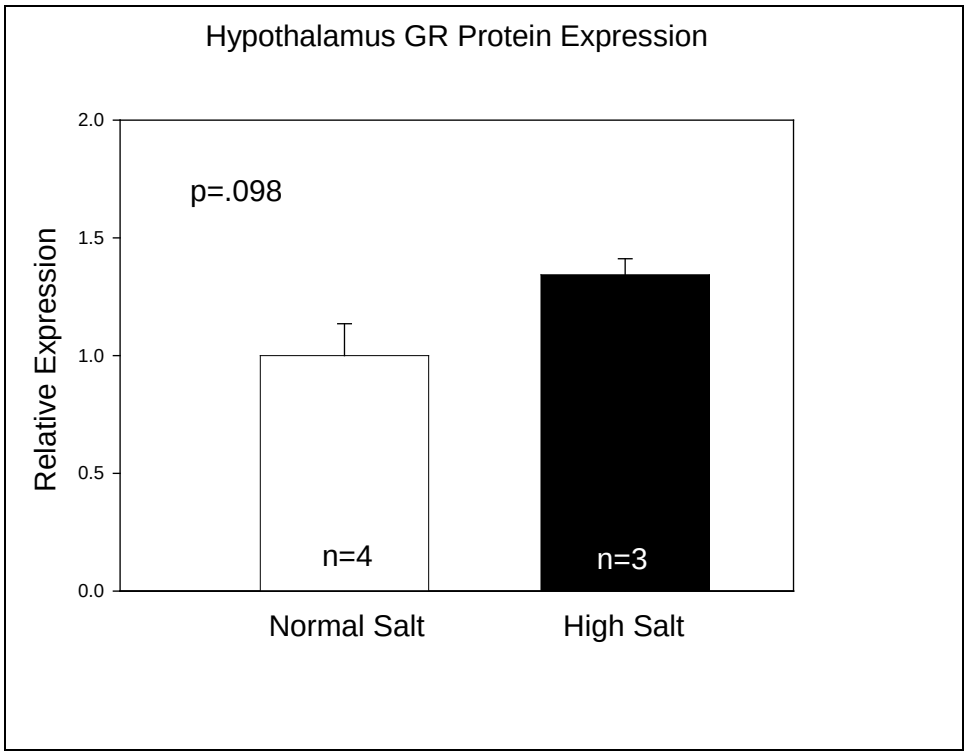


Figure 5


Hypothalamus GR Protein Expression
Relative Expression
2.0
1.5
1.0
0.5
0.0
p=.098
n=4
n=3
Normal Salt
High Salt

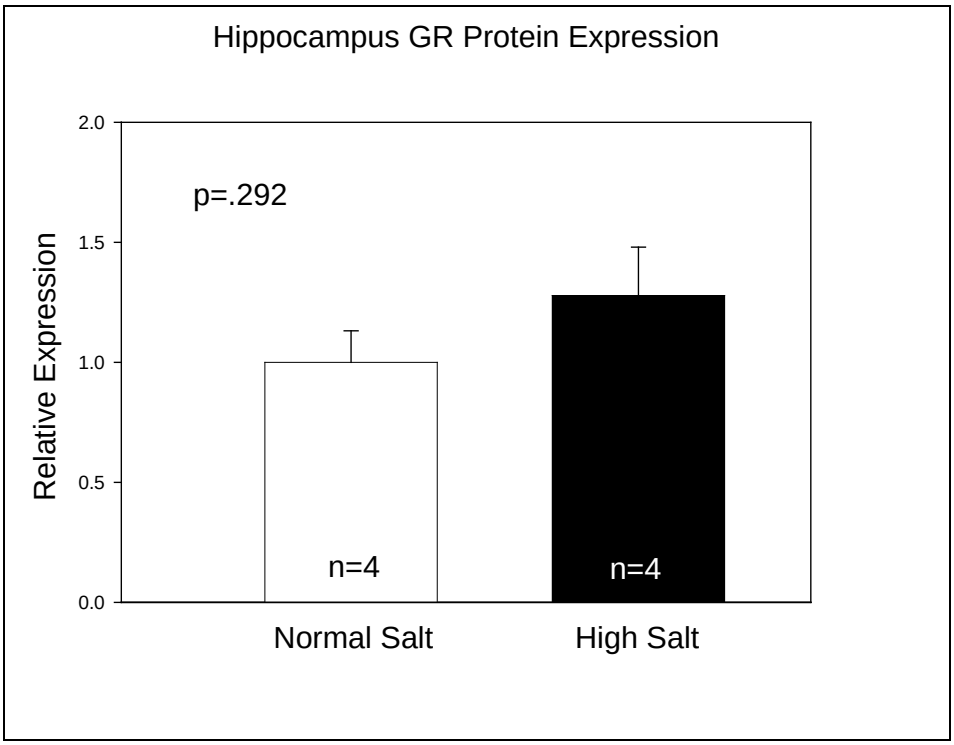
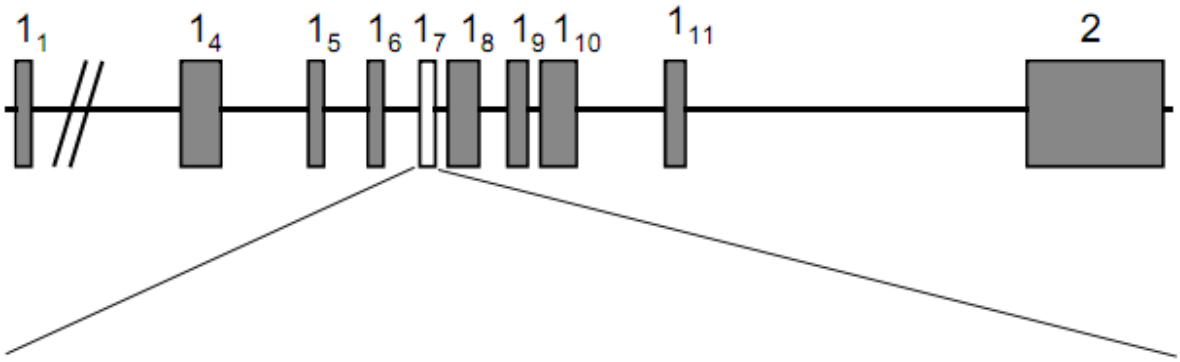

Hippocampus GR Protein Expression
Relative Expression
2.0
1.5
1.0
0.5
0.0
p=.292
n=4
n=4
Normal Salt
High Salt

Figure 6



tctgcgctcccctccccaggcctccccagagggcgtgtctgcagtcctgccccgagagcaagcggccagggctctgcggcaccgtttccg
tgccatcctgtagcccctctgctagtgtgacacactt[1]**cg**[2]**cg**caactc[3]**cg**cagttgg[4]**cg**gg[5]**cg**[6]**cg**gaccacccctg[7]**cg**gctctgc[8]**c**
ggctggctgtcaccct[9]**cg**ggggctctggctgc[10]**cg**accca[11]**cg**ggg[12]**cg**ggctcc[13]**cg**ag[14]**cg**gttccaagcct[15]**cg**gagctgg
g[16]**cg**ggggg[17]**cg**ggagggagcctgggagaagagaaactaaagaaactcggtttccc

Figure 7

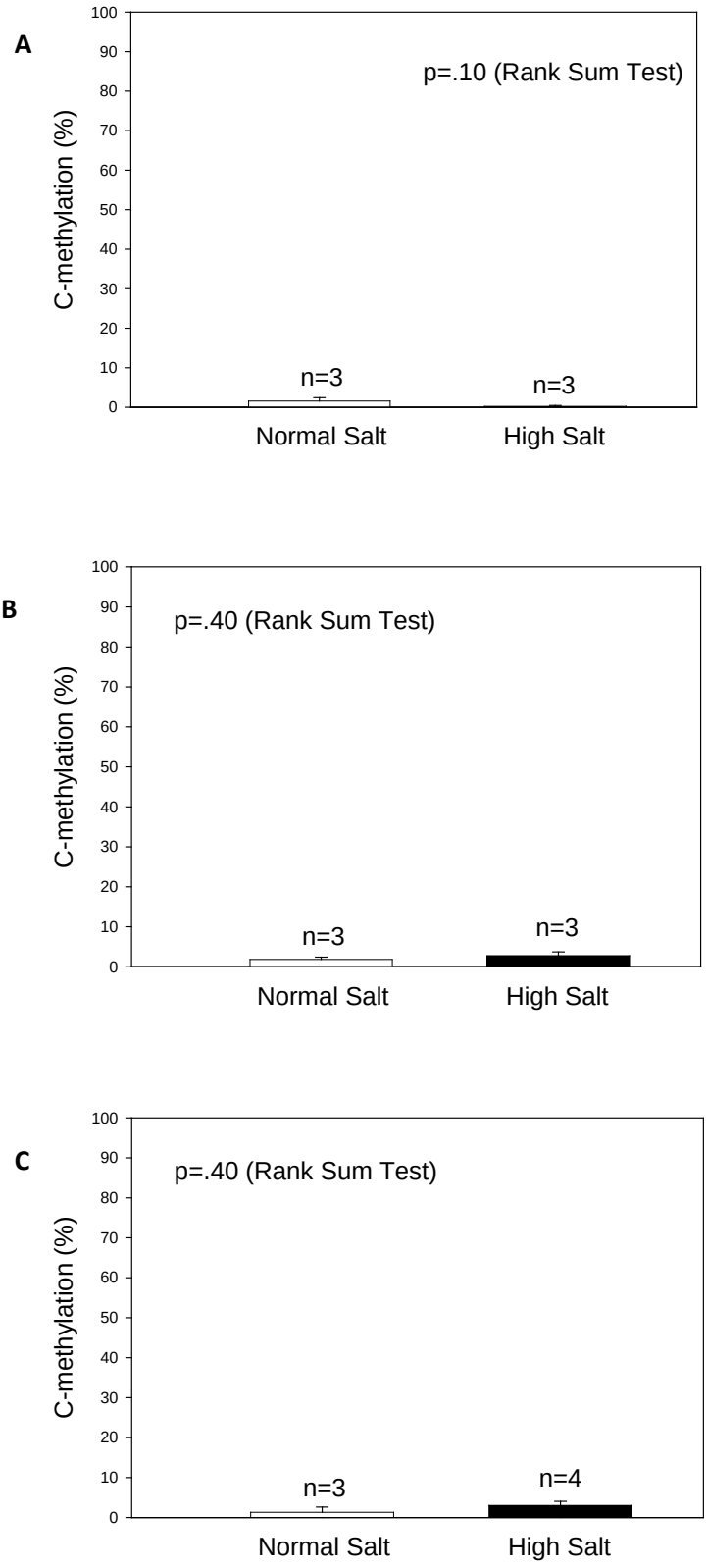

A
p=.10 (Rank Sum Test)
C-methylation (%)
n=3
n=3
Normal Salt
High Salt
B
p=.40 (Rank Sum Test)
C-methylation (%)
n=3
n=3
Normal Salt
High Salt
C
p=.40 (Rank Sum Test)
C-methylation (%)
n=3
n=4
Normal Salt
High Salt

Figure 8

CURRICULUM VITAE

Clinton Lee Johnson

1. EDUCATION

MS	Brigham Young University	2011	Physiology and Developmental Biology
BA	Brigham Young University	2010	Korean/Biology

2. HONORS AND REWARDS

University Scholarship Recipient	2007-2011
Dean's List of Humanities	2007

3. WORK EXPERIENCE

Apr.-June 2011 — Teaching Assistant, PDBio 365 (Pathophysiology)
Brigham Young University, Provo, UT
Held weekly office hours and help sessions for one-on-one tutoring. Created and presented reviews for exams. Graded various homework assignments.

Jan.-Apr. 2011 — Research Assistant
Brigham Young University, Provo, UT
Mentored undergraduate students. Performed various lab techniques including PCR; RNA and DNA isolation; western blot; gel electrophoresis; DNA CT conversion; and plasmid cloning.

Aug.-Dec. 2010 — Teaching Assistant, PDBio 305 (Physiology lab)
Brigham Young University, Provo, UT
Taught four lab classes. Held weekly help sessions and office hours.

Nov. 2008-July 2010 — Certified Nursing Assistant
Country View Manor, Provo, UT
Performed direct care for residents of the facility. Assisted in activities of daily living.

Sept. 2008- Apr. 2011 — Teaching Assistant, KOREA 490R (Hanja)
Brigham Young University, Provo, UT
Taught two classes and instructed students in Korean Hanja (Chinese characters). Wrote and graded exams. Designed homework assignments and class activities. Held personal help sessions.

Aug.-Dec. 2006 English Tutor
Brigham Young University, Provo, UT
Taught English to Hyundai middle managers from South Korea. Taught grammar, vocabulary, idioms, conversation, and culture.

4. VOLUNTEER SERVICE

Jan.-Mar. 2010 Student Mentor
Spring Creek Elementary School, Provo, UT
Gave guidance to 6th grade students in building a robot for the BYU robotic competition.

Feb.-Aug. 2009 ER volunteer
Orem Community Hospital, Orem, UT
Assisted patients to the exam room and took vital signs. Ran specimens to the lab. Comforted patients and cleaned the exam rooms.

Sept. 2007-Feb. 2009 Nursing Home Volunteer
Country View Manor, Provo, UT
I Ran a bingo game every week for residents. Helped residents with social skills. Assisted residents with disabilities to participate in activities.

June 2006 Medical Volunteer
Reach the Children, Nairobi, Kenya
Assisted in giving medical care to orphaned children. Taught simple first-aid and hygiene classes.

Oct. 2003-Oct. 2005 Proselytizing Missionary
Church of Jesus Christ of latter Day Saints, South Korea
Taught the gospel of Jesus Christ. Volunteered at a center for the handicapped, a hospital preparing medical supplies, and taught English at an elementary school.

5. MEETINGS ATTENDED

2010 Experimental Biology
Author on abstract and presented poster