Central Sodium Sensing: Implications for Blood Pressure Regulation

NCT Number: NCT05480722

August 19, 2025

STUDY PROTOCOL

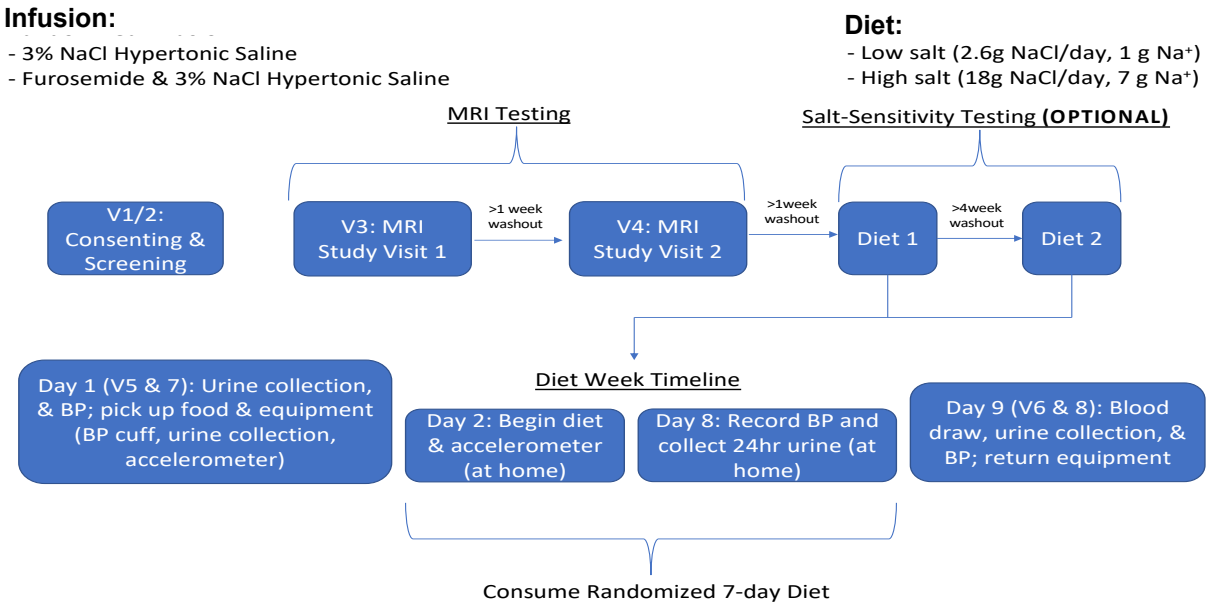

Figure 1. Study Design

All subjects will complete 8 visits (see above). Visits 1, 3, and 4 will be at the UD Center for Biomedical and Brain Imaging (CBBI). Visits 2, and 5-8 will take place at UD's Science, Technology & Advanced Research (STAR) Campus.

VISIT 1: INFORMED CONSENT, CBBI (60 MINUTES)

Informed consent will be obtained in person by a trained member of the research team at CBBI. All procedures and aspects related to the study including any potential risks will be described to the subject. The subject will read the informed consent form and the investigator will answer any questions that the subject may have prior to obtaining the subject's written consent. As documentation of the informed consent the subject and the investigator performing the consent will sign the consent document. A copy of the signed consent form will be given to the subject. After giving consent, subjects will lie in the mock scanner for at CBBI to screen for claustrophobia. Lastly, subjects will be given a personal food information form and 3-day diet recall to record food intake (2 weekdays and 1 weekend). These will help our dietician prepare food for the controlled feeding portion of this study. Subjects must complete these forms before the Nurse Managed Primary Care Center screening (visit 2). Subjects will not be excluded for food allergies

or preferences, but we will exclude individuals with very specific dietary restrictions such as celiac disease, gluten sensitivity, if an individual is vegan, or indicates an eating disorder. We can accommodate vegetarians who eat dairy and eggs.

VISIT 2: SCREENING, STAR CAMPUS (60 MINUTES)

All participants will be screened at the Nurse Managed Primary Care Center in the Health Sciences Complex of STAR. This initial screening visit lasts approximately 1 hour and involves answering an MRI screening form and a medical history questionnaire. Additionally, nurses will assess resting blood pressure, 12-lead electrocardiogram, height and weight, and obtain a venous blood and urine sample. The blood and urine sample will assess liver and kidney function, a lipid profile (i.e., cholesterol), a complete blood count, glucose, sodium, potassium, and chloride. Approximately 2 teaspoons of blood will be sampled. Investigators will review all information. Individuals with any signs or symptoms of disease (example: history of angina, chest pain, dizziness, or shortness of breath, etc.) will be excluded. Individuals with blood work within normal limits, and a resting electrocardiogram within normal limits will be accepted into the study. The results from the screening are reviewed by the registered nurse to determine if participants qualify for the study. Participants will receive a copy of the data collected on their vital statistics. Women taking hormonal or non-hormonal contraceptives will not be excluded. Additional exclusion criteria include allergy to sulfa drugs (Lasix/furosemide), claustrophobia, low blood pressure (systolic blood pressure <100mmHg and/or diastolic blood pressure <60 mmHg), high blood pressure (defined here as systolic blood pressure >130mmHg and/or diastolic blood pressure >80 mmHg), low potassium levels in the blood (<3.5 mmol/L), a history of cancer, diabetes, or any other chronic disease; a history of any heart disease; use of tobacco or nicotine products; nursing mothers; or a body mass index less than 18.5kg/m^2 or greater than 30kg/m^2. Certain medications may also result in exclusion from participating; this will be determined by the NMPCC in consultation with the primary investigator on an individual basis. If one of the test results is abnormal, individuals will be referred to their personal provider.

VISITS 3 & 4: fMRI SCAN (2 hours/visit; CBBI)

Subjects will be asked to log food and water intake for the 3 days leading up to the MRI visits on the AHA Sodium Tracker. Subjects will be asked to consume a diet containing 2,300 mg of sodium each of these days. Subjects will consume their own food. Food will NOT be provided by the dietician for the 3 days leading up to the MRI visits. Subjects will be instructed to refrain from taking over the counter medications for 48 hours, and from vigorous aerobic exercise or alcohol for 24 hours prior to MRI scans. Subjects will come into the study visit after at least a four-hour fast. The MRI studies must be completed within 3 months of the screening. If we are unable to schedule both MRI visits within 3 months of screening, subjects will be asked to come to the Cardiovascular Physiology Lab again before the MRI visit for a blood draw to check if electrolyte levels are normal. The second MRI data collection must be performed no sooner than 1 week from the first MRI data collection.

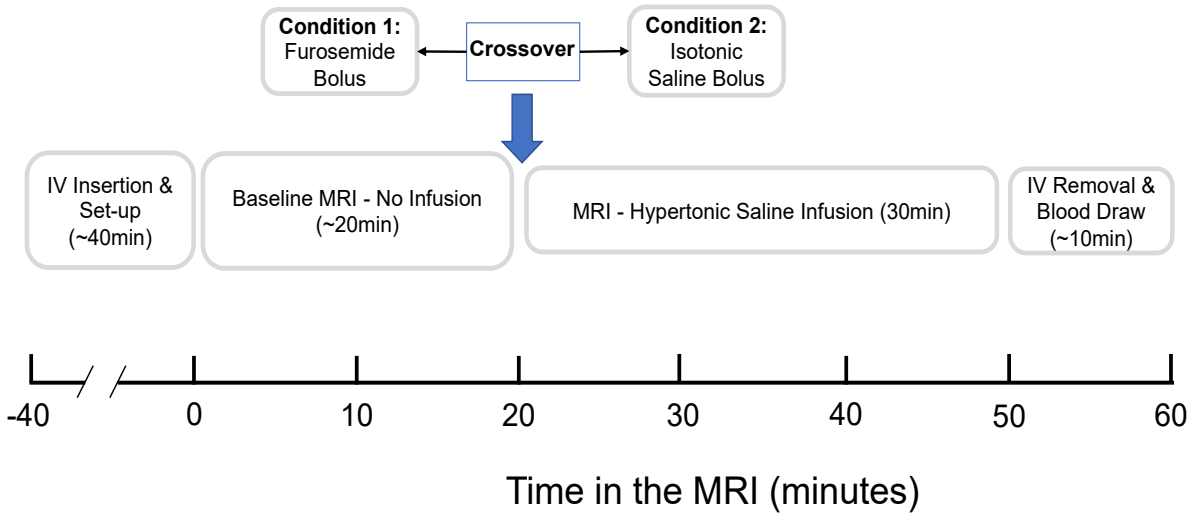

Figure 2: Timeline of Experimental Protocol

Urine Sample Collection and Absorbent Brief or Condom Catheter (men only)

All subjects will be asked to provide a urine sample. We will measure electrolytes, osmolality, and specific gravity as well as perform a pregnancy test for women. Only women with a negative pregnancy test will be allowed to continue with the study. If the test is positive, the participant will be informed and referred to their primary care

provider. After subjects provide a urine sample, they will be asked to change into scrubs and put on the absorbent brief, both provided by the research team in a private changing room and bathroom. Subjects will be asked to urinate into the brief when they have the urge to urinate during the MRI. Male subjects will have the additional option of wearing a condom catheter instead of the absorbent brief. Likewise, male subjects will be asked to urinate while wearing the condom catheter when they have the urge to urinate during the MRI.

Catheter Insertion & Blood Sample

At CBBI, a registered nurse will insert one catheter into the arm of the participant, leaving a small flexible tube in their vein prior to the MRI scan. The catheter will be used for infusion of the saline and furosemide, and to draw blood samples. Blood samples will be obtained pre- and post-infusion to measure electrolytes, osmolality, hematocrit, and hemoglobin. Some of the blood sample will be stored in our -80C freezer (indefinitely) for future assessment of renin angiotensin aldosterone system hormones, inflammatory markers, and reactive oxygen species. About 40 mL of blood will be sampled from the intravenous catheter, pre- and post-infusion (80 mL total). The catheter will be removed at the end of the study.

Furosemide Administration

Each subject will undergo two separate MRI study visits (visit 3 and visit 4), one with and one without NKCC2 antagonism. Furosemide (NKCC2 antagonist) will be purchased through the Nurse Managed Primary Care Center from Heritage Pharmaceuticals through McKesson (link here). NKCC2 antagonism will be achieved by administering a bolus of furosemide (40mg in 4mL of 0.9% NaCl isotonic saline) over a 2-minute period immediately prior to the hypertonic saline infusion. This timing will achieve peak NKCC2 blockade. A bolus of 0.9% NaCl (4mL) will be administered over a 2-minute period during the control infusion, and the participant will be blinded to the condition. A registered nurse, under the off-site supervision of a cardiologist will administer the bolus of furosemide and administer the hypertonic saline infusion.

Hypertonic Saline Infusion

A 3% NaCl hypertonic saline will be infused at a rate of 0.15 $ml \cdot kg^{-1} \cdot min^{-1}$ into the participant's arm for 30 minutes total while participants are in the MRI scanner. The hypertonic saline will be purchased from a manufacturer and will be prepared by the registered nurse. The volume of hypertonic saline in the tubing will be closely monitored by the registered nurse to ensure that participants are receiving the correct amount of saline. The volume infused and infusion rate is similar to previous studies infusing saline, which we have previously published (Farquhar et al AJP-Heart 2006). The 3% hypertonic saline is expected to raise blood sodium concentration and plasma osmolality approximately 1-2%. Blood samples will be taken pre- and post-infusion to measure changes in blood sodium concentration and osmolality.

MRI Scan. The MRI scan will take approximately 1 hour (Figure 2). The subject will be free to stop at any time, either by indicating their desire to do so directly to the investigator or by squeezing a ball while in the MRI scanner that alerts the technologist who is running the machine.

- **MRI Screening Form.** Participants will again fill out the MRI screening form prior to the scan to ensure they can safely undergo MRI.
- **Structural and BOLD MRI.** During all scans the subjects will wear disposable earplugs and headphones to reduce scanner noise. Participants may also be asked to wear a pulse oximeter on their finger to measure heart rate or a respiratory belt around their chest to measure breathing. Both devices are provided by Siemens with the MRI scanner, are MR-safe and MR-compatible, and interface with the MRI scanner software. Participants will also wear an FDA-approved MRI compatible blood pressure cuff on their middle finger throughout the scan that measures blood pressure through a Pulse Decomposition Analysis. This device is made through BioPac Systems and is MR-safe and MR-compatible.

OPTIONAL COMPONENT OF STUDY (VISITS 5-8)

VISITS 5 & 7: BLOOD DRAW, URINE COLLECTION, BP; & FOOD & EQUIPMENT PICKUP, STAR (30min/visit)

Salt Sensitivity Assessment: Subjects will consume a low (2.6g NaCl/day, 1g Na^+) and high (18g NaCl/day, 7g Na^+) sodium diet for 7 days each (randomized, washout between diets). Twenty-four-hour BP (SunTech Oscar2) and urine, to confirm diet compliance, will be collected on day 8 of each diet week (Figure 2). A change of 24 hr mean BP of > 5 mmHg, from the low to the high sodium condition will be considered a salt sensitive response.

Participants will report to the CV lab of STAR on day 1 (Figure 1) of each randomized diet to 1) have a venous blood draw performed on them by a research team member trained in phlebotomy; 2) provide a urine sample; 3) have brachial blood pressure measured; and 4) pick up food, a 24hr BP cuff, and urine collection supplies. Blood samples will be obtained to measure electrolytes, osmolality, hematocrit, and hemoglobin. Some of the blood sample will be stored in our -80C freezer (indefinitely) for future assessment of renin angiotensin aldosterone system hormones, inflammatory markers, and reactive oxygen species. About 40 mL of blood will be collected from the butterfly needle insertion. Urine samples will be obtained to measure electrolytes, osmolality, and specific gravity as well as a pregnancy test for women. Diets will be prepared by a registered dietician on staff. These diets will be separated by a minimum 4-week washout, but no greater than 6 months. Participants will drink water ad libitum but quantities will be recorded. Participants during different dietary control phases may be provided with Gatorade, juices and milk; in addition, the dietician will provide a detailed diet menu to allow for additional beverages such as coffee, decaffeinated coffee and/or tea that will not be provided to the participants. Physical activity will also be assessed using a physical activity monitor to evaluate activity patterns during each period of the controlled feeding study. Prior to starting the controlled salt diet, we will confirm that all female subjects are not pregnant by performing an over-the-counter pregnancy test. To accomplish this, participants will have to provide a small urine sample.

VISITS 6 & 8: BLOOD DRAW, URINE COLLECTION, BP; & EQUIPMENT DROP-OFF, STAR (30min/visit)

Participants will report to the CV lab of STAR on day 9 (Figure 1) of each diet to 1) have a venous blood draw performed on them; 2) provide a urine sample; 3) have brachial blood pressure measured; and 4) drop off any left over food, the 24hr BP cuff, accelerometer and urine collection supplies. Blood samples will be obtained to measure electrolytes, osmolality, hematocrit, and hemoglobin. Some of the blood sample will be stored in our -80C freezer (indefinitely) for future assessment of renin angiotensin aldosterone system hormones, inflammatory markers, and reactive oxygen species. About 40 mL of blood will be collected from the butterfly needle insertion. Urine samples will be obtained to measure electrolytes, osmolality, and specific gravity as well as a pregnancy test for women.

Alternate order of MRI (Visits 3 and 4) and Salt Sensitivity Testing (Visits 5-8).

Subjects will be given the option to instead do the salt sensitivity testing (visits 5-8) first, followed by the MRI study visits (visits 3 and 4) if they have previously volunteered in an MRI study at the University of Delaware (and were not claustrophobic) or must begin with the salt sensitivity testing due to the logistics of scheduling.

STUDY POPULATION AND RECRUITMENT

We will recruit 50 normotensive men and women (18-45 years old), from the community surrounding the University of Delaware. Our study sample will be representative of the general population, including all races/ethnicities. Our strategies will include: Nextdoor app, classifieds, flyers posted locally, and targeted social media ads using ‘bump’ advertisement. The study will include all healthy participants with blood pressure between 100/60 to 130/80 mmHg. Individuals will not have any chronic diseases.

Participants excluded from the study will include individuals with known cardiovascular disease, high blood pressure (defined here as systolic blood pressure >130mmHg and/or diastolic blood pressure >80 mmHg), low blood pressure (defined here as systolic blood pressure <100mmHg and/or diastolic blood pressure <60 mmHg), an

allergy to sulfa drugs (Lasix/furosemide), low potassium in the blood (hypokalemia, <3.5 mmol/L), metabolic diseases, cancer, kidney disease, a body mass index (BMI) outside of the range of 18.5 and 30 kg/m^2, nicotine product consumer, pregnant or planning to become pregnant, and other chronic diseases. Participants with any conditions which would contra-indicate MRI: implant of pacemakers or pacemaker wires; artificial heart valve; brain aneurysm surgery; middle ear implant; non-removable hearing aid or jewelry; braces; cataract surgery or lens implant; implanted mechanical or electrical device; foreign metallic objects in the body such as bullets, BBs, shrapnel, or metalwork fragments; claustrophobia; uncontrollable shaking or the inability to lie still for 1.5 hours will be excluded.

RISKS AND BENEFITS

There are no known risks associated with obtaining height, weight, resting blood pressure, or urine analysis to test for pregnancy.

Catheter Insertion & Butterfly Needle Insertion for Blood Draw

Participants may have pain or bruising at the site where the catheter or butterfly needle is placed in the arm, and there is a small risk of infection. Fainting sometimes occurs during or shortly after blood is drawn.

Condom Catheter

The condom catheter is secured on the male penis by a self-adhesive latex. The self-adhesive latex may increase male participant discomfort when removing the condom catheter. Additionally, there is a risk of an allergic reaction to the latex if male participants are allergic to latex.

Hypertonic Saline Infusion

The risks associated with infusing saline in the vein are low but can include damage to the vein, blood dilution, spasm or contraction of the vein, edema, high blood pressure, excess fluid in the lungs or brain, volume overload (too much fluid in the body), and heart failure. Adverse symptoms of these conditions include local arm discomfort where the

catheter was inserted, shortness of breath, a headache, and/or fluid accumulation in the arms, legs, or lungs.

None of these adverse symptoms are expected in healthy adults. Blood pressure may increase during the hypertonic saline infusion. Therefore, hypertonic saline will not be administered if resting blood pressure on the day of testing is above 140/90 mmHg. If blood pressure goes above 170/90 mmHg, we will stop the infusion. A controlled infusion of 3% NaCl has been safely performed in our lab and other labs.

Hypertonic saline infusion studies have been previously used in our lab

1. Greaney JL, Ray CA, Prettyman AV, Edwards DG, Farquhar WB. Influence of increase plasma osmolality on sympathetic outflow during apnea. *Am J Physiol Regul Integr Comp Physiol*, 299: R1096. 2010.
2. Wenner MM, Rose WC, Delaney EP, Stillabower ME, Farquhar WB. Influence of plasma osmolality on baroreflex control of sympathetic activity. Am J Physiol Heart Circ Physiol, 293: H2313-H2319. 2007.
3. Farquhar WB, Wenner MM, Delaney EP, Prettyman AV, Stillabower ME. Sympathetic neural responses to increased osmolality in humans. *Am J Physiol Heart Circ Physiol,* 291: H2181-H2186. 2006.
4. Babcock MC, Brian MS, Watso JC, Edwards DG, Stocker SD, Wenner MM, Farquhar WB. Alterations in dietary sodium intake affects cardiovagal baroreflex sensitivity. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, ajpregu.00002. 2018.

Hypertonic saline infusion studies performed in other labs

1. Stachenfeld NS, DiPietro L, Palter SF, and Nadel SR. Estrogen influences osmotic secretion of AVP and body water balance in postmenopausal women. *Am J Physiol* Jan; 274(1):R187-95, 1998.
2. Stachenfeld NS, Mack GW, Takamata A, DiPietro L, and Nadel ER. Thirst and fluid regulatory responses to hypertonicity in older adults. *Am J Physiol* Sep;271(3 pt 2): R757-65), 1996.

Furosemide

There are several risks of infusing furosemide although the adverse reactions are uncommon. Furosemide is a widely used medication. References 1 (n=199) & 3 (n=8) below used an identical dosage that we propose (40mg). The infusion of furosemide could lead to gastrointestinal, allergic, central nervous system, blood and heart reactions. Blood pressure may decrease after the furosemide bolus. If blood pressure goes below 90/50 mmHg after furosemide is given, we will stop the study visit. Symptomatic hypotension leading to dizziness or fainting may also result. Furosemide is a diuretic and increases the urge to urinate and can make participants feel thirsty. Because of this, it is likely that participants will need to use the restroom during the MRI scan. When subjects need to use the restroom during the MRI scan, they will be asked urinate into the absorbent brief they will be wearing. This is a normal response, but it may cause a feeling of embarrassment. If participants choose to come out of the MRI scan to use the restroom, the study will be concluded because the effect of the hypertonic saline and furosemide will have worn off by the time the participant is put back into the MRI. Participants will still be compensated and allowed to proceed with the other study visits.

Pregnancy: Furosemide falls under Pregnancy Category C. At higher than maximal recommended human doses, rodent studies have shown unexplained fetal harm. There are no adequate and well-controlled studies in pregnant women. Although there are no established guidelines at this time regarding furosemide and pregnancy, participants will be informed that there is risk. If the participant may be pregnant or if the participant does not want to expose themselves to this risk, they will be excluded from participating in this study. Female subjects will only be permitted to participate upon a negative pregnancy test.

Furosemide infusion studies performed in other labs

1. Chun TY, Bankir L, Eckert GJ, Bichet DG, Saha C; Zaidi S, Wagner MA, Pratt JH. Ethnic differences in renal responses to furosemide. *Hypertension*, 52(2):241-8. 2008.

2. Musso CG, Reynaldi J, Vilas M, De Miguel R, Imperiali N, Algranati L. Fractional excretion of K, Na, and Cl following furosemide infusion in healthy, young and very old people. *Int Urol Nephrol.* 42:273-277. 2010
3. van Meyel JJ, Smits P, Russel FG, Gerlag PG. Diuretic efficiency of furosemide during continuous administration versus bolus injection in healthy volunteers. *Clinical Pharmacology and Therapeutics*. Apr;51(4):440-444. 1992.

Magnetic Resonance Imaging (brain structure and activation):

MRI does not use any ionization radiation and it does not use radioactivity, however, there are risks associated with MRI scans. Potential risks are listed below.

- **Metal**: The MRI scanner produces a constant strong magnetic field, which may cause any metal implants, clips, or implanted medical devices within the body to shift position or malfunction. Participants will not be allowed to participate in this study if they have any implanted metal, clips or devices. Participants will be screened to make sure that it is safe for them to enter a strong magnetic field. Participants will be asked to provide as much information as they can, for example if they had surgery in the past, so that we may decide whether it is safe for them to be a participant. Metallic objects brought into the MRI environment can become hazardous projectiles and can also interfere with the data quality. To minimize this risk, metal earrings, other piercings, necklaces and any other metal in contact with their body must be removed prior to the study. Participants must also remove all items from their pockets, including coins, electronics (including cell phones and hearing aids) and wallets. Participants must remove belts with metal buckles, and they may be asked to change into a gown that we will provide if their clothing contains significant metal, including metal underwire bras.
- **Pregnancy**: Exposure to MRI scanning might be harmful to an unborn child. Although there are no established guidelines at this time regarding MRI and pregnancy, participants will be informed that there is a possibility of a yet undiscovered pregnancy related risk. If the participant may be pregnant or if the participant does not want to expose themselves to this risk, they will be excluded from participating in this study.

- **Inner ear damage**: MRI scanning produces loud noises that can cause damage to the inner ear if appropriate hearing protection is not used. Earplugs and/or headphones will be provided to protect participant's ears.
- **Claustrophobia**: When a participant is inside the MRI scanner, the "bore" of the scanner will surround the part of their body that is being scanned. For example, if a participant was getting an MRI of their knee, the participant would be positioned so that their knee was centered in the bore of the scanner. In the case of fMRI, we are interested in brain activity, and the participant's head will be centered inside a close-fitting scanning coil positioned in the bore of the scanner. If the participant feels anxious in confined spaces, they will have the option to discontinue the study. We will have subjects try our "mock" scanner to evaluate their comfort level with the enclosed space of the magnet bore. If the participant decides to participate and begins to feel claustrophobic, they will be able to tell us via the intercom or the squeeze ball and we will discontinue the study immediately.
- **Burns**: In rare cases, contact with the MRI transmitting and receiving coil, conductive materials such as wires or other metallic objects, or skin-to-skin contact that forms conductive loops may result in excessive heating and burns during the experiment. The operators of the MRI scanner will take steps, such as using foam pads, when necessary, to minimize this risk. Tattoos with metallic inks can also potentially cause burns. In addition, please let the MRI operator know immediately if you experience any heating or burning sensations during a scan. The scanning session will be stopped as soon as the participant tells the operator.
- **Nerve or muscle stimulation**. While the scanner is operating, there is a small chance that the rapidly changing magnetic fields could cause a slight tingling sensation or a muscle twitch, usually felt in the upper arms or torso. While these sensations may be startling, they are not dangerous or a health risk, and they have no lasting consequences. The sensations should stop when the scan ends. Because these sensations may nevertheless be distracting or even possibly uncomfortable, participants will be asked to squeeze the signal bulb to alert the scanner operator if they feel tingling or muscle twitching, and we will immediately stop the scan.

Participants will then have the opportunity to choose to withdraw from the study or to continue.

- **Other Risks**. Besides the risks listed above, there are no other known risks from the magnetic field or radio waves at this time. Although functional MRI scanning has been used for more than 20 years, long-term effects are unknown.

Seven-day diets:

There are minimal risks associated with consuming a 7-day low salt diet and 7-day high salt diet. During the high salt diet, blood pressure may rise to higher-than-normal levels, and subjects might experience slight hand swelling, frequent urination from increased water intake, bloating, nausea and/or vomiting during the dietary salt manipulation. There is no increased risk for women regardless of phase of menstrual cycle and diets should not affect menstruation.

7-day accelerometer, 24-hour blood pressure monitoring & urine collection:

There are no known risks to wearing an accelerometer or continuous blood pressure monitor on the upper arm. During waking hours, blood pressures are taken every 20 minutes, while at night they are taken every 30 minutes. Minor discomfort may be experienced when the cuff inflates, and it may wake subjects during sleep. The 24-hour blood pressure cuff and 24-hour urine collection may disrupt normal daily living. There are no known risks to providing a urine sample.

STEPS TAKEN TO MINIMIZE RISK

Catheter Insertion & Butterfly Needle Insertion for Blood Draw

Standard safety precautions will be taken when drawing blood samples and placing the IV catheter and butterfly needle. To minimize the risk of infection associated with blood collection, only sterile, single use catheters and butterfly needles will be used. To minimize the risk of bruising, proper blood collection technique will be utilized.

Condom Catheter

There is nothing we can do to minimize the risk of discomfort when removing the condom catheter. We will ask male participants if they have a known latex allergy. If they are allergic to latex, they will not be permitted to use the condom catheter and will have to use the absorbent brief.

Hypertonic Saline & Furosemide Infusion

The risks of the hypertonic saline and furosemide infusions will be minimized by having proper medical oversight of the procedure. A registered nurse, under the supervision of a cardiologist, will administer the infusions and monitor the participant throughout the entire protocol. We are using previously established hypertonic saline and furosemide doses that have shown to be safe for research (see references above). We will not administer furosemide if blood pressure is too low (hypotensive, <100/60 mmHg), if subjects are allergic to sulfa drugs (Lasix/furosemide), or if subjects have low potassium in the blood at their screening visit (hypokalemia, <3.5 mmol/L). Furosemide is a diuretic and increases the urge to urinate and can make participants feel thirsty. When subjects need to use the restroom during the MRI scan, they will be asked to urinate into the absorbent brief they will be wearing. This is a normal response, but it may cause a feeling of embarrassment.

Following the conclusion of the infusion protocols, participants will be given sanitation wipes and asked to change back into their clothing and remove and dispose of the absorbent brief in the private changing room and bathroom. They will also be given water and a snack and will remain in the lab for at least 45 minutes for observation by the research nurse. We will leave the IV catheter in the arm and assess blood pressure and electrolytes during this time, to ensure the values are within clinically acceptable limits prior to the participant leaving the facility. In the unlikely case the person has symptoms, an abnormal blood pressure (e.g., < 90/50 mmHg or > 170/90 mmHg), and/or abnormal electrolyte levels (e.g., potassium level (< 3.5 mmol/L), the registered nurse will determine the appropriate course of action. The course of action will be determined by our post furosemide blood pressure and potassium decision charts (Figure 3). Both decision flow

charts will be followed to determine appropriate course of action after the infusion. Subjects may be asked to remain at the MRI facility for further blood pressure and electrolyte observation (up to 2 hours after infusion). The additional blood draw (4mL) will be through the IV catheter already in place. Subjects may also be encouraged to eat a diet high in potassium for a few days if potassium is low (diet suggestions attached). In the highly unlikely case of abnormal electrolytes and symptoms, subjects may be referred to their primary care physician or sent to a local emergency department via ambulance. We do not expect any adverse effects in the well-screened subjects that will be tested as part of this protocol.

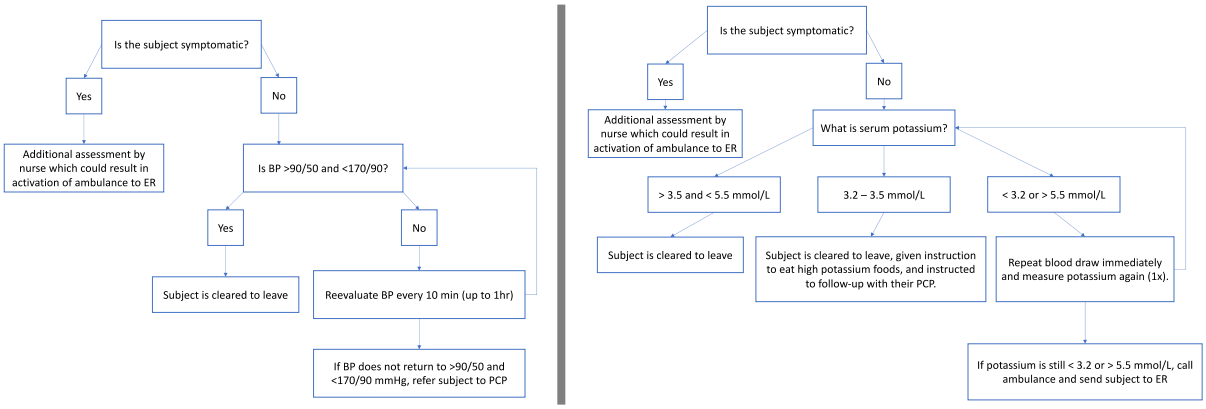

Figure 3 Decision Charts. Post furosemide blood pressure decision chart (left). Post furosemide potassium decision chart (right). Both decision flow charts will be followed to determine appropriate course of action after the infusion.

Magnetic Resonance Imaging (brain structure and activation)

The fMRI protocol will be performed using an MRI scanner employing pulse sequences and hardware that have been approved by the FDA for human clinical use. The field strength is 3 Tesla and all relevant operating characteristics (RF power deposition, rate of change of the field gradients, coil design) fall within the limits of FDA guidelines for NMR exposure. Participants will be carefully screened to exclude those who may have metal in or on their bodies that cannot be removed (e g., bullets, metal filings, body piercings, etc.). MR facility rules strictly forbid staff from entering the magnet room carrying metal objects. The risk of claustrophobia is minimized by screening subjects for

self-reported claustrophobia and making sure the subject is lying comfortably with head and neck supported and providing ear protection with headphones, a mirror to see out, a button to signal distress, and an intercom. Scan time will be kept to a minimum. Female participants will be given a urine pregnancy test immediately before the scanning period, and those with a positive result will not be scanned. Participants are given a squeeze ball to use in case of an emergency. Participants are informed that if they experience any unpleasant sensations or are otherwise uncomfortable, they can alert the MRI technologist via the squeeze ball and the technologist will stop the scan immediately.

With regard to subject confidentiality, all records will be coded, stored in a locked cabinet, and kept strictly confidential. Samples stored in the freezer will all be coded; names will not appear on samples. Our database will be password protected, as is the computer that the database resides on. Subject names do not appear in the database, just a subject ID number. The list linking the ID number with the subject name is kept in a separate, locked filing cabinet. Thus, breaches of confidentiality are very unlikely.

University policy requires that all study personnel complete human subjects training. Thus, the PIs, study coordinator, and graduate students involved in this data collection have all had this training. While the risk of medical emergency is very low, all subject personnel are certified in CPR. The Nurse Managed Health Center is located across the hallway from the research laboratory. An automated external defibrillator is located on site, and emergency numbers are posted on the phone and in the laboratory. Christiana Care Hospital, a 913-bed teaching hospital, is located 4 miles from the University.

MRI ACQUISITION AND ANALYSIS AND STATISTICS

MRI Acquisition

We will use a 3T MRI Siemens Prisma Scanner and 64-channel head coil to obtain all brain images. Padding will be placed around the participant's head to minimize head motion. Participants will be instructed to keep both their head and the rest of their body as still as possible.

All participants will undergo a T1-weighted anatomical scan [repetition time (TR) = 2080 ms; echo time (TE) = 4.6 ms; field of view = 210 x 210 mm; voxel size = 0.7 mm^3; slice thickness = 0.7 mm] followed by two BOLD fMRI scans (baseline & infusion). Functional scans will be acquired using a multi-band gradient-echo echo-planar imaging (EPI) sequence [TR = 829 ms; TE = 40 ms; flip angle = 52°; field of view = 208 x 208 mm; voxel size = 2.0 mm^3; slice thickness = 2.0 mm]. During functional scans, participants will be instructed to keep their eyes open and fixated on a white cross (on a black screen) to minimize the effects of eye motion and ensure the reliability and consistency of our results. These sequences are identical to those used in our group's pilot study.

MRI Analysis

The preprocessing steps described here were also utilized in our group's pilot study. Functional MRI images will be converted and analyzed using several software packages including MRIcroGL, AFNI (Analysis of Functional NeuroImages) and FSL (FMRIB Software Library). Initially, the 30-minute infusion fMRI scans will be split into an early phase (0-15 minutes) and late phase (15-30 minutes). Processing steps include: 1) de-spiking of the resting-state fMRI scans to eliminate outliers, 2) slice-timing correction of the fMRI scans, 3) 3D rigid motion correction to a reference frame to correct for head motion in the resting-state data (we removed time points with head motion > 0.5 mm), 4) cleaning the fMRI data using ANATICOR (accounting for the 6 motion parameters, white matter, and CSF signal), 5) band-pass filtering (0.008 to 0.10 Hz) (42), 6) skull-stripping of the T1-anatomical scan using FSL, 7) co-registration (i.e., alignment) of the T1-anatomical and functional scans, 8) normalization of all scans to the 1mm MNI152 template, and 9) spatial smoothing of the resting-state data using a Gaussian kernel of full-width half maximum (FWHM) = 4mm. These smoothing dimensions align with recommendations to choose a Gaussian kernel of FWHM double the voxel size (which was 2 mm^3 for this study) and are appropriate for smaller brain structures like the SFO and OVLT.

The ROIs for this study will be defined as follows. The SFO and OVLT will be defined as a 2-mm radius spheres based on previous studies. The Montreal Neurological Institute

(MNI) coordinates corresponding to the center of each ROI are: SFO: x = 0 mm, y = 2 mm, z = -6 mm and OVLT: x = 0 mm, y = 2 mm, z = -12 mm. The location of these seeds has been verified using Duvernoy's Atlas of the Human Brainstem and Cerebellum and these ROIs are consistent with those used in a pilot study conducted in our lab.

Following pre-processing, we will conduct seed-to-seed functional connectivity analyses to assess the synchronization of the spontaneous low frequency fluctuations in the BOLD signal between our ROIs. We will calculate functional connectivity between the SFO and OVLT. Pearson correlations will be computed between the ROIs for the seed-to-seed functional connectivity analysis. Pearson correlations will be converted to Z-scores using a Fisher's transform which will be statistically analyzed, as in our pilot study.

Statistical Analysis

Normality will be verified using Shapiro-Wilk tests. Normally distributed baseline characteristics will be reported as frequences and mean standard deviation. Two-way repeated measures analysis of variance (ANOVA) models will be used to assess changes in functional connectivity between baseline, the early phase (0-15 min) of the infusion, and the late phase (15-30min) of the infusion with and without furosemide prior. If data are non-normally distributed, aligned rank transformations (ARTs) will be conducted prior to performing two-way repeated measures ANOVAs. ARTs will be completed using ARTool2 (version 2.2.2) or ARTool integrated into R. Significant main effects of time and time*sex interactions will be followed with pairwise post-hoc comparisons corrected for multiple comparisons.