Study Protocol and Statistical Analysis Plan NCT03854539 August 27, 2020

Protocol. Participants. Healthy male and female adult participants (ages 18+) will be recruited from the Arkansas Tech University psychology student test pool and may receive course credit for participating. Participants fill out a questionnaire screening for mental disorders and use of substances which interfere with accurate assessment of salivary alpha amylase. The questionnaire also includes items on age, ethnicity and gender. In both experiments participants will be randomly assigned to groups with stimulation order determined using a Latin square design. Protection of human subjects. The IRB protocol is under review. Women and minorities are included and their data will be analyzed in accordance with the described data analysis plan. Stimulation procedures. We will apply sham stimulation and tVNS using identical stimulation parameters with the exception of electrode placement. *Electrode Placement.* Participants in *tVNS* condition groups will receive stimulation via electrodes attached on the anterior and posterior ear at the concha, an area richly innervated with vagal fibers [10]. *Sham-stimulated* participants will be stimulated with identical stimulus parameters as the tVNS group with the electrodes placed on the helix region. The helix region of the ear is thought to be free of vagal or other cranial nerve fibers. *Non-stimulation* participants will receive no stimulation. In the nonstimulation condition electrodes will be applied randomly to one of the above locations without electrical stimulation. The purpose of this group is to control for the possibility that mild stimulation will increase arousal through non-vagal pathways. *Stimulation parameters.* In the first study, to establish a stimulation intensity effect curve, participants will receive 0.0, 0.25, 0.5, or 1.0mA stimulation either to the concha or helix region (sham stimulation). The stimulus will consist of trains of 30Hz, with 0.5mS pulse width. Trains will alternate 30 seconds on and 30 seconds off for five minutes. In a second study to determine the relative effects of intermittent vs constant stimulation, participants will receive helix (sham) or concha (tVNS) stimulation at the 0.5mA level. Intermittent stimulation will consist of trains of 30Hz, with 0.5mS pulse width. Trains will alternate 30 seconds on and 30 seconds off for ten minutes. Constant stimulation will be identical with the exception that instead of alternating on-off periods, the stimulation will be a single five minute on block (holds total number of impulses equal). Salivary Alpha Amylase. Saliva samples will be collected using the passive drool method, considered to be the superior method for collection of alpha amylase samples [11]. Participants allow drool to pool inside the oral cavity and use a straw to provide 2mL of saliva into a cryogenic sample container. Following collection samples are placed on ice and then frozen until assay. *Salivary alpha amylase assay*. Samples are assayed using kinetic reaction assay, without modifications to the manufacturer's recommended protocol (Salimetrics, State College, PA) will be used. Details on this assay can be found in Gordis *et al* [11]. For sAA, sample test volumes are 10 µl and the lower limit of sensitivity is 0.4 U/mL. Samples are tested in duplicate with the average of the duplicates used for analysis. Duplicates exceeding 10% variance will be re-assayed. As a secondary measure, heart rate will be monitored by ECG and continuously recorded (3 lead) throughout the experiment using an iWorx TA device. Recoding electrodes are placed on the right and left clavicles andcostal margin. Heart rate will be used to confirm sympathetic induction and effects of heart rate variability may later be investigated as a primary measure in future studies.

Data analysis Within-subject percent change from immediately preceding baseline scores will be calculated for each level of stimulation ([baseline/stimulation] *100). Statistical analysis of percent changes in salivary alpha amylase concentrations by stimulation group will be performed with one-way repeated measures ANOVA with Bonferroni correction for multiple comparisons of each stimulation level to the sham stimulation level. Statistical analysis to determine changes in baseline concentration of salivary alpha amylase within each intensity will be conducted using repeated measures ANOVA with Bonferroni correction for

multiple <comparisons. A significance level of p <0.05 will be used for all statistical tests conducted. Within-subject percent change from immediately preceding baseline scores will be calculated foreach level of stimulation ([baseline/stimulation] *100). Statistical analysis of percent changes in salivary alpha amylase concentrations by stimulation group will be performed with one-way repeated measures ANOVA with Bonferroni correction for multiple comparisons of each stimulation level to the sham stimulation level. Statistical analysis to determine changes in baseline concentration of salivary alpha amylase within each intensity will be conducted using repeated measures ANOVA with Bonferroni correction for multiple <comparisons. Asignificance level of p <0.05 will be used for all statistical tests conducted.