Statistical analysis plan

1. Introduction

This analysis plan is for the study “Modulating reward function using focused ultrasound aimed at the nucleus accumbens in healthy adults: a neurophysiological and behavioural randomized crossover comparison over three conditions”.

2. Analysis population

The primary analyses will include all randomized participants who complete at least one intervention session and provide at least one valid observation for the relevant outcome. Participants will be analysed according to the condition received during each session. Outcome-specific observations that fail prespecified quality criteria will be excluded, while remaining valid observations from the same participant will be retained.

Inclusion criteria

- Healthy individuals between 18 and 60 years of age.
- Signed informed consent sheet.
- Sufficient understanding of the Swedish language to be able to comprehend the information given about the project and take part in the interventions.

Exclusion criteria

- Previous or current neurologic disorder or serious mental illness.
- Previous or current diagnosis of depression.
- Previous or current alcohol or drug addiction/dependency.
- Currently on psychofarmacologic treatment for a psychiatric condition.
- Contraindications for MRI and TUS, including metal in the body, metal implant~~ate~~, metal fragments after working with metals or serious claustrophobia.
- Pregnancy - current or planned in the near future.
- Physically incapable of sitting still in a chair with neck support for up to 2 hours.
- Physically incapable of laying still on the back for up to 1 hour.

3. Descriptive analyses

1. Age
2. Handedness
3. Education
4. Gender

4. Randomisation and blinding in the analysis stage

Participants will be randomly assigned to one of three Latin-square sequences counterbalancing the order of the three conditions. Thus, each participant completes all three conditions, but in different orders depending on the assigned sequence (ABC, BCA, or CAB), ensuring that each condition appears equally often in each ordinal position across the sample.

5. Outcome measures

Primary outcomes

I. *Differences between conditions in average amplitude of the ERP component Reward Positivity (RewP) measured using a 32 channel EEG system in conjunction with a Doors task at post-intervention.*
II. *Differences between conditions and blocks in response bias measured using a Probabilistic Reward Task (PRT) with two blocks at post-intervention.*

Secondary Outcomes

I. *Difference between conditions in average amplitude of the Stimulus Preceding Negativity (SPN) ERP component measured using a 32 channel EEG system during anticipation phase of door task at post-sonication for every condition*
II. *Differences in changes in EEG spectral analysis of theta power, individual alpha band frequency and aperiodic activity between conditions measured using a 32 channel EEG system during rest at pre vs post interventions.*
III. *Differences in pupil dilation between conditions measured using eye tracker during psychological paradigms at post-intervention.*
IV. *Evaluation of experimental blinding and double-blinding.*
V. *Differences between conditions in reward-dependent response repetition (win-stay behaviour) during the Probabilistic Reward Task.*

6. Pre-processing of data

ERP data

All pre-processing of ERP-data will be done in MNE Python and will be based on the pyprep pipeline. Both the RewP and the SPN are collected in conjunction with the Doors task. Data will be resampled to 256 Hz and band-pass filtered between 0.1 and 10 Hz.

Continuous artifact attenuation will be performed using an ASR-like algorithm (e.g. PREP/pyPREP or autoreject for channel/segment repair). Individual Component analysis (ICA), labeling and rejection will be applied with a ICLabel probability threshold = 0.90 for eye/muscle components. Removed channels are interpolated. Data is then re-referenced using the REST reference, or if unavailable, the average reference.

Finally, the data will be epoched around the feedback onset for the RewP (-200 to 800 ms) and around the button press for the SPN (-2000 to 3000ms). Baseline correction will then be performed at -2000 to -1700 ms prior to the button press for the SPN and at -200 to 0 ms prior to feedback onset for the RewP. Epochs with residual artifacts exceeding ±150 µV in the 0–600 ms window will be rejected.

Participants will not be excluded from the entire RewP analysis solely because data from one session fail the EEG quality criteria. Instead, EEG data quality will be assessed separately for each session. A session-specific observation will be excluded if fewer than 50% of the relevant epochs, or fewer than 20 epochs in total, remain after preprocessing. Participants may contribute data from their remaining valid sessions to the linear mixed-effects model.

For analyses comparing win and loss trials, A valid session must contain at least 20 artifact-free epochs in total and artifact-free epochs from both win and loss feedback categories. The number of retained trials in each feedback category will be reported descriptively.

Resting state EEG data

Resting-state EEG pre-processing will follow standard pipelines used in contemporary MNE workflows: raw data will be imported, notch- and bandpass-filtered, noisy channels identified and interpolated, continuous data cleaned using ICA-based ocular and muscle component removal, re-referenced to the average, and segmented into artifact-free epochs. Segments failing amplitude or noise criteria will be removed. Resting-state recordings containing less than two minutes of artifact-free data will be excluded, while the participant's remaining valid recordings will be retained. Cleaned data will be used for spectral and aperiodic analysis.

Eye tracker data

Pupillometry data will be pre-processed by identifying and removing blinks, interpolating short periods of missing data, filtering, down-sampling, epoching, baseline correction, and trial rejection. Blinks will be defined as consecutive data loss lasting 80–500 ms. Blink periods, including participant-specific margins of up to 50 ms before and 100 ms after each blink, will be marked as missing. Missing segments of no more than 500 ms will be reconstructed using linear interpolation, whereas longer gaps will remain missing.

The data will be smoothed using a 150-ms moving-window median filter and down-sampled from 1200 Hz to 20 Hz. Feedback-locked epochs will be defined, and pupil size will be baseline-corrected separately for each trial by subtracting the mean pupil size during the interval from 250 ms before to 150 ms after feedback onset. Trials containing more than 30% missing data will be excluded.

Mean and minimum baseline-corrected pupil size will be calculated within a 500–3000 ms post-feedback analysis window. Measures will be extracted separately for all feedback trials and for rewarded, correct-but-unrewarded, and incorrect trials.

7. Primary analyses

The two primary outcomes will be analysed using linear mixed-effects models. All available valid observations will be included, and missing outcome values will not be imputed. Participants with partially missing outcome data may contribute observations from their remaining valid sessions. The models therefore assume that missing outcome data are missing at random, conditional on the variables included in the models.

All models will include a participant-specific random intercept to account for repeated observations within participants. Condition and other categorical predictors will be entered as fixed effects. Session period (1, 2, or 3) will be included as a categorical fixed effect in all primary models.

Model assumptions will be assessed by inspecting residual distributions, residual-versus-fitted plots, homoscedasticity, and influential observations. All valid observations will be retained in the primary analyses. Analyses excluding observations identified as strongly influential based on model diagnostics will be conducted as sensitivity analyses. If substantial violations of model assumptions remain, an appropriate transformation or robust mixed-effects modelling approach will be considered.

Statistical tests will be two-tailed. Estimated marginal means, model-based contrasts, 95% confidence intervals, and p-values will be reported. A Holm–Bonferroni correction will be applied across the two prespecified primary tests to control the family-wise error rate at $\alpha = 0.05$. Follow-up comparisons within each outcome will be corrected separately using the Holm method.

I. Differences between conditions in average amplitude of the ERP component Reward Positivity (RewP) measured using a 32 channel EEG system in conjunction with a Doors task at post-intervention.

Mean ERP amplitude at Fz between 250 and 350 ms will be calculated separately for win and loss trials within each valid session in the Doors Task. A linear mixed-effects model will include Condition, Feedback type, their interaction, and Session period as fixed effects, with a random intercept for participant:

$$ERP\ amplitude \sim Condition \times Feedback\ type + Session\ period + (1 \mid Participant)$$

The primary test will be the Condition × Feedback type interaction. Significant interactions will be followed by Holm-adjusted pairwise comparisons of model-estimated win-minus-loss contrasts between conditions.

II. Differences between conditions in reward responsiveness (response bias) measured using a Probabilistic Reward Task (PRT) with two blocks at post-intervention.

Differences between conditions in reward responsiveness will be assessed using response bias measured during the two blocks of the Probabilistic Reward Task after each intervention. Response bias, which is a measure of reward sensitivity, is calculated using the following formula:

$$\text{Response Bias:} \quad \log b = \frac{1}{2}\log\left(\frac{Rich_{correct} * Lean_{incorrect}}{Rich_{incorrect} * Lean_{correct}}\right)$$

A linear mixed-effects model will be fitted with response bias as the dependent variable. Condition (TUS-NAcc, TUS-LV, and TUS-sham), block (Block 1 and Block 2), and their interaction will be included as fixed effects. Session period will additionally be included as a fixed effect, and participant will be included as a random intercept:

$$\textit{Response bias} \sim \textit{Condition} \times \textit{Block} + \textit{Session period} + (1 \mid \textit{Participant})$$

The primary PRT test will be the Condition × Block interaction, which tests whether the change in response bias from Block 1 to Block 2 differs between the intervention conditions. If the interaction is statistically significant, the model-estimated change from Block 1 to Block 2 will be calculated separately for each condition. These changes will then be compared pairwise between TUS-NAcc, TUS-LV, and TUS-sham. Holm-adjusted p-values will be used for the three follow-up comparisons.

8. Secondary analyses

Continuous secondary outcomes will generally be analysed using linear mixed-effects models, whereas binary outcomes will be analysed using generalized linear mixed-effects models with an appropriate link function. All available valid observations will be included, and observations failing outcome-specific quality criteria will be excluded at the recording or session level rather than resulting in exclusion of the participant from the entire analysis. Participant will be included as a random intercept, and session period will be included as a fixed effect. Model assumptions will be evaluated using the same diagnostic procedures as for the primary analyses.

Secondary analyses will use $\alpha = .05$ without correction across the different secondary outcomes and will be interpreted cautiously. Follow-up pairwise comparisons within an outcome will be adjusted using the Holm method.

I. Stimulus Preceding Negativity (SPN) ERP component measured using a 32 channel EEG system during anticipation phase of door task at post-sonication for every condition

Mean SPN amplitude will be calculated over a predefined centroparietal region of interest comprising CPz, Pz, CP1, CP2, P1, and P2 during the −200 to 0 ms interval preceding feedback onset in the Doors Task.

A linear mixed-effects model will be fitted with mean SPN amplitude as the dependent variable. Condition will be included as a fixed effect, session period will be included as an additional fixed effect, and participant will be included as a random intercept:

SPN amplitude ~ Condition + Session period + (1 | Participant)

If the overall effect of Condition is statistically significant, estimated marginal means will be used to conduct the three pairwise comparisons between conditions. These comparisons will use Holm-adjusted p-values.

II. Change in EEG spectral analysis of theta power, individual alpha band frequency and aperiodic activity measured using a 32 channel EEG system during rest at pre vs post interventions.

Resting-state EEG will be recorded using a 32-channel EEG system during a 4 minutes eyes-open and 4 minutes eyes closed condition both pre- and post-intervention. Following pre-processing, continuous EEG will be segmented into non-overlapping epochs and subjected to spectral decomposition. Power spectral density (PSD) will be estimated using Welch's method. The following outcome measures will be extracted per participant:

1. **Theta power**, averaged across all epochs.
2. **Individual Alpha Frequency (IAF)**, defined as the peak frequency within 8–13 Hz for each individual.
3. **Aperiodic activity (1/f component)**, estimated using FOOF. FOOOF will be run in aperiodic-only mode (no peak fitting), extracting slope and intercept

For each spectral outcome, separate linear mixed-effects models will be fitted to the pre- and post-intervention values. Condition (TUS-NAcc, TUS-LV, and TUS-sham), time (pre-intervention and post-intervention), and their interaction will be included as fixed effects. Session period will additionally be included as a fixed effect, and participant will be included as a random intercept:

$$EEG\ outcome \sim Condition \times Time + Session\ period + (1 \mid Participant)$$

The primary effect of interest within each model will be the Condition × Time interaction, which tests whether the pre-to-post change differs between intervention conditions. If the interaction is statistically significant, model-estimated pre-to-post changes will be compared between conditions using Holm-adjusted pairwise contrasts.

Data quality will be assessed separately for each resting-state recording. Recordings containing less than two minutes of artifact-free data will be excluded, while the

participant's remaining valid recordings will be retained in the analysis.Separate models will be fitted for eyes-open and eyes-closed recordings.

III. Differences in pupil dilation between conditions measured using eye tracker during psychological paradigms at post-intervention.

Mean and minimum baseline-corrected pupil size within the predefined analysis window will be analysed in two separate trial-level linear mixed-effects models in the Doors Task. Each model will include stimulation condition (TUS-NAcc, TUS-LV, and TUS-sham), reward condition (win and loss), and their interaction as fixed effects. Session period and trial number will be included as covariates to account for period effects and systematic changes over the course of the task. Random intercepts will be included for participant and session nested within participant:

$$Pupil\ size \sim Condition \times Reward\ condition + Session\ period + Trial\ number + (1 \mid Participant/Session)$$

Incorrect trials will be excluded because their expected low and uneven frequency would provide unreliable group-level estimates. All remaining valid trials will be included without imputation. Models will be fitted using restricted maximum likelihood, with Satterthwaite approximation for degrees of freedom. The primary effect of interest will be the Condition × Reward condition interaction. Significant interactions will be followed by Holm-adjusted model-based contrasts comparing the reward-related pupil response between stimulation conditions.

IV. Evaluation of experimental blinding

After each session, participants and operators will independently guess whether the participant received TUS-NAcc, TUS-LV, or TUS-sham and rate their confidence in the guess on a 1–10 scale, where 1 indicates complete uncertainty and 10 indicates complete certainty.

Guesses will be summarized using confusion matrices and percentages of correct guesses, separately by actual condition and session period. Participant and operator guesses will be analysed separately using multinomial mixed-effects logistic regression, with guessed condition as the dependent variable and actual stimulation condition and session period as fixed effects. Participant will be included as a random intercept:

$$Guessed\ condition \sim Actual\ condition + Session\ period + (1 \mid Participant)$$

For operator guesses, operator identity will additionally be accounted for as a fixed or random effect, depending on the number of operators.

As a complementary analysis, guesses will be coded as correct or incorrect and analysed using binomial mixed-effects logistic regression. The model-estimated probability of a correct guess will be compared with the chance level of one-third.

Confidence ratings will be analysed using an ordinal mixed-effects model with actual condition, session period, and guess accuracy as fixed effects and participant as a random intercept:

Confidence ~ Actual condition + Session period + Guess accuracy + (1 | Participant)

This analysis will assess whether confidence differs between conditions or sessions and whether correct guesses are associated with greater confidence. Participant and operator confidence ratings will be analysed separately.

V. *Reward-dependent response repetition (win-stay behaviour) in the Probabilistic Reward Task*

Win-stay probability will be defined as the probability of repeating the response made on trial t on trial t+1 following a rewarded trial. Win-stay probability will be calculated separately for each participant, stimulation condition, and block. Differences in win-stay probability between stimulation conditions will be analysed using a linear mixed-effects model with stimulation condition, block, and session period as fixed effects and participant as a random intercept:

Win-stay probability ~ Condition + Block + Session period + (1 | Participant)

The primary effect of interest will be the effect of Condition, testing whether reward-dependent response repetition differs between TUS-NAcc, TUS-LV, and TUS-sham. If the overall effect of Condition is statistically significant, model-estimated win-stay probabilities will be compared pairwise between conditions using Holm-adjusted p-values.

As a sensitivity analysis, trial-level response repetition following rewarded trials will be analysed using a binomial mixed-effects logistic regression including stimulus type on trial t+1 and response type on trial t as covariates, to assess whether any condition effect is robust to perceptual stimulus characteristics and the asymmetric reward contingencies of the PRT.-adjusted pairwise contrasts of the reward-related repetition effect between conditions.

9. Additional exploratory analyses

The following exploratory analyses will be conducted:

I. *Associations between pre-to-post changes in EEG microstate measures and changes in the primary outcomes*
II. *NAcc structural and functional connectivity measured using rsfMRI and DWI at baseline as a predictor of primary outcome measures in the TUS-NAcc condition.*
III. *Baseline excitatory tone (Glx) of the ventral striatum measured using MRS at baseline as a predictor of effects on primary outcome measures in the TUS-NAcc condition.*
IV. *Self-assessed reward responsiveness measured using SHAPS and PVSS-21 at pre-interventions and baseline as a predictor effects on main outcome measures in the TUS-NAcc condition.*
V. *Evaluation of the effect of stimulus type (visual properties of long vs short mouth, not lean vs rich) on response bias in the probabilistic reward task.*

Baseline predictors will be examined as moderators by adding each predictor separately to the primary mixed-effects models. Associations involving change measures, including EEG microstate changes, will be examined using separate exploratory regression or mixed-effects models appropriate to the structure of the outcome.

For RewP, the predictor and its interactions with Condition and Feedback type will be added to the primary RewP model. The Condition × Feedback type × Predictor interaction will be used to examine whether the predictor moderates condition-related differences in RewP:

$$ERP\ amplitude \sim Condition \times Feedback\ type \times Predictor + Session\ period + (1 \mid Participant)$$

For response bias, the predictor and its interactions with Condition and Block will be added to the primary PRT model. The Condition × Block × Predictor interaction will be used to examine whether the predictor moderates condition-related differences in the development of response bias:

$$Response\ bias \sim Condition \times Block \times Predictor + Session\ period + (1 \mid Participant)$$