Neuroimaging Data Collection

1. **Neuroimaging pre-screening and institute screening**

- A brief interview will be conducted at recruitment stage regarding the MRI contraindications:
  (1) Any previous reports of traumatic brain injury;
  (2) Any implanted devices or foreign bodies, including cardiovascular device, nerve stimulator, tooth brace, or others.
- A confirmatory interview for MRI exclusion criteria will be conducted to every participant on the day of MRI scan, with the same criteria mentioned above.
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2. **Training programs prior to MRI scans**

- Home training program: Carergivers will be asked to help acclimatise the participants to using ear plugs and sleeping on their backs. Home training started 2 weeks prior to the MRI visit.
- On-site training: 1) participants will be shown around the MRI scanning room to familiarise themselves with the new environment and various MRI sounds for 2-3 hours, prior to the actual scans; 2) participants will be brought to the MRI scanning room, they will sit/lie on MRI scanner for around 10 min to become acclimatised to the MRI scanner and the room temperature, before falling asleep.

3. **Demographic and sleeping habits information collection**

- Demographic information: weight, height and head circumference of the participants will be collected on the day of MRI scan
- Sleeping habits information: typical timing for waking up in morning and sleeping at night, whether or not taking an afternoon nap, as well as bed timing last night will be collected

4. **MRI and MRS pre-scanning**

- To ensure the participant not wearing any metal stuff and to remove all of them before entering the scanning room.
- Giving the young age and clinical features of our participants, sedation (50 mg/kg Chloral Hydrate at a maximum dose of 1 gram, administered rectally) will be offered

before the MRI/MRS scanning when it is necessary. The information including the time when the drug is administrated and the time when participant falling asleep will be recorded.

- 10-15 minutes after falling asleep, the participant will be transferred to the scanning room and placed on the MR scanning bed. Earplugs, earphones, and extra foam padding will be provided to reduce the sound of the scanner during the scan. Respiration and pulse will be closely monitored.

5. Imaging data acquisition

The MRI scan appointment will be scheduled with the caregiver one week before the day of the scan. One day before the scheduled scan date, a reminder phone call will be made to the caregiver. Participants will be scanned using a Siemens Verio 3.0-Tesla MRI scanner (Siemens Medical Solutions, Munich, Germany) with 32-channel head coil and four-channel neck coil. The MRI scans will be scheduled between 14:00pm and 15:00pm. The follow-up scan will be scheduled around 14:00pm to 15:00pm on the last day of the 3-month treatment period.

- **The overview of imaging session**

Neuroimaging sequences		
No	**Item**	**Duration**
1	Localizer	0:13
2	T1	4:18
3	T2	2:06
4	T2-Flair	1:20
5	MRS, Insular Cortex	13:52
6	MRS, Orbital Frontal Cortex (optional)	13:52

- **T1-weighed structural imaging**

A high-resolution anatomical T1-weighted magnetization-prepared rapid gradient echo (MPRAGE) image will be collected with parameters setting as 192 sagittal slices, voxels = 1 x 1 x 1 mm, repetition time = 2,300 ms, echo time = 2.28 ms, inversion time = 1100 ms, flip angle = 8°, field of view = 192 x 192 x 192 mm.

- **MRS imaging**

For neurotransmitter measurements (GABA, glutamate, etc.), Mescher-Garwood point-resolved spectroscopy (MEGA-PRESS) scans will be applied with on-/off-resonance frequency = 1.9/7.5 ppm using TR/TE = 1500/68 ms. The neurotransmitter measurements would be obtained within a volume of interest (VOI) of two brain regions including insular cortex and orbital frontal cortex. The insular cortex VOI is the required while the orbital frontal cortex VOI is optional if the participants sleep steady after insular cortex VOI scanned. The insular cortex VOI (20 x 40 x 20 mm) is placed along the anterior-posterior direction of the right insular cortex, which covered the anterior and posterior limit of the insula[1]. The placement of orbital frontal cortex VOI (20 x 25 x 15 mm) is placed in the right medial orbital frontal cortex using the landmarks of previous studies as the reference[2, 3]. To ensure the consistency of VOI positioning in the longitudinal experiments, we use the first scanned VOI of each participant as a reference to locate the same VOI in the follow-up scan.

6. Imaging preprocessing

- **MRI data preprocessing**

T1 weighed structural image would be used for volumetric or surface analysis. For voxel-based morphometry, T1-weighted images will be processed in the SPM8 (http://www.fil.ion.ucl.ac.uk/spm/) using the VBM8 toolbox, incorporating the DARTEL toolbox. The standard optimized method of iterative tissue segmentation and spatial normalization, using both linear (12-parameter affine) and non-linear transformations, will be performed. Data quality will be checked using VBM8 with the sample homogeneity option. To make the residuals in later analyses conform more closely to a Gaussian distribution and to account for individual differences in brain anatomy, the modulated grey matter (GM)

images in the MNI space will be smoothed with an isotropic Gaussian kernel of 6/8 mm full width at half maximum.

For surface analysis, the cortical thickness and surface area will be estimated from the T1 structural images using FreeSurfer software (http://surfer.nmr.mgh.harvard.edu/). The T1-weighted images will be used to segment cerebral white matter (WM) and GM. Visually inspection of the segmentation will be performed. A two-dimensional tessellated mesh consisting of over 300,000 vertices will be constructed over the white matter surface to distinguish the gray–white matter interface. This mesh will be then expanded outward to meet the grey matter and pial surface boundary. Cortical thickness at each vertex is measured based on the difference between the positions of equivalent vertices in the pial and gray-white matter surfaces. Surface area of each vertex is measured at the pixel level and calculated as the average of the area of the tessellated triangles touching that vertex. There measures will be initially estimated in native space. To conduct group analysis at vertex level, we resample each subject's data into a common space –fsaverage space and smoothed on the surface using a Gaussian smoothing kernel with a series of full-width half-maximum.

- **MRS data preprocessing**

For reliable quantification of the GABA signal, we use LCModel software with a simulated MEGA-PRESS basis set to fit the MRS data and determined the N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG), γ-aminobutyric acid (GABA), glutamate and glutamine (Glx), glutathione (GSH) concentrations using the difference spectra. Each spectrum will be reviewed and the quality control parameters from LCModel will be applied to ensure an acceptable signal-to-noise ratio (SNR) for the MRS voxel. Participants with SNR ≤ 15, full-width at half maximum ≥ 0.05 ppm, and Cramer–Rao lower bounds in the fitted spectrum equal to or higher than 20% for GABA are excluded from further analysis. GABA metabolite concentrations are expressed in institutional units and normalized by the NAA+NAAG concentration within the VOI. In addition, due to the difference in tissue composition of each VOI (partial volume effect), tissue correction is performed for GABA-edited MRS to adjust GABA measurements. We segment the T1-weighted images into GM, WM, and cerebrospinal fluid using SPM8 to compute the volume fraction of each tissue

component covered within the VOI. The corrected GABA metabolite concentration will be calculated with the following equation: $Metabolite_{corrected} = Metabolite_{raw}/NAA_{observed} \times (1/[frGM + 0.5 \times frWM])$.

GWAS analysis protocol

Genotyping and quality control

Genomic DNA is extracted from peripheral blood of participants and collected at baseline before treatment. Individuals will be genotyped with the Affymetrix CytoScan HD array. Systematic quality control steps will be conducted on the raw genotyping data. The exclusion criteria for SNPs are minor allele frequency less than 5%, genotyping call rate less than 95%, genotype frequency that deviated from Hardy–Weinberg equilibrium ($P < 1 \times 10^{-7}$). Individuals with overall genotyping rate less than 95% are excluded. Samples will be excluded if heterozygosity rates for autosomal chromosomes are greater than six standard deviations from the mean. We will also conduct further exclusions for those with abnormal heterozygosity rate on the X chromosome. Unexpected duplicates and first-degree and second-degree relatives are removed based on identity-by-state estimates calculated in PLINK. SNPs pass quality control measures will be used in the following analysis.

Genetic associations

We have selected genes that are known to have a role in either glutamate (hsa04724) or GABA (hsa04727) signaling for an overview of genes from the KEGG pathways (https://www.genome.jp/kegg/pathway.html). We are going to focus on 203 genes, among which 114 are involved in Glutamatergic signaling and 89 in GABAergic signaling. SNPs on these genes will be selected from the quality-controlled genome-wide data set.

- *Candidate gene-wide association study:*

  Linear regressions will be performed in bumetanide and placebo group separately, using behavioral changes in the total score of childhood autism rating scale (CARS) after three-month treatment as a dependent variable and the additive dosage of each SNP on the candidate genes as an independent variable of interest, while considering the covariates including age, sex, and intelligent status. Adjustment for multiple comparisons will be performed with the false discovery rate approach.

  We are also going to test if any SNP can serve as a moderator of the treatment effect

using the following model “change of severity ~ treatment * SNP + treatment + SNP + baseline severity”, where the severity of ASD symptoms can be assessed by the CARS total score. A similar model can also be applied to test if any SNP can moderate the treatment effect on glutamate or GABA concentration. Adjustment for multiple comparisons will be performed with the false discovery rate approach.

- *Polygenic risk score:* Polygenic risk scores (PRSs) for ASD (PRS_{ASD}) will be calculated using the PRSice software (http://prsice.info/). To generate PRS_{ASD} with these candidate genes, we will use ASD GWAS summary data from the Psychiatric Genomics Consortium (https://www.med.unc.edu/pgc/). The shared SNPs with the smallest *p*-value for each linkage disequilibrium block (excluding SNPs with an $r^2<0.1$ in 250 kb windows) are retained after clumping; and the PRS_{ASD} will be calculated at the *p*-value threshold of 0.01, 0.05, 0.1 and 0.5.

  We are also going to test if there is any SNP can serve as a moderator of the treatment effect using the following model “change of severity ~ treatment * PRS + treatment + PRS + baseline severity”. A similar model can also be applied to test if any SNP can moderate the treatment effect on glutamate or GABA concentration.

Metabolomic data analysis

1. Sample collection and Targeted metabolomic analysis

Plasms sample of participants and collected at baseline before and after treatment. Samples will be stored at –80°C until processed. To identify and quantify metabolite concentrations, plasma samples will be measured using the AbsoluteIDQ p150 targeted metabolomics kit (Biocrates Life Sciences AG, Innsbruck, Austria), and a Waters Xevo TQ-S mass spectrometer coupled to an Acquity H LC system (Waters Corporation, Milford, MA, USA). The kit provides absolute concentrations for acylcarnitines, amino acids, biogenic amines, hexose, glycerophospholipids and sphingolipids. Plasma samples will be prepared according to the manufacturer's instructions. To diminish analytical bias within the entire analytical process, the samples will be analyzed in group pairs but the groups will be analyzed randomly. The QC samples, calibrators, and blank samples will be analyzed across the entire sample set.

2. Statistical analysis

The raw data files generated by UPLC-MS/MS will be processed using the MassLynx to perform peak integration, calibration, and quantitation for each metabolite. All metabolite data will be first checked for missing values (none at >20% missing abundances) and subjected to imputation by the k-nearest neighbor algorithm. Data will be then log2 transformed and scaled to unit variance prior to statistical analyses. Principal components analysis will be applied to identify outliers, followed by PLS-DA to assess the ability of the metabolites to differentiate before and after treatment.

To test the effect of the 3-month bumetanide treatment, the linear mixed-effects models (R package nlme) are going to be applied to each metabolite while adjusting for age, gender, and CARS total score at baseline with a random effect for each subject. The false discovery rate (Benjamini–Hochberg method) is going to be used to correct for the multiple comparisons.

EEG acquisition and statistical analysis protocol

1. EEG participant pre-screening

ASD subjects who have the willingness to wear the EEG cap will participant in this part.

2. EEG recordings

- EEG will be recorded using the high-density 128 channel geodesic nets with Ag/AgCl electrodes (Electrical Geodesics, Inc., Eugene, OR, USA). Sensor nets of different sizes are employed, and the one that most closely corresponded to the child's head circumference is used.
- During acquisition, impedances are kept below 50KΩ. The sampling rate is 1000 Hz. The Cz electrode is used as the reference. Data are filtered online with an analog band pass elliptical filter between 0.1 to 100 Hz.
- EEG data are acquired while the subjects sat on the chair or seated on their caregiver's laps, in a quiet room with a dim lit. Then, the subjects will be offered some toys or shown a favorite video during placement of the EEG net in order to make the recording session as comfortable and enjoyable as possible for each subject.
- The experimental procedure will be carefully explained to the parents and the subject with the help of picture card.
- **Task 1 Sustained visual attention**

Sustained visual attention attracted by: 1) soap bubbles presented by an experimentator at about 1 meters distance from the subject; and 2) computer presentation of moving fish ('Aquatica' screen saver). Each type of stimuli will be presented for about 2 minutes[1].

- **Task 2 Joint play**

The subject will be presented with sets of toys at a table. Toys consist of dolls, doll furniture, blocks. The entire play interaction lasts approximately 10–15 minutes. The subject's play behaviors are videotaped and later coded [2].

Reference：

[1] Orekhova E V , Stroganova T A , Nygren G , et al. Excess of High Frequency Electroencephalogram Oscillations in Boys with Autism[J]. Biological Psychiatry, 2007, 62(9):1022-1029.

[2] Kasari C , Freeman S , Paparella T . Joint attention and symbolic play in young

children with autism: a randomized controlled intervention study[J]. Journal of Child Psychology and Psychiatry, 2006, 47(6):611-620.

- The experimental session is videotaped to enable the researcher to control stimulus presentation and to provide a record of the subjects' movement and behaviour during the task.

3. EEG preprocessing

EEG data will be processed with EEGLAB.

- EEG data will be digitally filtered (0.5~40Hz) and re-referenced using an average reference that is applied after having excluded channels in close proximity to the eyes.
- Data will be visually inspected, movement and electrical artifact (as evidenced by large amplitude fluctuations that exceeded +100 μV) are removed.
- Data segments will be a minimum of 2 seconds long for inclusion in further analysis.
- Absolute and relative power indices will be computed for the delta (1–4 Hz), theta (4–8 Hz), alpha (8–13 Hz), and beta (13–30 Hz) frequency bands, as well as the total power of the EEG (1–30 Hz). Coherence indices are computed between every two electrode pairs.

4. EEG data analysis

The treatment effects of EEG measurements will be assessed using a mixed-effect model. In this model, we assumed individualized random intercepts, and tested the treatment effect by the interaction term, treatment (0, placebo; 1, bumetanide) × time (0, baseline before treatment; 1, month 3 after treatment); sex, age and intellectual assessment are controlled as covariates. The normality of the model residuals will be assessed with the Shapiro–Wilk normality test, and homogeneity of variance across groups will be evaluated with Levene's test. If at least one of the two tests are significant, a permutation-based mixed-effects model is established by 3000 random permutations of the group label using the permlmer function in R package "predictmeans". Adjustment for multiple comparisons will be performed with the false discovery rate approach.