Supplementary Methods

Methods S1: Cohorts

S1A. NeuroIMAGE II cohort

NeuroIMAGE II is a follow-up of NeuroIMAGE [1], which is the Dutch follow-up of the IMAGE study on ADHD performed between 2003-2006 (as described previously in [2, 3]). Families with at least one child with combined subtype ADHD and at least one biological sibling (regardless of ADHD diagnosis) were recruited, in addition to control families with at least one child, with no formal or suspected ADHD diagnosis in any of the first-degree family members.

Inclusion criteria for participants were: between 8-30 years, of European Caucasian descent, an IQ ≥ 70, and no diagnosis of autism (as determined in the preceding IMAGE project [4] using criteria of the DSM-IV), epilepsy, general learning difficulties, brain disorders and known genetic disorders (such as Fragile X syndrome or Down syndrome), and no contraindication to MRI scanning (e.g. implanted metal or medical devices, or possible pregnancy). Participants were asked to withhold use of psychoactive drugs for 48 hours before measurement.

The NeuroIMAGE follow-up study had a comprehensive assessment protocol encompassing questionnaires, a diagnostic interview and several neurocognitive measures from all family members, as well as an extensive MRI scanning protocol in participants. During the testing day, participants were motivated with short breaks, and at the end of the day, they received a reward of €50,-. Participants gave written informed consent (and their parents when <18 years old) and the study was approved by the regional medical ethics committee.

To determine ADHD diagnoses at the follow-up measurement in the NeuroIMAGE study, all participants in the study were similarly assessed using a semi-structured diagnostic interview. For participants using medication, ratings were done of children's functioning off medication. All participants were administered the Dutch translation of the Kiddie Schedule for Affective Disorders and Schizophrenia (K-SADS) Present

and Lifetime Version [5], carried out by trained professionals. Both the parents and the child, if ≥ 12 years old, were interviewed separately and were initially only administered the ADHD screening interview. Participants with elevated scores on any of the screen items were administered the full ADHD section. Participants with a symptom count of ≥ 6 symptoms of either hyperactive/impulsive behaviour or inattentive behaviour were diagnosed with ADHD, provided they: a) met the DSM-IV criteria (American Psychiatric Association, 2000) for pervasiveness and impact of the disorder (measures derived from the K-SADS), b) showed an age of onset before 12 (following the proposed changes for the DSM-V; see [6]), derived from the K-SADS. Criteria were slightly adapted for young adults (≥ 18 years), such that a symptom count of 5 symptoms on either hyperactive/impulsive or inattentive scale was sufficient for a diagnosis [7]. Young adults were considered unaffected when they received ≤ 2 symptoms on the combined symptom counts. Inconsistent cases were evaluated by a team of trained experts (consisting psychiatrist JB and 8 psychologists), in order to derive a consensus diagnosis.

S1B. BIG cohort

The remaining microbiome participants (n = 39) were selected from the Brain Imaging Genetics (BIG) study. The study sample consisted of healthy adult volunteers taking part in the diverse studies conducted at the Donders Institute for Brain, Cognition and Behaviour in Nijmegen, The Netherlands [8]. Participants of the BIG cohort were of Caucasian origin, between 18 and 36 years of age and right-handed. No diagnostic assessment was performed, but participants had no self-reported neurological or psychiatric history. Participants gave written informed consent and the study was approved by the regional medical ethics committee.

S1C. Inclusion criteria microbiome part

Participants of the BIG cohort (control subjects) could take part in the microbiome study if they were older than 18 years, not pregnant (for women), did not have chronic or acute diseases at the time of assessment, did not use chronic or acute medication (including antibiotics) during the last month before the study, and were of Western-European descent. Retrospectively, we discovered that one participant used medication and one participant was not from Western-European descent but from northern-African descent. Our analysis of differences in gut microbiome metabolic potential (with PICRUSt) did not change qualitatively when excluding these two controls; i.e. the cyclohexadienyl dehydratase (CDT) difference between ADHD and controls (see main Results) remained significant (p = 0.044). None of the fMRI analyses included BIG controls.

Methods S2: fMRI parameters and analyses

S2A. fMRI data acquisition

Whole-brain imaging was performed on a 1.5T MR scanner (Magnetom Avanto, Siemens Medical Systems, Erlangen, Germany). BOLD sensitive functional images were acquired using a T2*-weighted multi-echo EPI sequence (TR: 2.67 s; TEs for 5 echoes: 7.7 ms, 17.3 ms, 27.0 ms, 36.6 ms, and 46.3 ms). We used a multi-echo EPI sequence to reduce image distortion and increase BOLD sensitivity in our regions of interest which are typically affected by strong susceptibility artifacts, such as the ventral striatum [9]. One volume consisted of 37 axial slices (voxel size: 3.5 x 3.5 x 3.0 mm3; interslice gap: 0.5 mm; field of view: 224 mm; flip angle: 90 degrees). All images were acquired in a single run comprising 20 min. Visual stimuli were projected on a screen and were viewed through a mirror attached to the head coil. In addition to the acquisition of functional images, a high-resolution T1-weighted magnetization prepared rapid gradient-echo (MP-RAGE) anatomical scan was obtained (176 sagittal slices, TR: 2.73 s, TE: 2.95 ms, voxel size: 1.0 x 1.0 x 1.0 mm3, field of view: 256 mm).

S2B. fMRI preprocessing

Echo-time (TE) weighted summation was then used to combine all five echoes into a single data set. Realignment parameters were estimated from the combined TE-images using a least squares approach and a 6 parameter (rigid body) spatial transformation (Friston *et al*., 1995). During subsequent slice timing correction, the time-series for each voxel were realigned temporally to acquisition of the middle slice. Anatomical images were spatially co-registered to the mean of the functional images and segmented using a unified segmentation approach as implemented in the VBM8 toolbox in SPM (http://www.neuro.uni-jena.de/vbm/). The resulting transformation matrix and warp field were then used to normalize the anatomical and functional images into the common MNI152 reference space. Normalized images were spatially smoothed with an isotropic 6 mm full-width-half-maximum (FWHM) Gaussian kernel.

S2C. fMRI statistical analysis

The first level model included 2 regressors for reward cues (high, low) and 4 regressors for the targets (high_congruent, high_incongruent, low_congruent, low_incongruent). All regressors of interest were modeled as an impulse response function (duration = 0) convolved with a canonical haemodynamic response function. Regressors of non-interest included: the 15-second breaks, missed targets (no button response), and 24 motion parameters to optimally control for motion effects (i.e. the linear and quadratic effects of x, y, z, pitch, roll, and yaw movement). Functional scans were high-pass filtered (128 seconds) to remove low-frequency confounds such as scanner drifts. Parameter estimates for all regressors were obtained by maximum-likelihood estimation, modeling temporal autocorrelation as an autoregressive AR(1) process. Contrast images from the first level were entered into second level random effects analyses. To further account for motion, we calculated a summary motion score for every subject, as the

sum of the root-mean-square value of subjects' framewise-displacement parameters (x, y, z in mm & pitch, roll, and yaw in degrees) [10]. This score was greater for ADHD cases than controls ($t(85)=-2.66$, $p = 0.009$), which is why we added this individual summary motion score in all second level analyses as covariate of non-interest.

S2D. Region of Interest (ROI)

As our ventral striatal ROI, we used the anatomically-defined bilateral nucleus accumbens region from the Hammersmith atlas (www.brain-development.org [11]). We extracted the mean beta-weights for every participant with MarsBar [12]. The regionally averaged betas were used to assess the effects of ADHD diagnosis on reward anticipation responses using an independent-sample t-test in SPSS version 22 (IBM Corp. IBM SPSS Statistics, Armonk, New York, USA).

S2E. Psychostimulant predictor in multiple regression

We ran a multiple regression analysis with reward anticipation responses in the ventral striatum as dependent. In addition to the functional microbiome measure, one of the other predictors was long-term medication use. For this predictor, we calculated the duration of psychostimulant use minus the period not using psychostimulants anymore (if any), and multiplied this with the instant dose. For the sustained-release Concerta, we calculated the instant dose by multiplying the daily dose with 0.278, and added this to the immediate-release Ritalin dose (if any). This duration x dose amount was used as the medication predictor in the multiple regression analysis. Out of the 28 subjects in this multiple regression analysis, six were (n = 5) or had been (n = 1) using medication for ADHD; all six were using Ritalin and/or Concerta.

Methods S3: Microbiome sequencing and analyses

S3A. 16S marker gene amplification, sequencing and data acquisition

According to Jaeggi and colleagues [13], the V3-V6 region of the 16S rRNA gene was amplified by PCR using the following universal primers: (i) forward primer, 5'-*CCATCTCATCCCTGCGTGTCTCCGACTAG*NNNNNN**ACTCCTACGGGAGGCAGCAG**-3' (the italicised sequence is the 454 Life Sciences primer A, and the bold sequence is the broadly conserved bacterial primer 338F; NNNNNN designates the sample-specific six-base barcode used to tag each PCR product, see Table S1); (ii) reverse primer 5'-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG***CRRCACGAGCTGACGAC**-3' (the italicised sequence is the 454 Life Sciences primer B, and the bold sequence is the broadly conserved bacterial primer 1061R).

The PCR amplification mixture contained: 1 µL faecal DNA, 1 µL bar-coded forward primer, 15 µL master mix (1 µL KOD Hot Start DNA Polymerase (1 U/µL; Novagen, Madison, WI, USA), 5 µL KOD-buffer (10×), 3 µL MgSO4 (25 mM), 5 µL dNTP mix (2 mM each), 1 µL (10 µM) of reverse primer) and 33 µL sterile water (total volume 50 µL). PCR conditions were: 95°C for 2 min. followed by 35 cycles of 95°C for 20 sec., 55°C for 10 sec., and 70°C for 15 sec. The approximately 750 bp PCR amplicon was subsequently purified using the MSB Spin PCRapace kit (Invitek, Hayward, CA) and the concentration was checked with a Nanodrop 1000 spectrophotometer (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA). A composite sample for pyrosequencing was prepared by pooling 200 ng of these purified PCR products of each sample. The pooled sample was purified using the Purelink PCR Purification kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA), with high-cut-off binding buffer B3, and submitted for pyrosequencing of the V3-V4 region of the 16S rRNA gene on the 454 Life Sciences GS-FLX platform using Titanium sequencing chemistry (GATC-Biotech, Germany).

S3B. Microbiome sequencing data analysis and bioinformatics workflow

Reads were filtered for chimeric sequences using the UCHIME algorithm version 4 [14]. Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure as implemented in QIIME 1.2. Figures resulting from these clustering analyses were generated using the interactive tree of life (iTOL) tool [15]. The Ribosomal Database Project classifier version 2.3 was performed for taxonomic classification of the sequence reads [16]. Alpha diversity metrics (PD whole tree, Chao1, Observed Species and Shannon) were calculated by bootstrapping 1126 reads per sample, and taking the average over four trials. For visualization of the differential microbiome, Cytoscape software version 3.1 [17] was used together with in-house developed Python scripts for generating the appropriate input data deriving from the QIIME analysis.

Note that due to technical limitations in the resolution of 16S marker gene sequencing, OTU (Operational Taxonomic Unit) calling on the level of species should be interpreted with caution (Table S2 and Figure 3).

S3C. Microbiome-derived function prediction

We predicted the presence of Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (K numbers; note that K numbers can represent orthologs with multiple enzymatic functions and vice versa) [18] and subsequent functional and metabolic pathways using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; version 1.0.0) [19]. PICRUSt requires closed-reference OTU picking in QIIME, for which the Greengenes reference collection version 13.5 (May 2013) was used [20]. For additional calculation of relative abundances of pathways and KEGG Orthologs (i.e. the candidates), and for downstream statistics, in-house Python scripts were used.

References Supplementary Methods

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