SUPPLEMENTARY MATERIALS AND METHODS

Amelioration of Autism-like Social Deficits by Targeting Histone Methyltransferases EHMT1/2 in *Shank3*-deficient mice

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Animals, Human Postmortem Tissues, and Reagents

Two lines of *Shank3*-deficient mice, *Shank3*$^{+/\Delta C}$ and *Shank3*$^{e4-9}$, were used in this study (see **Suppl. Fig. 9** for knockout verification). Mice were group-housed with ad libitum food accessibility in the 12-hr light-dark cycle (light: 6am-6pm; dark: 6pm-6am). Mice of different genotypes were randomly assigned to drug/saline groups. Mice for biochemistry and electrophysiology studies were sacrificed at the same time of the day (9:00am-11:00am). All the i.p. injections were done in the afternoon (3:00pm). Researchers were blind to both genotypes and treatments during experiments.

Frozen human postmortem tissues (Brodmann's Area 9) from autism patients and age-matched healthy controls were provided by NIH NeuroBioBank. Detailed information about these ASD patients is included in **Suppl. Table 6**. Upon arrival, tissue was stored in a -80°C freezer until used for RNA and protein extraction. UNC0642 (Tocris) or BIX01294 (Tocris) was prepared by dissolving in DMSO or saline stock, and then diluted with saline before use (DMSO concentration of working solution: <0.2%).

Quantitative Real-time RT-PCR

As previously reported[25,26], total RNA was isolated from mouse PFC punches using Trizol reagent (Invitrogen) and treated with DNase I (Invitrogen) to remove genomic DNA. Then the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) was used to reverse-transcribe mRNA into cDNA, followed by the treatment with RNase H (2 U/ l) for 20 min at 37°C. Quantitative real time RT-PCR was performed using the iCycler iQ™ Real-Time PCR Detection System and iQ™ Supermix (Bio-Rad), according to the manufacturer's instructions. In brief, GAPDH was used as the housekeeping gene for quantitation of the expression of target genes in samples from WT vs. Shank3$^{+/\Delta C}$ mice treated with UNC0642 or saline control. Fold changes in the target genes were determined by: Fold change = $2^{-\Delta(\Delta C_T)}$, where $\Delta CT = C_{T(target)} - C_{T(GAPDH)}$, and $\Delta(\Delta C_T) = \Delta C_{T(treated\ group)} - \Delta C_{T(WT+saline)}$. C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence reaches 10x the standard deviation of the baseline. A total reaction mixture of 20 μl was amplified in a 96-well thin-wall PCR plate (Bio-Rad) using the following PCR cycling parameters: 95°C for 5 min followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec. Primers for all the target genes are listed in **Supplemental Table 7**.

Western Blotting of Nuclear and Total Proteins

Nuclear extracts from mouse brains and human postmortem brains were prepared according to the manufacturer's instructions (Life Technologies) with modifications. Briefly, 8 PFC punches (diameter: 2 mm) from fresh mouse slices (300 μm) per animal were collected, and then homogenized with 500 μl hypotonic buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 1 mM PMSF, with cocktail protease inhibitor). The homogenate was incubated on ice for 15 min, followed by centrifugation at 3,000 x g, 4°C for 10 min. The nuclear pellet was resuspended in 50 μl nuclear extract buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 10% glycerol, 1 mM PMSF, with cocktail protease inhibitor) and incubated on ice for 30 min with vortex periodically to re-suspend the pellet. After centrifugation, the supernatant for nuclear fractions was collected, boiled in 2 × SDS loading buffer for 5 min and then separated on 12% SDS-polyacrylamide gels. Western blotting experiments for nuclear proteins were performed with antibodies against H3K9me2 (1:500, Cell Signaling, 4658), H3K4me3 (1:1000, Cell Signaling, 9751), H3K27me3 (1:1000, Cell Signaling, 9733), total H3 (1:1000, Cell Signaling, 4499), EHMT1 (1:500, Millipore, 09-078), EHMT2 (1:500, Abcam, ab180815), and β-catenin (1:1000, CST, #2698). For total protein Western blotting, brain samples were homogenized in 1% SDS. Antibodies used include: NR1 (1:500, NeuroMab, 75-272), NR2A (1:500, Millipore, 07-632), NR2B (1:500, Millipore, 06-600), GluR1 (1:500, NeuroMab, 75-327), GluR2 (1:500, NeuroMab 75-002), Shank3 (1:500, NeuroMab, 75-344, clone N367/62), Arc (1:1000, abcam, ab23382) and tubulin (1:10,000, Sigma, T9026). All the antibodies have been validated in previous literatures and in our lab. WB images were taken from different membranes when the tested proteins had similar molecular weights.

Behavioral Testing

Social preference test: The three-chamber social preference test used a rectangular Plexiglas arena (L: 101.6 cm, W: 50.8 cm, H: 50.8 cm) divided into 3 chambers with the retractable doorways allowing for access to side chambers[18,19]. Briefly, each mouse was placed in the center chamber to habituate for 10 min, followed by an additional 10-min exploration with free access to all 3 chambers of the apparatus. Two enclosures (an inverted pencil cup, D: 10.2 cm, H: 10.5 cm) were placed in the center of each side chambers, with an upright plastic drinking cup placed on top of each enclosure to prevent the test mouse from climbing on top. After 2 days of habituation, the social preference test was performed. The test contained two phases. In phase 1, two identical nonsocial stimuli (folded black papers) were placed under each enclosure. In phase 2, a nonsocial (NS) stimulus (a wood block) and a social (SOC) stimulus (age- and sex-matched mouse of the same strain) were placed under each enclosure,

and the locations of the NS and SOC stimuli were counterbalanced. The test animal was placed in the center chamber to freely explore the apparatus for 10 min in each phase, with a 5-min interval in between. The chamber was cleaned with 75% Ethanol after each phase. Interaction time was counted based on “investigating” behaviors of the test animal to each stimulus. In some experiments, an experimentalist measured the time that the test animal spent on actively seeking and sniffing the stimulus. In other experiments, a computer running the Any-maze behavior tracking software (Stoelting, Wood Dale, IL) measured the time that the test animal spent in proximity to the capsule (distance of animal head to cup edge: ≤3.5 cm). Data generated manually and automatically were consistent, so they were pooled. Preference scores were calculated, where time spent with one stimulus was subtracted from the time spent with the other stimulus and divided by the total time spent exploring both stimuli.

Social approach test: Each mouse was habituated in an apparatus (L: 67.7 cm, W: 50.8 cm, H: 50.8 cm) containing an enclosure (an inverted pencil cup) for 10 min, then was returned to the home cage. After cleaning the apparatus, a social stimulus (an age- and sex-matched mouse) was placed inside the enclosure. The test mouse was put back into the apparatus to explore for 10 min. The time spent on interacting with the social stimulus was measured.

Rota-rod test: To assess motor coordination and balance, an accelerating rota-rod (SD instruments, San Diego, CA) was used. Mice were placed on a cylinder, which slowly accelerated from 4 to 40 revolutions per minute over a 5-min test session. The task requires mice to walk forward in order to remain on top of the rotating cylinder rod.

Self-grooming: Mice were scored for spontaneous grooming behaviors when placed individually in a clean cage[18]. The cage was lined with a thin layer of bedding (~1 cm) to reduce neophobia, but prevent digging, a potentially competing behavior. Prior to the testing period, animals were allowed to habituate to the novel environment for 10 min. Each mouse was recorded for 10 min to get the total time spend in grooming.

Locomotion test: Locomotor activity was measured in a rectangular apparatus (40×40×30 cm) equipped with photo beam monitors (AccuScan Instruments). Total travel distance were recorded during the 30-min test session.

Elevated plus maze test: Mice were placed in the center of a white Plexiglas elevated plus maze (each arm was 38 cm long and 7.6 cm wide, with 28 cm high black Plexiglas walls on closed arms) and allowed to explore for 10 min. The test was conducted in dim white light. Mice were monitored using Any-maze Software, and the time spent in the open arms and the number of entries into the open arms were measured.

Viral Gene Transfer

The shRNA oligonucleotide targeting mouse *Ehmt1*, *Ehmt2* or *Arc* sequence was inserted to the lentiviral vector pLKO.3G (Addgene), which contains an eGFP reporter. For the production of lentiviral particles, a mixture containing the pLKO.3G shRNA plasmid, psPAX2 packaging plasmid and pMD2.G envelope plasmid (Addgene) was transfected to HEK-293FT cells using lipofectamine 2000. The transfection reagent was removed 12-15 hours later, and cells were incubated in fresh DMEM (containing 10% FBS + penicillin/streptomycin) for 24 hrs. The media harvested from the cells, which contained lentiviral particles, was concentrated by centrifugation (2,000 × g, 20 min) with Amicon Ultra Centrifugal Filter (Ultracel-100K, Millipore). The concentrated virus was stored at -80°C. EHMT1 and EHMT2 shRNA lentiviruses were mixed (1:1) before use. Arc CRISPR activation lentiviral particle was purchased from Santa Cruz Biotech. *In vivo* delivery of viral suspension (1 µl) was achieved by stereotaxic injection bilaterally into PFC with a Hamilton syringe (needle gauge 31) as previously described[18,19]. Electrophysiological experiments and behavioral testing were performed after 10 days of viral expression.

Immunohistochemistry

Mice were anesthetized and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). Whole brains were immediately removed, post-fixed at 4°C for 24 hours and coronally cut into 40 µm slices. Slices containing PFC or other brain regions (striatum and hippocampus) were blocked for 2 hours in PBS containing 3% BSA and 0.3% Triton. After washing, slices were incubated with the primary antibody against GFP (1:1000, Millipore, 3080), H3K9me2 (1:50, CST, D85B4), NeuN (1:100, Millipore, MAB377), EHMT1 (1:100, Millipore, 09-078) or EHMT2 (1:100, Abcam, ab185050) for 48 hours at 4°C. After washing 3 times (15 min each, with gentle shaking) in PBS, slices were incubated with secondary antibody (Alex488, Invitrogen, A21202, 1:1000) for 1 hour at room temperature, followed by 3 washes with PBS. Slices were mounted on slides with VECTASHIELD mounting media (Vector Laboratories). Low magnification images were acquired using a 10X objective on a Zeiss AxioImager Fluorescence microscope. High magnification images were acquired using a 60x objective Zeiss LSM710 confocal microscope. All specimens were imaged and analyzed under identical conditions using Image J software.

Electrophysiological Recordings

Whole-cell voltage-clamp recording technique was used to measure synaptic currents in layer V pyramidal neurons of prefrontal cortical slices, as previously described [19,26-28]. Mice were anesthetized with Halothane (Sigma) inhalation. Brains were immediately removed, iced and cut into 300 µm slices by a Vibratome (Leica VP1000S). Mouse brain slices were then positioned in a perfusion chamber attached to the fixed stage of an upright microscope (Olympus) and submerged in continuously flowing oxygenated ACSF (in mM: 130 NaCl, 26 $NaHCO_3$, 3 KCl, 2 $MgCl_2$, 1.25 NaH_2PO_4, 1 $CaCl_2$, 10 Glucose, pH 7.4, 300 mOsm). Bicuculline (20 µM) and CNQX (20 µM) were added in NMDAR-EPSC recordings. Bicuculline and D-APV (50 µM) were added in AMPAR-EPSC recordings. Patch electrodes contained internal solution (in mM): 130 Cs-methanesulfonate, 10 CsCl, 4 NaCl, 10 HEPES, 1 $MgCl_2$, 5 EGTA, 2.2 QX-314, 12 phosphocreatine, 5 MgATP, 0.2 Na_2GTP, 0.1 leupeptin, pH 7.2-7.3, 265-270 mOsm. Layer V mPFC pyramidal neurons were visualized with a 40X water-immersion lens and recorded with the Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA). Evoked EPSC were generated with a pulse from a stimulation isolation unit controlled by a S48 pulse generator (Grass Technologies, West Warwick, RI). A bipolar stimulating electrode (FHC, Bowdoinham, ME) was placed ~100 µm from the neuron being recorded. For NMDAR-EPSC, the cell (clamped at -70 mV) was depolarized to +40 mV for 3 s before stimulation to fully relieve the voltage-dependent Mg^{2+} block. Membrane potential was maintained at -70 mV for AMPAR-EPSC recordings. For input-output responses, EPSC was elicited by a series of pulses with different stimulation intensities (50-90 µA) delivered at 0.033 Hz. Data analyses were performed with Clampfit (Molecular Devices, Sunnyvale, CA), Kaleidagraph (Synergy Software, Reading, PA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

RNA Sequencing and Analysis

PFC samples were obtained from three WT mice, three saline-treated $Shank3^{+/\Delta C}$ mice and three UNC0642-treated $Shank3^{+/\Delta C}$ mice. We generated strand-specific RNA libraries from 1 µg purified RNA using TruSeq stranded total RNA plus Ribo-zero kits (Illumina). The sequencing was performed at the Genomics and Bioinformatics Core of State University of New York at Buffalo. Single end reads per sample were obtained using the HiSeq 2500 platform from Illumina. Reads were first trimmed using Cutadapt to remove the 3' end adapters and trailing sequences, followed by aligning to mouse RefSeq mRNAs using TopHat2. Transcript counts were estimated using HTSeq. Differences in gene expression levels between samples were assessed with edgeR and calculated as log2 fold change. Functional protein classification analyses were undertaken using Panther. Enrichment analyses of differentially expressed

genes were undertaken using gene sets derived from the Biological Process Ontology from DAVID (https://david.ncifcrf.gov/). For comparisons to ASD-susceptibility genes, we used the list on the SFARI database.

Chromatin Immunoprecipitation (ChIP)

ChIP samples were prepared as previously described with modifications[19,26,29]. Briefly, eight PFC punches from 300 μm brain slices of each mouse were collected, cross-linked with 1% formaldehyde for 12 min and quenched by the addition of glycine at a final concentration of 0.125 M for 5 min before freezing at -80°C. For the ChIP assay of β-catenin binding to *EHMT1/2* promoter regions, chromatin was solubilized and extracted by cell and nuclear lysis buffer, and sheered using a Bioruptor 300 (Diagenode) at 4°C with high sonication intensity (30 s on/30 s off for 10 min, repeat for 3 times with 10-min pause each time). For the ChIP assay of H3K9me2 occupancy at gene promoters, chromatin was extracted by 1% SDS lysis buffer, followed by the sheering using a Fisher Scientific Sonic Dismembrator Model 300 at 28% of power (ten 15-s pulses with 30-s pause between pulses). These procedures resulted in DNA fragment sizes of 200-500 bp. After centrifugation, ~10% of the supernatant was saved for input control. For β-catenin ChIP, magnetic sheep anti-rabbit beads (Invitrogen) were incubated with anti-β-catenin antibody (CST, #8480, 10 µl per reaction) at 4 °C overnight on a rotator. Following washing of the magnetic bead/antibody complex, 70 µl (magnetic bead/antibody complex slurry) was incubated with the sheared chromatin sample for 16 hrs at 4 °C. For H3K9me2 ChIP, to reduce nonspecific background, the supernatants were diluted in ChIP dilution buffer and pre-cleared with 80 µl of salmon sperm DNA/protein A agarose-50% slurry (Millipore, 16-157) for 30 min at 4°C with agitation. The pre-cleared supernatant was incubated with antibodies against H3K9me2 (abcam, ab1220, 10 µl per reaction) overnight at 4°C under constant rotation, following by incubation with 60 µl of Salmon Sperm DNA/Protein A agarose-50% Slurry for 1 hr at 4°C. After washing, bound complex was eluted from the beads by incubating with 150 μl elution buffer for twice at room temperature. After reversing crosslinks in 65°C for 4 hours, proteins and RNA were removed using proteinase K (Invitrogen) and RNase (Roche), respectively. Then immunoprecipitated DNA and input DNA were purified by PCR purification kit (Qiagen). Normal mouse IgG immunoprecipitations using a mouse monoclonal anti-IgG antibody were carried out to control for appropriate enrichment of signal amplification.

Purified DNA was subjected to qPCR reactions with primers against mouse *Ehmt1* (Forward, +371 bp ~ +351 bp relative to TSS, 5'- ACAAAAGGGTGGTCAGCCAG -3'; Reverse, +207 bp ~ +187 bp relative to TSS, 5'- AGCTGCTCTGTTTCCCGTTT -3'), *Ehmt2* (Forward,

+379 bp ~ +359 bp relative to TSS, 5'-CTTCCGTGAGTCTCTGTCGC -3'; Reverse, +266 bp ~ +246 bp relative to TSS, 5'- GGGAACACGTGTAGCCTGAG -3'), *Arc* (Primer 1: Forward, +5146 bp ~ +5126 bp relative to TSS, 5'-GCCATCAGGTGGGGAAAATG-3'; Reverse, +4984 bp ~ +4965 bp relative to TSS, 5'-GAGGGCCAGAAACCTATCCA-3'. Primer 2: Forward, +6000 bp ~ +5980 bp relative to TSS, 5'- GCAGATGCACACAAGATAGACC-3'; Reverse, +5885 bp ~ +5865 bp relative to TSS, 5'- CACCCTCCTGTTCAGTTGAC-3'. Primer 3: Forward, +2265 bp ~ +2245 bp relative to TSS, 5'- AAATGCAGTGGTCAGAGGGT-3'; Reverse, +2137 bp ~ +2117 bp relative to TSS, 5'- GATGAGCAGGCTGGAGAGTG-3'), *Grin1* promoter (Forward, +2560 bp ~ +2539 bp relative to TSS, 5'- GCCACATTTGTCCATGAACTG-3'; Reverse, +2467 bp ~ +2447 bp relative to TSS, 5'- CCCATGCTACTCCACTGCTC-3'), *Grin2a* promoter (Forward, +6912 bp ~ +6892 bp relative to TSS, 5'- ACCAAAGACAGGAGCCTGGA-3'; Reverse, +6724 bp ~ +6704 bp relative to TSS, 5'- TGGGTAAGCATTCGACCGAT-3'), *Grin2b* promoter (Forward, +2487 bp ~ +2467 bp relative to TSS, 5'- AGGTGGGTGTCACAAGTCTG-3'; Reverse, +2309 bp ~ +2289 bp relative to TSS, 5'- CCCTGCCCACTCTTTCATGG-3'). Primers from multiple sties relative to TSS (proximal or distal promoter regions) were designed and pre-tested in both input and ChIP samples. Only those with solid signals in both input and ChIP samples were chosen for this experiment.

Primary Neuronal Culture

Rat PFC cultures were prepared by modification of methods described previously[27]. Briefly, the PFC was dissected from 18-day-old rat embryos, and cells were dissociated using trypsin and titrated through a Pasteur pipette. Neurons were plated on coverslips coated with poly-L-lysine in DMEM with 10% fetal calf serum at a density of 1×10^5 cells/cm^2. Neurons attached to the coverslip within 24 hours. Then the medium was changed to Neurobasal with a B27 supplement. Two to three days after plating, 5 μM AraC was added to inhibit glial growth. Subsequently, half of the medium was changed to a conditional medium once a week. Neurons were maintained for 2–3 weeks before testing the knockdown effect.