Methamphetamine Down-Regulates Striatal Glutamate Receptors Via Diverse Epigenetic Mechanisms

Supplemental Information

Supplemental Methods & Materials

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analyses

Unpooled total RNA (1 μg) isolated from striatal samples (n = 6 per group) was reverse-transcribed with oligo dT primers using Advantage RT-for-PCR kit (Clontech). qRT-PCR experiments were done using Roche LightCycler (Roche Diagnostics) using iQ SYBR Green supermix (Bio-Rad). For all qPCR experiments, individual data were normalized using the corresponding OAZ1 (ornithine decarboxylase antizyme 1) mRNA level. OAZ1 showed very stable expression in the mouse based on their analyses of 2,543 tissue samples hybridized to Affymetrix Mouse GeneChips after exposure to various experimental manipulations. OAZ1 expression was also very stable after METH injections. The results are reported as fold changes calculated as the ratios of normalized gene expression data for the METH group in comparison to the control group. The primers for RT-PCR were synthesized at the Synthesis and Sequencing Facility of Johns Hopkins University (Baltimore, MD, see Table S2).

SubCellular Fractionation

Samples were homogenized separately in 10% w/v of ice-cold 10 mM HEPES buffer (pH 7.4) containing 10 mM KCl, 1.5 mM $MgCl_2$, 1%-Igepal CA-630 supplemented with a Roche protease inhibitor cocktail tablet. The homogenate was centrifuged for 5 min at 14,000 x g to produce pellet P1 (nuclear fraction). The nuclear fraction was resuspended in a nuclear lysis buffer (20 mM HEPES, 25% glycerol, 840 mM NaCl, 1.5 mM $MgCl_2$, 0.4 mM EDTA). The supernatant were then centrifuged at 100,000 x g for 60 min to give a high speed pellet P2 (membrane fraction) and the high speed supernatant S (cytosolic fraction). The membrane fraction was solubilized in SML buffer (2% sodium monolaurate, 2 mM MgCl2, 2 mM phenylmethylsulfonyl fluoride (PMSF) in PBS). After 1 h incubation on ice, the detergent-insoluble material was removed by centrifugation. The protein concentrations of the supernatant

membrane fractions obtained by this method were determined by a BCA assay (Thermo Fisher Scientific) and the fractions were directly used for western blotting.

Immunoblot Analysis

Western blot analyses were carried out from striatal protein lysates (n = 6). Cytoplasmic and membrane protein fractions were obtained according to protocol described above. Protein concentration of lysates was determined with the Bio-Rad Dc Protein assay reagent (Bio-Rad). The lysates were then denatured with sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 50 mM DTT) at 100°C for 5 min, and subjected to SDS-PAGE. Proteins were electrophoretically transferred to Hybond-PTM membrane (Amersham) and incubated overnight at 4°C with specific antibodies listed in the main text. After incubation, the blots were washed with TBS (0.1% Tween-20). The membranes were then incubated with HRP-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibody for 1 h at room temperature. To confirm equal protein loading, the blots were re-probed with α-Tubulin antibody (1:4000, 2-h at room temperature; Sigma). Electrochemiluminescence (ECL) plus chemiluminescent reagents (GE Healthcare) were used to detect protein expression. Signal intensity was measured with Carestream Molecular Imaging software. Experiments were done twice.

Co-immunoprecipitation

Briefly, the rat striatum was dissected out, minced to 1 mm pieces and were added to eppendorf tubes containing ice-cold artificial cerebrospinal fluid (aCSF) containing BS^3 [bis (sulfosuccinimidyl) suberate] (Thermo Fisher Scientific). The tubes were gently rotated for 30 min at 4°C. Cross-linking was terminated by quenching the reaction with 100 mM glycine (10 min, 4°C). The slices were pelleted by brief centrifugation and the supernatant was discarded. Pellets were resuspended in ice-cold lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 0.5% Triton X-100, 10 ug/ml aprotinin, 5 ug/ml leupeptin, 1 mM sodium orthovanidate, and 1 mM PMSF and gently homogenized. For immunoprecipitation, 300 ul (750 ug) of striatal tissue lysate were pre-cleared by protein G-Sepharose beads (GE Healthcare), 30 ul were taken out as “input” protein and the remaining was incubated overnight with 3 ug of anti-REST antibody (Millipore), anti-CoREST antibody (BD Transduction Labs) or anti-MeCP2 antibody (Abcam) at 4°C. Afterwards, 30 ul of Protein G-Sepharose beads were added and mixed gently at 4°C for 2

h. Beads were washed five times with RIPA buffer (50 mM Tris-HCl- pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail tablet) and the proteins were eluted by adding 30 ul of 2X SDS-PAGE sample buffer and heating at 95°C for 10 min. Samples were size-separated by electrophoresis in SDS-containing (4-12%) polyacrylamide gels (NuPAGE Bis-Tris gels, Life Technologies) and transferred to PVDF membranes. Membranes were blocked in TBST (TBS plus 0.1% Tween) containing 5% skim milk powder. Blots were then incubated with primary antibodies recognizing anti-HDAC1 or anti-HDAC2 antibody (1:1000, Santa Cruz Biotechnology). After washing, blots were incubated with either an anti-rabbit HRP-conjugated antibody or anti-mouse HRP-conjugated antibody (1:10,000, GE Healthcare) for 1 h at room temperature. To reduce heavy and light chain background signals, blots were incubated at room temperature with a 1:5000 dilution of protein A-HRP (GE Healthcare), prepared in TBST (0.1% Tween). Blots were washed and were developed with ECL prime chemiluminescent reagents (GE Healthcare).

Chromatin Immunoprecipitation (ChIP) Assay

Striatal tissue was rapidly removed from rat brains, minced to ~1 mm-sized pieces, and immediately cross-linked in 1% formaldehyde for 15 min at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125M. The tissue was washed five times in cold PBS containing the proteinase inhibitors in the Roche protease inhibitor cocktail tablet (Roche Diagnostics) and 1 mM PMSF (Sigma). Tissues were then rapidly frozen on dry ice. The fixed striatal tissue was resuspended in SDS lysis buffer (Millipore) containing Roche protease inhibitor cocktail and 1 mM PMSF and homogenized twice for 10 s.

Each sample was transferred to TPX plastic tube (Diagenode) and sonicated for 15 cycles of 30 s Time ON and 30 s Time OFF using the Bioruptor (Diagenode). Afterwards, the fragmentation was checked by gel analysis to confirm the sheared range of 300-600 bp. Dynabeads (Life Technologies) were incubated with 5 µg of antibodies directed against either acetylated H4K5 (H4K5ac, 07-327), acetylated H4K12 (H4K12ac, 07-595), acetylated H4K16 (H4K16ac, 07-329), REST (09-019), CoREST (07-455), (Millipore), MeCP2 (ab2828); HDAC1 (ab46985), or HDAC2 (ab12169) (Abcam). As a control, the striatal tissue was also incubated with 5 µg nonimmune rabbit IgG (IgG, 12-370) (Millipore) overnight at 4°C.

Equal amounts of chromatin lysate (60 µg) were diluted with ChIP dilution buffer (Millipore) to a final volume of 1.5 ml. One hundred microliters of the pre-immunoprecipitated lysate were saved as "input" for later normalization. The chromatin lysate was then immunoprecipitated with appropriate antibodies overnight at 4°C. The beads were sequentially washed once with low salt, high salt, LiCl, and TE according to the manufacturer's instructions.

The DNA-protein complex was then eluted from the beads with 500 µl of $NaHCO_3$/SDS elution buffer. DNA and proteins were dissociated at 65°C for 4 h under high-salt conditions, followed by RNase A treatment for 30 min at 37°C and proteinase K treatment for 1 h at 55°C. The DNA was then extracted with phenol/chloroform, precipitated with ethanol, and finally resuspended in 80 µl of 10 mM Tris pH 8.0.

qPCR was performed with ChIP specific primers for GluA1, GluA2 and GluN1 promoters (around transcription start sites) and also for the distal CpG rich sequence (23Kb upstream) for GluA1 (Table S2). qPCR was performed on ChIP-derived DNA samples using Roche LightCycler (Roche Diagnostics), with iQ SYBR Green (Bio-Rad) monitoring. All PCR reactions were performed in duplicate and included negative controls (no DNA). LightCycler software was used to calculate standard curves calculated using serial dilutions (100–0.1 ng) of input genomic DNA.

Methylated DNA Immunoprecipitation (MeDIP) and Hydroxymethylated DNA Immunoprecipitation (hMeDIP)

Genomic DNA was isolated from striatal tissue by overnight Proteinase K treatment, phenol-chloroform extraction, ethanol precipitation and RNase digestion. Subsequently 300 µl fractions of DNA (20 ug) were sheared by ultrasonic treatment using the Diagenode Bioruptor (12 cycles, 30 s "ON", 30 s "OFF") to obtain a fragment size between ~200–600 bp. After denaturation (10 min at 95°C), DNA (5 ug) was then immunoprecipitated overnight at 4°C using 5 µl of mouse monoclonal anti-5meC antibody 33D3 (Millipore, MABE146) for MeDIP assay or 5 µl of rabbit polyclonal anti-5hmC antibody (ActiveMotif, 10310001) for hMeDIP assay in a final volume of 500 µl IP buffer (10 mM sodium phosphate (pH 7.0), 140 mM NaCl, 0.05% Triton X-100). We incubated the mixture with 80 µl of Dynabeads (Life Technologies) overnight at 4°C and washed it three times with 700 µl of IP buffer. We then treated the beads with proteinase K for 3 h at 50°C and recovered the methylated or hydroxymethylated DNA by phenol-chloroform extraction

followed by ethanol precipitation. For later comparison with immunoprecipitated DNA, sheared "input" DNA samples were collected prior to immunoprecipitation.

qPCR on MeDIP and hMeDIP Samples

We carried out qPCR reactions with 20 ng of input DNA and immunoprecipitated methylated or hydroxymethylated DNA. For qPCR reactions, we used the iQ SYBR Green PCR master mix (Bio-Rad) and Roche thermal cycler (Roche Diagnostics). Reactions were done in duplicates and standard curves were calculated on serial dilutions (100–0.1 ng) of input genomic DNA. To evaluate the relative enrichment of target sequences after MeDIP or hMeDIP, we calculated the ratios of the signals in the immunoprecipitated DNA versus input DNA.

Slice Preparation

Rats were anesthetized with 40 mg/kg pentobarbital (i.p.) and transcardially perfused with ~30 ml of ice cold (~ 4°C) modified aCSF at a rate of ~ 20 ml/min. The modified aCSF for perfusion contained (in mM): 225 sucrose, 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 4.9 $MgCl_2$, 0.1 $CaCl_2$, 26.2 $NaHCO_3$, 1.25 glucose, 3 kynurenic acid, and 1 mM ascorbic acid. After perfusion, the brain was quickly removed and placed into ice-cold aCSF for 1–2 min. Coronal sections containing the dorsal striatum (250 μm) were prepared with a vibratome (Leica, Nussloch, Germany). Slices were placed in a holding chamber (containing aCSF with 1 mM ascorbic acid added 15 min before brain dissection) and allowed to recover for at least 30 min before being placed in the recording chamber and superfused with a bicarbonate-buffered solution saturated with 95% O_2 and 5% CO_2 and containing (in mM): 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 1.3 $MgCl_2$, 2.4 $CaCl_2$, 26.2 $NaHCO_3$, and 11 glucose (at 32°C–34°C).

Electrophysiology

Picrotoxin (100 μM) was present throughout the experiment to block inhibitory synaptic transmission. Excitatory postsynaptic currents (EPSCs) were evoked using a single stimulating electrode positioned ~ 100 μm dorsal to the recording neuron. Input-output curve was obtained by increasing the strength of stimulation. Evoked EPSCs were obtained using stimulation of 0.05, 0.1, 0.2 and 0.4 mA stimulation. Data was normalized to 100% of maximal evoked EPSC. AMPAR miniature EPSCs (mEPSCs) were recorded in cells voltage-clamped at −70 mV and in

the continual presence of Tetrodotoxin (500 nM). Detection criteria were set at >7 pA. mEPSC traces were recorded while sampling every 10 μs. The images were filtered at 1 kHz.

In the recordings in which AMPA/NMDA receptor response ratios were determined, the internal solution contained 3 mM QX-314 and cells were held at +40 mV. AMPAR-mediated currents were isolated with the selective NMDAR antagonist AP5. The NMDAR-mediated current was then digitally obtained by taking the difference current before and after AP5 application.

Table S1. Schedule of Chronic METH Treatment

	Monday	Tuesday	Wednesday	Thursday	Friday
Week 1					
9:30					
10:00	0.5 mg/kg	1 mg/kg	1 mg/kg	1.5 mg/kg	
11:00					
12:00			1 mg/kg	1.5 mg/kg	
13:00					
14:00			1 mg/kg	1.5 mg/kg	
15:30					
16:00	0.5 mg/kg	1 mg/kg	1 mg/kg	1.5 mg/kg	
Week 2					
9:30					
10:00	1 mg/kg	1.5 mg/kg	2 mg/kg	2.5 mg/kg	
11:00					
12:00	1 mg/kg	1.5 mg/kg	2 mg/kg	2.5 mg/kg	
13:00					
14:00	1 mg/kg	1.5 mg/kg	2 mg/kg	2.5 mg/kg	
15:30					
16:00	1 mg/kg	1.5 mg/kg	2 mg/kg	2.5 mg/kg	
Week 3					
9:30		Dissection			
10:00	3 mg/kg				
11:00					
12:00	3 mg/kg				
13:00					
14:00	3 mg/kg				
15:30					
16:00	3 mg/kg				

The rats were treated with saline or METH for two weeks as described above. Rats were euthanized 16 h after the last drug injection.

Table S2. Primer Table

Primer Name	Up Sequence	Down Sequence
RT-PCR		
GluA1/Gria1	GGC AAA TAC GCC TAC C	ACT CGA TTA AGG CAA CC
GluA2/Gria2	TCC TAC ACG GCT AAC TT	GCT CGA TGT ACT CGT TC
GluN1/NR1	AAA ACA CAA TTA CGA GAG C	CCT GAT ACC GAA CCC A
Drd1a	CAG GAC GTA TGC CAT TTC	CAA TTC TTG GCA TGG ACT
Drd2	GTG TGT TCA TCA TCT GCT	GAA CTC GAT GTT GAA GGT
Gfap	CGG AGT ATC GCC TAG AC	ACT TTC AGC TCC ATT TCT T
ChIP-PCR		
GluA1	GGA GGA GAG CAG AGG GAG AG	TTC CTG CAA TTC CTT GCT TG
GluA1 (-23K)	TCG ACC CTC GCA CCT AC	GCG GAA GGA GAG GCA AT
GluA2	GCG GTG CTA AAA TCG AAT GC	ACA GAG AGG GGC AGG CAG T
GluN1	AGT GGC GTT GAG CTG TA	CCC AAG ATC GTC AAC ATC GG

ChIP, chromatin immunoprecipitation; RT-PCR, real-time polymerase chain reaction.

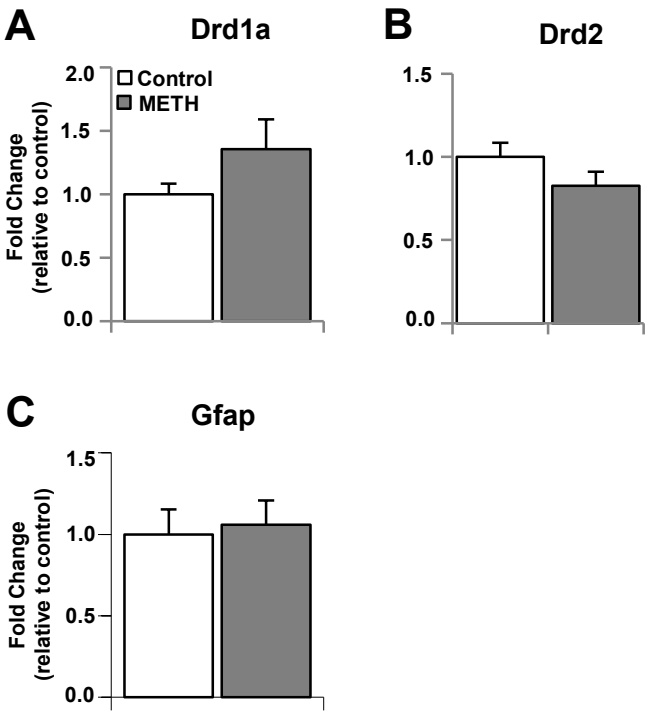

Figure S1. Chronic METH caused no changes in striatal (**A**) D1 DA receptor (**B**) D2 DA receptor and (**C**) Gfap mRNA levels in the rat. RT-PCR was conducted as described in the text using specific primers (see Table S2). Values (n = 8 rats per group) represent means ± SEM of fold changes relative to the controls. DA, dopamine; METH, methamphetamine; RT-PCR, real-time polymerase chain reaction.

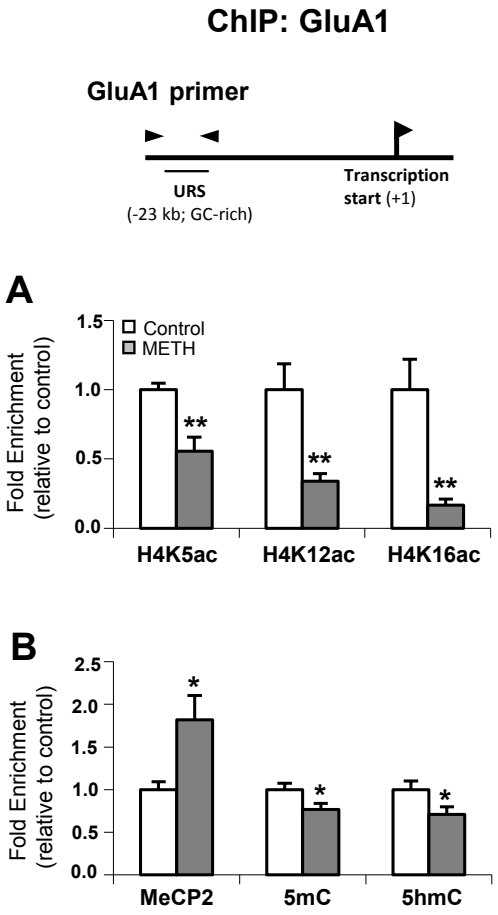

Figure S2. Chronic METH promotes hypoacetylation of H4K5, H4K12 and H4K16 (**A**) and increased enrichment of MeCP2 (**B,** first bar graph) in the CpG rich upstream region (-23 Kb) of AMPA GluA1. ChIP assays (n = 8 rats per group) were performed using striatal tissues from control and METH-treated rats. Quantitative PCR was conducted as described in the text using specific ChIP primers directed at the GluA1 CpG rich upstream region (-23 Kb) (see Table S2). (**B**) Denatured genomic DNA of ~200 – 600 bp (generated by sonication) was incubated with an antibody directed against 5mC (**B**, second bar graph) or 5hmC (**B**, third bar graph), in order to isolate methylated or hydroxymethylated DNA by immunoprecipitation. Relative enrichment of 5mC and 5hmC in the bound over input fractions was calculated by real-time PCR. Values represent means ± SEM of fold enrichment relative to the controls. Statistical significance was determined by unpaired Student's t-test. Key to statistics: *$p < 0.05$; **$p < 0.01$ vs. control group. ChIP, chromatin immunoprecipitation; METH, methamphetamine; PCR, polymerase chain reaction.

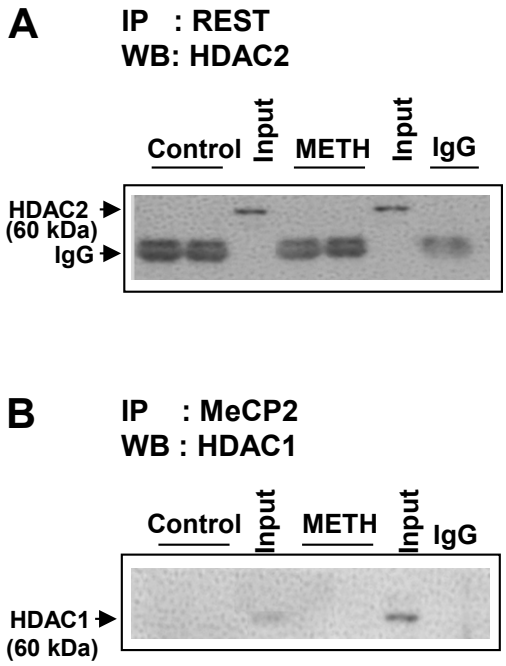

Figure S3. Chronic METH did not cause any changes in (**A**) REST and HDAC2, and of (**B**) MeCP2 and HDAC1 interactions measured by co-immunoprecipitation. Immunoprecipitates were prepared from striatal nuclear extracts of control and METH-treated rats using antibody against anti-REST, anti-MeCP2, and recovery of HDAC2 and HDAC1 was determined by western blot assay. The levels of HDAC1 and HDAC2 from non-specific IgG are indicated. Input levels (5%) of HDAC1 and HDAC2 are shown for comparison. METH, methamphetamine.

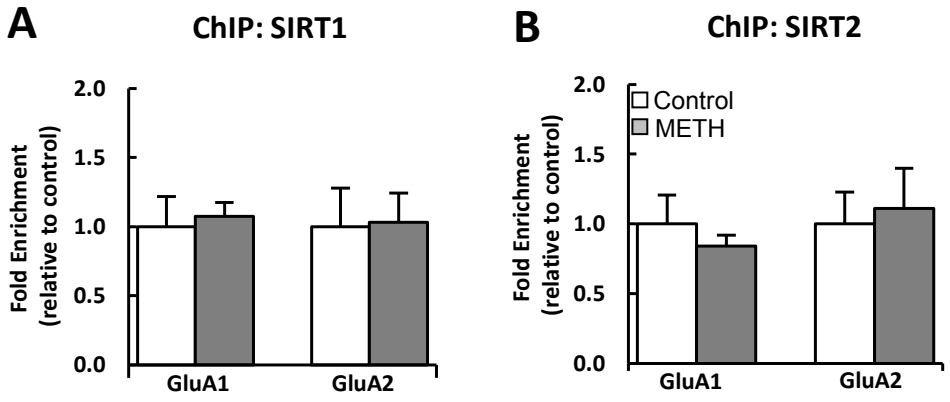

Figure S4. Chronic METH did not alter (**A**) SIRT1 and (**B**) SIRT2 binding in promoter regions of either GluA1 or GluA2. ChIP assays (n = 8 rats per group) were performed using striatal tissues from control and METH-treated rats. Quantitative PCR was conducted as described in the text using specific ChIP primers directed at the TSSs of GluA1and GluA2 (see Table S2). Values represent means ± SEM of fold enrichment relative to the controls. ChIP, chromatin immunoprecipitation; METH, methamphetamine; PCR, polymerase chain reaction; TSSs, transcription start sites.