Neuron, Volume *52*

Supplemental Data

Brain-Specific Phosphorylation of MeCP2

Regulates Activity-Dependent *Bdnf* Transcription,

Dendritic Growth, and Spine Maturation

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Supplemental Experimental Procedures

Plasmids

N-terminal Flag-tagged MeCP2 and derivatives were cloned into a dual-promoter vector such that mouse MeCP2 coding sequences (e1 or e2) are under the control of the CMV promoter (pA1-FgMeCP2). The pA1 vector also contains a second expression cassette in which GFP is expressed from the EF-1 promoter that allows the monitoring of transfection efficiency. Different mutations of MeCP2 were generated using the QuikChange site-directed mutagenesis kit (Stratagene). The MeCP2 LEMPRA (lentivirus-mediated protein-replacement assay) plasmid is a dual-promoter lentiviral vector constructed by inserting the U6 promoter-driven shRNA cassette 5' to the Ubiquitin-C promoter in the FUIGW plasmid (Lois et al., 2002), and inserting Flag-tagged shRNA-resistant MeCP2 under the control of the Ubiquitin-C promoter in FUIGW. The shRNA targeted either rat or mouse MeCP2 and was directed against the sequence 5'GTCAGAAGACCAGGATCTC-3'. The shRNA-resistant Flag-MeCP2 was generated by the introduction of five silent mutations into the sequence of MeCP2 targeted by the shRNA, indicated in the following by lower case letters: 5'GagcGAAGACCAaGAcCTC-3'. The following constructs have been previously described: caCaMKK, dnCaMKK, caCaMKI, dnCaMKI, dnCaMKIV (Schmitt et al., 2004; Wayman et al., 2004) and CaMKIIN (Chang et al., 1998); caCaMKII and caCaMKIV (Sun et al., 1994); and caAkt (Brunet et al., 1999).

Antibodies

The polyclonal antibody that recognizes MeCP2 regardless of its phosphorylation status at S421 (anti-total MeCP2) was generated by injecting New Zealand White rabbits (Covance Research Products) with the peptide C-PRPNREEPVDSRTP (Tufts Synthesis Facility, Boston, MA). The polyclonal antibody that recognizes S421-phosphorylated MeCP2 was generated by injecting rabbits with the peptide C-MPRGGpSLES. The antiserum was affinity-purified by application to a column that was conjugated with unphosphorylated MeCP2 S421 peptide. The flow-through was then passed over a second column that was conjugated to phosphorylated MeCP2 S421 peptide, and the affinity-purified anti-MeCP2 pS421 antibody was eluted. Additional information for antibodies used in this study is listed in Supplemental Table 1.

Dissociated Cell Cultures

HEK 293T cells were cultured in DMEM with10% fetal bovine serum. Long Evans rat E17-18 (Charles River) cortical neurons or hippocampal neurons were prepared as previously described (Xia et al., 1996) and maintained in either Basal Medium Eagle (Sigma) supplemented with 10% calf serum, 15 mM glucose, 1 mM L-glutamine, and 100 U/ml penicillin/streptomycin or Neurobasal with B27 supplement (Invitrogen), 1 mM L-glutamine, and 100 U/ml penicillin/streptomycin. Rat P1 hippocampal neurons were maintained in Neurobasal/B27. Cells were typically seeded at a density of 1-3x10^5cells/cm^2 on dishes coated with poly-D-lysine or polyornithine. Neuronal cultures were treated overnight in 1μM tetrodotoxin (TTX) to

reduce endogenous neuronal activity prior to stimulation (except cultures described in Figure 1F). Neurons were membrane depolarized with 55 mM extracellular KCl as previously described (Chen et al., 2003). Neurons were also stimulated with various pharmacological agents or treated with inhibitors that are described in more detail in Supplemental Table 1. 293T cells and neurons were transfected using the calcium phosphate method (Xia et al., 1996).

Organotypic Slice Culture

350 μm transverse slices of P5-7 hippocampus were prepared and cultured essentially as described in (Stoppini et al., 1991). Slices prepared under sterile conditions were cultured on nylon inserts (0.4 μm pore size, Millicell) in 6-well dishes containing 0.75 ml of antibiotic-free medium (20% horse serum/MEM) and incubated in 5% CO_2 at 37°C. Slice cultures were transfected using a Helios Gene Gun (Biorad) at 2DIV. For each bullet, up to 3 different plasmids comprising a total of 60 μg of DNA (3 μg LEMPRA construct, 5 μg GFP/Bcl-XL dual-promoter plasmid, pcDNA3 empty vector plasmid for filler) were coated onto12.5 mg of 1.6 μm gold particles, as per the manufactuer's protocol (Biorad). Although very high levels of wild-type or S421A mutant MeCP2 over-expression (~10-fold over-expressed) in pilot experiments had a detrimental effect on neuronal survival, the over-expression of wild-type or S421A-mutant MeCP2 at the levels used in the experiments described in this study (~4-fold) had no observable effect on the viability of transfected cells as assessed by nuclear staining (data not shown) or GFP levels (Fig. S4a). Slices were fixed at 7 DIV in 2.5% paraformaldehyde and 4% sucrose and processed for immunofluorescence as described below.

Immunohistochemistry

Immunohistochemistry was performed on slice cultures directly on the nylon culture membrane or on cryosections adhered to glass slides. Blocked slices or tissue sections were incubated with primary antibodies in blocking solution (0.25 μg/ml anti-total MeCP2; 0.25 μg/ml anti-MeCP2 pS421; 0.2 μg/ml anti-Flag) for 2 hours at room temperature or 4°C overnight. Alexa 488-, Cy2-, or Cy3-conjugated secondary antibodies were used at dilutions of 1:500 in blocking solution for fluorescence detection. Sections were sometimes counterstained with Hoechst 33342 (1 μg/mL) to visualize cell nuclei.

Imaging and Image Analysis

Images were acquired on a Zeiss LSM5 510 laser-scanning confocal microscope. For each experiment, images across all conditions were acquired with identical settings for laser power, detector gain, and amplifier offset with a pinhole diameter equivalent to one Airy unit for the 488 nm laser. Pinhole diameters for the 543nm and the 633 nm lasers were set such that optical slice thickness was conserved. Laser power and detector gain were set so that pixel intensities remained within the dynamic range. For dendritic analysis, 8-bit images were obtained using a 25X objective at 512x512 pixel resolution, and for dendritic spine analysis, 12-bit images were obtained using a 63X objective at 1024x1024 pixel resolution. Images were acquired as a z-stack, and a maximum intensity projection of each neuron was created from the z-stack using approximately 20-25 sections (1.5 μm/section) for 25X images or approximately 30 sections (1 μm/section) for 63X images. The indicated *n* in the text for each experiment represents 3-5 independent experimental trials. All imaging and analysis was performed with the experimenter blind to the condition. Occasionally, for illustrative purposes only, images were also collected in which one or more of the channels was allowed to have saturated pixels, but these images were never used for quantification.

Dendritic complexity was measured by Sholl analysis (Sholl, 1953). Quantification of spine density, length, and width was performed manually using Metamorph software. Three to five basal segments, each at least 75 μm in length, were analyzed from each pyramidal neuron. Spine length was measured as the distance of a straight line drawn from the junction of the spine with the dendritic shaft to the farthest tip of the spine head. Spine width was measured as the distance of a straight line drawn across the widest part of the spine head. Measurements of anti-Flag and anti-total MeCP2 immunofluorescence intensity and GFP intensity were made on z-projections using Metamorph software. Statistical significance was assessed by analysis of variance (ANOVA) or Student's t-test. Distributions of spine length and width were compared using the Kolmogorov-Smirnov test.

Seizure Induction

Seizures were induced in P19 juvenile Long Evans rats by intraperitoneal injection of pentylenetetrazole (Metrazole) at a dosage of 55 mg/kg and killed 10-min or 30-min post-injection by CO_2 aphyxiation to harvest the forebrain and hippocampus for the preparation of brain lysates. Seizures were induced in adult male CD1 mice by intraperitoneal injection of kainic acid at a dosage of 25 mg/kg. Three hours post-injection, mice were anesthetized with Avertin and intracardially perfused with PBS followed by 4% paraformaldehyde. Whole brains were immediately removed, post-fixed in 4% paraformaldehyde overnight (4°C), and frozen for the preparation of coronal brain sections at 20 μm.

Circadian Entrainment

C57BL/6J mice were entrained to a 12-hr:12-hr light:dark cycle (≥10 days) and then transferred to constant darkness. After two days in constant darkness, mice were either kept in darkness or exposed to a light-pulse for 2, 4, or 6 hours (250 Lux), all ending at circadian time 21 hours. Mice were anesthetized with ketamine/xylazine and perfused intracardially with PBS followed by 10% formalin. Brains were post-fixed in 10% formalin overnight (4°C), cryoprotected in 30% sucrose/PBS (4°C), and then cut on a cryostat into 20-μm coronal sections through the SCN.

Lentiviral Production and qRT-PCR

Lentiviruses were produced by co-transfection of HEK293T cells with the MeCP2 LEMPRA plasmid together with the helper plasmids Δ8.9 and VSV-G as previously described (Lois et al., 2002). Viruses were concentrated by ultracentrifugation 48-60 hours after transfection, and viral titers were determined by infection of HEK293T cells and were typically 3-6x10^6 pfu/μl. E18 rat hippocampal neurons cultured in Neurobasal/B27 (1DIV) were infected with the MeCP2 LEMPRA lentiviruses at 3x10^4 pfu/μl for 6 hours in BME/10%FBS supplemented with 0.6 μg/ml polybrene. Four days post-infection, neurons were depolarized for 2 hours, followed by repolarization and continued maintenance in conditioned medium. This depolarization process was included to promote the release of endogenous MeCP2 from DNA and allow the incorporation of Flag-MeCP2 into the chromatin. At 7 days post-infection, TTX-treated neurons were membrane depolarized for 3 hours as described above, and total RNA was harvested using an RNeasy mini kit (Qiagen). Reverse transcription was performed using SuperScript II (Invitrogen), and quantitative real-time PCR using ABI SYBR Green Master Mix was performed on an ABI Prism 7700 system following the manufacturer's instructions. Optimized quantitative RT-PCR primers used in this study are listed in Supplemental Table 2. Statistical signficance was assessed by Bonferroni corrected multiple comparison after ANOVA.

Protein Purification, Kinase Assay, and Southwestern Assay

MeCP2 was purified from brain nuclear extracts following protocols developed previously with the following modifications (Lewis et al., 1992). A Hi-Trap S column (Roche) was used to fractionate brain nuclear extracts prepared from P15 rat forebrains. Eluted fractions (500 – 700 mM KCl) containing MeCP2 were passed through an antibody column against total MeCP2. MeCP2 proteins were then enriched using Ni-NTA beads (Qiagen) from antibody column elute. Commassie-stained MeCP2 was subjected to tandem MS/MS analysis, and phosphorylated peptides were analyzed. For the *in vitro* kinase assay, wild-type or mutant Flag-MeCP2 proteins were expressed and purified from 293T cells using Flag-M2 agarose (Sigma). Aliquots of MeCP2-bound agarose beads were incubated *in vitro* with purified recombinant CaMKIIα (Calbiochem), CaMKI, or CaMKIV (BioMol) in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM ATP, 5 mM DTT, 50 μg/ml calmodulin, and 5 μCi γ^{32}P-ATP for 60 min at 30°C. The Southwestern assay was performed essentially as previously described in (Lewis et al., 1992).

Supplemental References

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857-868.

Chang, B. H., Mukherji, S., and Soderling, T. R. (1998). Characterization of a calmodulin kinase II inhibitor protein in brain. Proc Natl Acad Sci U S A *95*, 10890-10895.

Chen, W. G., Chang, Q., Lin, Y., Meissner, A., West, A. E., Griffith, E. C., Jaenisch, R., and Greenberg, M. E. (2003). Derepression of BDNF Transcription Involves Calcium-Dependent Phosphorylation of MeCP2. Science *302*, 885-889.

Lewis, J. D., Meehan, R. R., Henzel, W. J., Maurer-Fogy, I., Jeppesen, P., Klein, F., and Bird, A. (1992). Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. Cell *69*, 905-914.

Lois, C., Hong, E. J., Pease, S., Brown, E. J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science *295*, 868-872.

Schmitt, J. M., Wayman, G. A., Nozaki, N., and Soderling, T. R. (2004). Calcium activation of ERK mediated by calmodulin kinase I. J Biol Chem *279*, 24064-24072.
Sholl, D. A. (1953). Dendritic organization in the neurons of visual and motor cortices. J Anat *87*, 387-406.

Sholl, D.A. (1953). Dendritic organization in the neurons of visual and motor cortices. J Anat *87*, 387-406.

Stoppini, L., Buchs, P. A., and Muller, D. (1991). A simple method for organotypic cultures of nervous tissue. J Neurosci Methods *37*, 173-182.

Sun, P., Enslen, H., Myung, P. S., and Maurer, R. A. (1994). Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev *8*, 2527-2539.

Wayman, G. A., Kaech, S., Grant, W. F., Davare, M., Impey, S., Tokumitsu, H., Nozaki, N., Banker, G., and Soderling, T. R. (2004). Regulation of axonal extension and growth cone motility by calmodulin-dependent protein kinase I. J Neurosci *24*, 3786-3794.

Xia, Z., Dudek, H., Miranti, C. K., and Greenberg, M. E. (1996). Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J Neurosci *16*, 5425-5436.

Figure S1. A Southwestern In Vitro Binding Assay Shows that S421A-Mutant MeCP2 Binds Methylated DNA

Anti-Flag immunoprecipitates from HEK293T cells co-expressing Flag-tagged wild-type or S421A-mutant MeCP2 with constitutively active CaMKII were separated by SDS-PAGE, transferred, and renatured on a nitrocellulose membrane. The membrane was then either probed with an anti-Flag antibody (top) or incubated with radiolabeled methylated oligonucleotides spanning the -148 CpG site of *Bdnf* promoter III (bottom). Whereas S421-phosphorylated wildtype MeCP2 does not bind methylated DNA, S421A-mutant MeCP2 appears to bind methylated DNA with similar affinity as non-S421-phsosphorylated MeCP2.

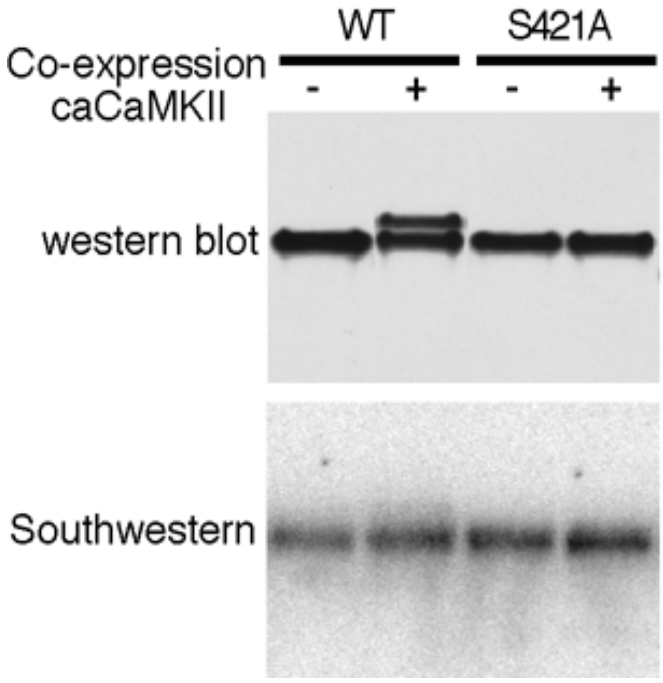


Figure S2. Dendritic Spine Density Is Not Affected by shRNA-Mediated MeCP2 Knockdown or Overexpression of Wild-Type or S421A-Mutant MeCP2

P5-7 organotypic hippocampal slices were biolistically transfected at 2DIV with LEMPRA-based plasmids encoding control vector, anti-MeCP2 shRNA only, wild-type, or S421A-mutant MeCP2. Slices were fixed, immunostained, and analyzed at 7 DIV. Quantification of spine density among the four conditions revealed no significant differences in average dendritic spine density (mean ± S.E.M., n=25-35 cells/condition, 3 or more independent experiments, $p>0.05$, ANOVA). Due to variations in absolute spine densities among different experimental repeats, average spine densities are reported as normalized to the average spine density of the control for each given experimental trial.

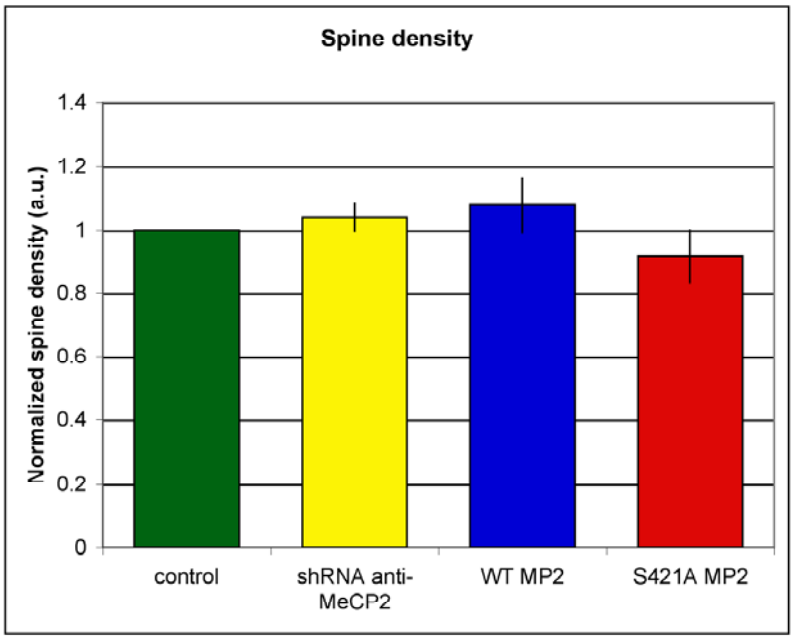


Figure S3. High-Magnification Images of Hippocampal Pyramidal Neuronal Nuclei Transfected with LEMPRA-Based Plasmids Encoding Control Vector, anti-MeCP2 shRNA Only, Wild-Type, or S421A-Mutant MeCP2

P5-7 organotypic hippocampal slices were transfected at 2DIV, and fixed and analyzed at 7 DIV. Note that the shRNA directed against MeCP2 provides efficient knockdown of endogenous MeCP2. In addition, exogenously wild-type and S421A-mutant MeCP2 are appropriately localized to the nucleus.

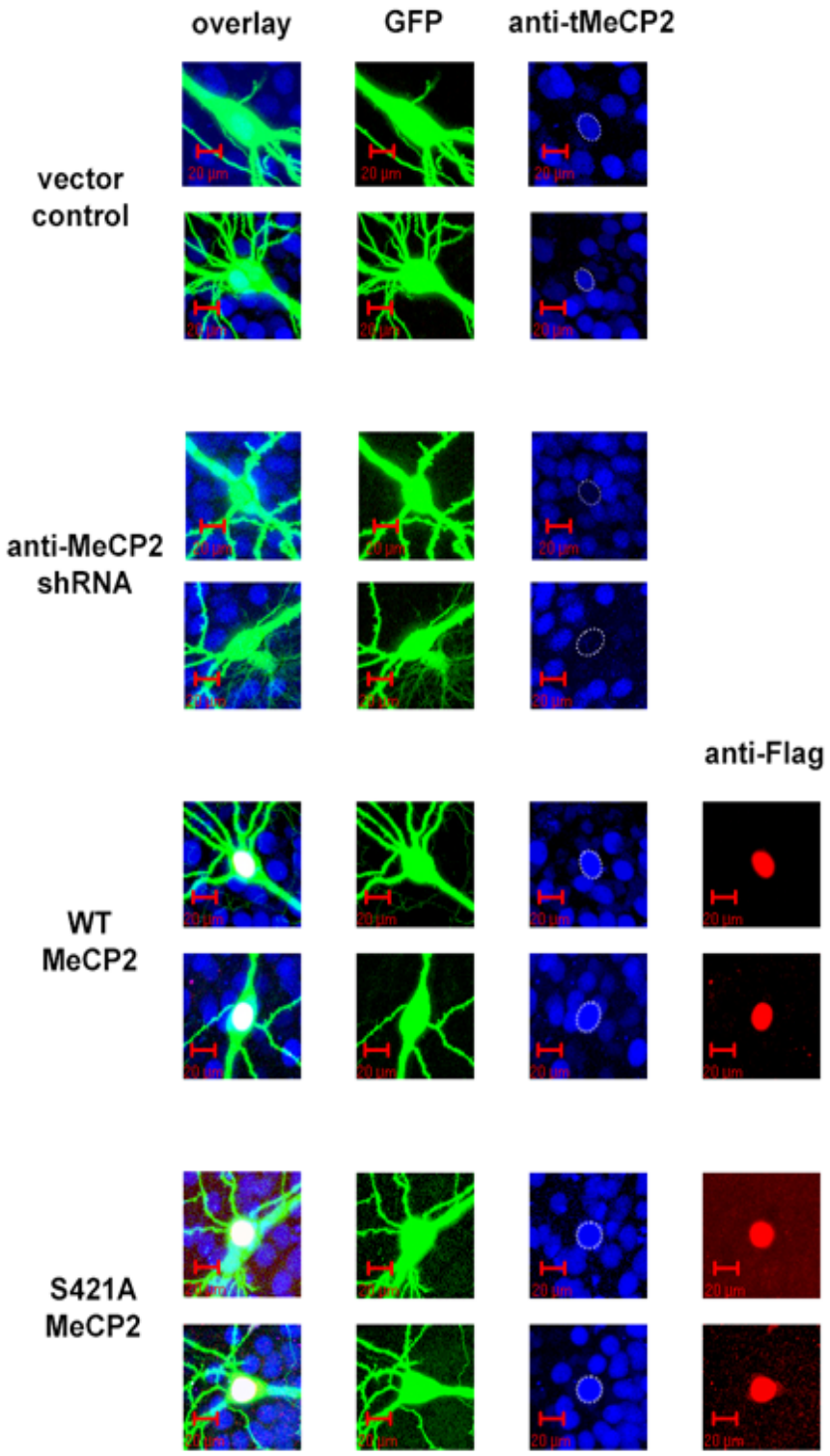


Figure S4. Quantification of GFP Intensity and Anti-Flag and Antitotal MeCP2 Immunostaining in Transfected Hippocampal Pyramidal Neurons in Organotypic Slice

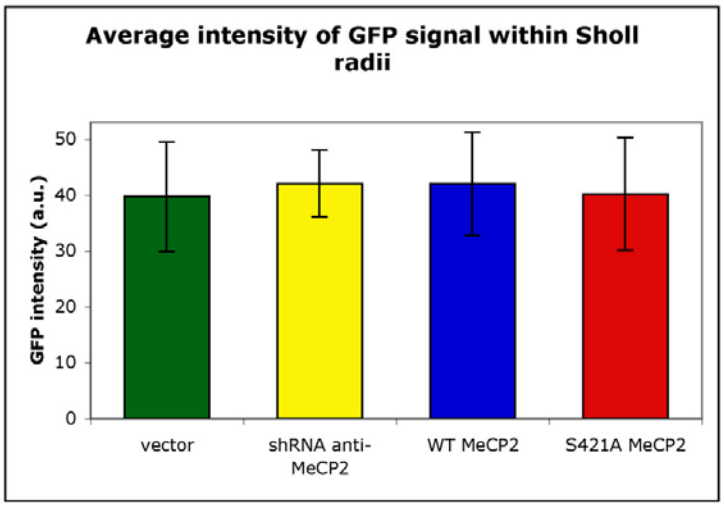
P5-7 organotypic hippocampal slices were biolistically transfected at 2DIV with LEMPRA-based plasmids encoding control vector, anti-MeCP2 shRNA only, wild-type, or S421A-mutant MeCP2. Slices were fixed and immunostained at 7 DIV, and maximum intensity z-projections of image stacks collected at 25X were analyzed using Metamorph software.

(A) Quantification of average GFP signal intensity. For each neuron, 12-15 line segments, each at least 40 μm in length, were drawn along both apical and basal dendrites within 120μm from the neuronal soma, and the average intensity of GFP signal was measured along each. These data were then averaged to obtain the average GFP signal intensity for each neuron. N=8-9 neurons/condition, $p>0.05$, ANOVA.

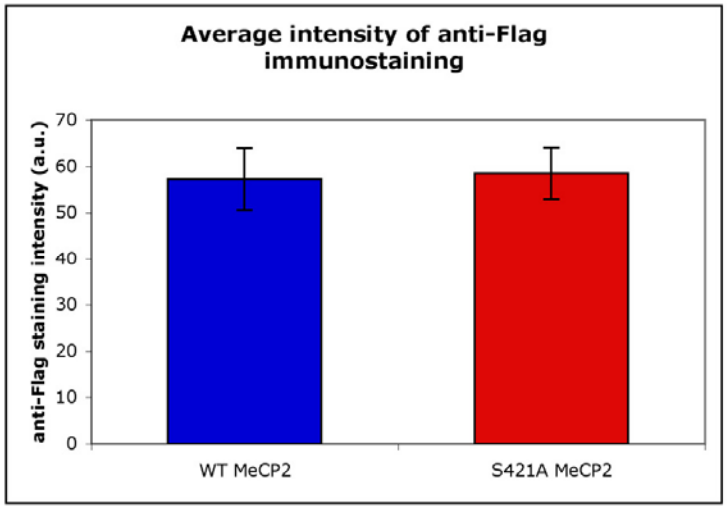
(B) Quantification of the average intensity of anti-Flag immunostaining in the nuclei of neurons expressing either wild-type or S421A-mutant MeCP2. N=22-25 neurons/condition, $p>0.05$, t-test.

(C) Quantification of the average intensity of anti-total MeCP2 immunostaining in the nuclei of neurons that were untreated, transfected with a vector control, an anti-MeCP2 shRNA only, wild-type MeCP2, or S421A-mutant MeCP2. N=22-35 neurons/condition. *$p<0.05$, shRNA v. untreated or shRNA v. control; **$p<0.05$, WT v. control or S421A v. control; $p>0.05$, WT v. S421A; ANOVA.

A


Average intensity of GFP signal within Sholl radii
GFP intensity (a.u.)
0
10
20
30
40
50
vector
shRNA anti-MeCP2
WT MeCP2
S421A MeCP2

B


Average intensity of anti-Flag immunostaining
anti-Flag staining intensity (a.u.)
0
10
20
30
40
50
60
70
WT MeCP2
S421A MeCP2

C

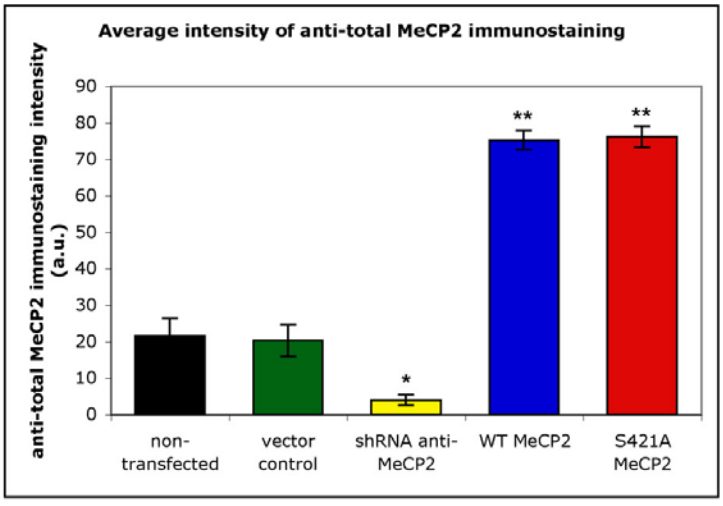
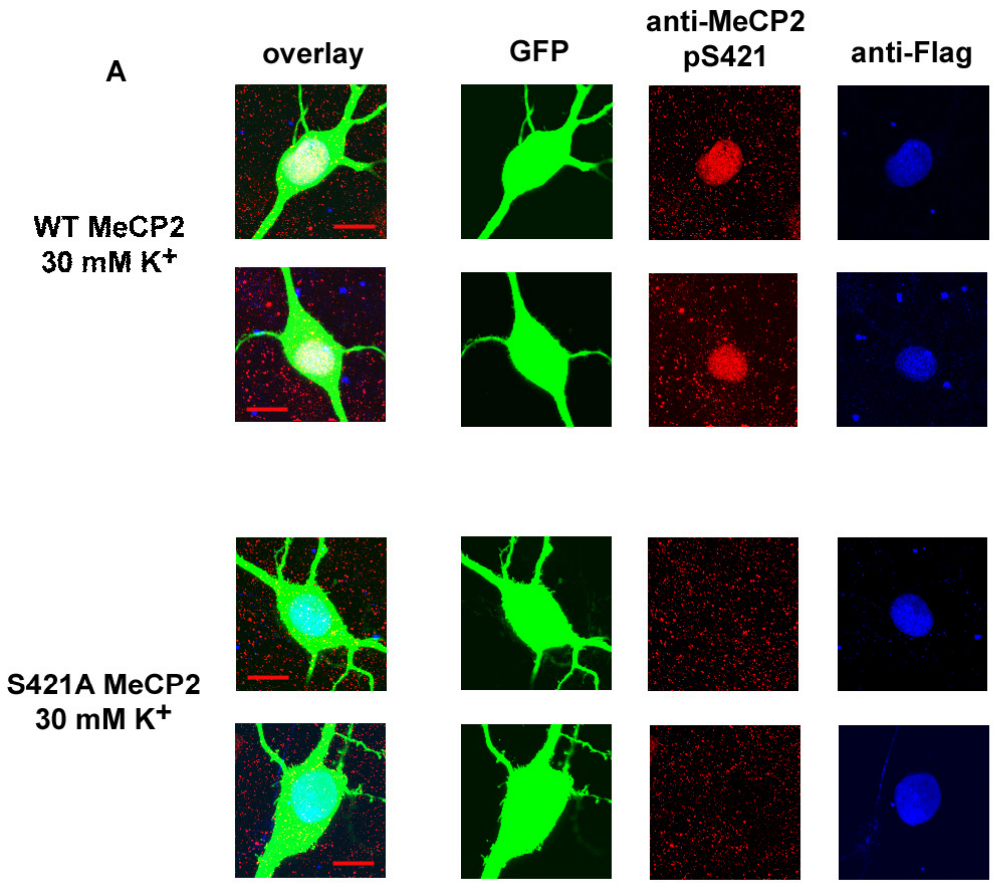
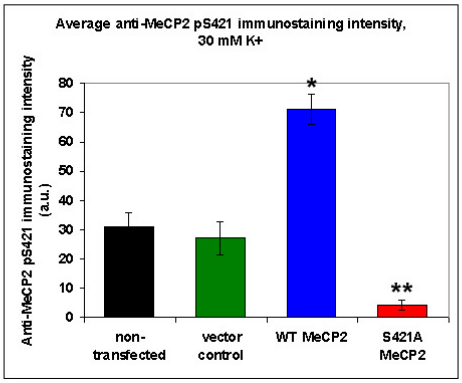

Average intensity of anti-total MeCP2 immunostaining
anti-total MeCP2 immunostaining intensity (a.u.)
0
10
20
30
40
50
60
70
80
90
*
**
**
non-transfected
vector control
shRNA anti-MeCP2
WT MeCP2
S421A MeCP2

Figure S5. Exogenously Expressed MeCP2 Is Efficiently Phosphorylated at S421 in Response to Activity
(A) High magnification images of hippocampal pyramidal neuronal nuclei transfected with LEMPRA-based plasmids encoding either wild-type or S421A-mutant MeCP2. P5-7 organotypic hippocampal slices were transfected at 2DIV. At 5 DIV, slice cultures were either membrane depolarized in 30 mM extracellular potassium chloride or stimulated with 40 μM glutamate for 3 hours, then fixed and immunostained with the anti-Flag and anti-MeCP2 pS421 antibodies. Efficient phosphorylation of exogenously expressed wild-type, but not S421A-mutant, MeCP2 is observed.
(B) Quantification of anti-MeCP2 pS421 antibody immunostaining in nuclei of neurons that are untreated or transfected with vector control-, wild-type MeCP2, or S421A-mutant MeCP2. *N*=12-15 neurons/condition, *$p<0.01$, WT v. control or WT v. untreated; **$p<0.01$, WT v. S421A; ANOVA.
(C) Quantification of anti-Flag antibody immunostaining in nuclei of neurons transfected with either wild-type or S421A-mutant MeCP2. *N*=12-15 neurons/condition, $p>0.05$, t-test.


A
overlay
GFP
anti-MeCP2 pS421
anti-Flag
WT MeCP2
30 mM K+
S421A MeCP2
30 mM K+


B
Average anti-MeCP2 pS421 immunostaining intensity, 30 mM K+
Anti-MeCP2 pS421 immunostaining intensity (a.u.)
0
10
20
30
40
50
60
70
80
*
**
non-transfected
vector control
WT MeCP2
S421A MeCP2

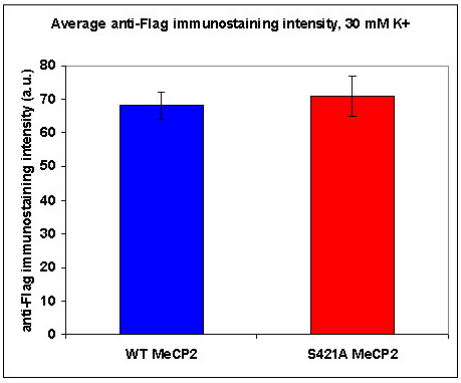

C
Average anti-Flag immunostaining intensity, 30 mM K+
anti-Flag immunostaining intensity (a.u.)
0
10
20
30
40
50
60
70
80
WT MeCP2
S421A MeCP2

Table S1. Phamacological Reagents and Antibodies

Name	Vendor	Working concentration
Bicuculline	Sigma	20 μM
Bisindolylmaleimide I	Calbiochem	10 μM
CNQX	Tocris	40 μM
D-APV	Tocris	100 μM
Forskolin	Calbiochem	10 μM
Glutamate	Sigma	10 μM
H89	Calbiochem	5 μM
K-252a	Calbiochem	0.2 μM
KN62	Calbiochem	5 μM
KN92	Calbiochem	5 μM
KN93	Calbiochem	5 μM
LY294002	Calbiochem	50 μM
Nimodipine	Calbiochem	5 μM
NMDA	Sigma	20 μM
PD98059	Calbiochem	10 μM
PMA	Calbiochem	100 ng/ml
Roscovitine	BioMol	50 μM
STO-609	Tocris	5 μM
Tetrodotoxin	VWR	1 μM
UO126	Calbiochem	10 μM
recombinant human BDNF	Peprotech	50 ng/ml
recombinant human CNTF	Peprotech	50 ng/ml
recombinant human EGF	Peprotech	50 ng/ml
recombinant human IGF	Peprotech	50 ng/ml
recombinant human NGF	Peprotech	50 ng/ml
recombinant human NT3	Peprotech	50 ng/ml
recombinant human NT4	Peprotech	50 ng/ml
recombinant human PDGF	Peprotech	50 ng/ml
Antibodies against		
total MeCP2	homemade	0.25μg/ml
MeCP2 S421	homemade	0.25μg/ml
beta Actin	abcam	0.2μg/ml
GAPDH monoclonal	abcam	0.2μg/ml
Flag M2 monoclonal	Sigma	0.2μg/ml
pCREB (S133)	Upstate	0.5μg/ml
pAkt (S473)	Upstate	0.5μg/ml
phospho-p44/42 MAPK	Upstate	0.5μg/ml
p44/42 MAPK	Upstate	0.5μg/ml

Table S2. qRT-PCR Primers Used in This Study

Gene	Forward primer	Reverse primer
rat *Bdnf* exon III	F-5'TGCCTAGATCAAATGGAGCTTCTC-3'	R-5'CCGATATGTACTCCTGTTCTTCAGC-3'
rat c-fos	F-5'CAGCTATCTCCTGAAGAGGAAG-3'	R-5' CTTCTCATCTTCAAGTTGAT-3'
rat *gapdh*	F-5' TCCATGACAACTTTGGCATCGTGG-3'	R-5' GTTGCTGTTGAAGTCACAGGAGAC-3'