Supplementary Information

In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9

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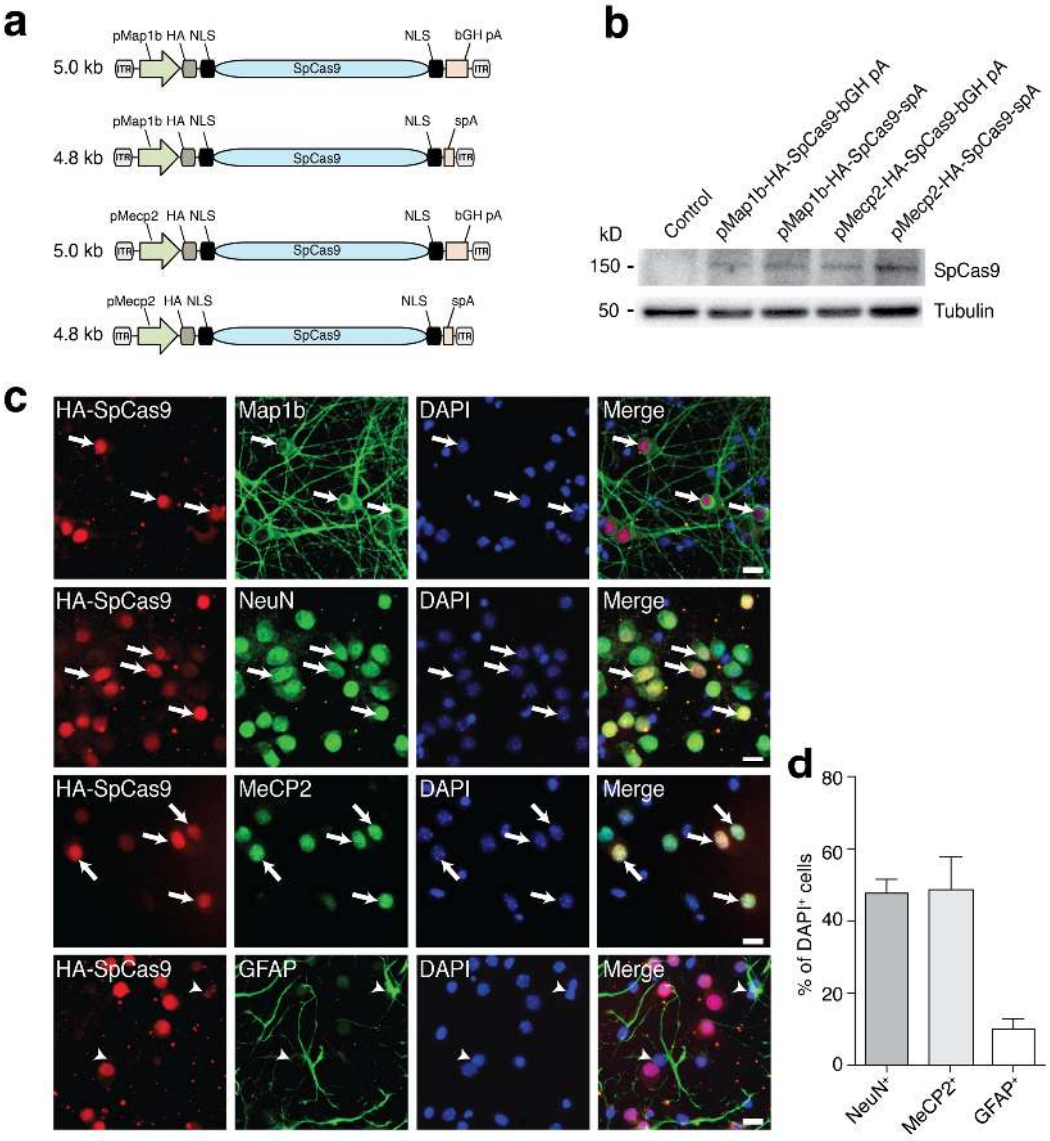
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Cloning and expression of HA-tagged SpCas9 (HA-SpCas9) for AAV packaging. (**a**) Schematic overview of different cloning strategies to minimize SpCas9 expression cassette size using short rat *Map1b* promoter (pMap1b), a truncated version of the mouse *Mecp2* promoter (pMecp2) and a short polyadenylation signal (spA). (**b**) Western blot analysis of primary cortical neuronal culture expressing HA-SpCas9 using different SpCas9 expression cassettes. (**c**) *Mecp2* promoter drives HA-SpCas9 (red) expression in neurons (Map1b, NeuN, MeCP2; arrows) but not in astroglia (GFAP; arrowheads). Nuclei were labeled with DAPI (blue). Scale bars, 20 μm. (**d**) Quantification of $NeuN^+$, $MeCP2^+$ and $GFAP^+$ cells in cortical primary neuronal culture.

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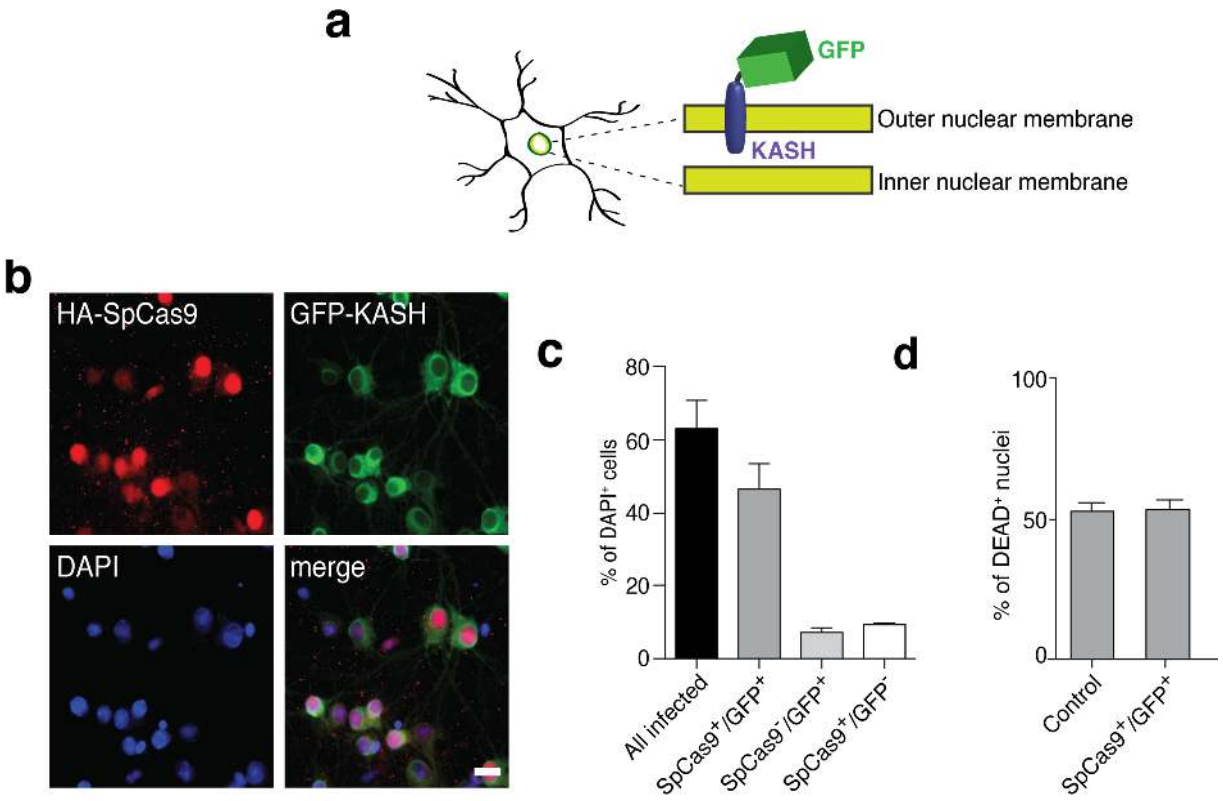

Supplementary Figure 2. Co-delivery of HA-SpCas9 and GFP-KASH into primary neurons. (**a**) Schematic overview of GFP-labeling. Enhanced green fluorescent protein (GFP) fused to the nuclear transmembrane KASH domain and integration of GFP-KASH to the outer nuclear membrane is illustrated. (**b**) Co-expression of HA-SpCas9 with GFP-KASH is shown. (**c**) Co-infection efficiency calculation (n=973 neurons from 3 cultures; error bars: s.e.m). (**d**) Cells were stained with LIFE/DEAD® kit 7 days after virus delivery. Quantification of $DAPI^+$ and dead ($DEAD^+$) cells (control n=518 $DAPI^+$ nuclei; SpCas9/GFP-KASH n=1003 $DAPI^+$ nuclei from 2 independent cultures; error bars: s.e.m).

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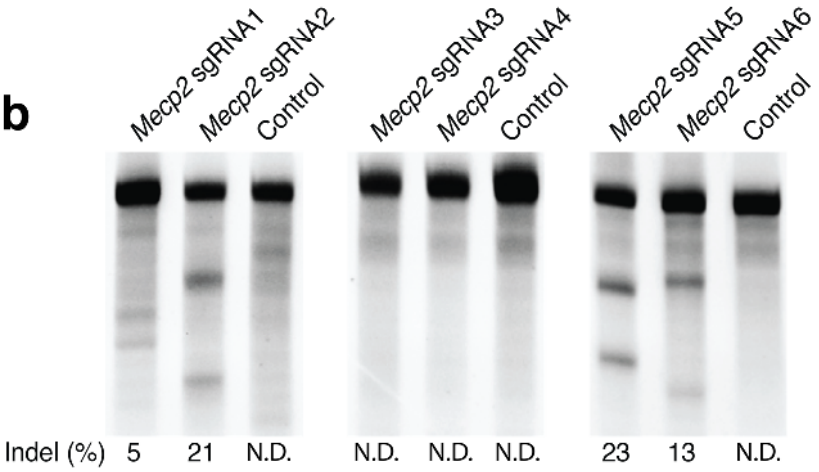
a

	Mecp2 target sequence	PAM
Target 1	CTGGGAGAGGGAGCCCCTCC	AGG
Target 2	AAAGGTGGGAGACACCTCCT	TGG
Target 3	TCCAACCTTCAGGCAAGGTG	GGG
Target 4	AGGAAGTCTGGCCGATCTGC	TGG
Target 5	CCATTCTGCAGAGCCAGCAG	AGG
Target 6	CTCTGAGGCCCTGGAGATCC	TGG

b



Supplementary Figure 3. Targeting of *Mecp2* in Neuro-2a cells. (**a**) *Mecp2* targeting sequences and corresponding protospacer adjacent motifs (PAM). (**b**) Evaluation of 6 *Mecp2* sgRNAs co-transfected with SpCas9 into Neuro-2a cells. Locus modification efficiencies were analyzed 48 h after transfection using SURVEYOR nuclease assay. Detected indel formation within *Mecp2* locus is indicated below.

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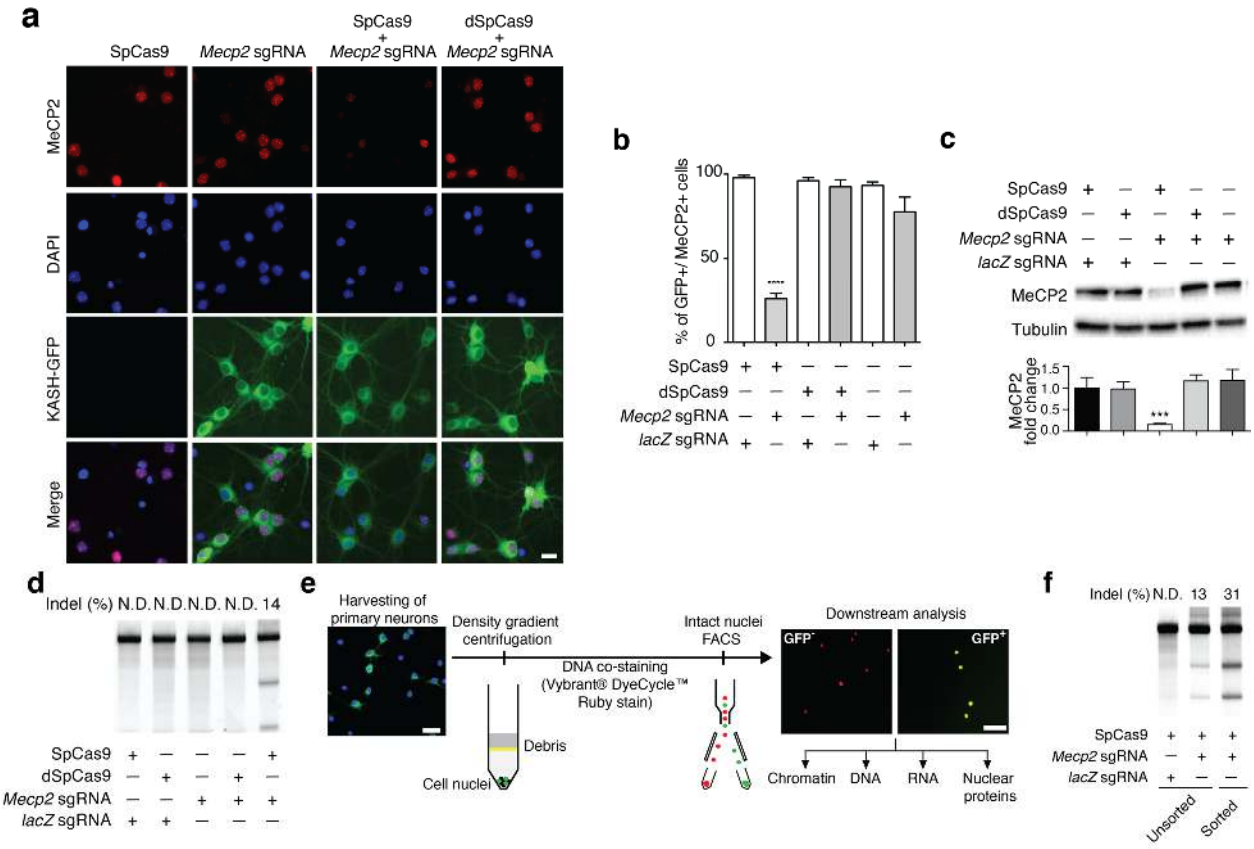

Supplementary Figure 4. CRISPR-SpCas9 targeting of *Mecp2* in primary cortical neurons. (**a**) Immunofluorescent staining of MeCP2 (red) in cultured neurons 7 days after *Mecp2* locus targeting. SpCas9 or dSpCas9, together with *Mecp2* sgRNA or control sgRNA (targeting bacterial *lacZ* gene) were used. KASH-GFP (green) indicates expression from SpGuide vector. Nuclei were labeled with DAPI (blue). Scale bar, 20 μm. (**b**) Quantification of MeCP2 positive nuclei in the targeted population of neurons (GFP^+). (**c**) Western blot of MeCP2 protein levels after CRISPR-SpCas9 targeting of *Mecp2* locus. Quantification of MeCP2 protein levels is shown below (*t*-test, ***$p<0.001$, n=6 from 3 cultures, error bars: s.e.m). (**d**) SURVEYOR nuclease assay showing indel formation in the *Mecp2* locus only in presence of *Mecp2* sgRNA and SpCas9. (**e**) Strategy for cell nuclei purification of GFP-KASH labeled cells in primary neuronal cultures. Scale bar: 50 μm. (**f**) SURVEYOR nuclease assay on targeted *Mecp2* locus in total and FACS-sorted population of nuclei from cultured neurons.

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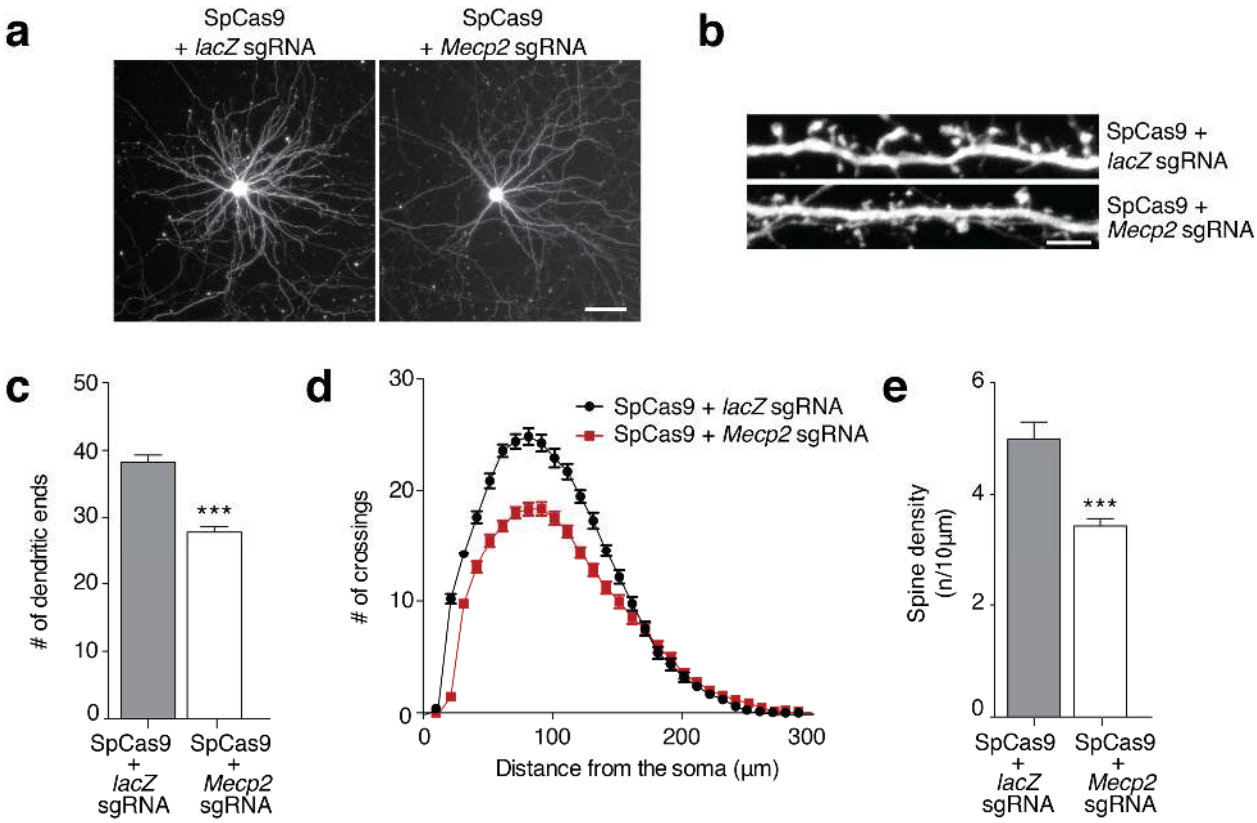

Supplementary Figure 5. Morphological changes in dendritic tree of neurons after SpCas9-mediated MeCP2 knockdown *in vitro*. (**a**) Reduced complexity of dendritic tree in neurons after CRISPR-SpCas9 targeting of *Mecp2* locus. Scale bar, 20 µm. (**b**) Changes in dendritic spines morphology in neurons targeted with SpCas9 and *Mecp2* sgRNA. Scale bar, 10 µm. Morphology of cells was visualized with co-transfection with mCherry construct. Cells for morphology analysis were chosen based on the result of MeCP2 staining. (**c**) Dendritic tree morphology assessed with number of dendritic ends and (**d**) Sholl analysis (*t*-test, ***p<0.0001, n=40 from 2 cultures). (**e**) Spine density quantification (*t*-test, ***p<0.0001, n=40 from 2 cultures, error bars: s.e.m).

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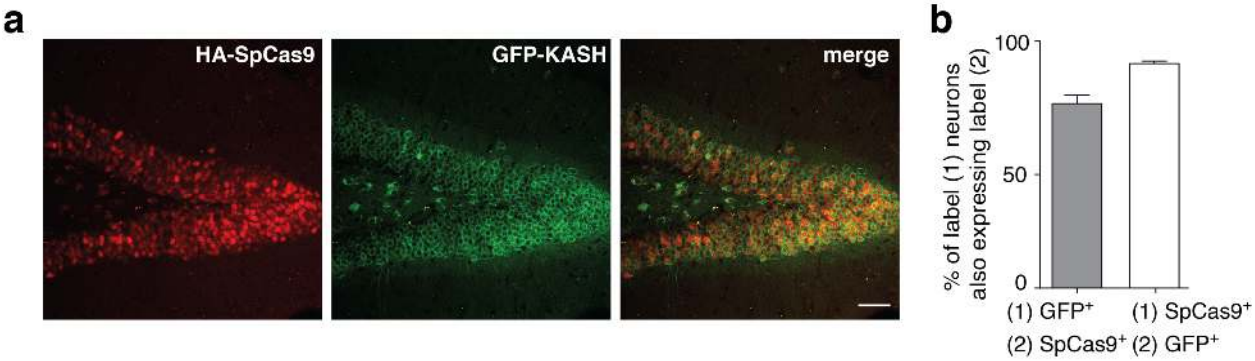
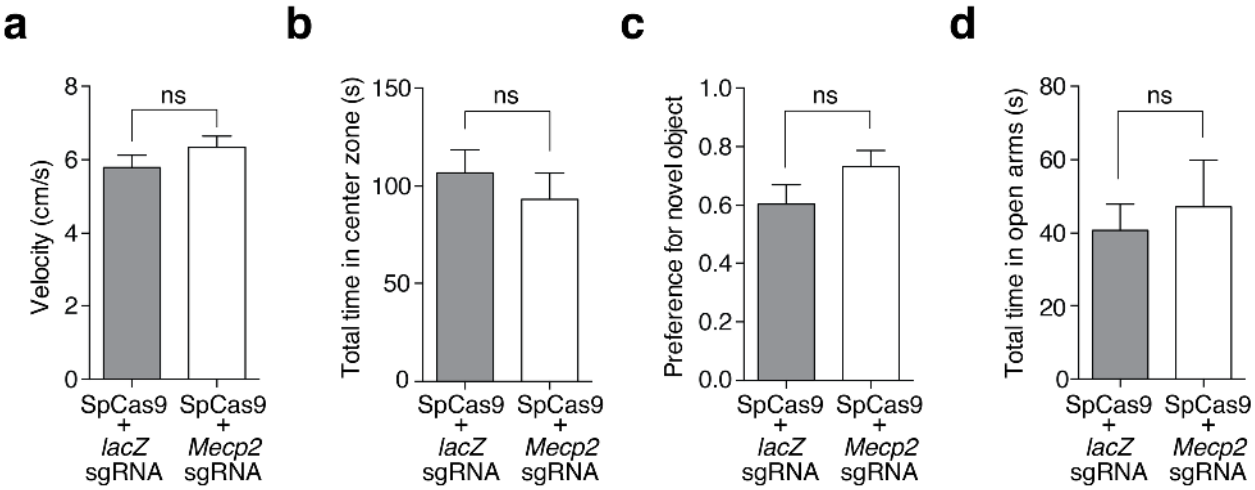


Supplementary Figure 6. Viral delivery of CRISPR/Cas9 into the mouse brain. (**a**) Expression of HA-Cas9 and GFP-KASH in the dorsal dentate gyrus (DG) of mouse hippocampus, 4 weeks after AAV delivery. Scale bar, 100 μm. (**b**) Quantification of cells efficiently targeted by the dual-vector Cas9-CRISPR system. Cells were co-stained with DAPI (not shown) and counted.



Supplementary Figure 7. Behavioral tests of mice after MeCP2 knockdown in the dorsal DG. (**a**) Velocity in the open field during 10 min test. (**b**) Total time spent in the center zone of arena during open field test (10 min trial). (**c**) Novel object recognition test, the discrimination ratio (interaction time with novel object vs. total time of interactions with novel and familiar objects) is shown. (**d**) Elevated plus maze; total time in open arms is shown (5 min trial) (n=7 male mice per group, 2 weeks after AAV injection).

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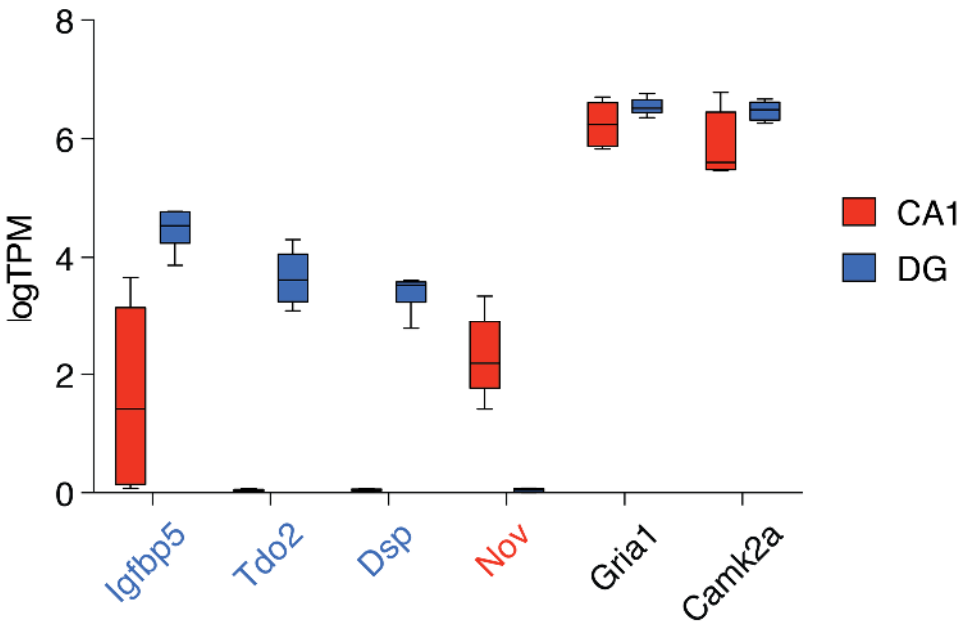

Supplementary Figure 8. Comparison of brain region specific neuronal markers. Dissection method purity verification for specific hippocampus regions by comparing the expression levels of the marker genes in dissected DG versus the adjacent Ammon's horn region (CA1) tissue. The distribution in expression levels (log(TPM+1) units, y-axis) of known neuronal markers is presented across six control samples of nuclei sorted from dissected dentate gyrus region (blue) and six control samples from the adjacent CA1 region (red) of control animals. Presenting known dentate gyrus markers (*Igfbp5*, *Tdo2* and *Dsp*), CA1 marker (*Nov*) and general neuronal markers (*Gria1* and *Camk2a*).

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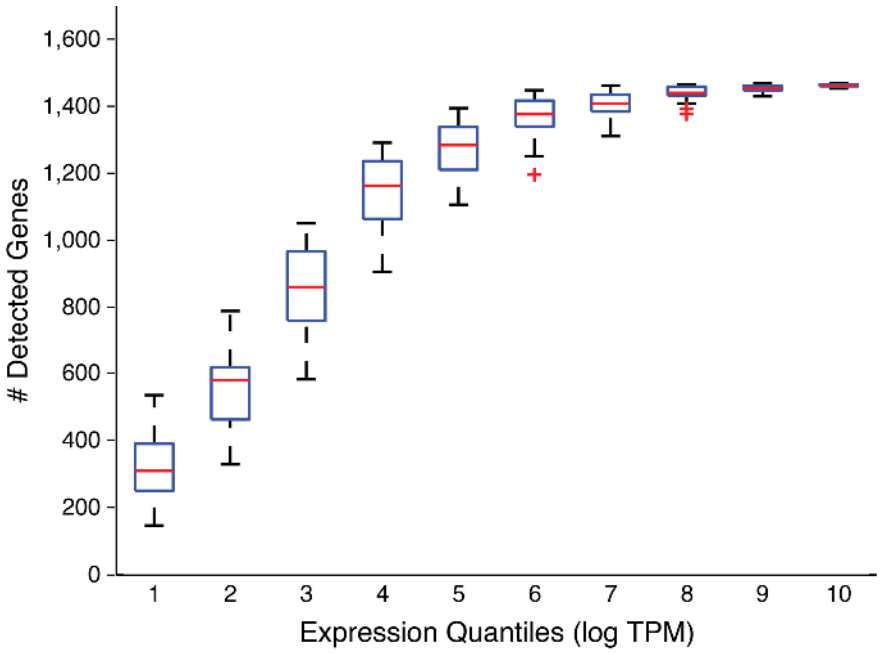

Supplementary Figure 9. **RNAseq of neuronal nuclei from control animals and SpCas9-mediated MeCP2 knockdown.** Box plot presenting the number of detected genes across the RNA-seq libraries (19 libraries each of 100 nuclei taken from control sgRNA or *Mecp2* sgRNA transduced nuclei; n=4 animals/group) per quantile of their mean expression level. All genes are divided to 10 quantiles by their mean log(TPM+1) expression level, then for each quantile the number of genes that are detected (log(TPM+1)>1) was counted in each sample.

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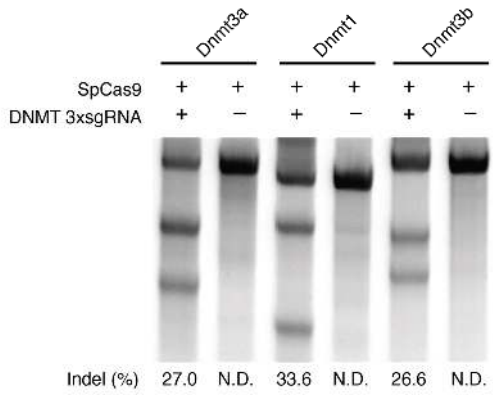
a

Target	Target sequence	PAM
Dnmt3a	TTGGCATGGGTCGCTGACGG	AGG
Dnmt1	CGGGCTGGAGCTGTTCGCGC	TGG
Dnmt3b	AGAGGGTGCCAGCGGGTATG	AGG

b



Supplementary Figure 10. Multiplex genome targeting of DNMT family members *in vitro*. (**a**) *Dnmt3a*, *Dnmt1* and *Dnmt3b* targeting sequences and corresponding protospacer adjacent motifs (PAM). (**b**) SURVEYOR nuclease assay analysis of Neuro-2a cells 48 hours after transfection with SpCas9 and DNMT 3xsgRNA vector targeting *Dnmt3a*, *Dnmt1* and *Dnmt3b* loci. Genome editing of all three targeted genes is shown.

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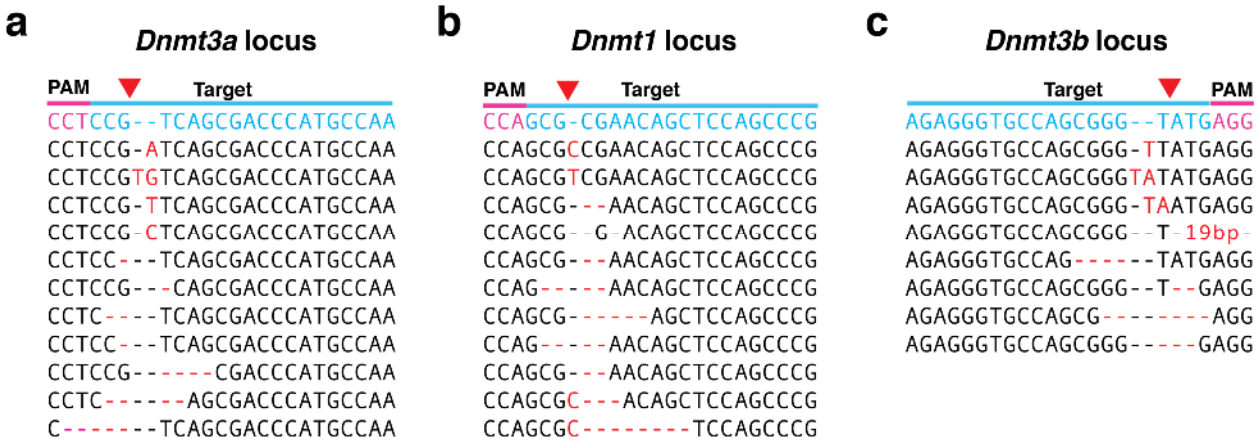
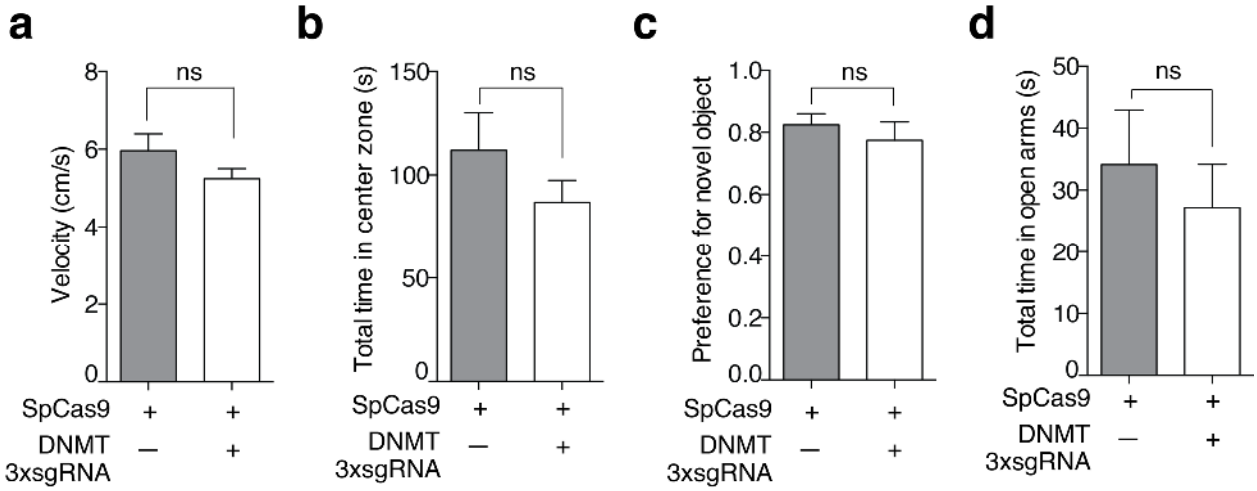


Supplementary Figure 11. Next generation sequencing of targeted *Dnmt3a*, *Dnmt1* and *Dnmt3b* loci. Examples of sequencing results of mutated *Dnmt3a* (**a**), *Dnmt1* (**b**) and *Dnmt3b* (**c**) loci after *in vivo* delivery of SpCas9 and DNMT 3xsgRNA into the mouse dentate gyrus. Green: wild-type sequence, red dashes: deleted bases, red bases: insertion or mutations. Red arrowheads indicate CRISPR-SpCas9 cutting site.



Supplementary Figure 12. Behavioral tests of mice after targeting DNMT loci in the DG. (**a**) Velocity in the open field during 10 min test. (**b**) Total time spent in the center zone of arena during open field test (10 min trial). (**c**) Novel object recognition test, the discrimination ratio (interaction time with novel object vs. total time of interactions with novel and familiar objects) is shown. (**d**) Elevated plus maze; total time in open arms in shown (5 min trial) (n=18 male mice per group, 8 weeks after AAV injection).

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SUPPLEMENTARY TABLES

Supplementary Table 1. Off-target analysis for DNMTs targeting

	Gene	GI	Potential off-target sequences	MLE (%)	SEM
Dnmt1	***Abca1***	NM_013454	GGAGCTGGAGCTGTTCACGTTGG	0.0000	0.00
	Mctp1	NM_030174	CGGGCAGCAGATGTTCGCGTAGG	0.0806	0.08
	Exd2	NM_133798	AGGGCTTGAGATGTTCGGGCTGG	0.0612	0.06
	Pik3r6	NM_001004435	CCGGCTGGGGCTGTCCTCGCTAG	0.0000	0.00
	Sobp	NM_175407	CGGGGTGCAGCTGCTCACGCCAG	0.0000	0.00
	Vac14	NM_146216	CTGGCGGGAGCTGGTCGCGTGAG	0.0083	0.00
Dnmt3a	***Efemp2***	NM_021474	TGAGCATGGGCCGCTGGCGGTGG	0.0050	0.01
	Bmpr1b	NM_001277217	ATGGCATAGGCCGCTGACAGAGG	0.0117	0.01
	Syce1	NM_001143765	TTGGCATGGTGAGCTGGCGGGGG	0.0067	0.00
	Atp8b3	NM_026094	TGGGCAGGGGTCTCTGAGGGCAG	0.0067	0.01
	Rdh11	NM_021557	TTGGCATGGGTCTCTTACCAAGG	0.0017	0.00
Dnmt3b	***Hecw2***	NM_001001883	ACATGGTTCCAGTGGGTATGTAG	0.0000	0.00
	Plekhg3	NM_153804	GGAGGTGGGCAGCGGGTATGTAG	0.0954	0.01
	Cdc25b	NM_001111075	AGAAGGTCCCCGCGGGCATGGAG	0.2421	0.12
	Top1mt	NM_028404	GGAGGGAACCAGCCGGTATGGGG	0.0167	0.01
	Sesn2	NM_144907	AGAGAGTGGCAGTGGGTAAGCAG	0.0000	0.00
	Ncan	NM_007789	AGAGGTGGCCAGCGGGCAGGAAG	0.0017	0.00
	Nacad	NM_001081652	TGAGGGGGCCAGCTGGGATGCAG	1.6254	0.76

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Supplementary Table 2. PCR primers used in the SURVEYOR assay

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Mecp2	GGTCTCATGTGTGGCACTCA	TGTCCAACCTTCAGGCAAGG
Dnmt3a	ATCCCTCCTCAGAGGGTCAGC	TACCTCATGCACAGCTAGCACC
Dnmt1	TTCGGGCATAGCATGGTCTTCC	GTTCTATTTCAGAGGGCTGATCCC
Dnmt3b	GTTCTGAGCCGCACAGTTTGG	GGATAAGAAGGGACAATACAGG

Supplementary Table 3. Primers used for on- and off-target genomic loci amplification

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Dnmt1	GCCGGGGTCTCGTTCAGAGCT	CTACCGCCTGCGGACATGGT
Dnmt3a	CCTGTCTCTCTGTCCTAGGGCTCC	CCGTTTGCTGATGTAGTAGGGGTCC
Dnmt3b	CCCACAGGAAACAATGAAGGGAGAC	CATCCTTCGTGTCTGAGGACTGGTC
Abca1	CCCTGACACCAGCTGTTCAGCAC	CTCTGGGTGACCACACACGATGC
Mctp1	GAGCAGGCAGAGCCGAGCAAG	GGAGAGCGTCCGCCAGGAG
Exd2	GGGTCTTGTTGTGAGTAGGGTGTG	GAAGCTCTCTTAACTACTGTTC
Pik3r6	CCTGGAATACTATTTCCACGCCG	CAGGCCCTAGCAGCGAGCAG
Sobp	GCAGCACACTCCACCCTCACAT	GGAAGGGGCTTTCCTCCGAGC
Vac14	CGGCGTCACGTGACCTGAGTAAC	GCTCCGACCCTGCTCTCCCA
Efemp2	GTGTCTGCCTCGCTCTGCTGC	CCTGTTCATCAGGCTCGTAGCCC
Bmpr1b	CTATCTGAAATCCACCACCTTAGACGC	CGATTGCTGGCTTGCCTTGAG
Syce1	GCCTGAGGGGGCCAGAGGT	GGTTCGCGTCCGCCCGCGTGAT
Atp8b3	GGGACTCCCCGGGTGGTG	GAGAGGTGGTCCTGTCGCCTATG
Rdh11	GACCCTGTGTTTCAAGTCTCTCTG	CCCAGCAGGTCACAGCTGACATC
Hecw2	GGCCATCCAGTACATTCAATACG	AGCACAGTATGTATTCTATAAAATAATACGAC
Plekhg3	GCAGAAGCCGTGACTCACAGCA	GTGGGAGGGGACAGAGACCATG
Cdc25b	CTTGTGCTTGTGATTCTGTCCTTACTGC	CCTTACCTGTTCCTCTTCCTTATCCAGC
Top1mt	CGAGAAGTCGATGCAGACACTTCAA	ATACCCAGTCCACATCCCTGCC
Sesn2	GCTGAAGACTGGCGAGCACAGCT	CCTCTGCATCTCCCTCAGGAAGTATT
Ncan	GACCTGAATGTTGTGGCTGAGAGTCC	GCCTCCTGTCCCCAGGTCCC
Nacad	CCCTCACGTTCCTGTCCAGCAA	CACTAGGCTTGGGCTGCCCTCT

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