Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice

Richard Z. Chen[1], Schahram Akbarian[1,3], Matthew Tudor[1,2] & Rudolf Jaenisch[1,2]

***Mecp2* is an X-linked gene encoding a nuclear protein that binds specifically to methylated DNA (ref. 1) and functions as a general transcriptional repressor by associating with chromatin-remodeling complexes[2,3]. *Mecp2* is expressed at high levels in the postnatal brain[1,4], indicating that methylation-dependent regulation of gene expression may have a crucial role in the mammalian central nervous system. Consistent with this notion is the recent demonstration that *MECP2* mutations cause Rett syndrome[5–8] (RTT, MIM 312750), a childhood neurological disorder that represents one of the most common causes of mental retardation in females[9–11]. Here we show that Mecp2-deficient mice exhibit phenotypes that resemble some of the symptoms of RTT patients. Mecp2-null mice were normal until 5 weeks of age, when they began to develop disease, leading to death between 6 and 12 weeks. Mutant brains showed substantial reduction in both weight and neuronal cell size, but no obvious structural defects or signs of neurodegeneration. Brain-specific deletion of *Mecp2* at embryonic day (E) 12 resulted in a phenotype identical to that of the null mutation, indicating that the phenotype is caused by Mecp2 deficiency in the CNS rather than in peripheral tissues. Deletion of *Mecp2* in postnatal CNS neurons led to a similar neuronal phenotype, although at a later age. Our results indicate that the role of Mecp2 is not restricted to the immature brain, but becomes critical in mature neurons. Mecp2 deficiency in these neurons is sufficient to cause neuronal dysfunction with symptomatic manifestation similar to Rett syndrome.**

A previous mouse chimeric study had indicated an essential role for *Mecp2* in development[12]. Consistent with early lethality of complete MECP2 deficiency is the predominance of RTT in girls[8,11,13] and the severe symptoms in the few identified boys carrying a *MECP2* mutation[6,14–19]. We generated a conditional *Mecp2* allele using the Cre-*loxP* recombination system. The *Mecp2* targeting construct (Fig. 1*a*) deleted exon 3, which encodes 116 amino acids including most of the methyl-CpG-binding domain. Mice carrying

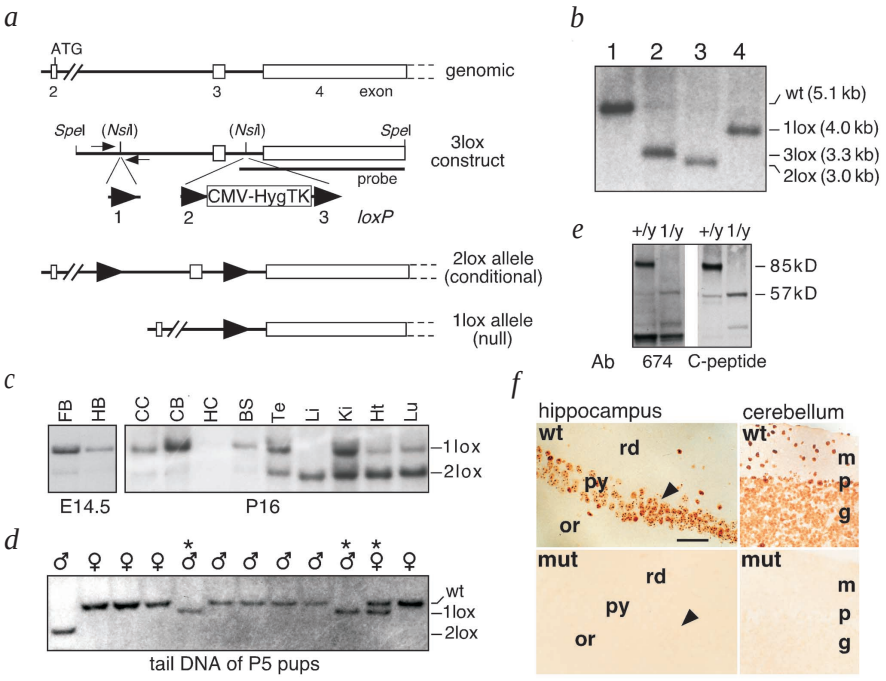

Fig. 1 Generation of Mecp2-deficient mice. ***a***, Targeting the *Mecp2* locus. Lines (top to bottom): genomic organization of the mouse *Mecp2* locus, structure of the conditional targeting construct (3lox construct), conditional (2lox) and mutant (1lox) *Mecp2* alleles. ***b***, Southern-blot analysis of the different *Mecp2* alleles. Digestion, *Eco*RI; probe, a 2.5-kb *Kpn*I-*Spe*I internal fragment as indicated in (*a*). Note that DNA was isolated from male ES cells, thus only one copy of a given *Mecp2* allele was present. Lanes 1–4, wild-type control, 3lox, 2lox and 1lox, respectively. ***c***, Activities of the Nestin-Cre transgene. Cre-mediated recombination (2lox→1lox) frequencies were determined by Southern-blot analysis of DNA isolated from the brain of an E14.5 *Mecp2*$^{2lox/y}$ embryo and tissues of a P16 *Mecp2*$^{2lox/y}$ mouse, both of which carried a Nestin-Cre transgene. FB, forebrain; HB, hindbrain; CC, cerebral cortex; CB, cerebellum; HC, hippocampus; BS, brainstem; Te, testis; Li, liver; Ki, kidney; Ht, heart; Lu, lung. ***d***, Germline activity of the Nestin-Cre transgene, which allowed transmission of a germline-recombined (1lox) *Mecp2*-null allele to offspring. Shown is Southern-blot analysis of tail DNA from pups that were produced from crossings between wild-type males and females carrying both *Mecp2*^{2lox} and a Nestin-Cre transgene. The Cre transgene can be activated during oogenesis resulting in about 30% offspring with a germline-deleted *Mecp2* allele. Asterisk, presence of *Mecp2*^{1lox}. ***e***, Western-blot analysis of protein samples prepared from whole brains of wild-type (+/y) and Mecp2-null (1/y) mice, using anti-Mecp2 antibodies against aa 1–305 (Ab 674; ref. 2) or a carboxy-terminal peptide (Ab C-peptide) of Mecp2. ***f***, Mecp2 immunohistochemical analysis of brain sections (hippocampus and cerebellum) from adult wild-type (wt) and Mecp2-null (mut) mice using the C-peptide anti-Mecp2 antibody, showing intense staining of neuronal nuclei in the wild-type brain sections and loss of immunoreactivity in the mutants. rd, stratum radiatum; py, stratum pyramidale; or, stratum oriens; m, molecular layer; p, Purkinje cell layer; g, granule cell layer. Arrowhead, boundary of hippocampal CA1/CA2 regions; scale bar, 50 μm.

[1]*The Whitehead Institute for Biomedical Research and* [2]*Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA.*
[3]*Department of Psychiatry, Massachusetts General Hospital, Boston, Massachusetts, USA. Correspondence should be addressed to R.J. (e-mail: jaenisch@wi.mit.edu).*

the *Mecp2*^{2lox} allele (Fig. 1*a*) were derived from independent mouse embryonic stem cells. For CNS-specific deletion, we used transgenic mice carrying a Nestin-Cre transgene, which causes over 90% recombination in the brain (Fig. 1*c*), beginning at E12 of gestation[20,21]. Both male (*Mecp2*$^{2lox/y}$) and female (*Mecp2*$^{2lox/+}$) pups carrying a Nestin-Cre transgene were normal at birth. We also obtained mice carrying a germline-recombined, mutant *Mecp2* allele (*Mecp2*^{1lox} allele; Fig. 1*a*,*d*), and found that hemizygous mutant males were normal.

Table 1 • Obesity in Mecp2-deficient mice

Mecp2 genotype	Number of mice	Over-weighted[b]	*P* value[c]
Germline *Mecp2* deletion (age: 40-60 days; number of litters[a]: 7)			
+/y	20	0	<0.001
1lox/y	17	8	
Nestin-Cre mediated *Mecp2* deletion (age: 40-60 days; number of litters[a]: 13)			
+/y	37	1	<0.002
2lox/y;Nestin-cre	18	6	
CamK-Cre93 mediated *Mecp2* deletion (age: > 10 weeks; number of litters[a]: 7)			
+/y	18	0	<0.001
2lox/y;CamK-Cre93	11	7	

[a]Only males in litters that had at least one mutant and two control mice were included. [b]Those with body weights of at least 3 standard deviations (+3SDs) above the mean value of the control littermates. It was found that in each case +3SDs represented at least 15% more body weight. [c]From χ^2 test.

To define the effects of exon 3 deletion on *Mecp2* expression, we compared RNA and protein from mutant and control brains. Northern-blot analysis detected a slightly smaller *Mecp2* transcript at a reduced level in mutant brain (data not shown). Western-blot analysis using anti-Mecp2 antibodies did not detect protein, but revealed peptides of smaller sizes in mutant and control brains (Fig. 1*e*). Immunohistochemical analysis did not detect Mecp2 immunoreactivity in mutant brains (Fig. 1*f*). When mice carrying the exon 3 deletion were compared with those with a complete *Mecp2* deletion[22], an identical phenotype (time of death, reduction in brain weight, neuronal cell size) was observed. We conclude that deletion of exon 3 results in a *Mecp2*-null mutation and that disruption of *Mecp2* does not lead to embryonic lethality.

Both Mecp2-null and Nestin-Cre conditional mutants seemed healthy for the first few weeks of age and were fertile, but developed abnormal behavior such as nervousness, body trembling, pila erection and occasional hard respiration at five weeks of age. A significant portion of the mutant mice (14/35; Table 1) became overweight and most exhibited signs of physical deterioration by eight weeks of age. At late stages of disease, mutants were hypoactive, trembled when handled, and often began to lose weight. Most mutants died at approximately 10 weeks without obvious correlation between physical deterioration and time of death. Heterozygous mutant females seemed normal for the first four months, but began to show symptoms such as weight gain, reduced activity and ataxic gait at a later age.

Autopsy failed to reveal any obvious alterations, except reduced brain size and weight in both Mecp2-null and Nestin-Cre conditional mutants (Fig. 2*a*). Smaller brain size has also been reported in RTT patients[23,24]. Comparison of mutant and control brain sections revealed no obvious abnormalities in brain architecture with one exception: cell bodies (Fig. 2*b–g*) and nuclei (Fig. 2 *h*,*i*) of mutant neurons in sections of hippocampus, cerebral cortex and

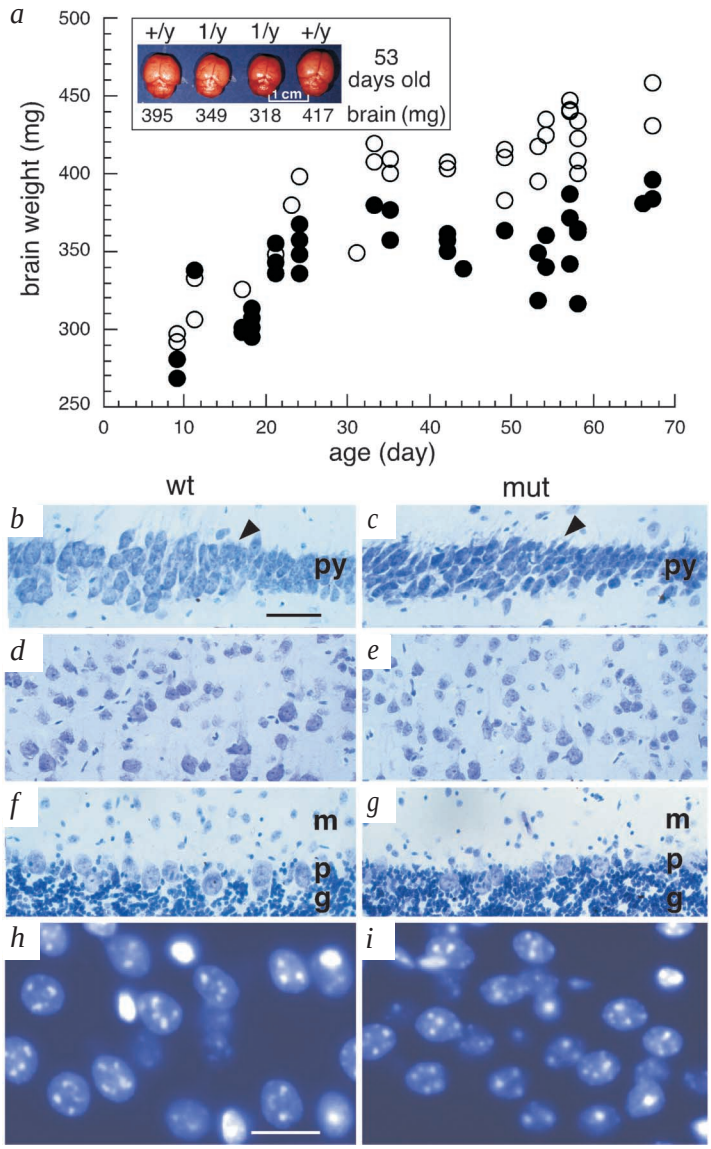

Fig. 2 Mecp2-deficiency leads to stagnation of brain growth and reduction of neuronal cell size. ***a***, Deceleration of brain growth in Mecp2-deficient mice. Filled circle, brain from mutant mice (Mecp2-null or Nestin-Cre conditional mutants, at roughly equal numbers); open circle, brain from wild-type littermates. Inserted box shows brain images of 2 mutant (1/y) and 2 wild-type (+/y) littermates at approximately 8 weeks, and their brain weights. ***b–i***, Smaller neurons and neuronal nuclei in Mecp2-deficient brains, as demonstrated by sections showing neurons (Thionine-stained) in CA1/CA2 regions (*b*,*c*), layer V of cerebral cortex (*d*,*e*) and folium II of cerebellum (*f*,*g*), and neuronal nuclei (DAPI-stained) in CA2 regions (*h*,*i*) of a wild-type (wt) and Mecp2-null (mut) brain. py, stratum pyramidale; m, molecular layer; p, Purkinje cell layer; g, granule cell layer. Arrowhead, boundary of hippocampal CA1/CA2 regions. Scale bars: *b–g*, 50 μm; *h*,*i*, 20 μm.

cerebellum were of smaller size and were more densely packed, consistent with findings in RTT patients[25,26]. To quantify the differences, we measured neuronal cell size by tracing cell contours on brain sections. Neurons in the CA2 region of mutant brains were 15–25% smaller compared with those of controls (Table 2).

To investigate whether deletion of *Mecp2* in postmitotic neurons would lead to neuronal dysfunction, we introduced a Cam kinase Cre transgene[21,27] (*CamK-Cre93*: a Cre transgene controlled by the Cam kinase promoter) into *Mecp2*^{2lox} mice. A *lacZ* reporter transgene[28] showed that *CamK-Cre93* became widely activated in the postnatal forebrain, hippocampus and brainstem (Fig. 3*a–e*), but only marginally in the cerebellum and not at all in glia[21,29] (data not shown). Southern-blot analysis showed that CamK-Cre93–mediated *Mecp2* recombination (*Mecp2*^{2lox}→*Mecp2*^{1lox}) started at the perinatal stage and reached a maximum of 60% at postnatal (P) 21 in the forebrain, but was barely detectable in the cerebellum (Fig. 3*f*). The time course of *Mecp2* deletion is in agreement with Cre activation in postmitotic neurons[21]. A 60% recombination frequency is consistent with Cre expression in only neurons and not in glia.

CamK-Cre93 conditional mutants were healthy for up to three months, when they began to show symptoms including gain of body weight (Table 1), ataxic gait and reduced nocturnal activity (Fig. 3*n*), similar to those seen in null and Nestin-Cre conditional mutants. At autopsy, the brain weight of CamK-Cre93 mutants was reduced (Fig. 3*g*). Histological analysis revealed smaller neuronal cell bodies in the cortex and hippocampus (Fig. 3*h–k* and Table 2), but not in the cerebellum, where Cre-mediated *Mecp2* deletion did not occur (Fig. 3*l*,*m*). We conclude that deletion of *Mecp2* in postnatal neurons results in a phenotype that seems to be similar, although delayed and less severe, to that seen in animals carrying a germline or a Nestin-Cre mediated deletion of the gene.

Table 2 • Reduction of neuronal sizes in Mecp2-deficient brains

	Body weight (g)	Brain weight (mg)	CA2 neuronal size (μm^2, n=50)
Germline *Mecp2* deletion (age: 9 weeks)			
Control 1	28.6	431	196.2±4.6
Control 2	28.8	458	203.3±4.2
Mutant 1	36.9	396	173.1±3.1*
Mutant 2	29.6	384	146.4±3.5*
Nestin-Cre mediated *Mecp2* deletion (age: 8 weeks)			
Control 1	27.8	441	209.1±6.6
Control 2	26.0	440	208.7±5.1
Mutant 1	31.2	387	145.1±3.4*
Mutant 2	34.6	342	155.6±4.2*
CamK-Cre mediated *Mecp2* deletion (age: 7.5 months)			
Control 1	41.7	490	211.9±3.9
Control 2	43.8	486	203.9±4.6
Mutant 1	55.9	424	145.3±3.0*
Mutant 2	53.8	413	142.1±2.6*

Brains in each comparison group were from littermates and all the brain samples in this table were fixed simultaneously in PBS-buffered 10% formalin (pH 7.4) and subsequently processed. Measurements were confined to neurons, cut through the level of the nucleolus, in the pyramidal layer of the CA2 region (see Methods). We also compared brains that were fixed in PBS-buffered 4% paraformaldehyde (pH 7.4; two mutants and two controls) or were from mice that had been perfused transcardially with PBS-buffered 10% formalin (pH 7.4; one mutant and one control). In those cases, we also observed consistent differences between mutants and their littermate controls although the absolute values varied with different fixation procedures. n, number of neurons measured. Asterisk, $P<0.001$, unpaired Student's *t*-test.

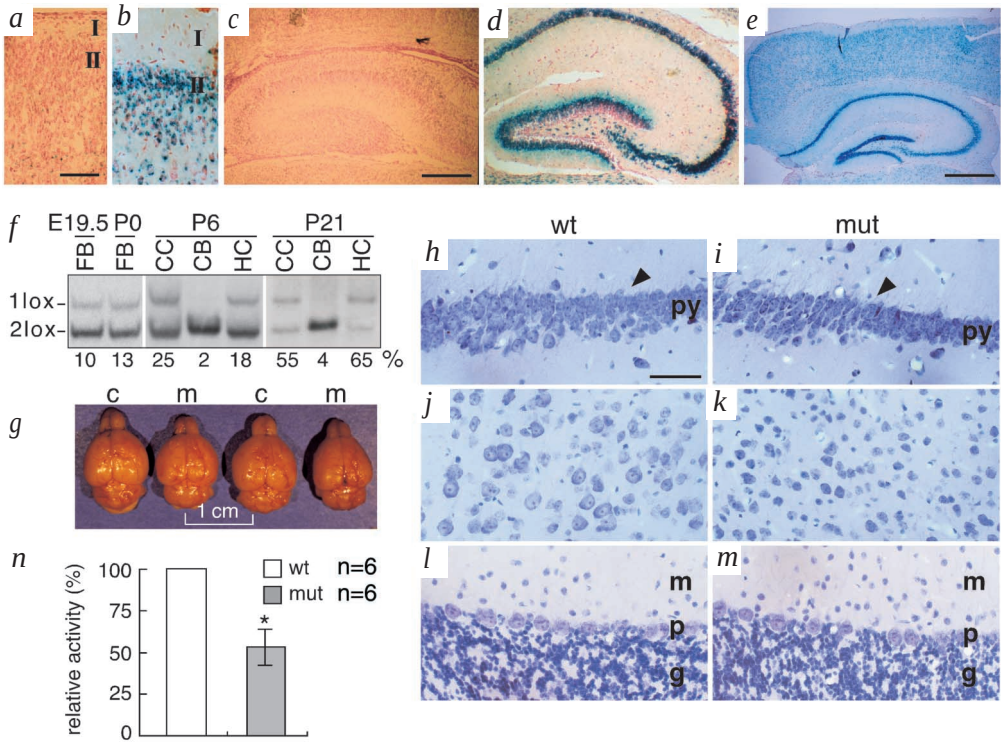

Fig. 3 CamK-Cre93–mediated deletion of *Mecp2* in postnatal neurons and phenotypes of the conditional mutants. *a–e*, CamK-Cre activity, as determined by a *lacZ* reporter transgene. Shown are brain sections stained with X-gal representing the cerebral cortex (***a***,***b***) and the hippocampus (***c***,***d***) of a P0 and a P21 brain, respectively, and the forebrain of a 4-month brain (***e***). I, layer I; II, layer II. Scale bars: *a*,*b*, 50 μm; *c*,*d*, 200 μm; *e*, 0.5 mm .***f***, CamK-Cre93–mediated *Mecp2* deletion as determined by Southern-blot analysis. The percentage in each lane indicates the frequency of Cre-mediated recombination (2lox→1lox). FB, forebrain; HB, hindbrain; CC, cerebral cortex; CB, cerebellum; HC, hippocampus. ***g***, Image of brains from two CamK-Cre93 conditional mutants (m) and two wild-type littermate controls (c) at 7.5 months of age. In total, we measured brains of six CamK-Cre93 conditional mutant mice and their wild-type littermates (at least two for each mutant) and found that in each case the mutant brain was of reduced size and weight compared with controls (on average, 11% reduction in brain weight; $P<0.001$, paired Student's *t*-test). ***h–m***, Smaller neurons in CamK-Cre93 conditional mutant brains. Note that neurons in the hippocampal CA2 regions (*h*,*i*) and layer V of cerebral cortex (*j*,*k*) are different between wild type and mutants, whereas those in the cerebellar regions (*l*,*m*) are similar. py, stratum pyramidale; m, molecular layer; p, Purkinje cell layer; g, granule cell layer. Arrowhead, boundary of hippocampal CA1/CA2 regions; scale bar, 50 μm. ***n***, Reduced nocturnal activity (as measured by infrared beam activated movement detector) in CamK-Cre93 conditional mutants. Each mouse was measured three times and the average was used for comparison. *$P<0.01$, paired Student's *t*-test.

In humans, the classic form of RTT is restricted to females, whereas most males carrying a *MECP2* mutation are thought to die pre- or perinatally[7,14–16]. Therefore, Mecp2 deficiency in mice causes a less severe phenotype than in humans, as hemizygous mutant male mice develop symptoms and die only as young adults, and heterozygous females remain healthy well into adulthood. Mutant mice frequently displayed tremor, heavy breathing and cold extremities indicating autonomic abnormalities that are also characteristic for human patients[11,13]. The change seen in mutant mice that is most consistent with the human condition is the smaller brain size and the general reduction of neuronal cell size. Thus, the similarities between brains of mutant mice and RTT patients indicate that deficiency of MECP2 leads to comparable brain and neuronal changes in both species.

A major unresolved issue in the pathogenesis of RTT is whether the disease is caused by dysfunction of postnatal neurons at a time when symptoms become apparent or whether it is a prenatal developmental disorder with postnatal phenotypic manifestation in the CNS. The phenotype seen in conditional mutant mice is relevant for the etiology of RTT in several aspects. Nestin-Cre–mediated deletion of *Mecp2* in the brain indicates that the primary cause of the phenotype is due to brain malfunction rather than to Mecp2 deficiency in peripheral tissues. Because CamK-Cre93–mediated deletion of *Mecp2* occurs mostly in postmitotic neurons, two additional conclusions can be drawn: (i) the phenotype is caused by Mecp2 deficiency in neurons rather than in glia; and (ii) deficiency of Mecp2 in postmitotic neurons is cell autonomous and sufficient to cause disease. The last result is of potential clinical significance because it may indicate that RTT is caused by defects in neuronal physiology that are not due to abnormal brain development, but rather to the absence of a continuous function of MECP2 in mature neurons. MECP2 may have an ongoing and critical role in mature CNS neurons and only prolonged deficiency may lead to the neuronal dysfunction characteristic for the disease. If this is correct, therapeutic strategies could be aimed at preventing postnatal defects of MECP2-deficient neurons from developing, rather than at correcting neuronal insufficiency that existed before birth.

The CNS-specific phenotype of Mecp2 deficiency is unexpected considering that the gene is expressed ubiquitously and thought to act as a general repressor of transcription. It remains a major challenge to understand the mechanisms of Mecp2-mediated changes in chromatin conformation and gene expression, and how the changes may relate to the brain-specific phenotype of RTT patients. Expression profiling of brain and peripheral tissues from the mutant mice by microarray analysis will likely facilitate defining the molecular changes causing RTT.

Methods

Targeting the *Mecp2* locus. We isolated a 5.5-kb *Spe*I *Mecp2* genomic fragment containing exon 3 and part of exon 4 from a 129/Sv genomic library and used it to construct a conditional *Mecp2* targeting vector (Fig. 1*a*). A *loxP* site and a CMV-HygTK double-selection cassette flanked by *loxP* sites were sequentially inserted into introns 2 and 3, respectively (Fig. 1*a*). The targeting vector (pMecp2-3lox) was linearized with *Not*I and transfected into 129/Sv and B6129F1 ES cell lines. We screened ES clones by Southern-blot analysis of genomic DNA digested with *Eco*RI, using a 2.5-kb *Kpn*I-*Spe*I internal fragment as the probe. The presence of the 5′ lox site (*loxP* 1) was confirmed by PCR using primers flanking the intron 2 *Nsi*I site. With both ES cell lines, the pMecp2-3lox construct demonstrated high targeting frequencies (20% or higher), and approximately 25% of the targeted clones contained the 5′ lox site. The CMV-HygTK cassette in the homologously targeted ES clones was removed by transient transfection of a Cre recombinase expression vector, giving rise to a conditional *Mecp2* allele in which exon 3 is flanked by *loxP* sites ($Mecp2^{2lox}$ allele; Fig. 1*a*). Independent $Mecp2^{2lox}$ ES cells derived from both original ES cell lines were injected into BALB/c host blastocysts to generate chimeras, which were subsequently crossed with BALB/c mice to derive mice bearing a germline-transmitted $Mecp2^{2lox}$ allele.

Generation of Mecp2-null and brain-specific conditional mutant mice. Mice used here were of mixed genetic backgrounds (129, C57BL/6 and BALB/c). We crossed male mice carrying either a Nestin-Cre (refs. 20,21) or a CamK-Cre (refs. 21,27; line 93) transgene with females either heterozygous or homozygous for $Mecp2^{2lox}$ to produce male ($Mecp2^{2lox/y}$;*Cre*) or female ($Mecp2^{2lox/+}$;*Cre*) conditional mutants. Nestin-Cre was found to be active in both the male and the female germ line. As $Mecp2^{2lox/y}$ males carrying a Nestin-Cre transgene, although fertile, developed disease and died as young adults, we crossed $Mecp2^{2lox/+}$;Nestin-Cre females with wild-type males to produce the first generation of mice carrying the germline-recombined *Mecp2*-null allele ($Mecp2^{1lox}$ allele). Subsequently, we used $Mecp2^{1lox/+}$ females, which were healthy for several months, for transmitting $Mecp2^{1lox}$. RNA and protein samples were prepared from tissues of mutant and control mice, and northern- and western-blot analyses were carried out as described[21].

Histology, immunohistochemistry and neuronal size measurements. Brains were immersion-fixed for 20 h in 10% formalin or 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), cryoprotected in 30% sucrose and then frozen on dry ice. For histological analysis, series of sagittal sections (10–20 μm) were slide-mounted and stained with Thionine (Sigma). For immunohistochemistry, sections were incubated with a rabbit antiserum against the C terminus of mouse Mecp2 (Upstate), then virtualized with a biotinylated goat anti-rabbit secondary antibody and an immuno-peroxidase stain (Vector). To measure neuronal cell size, neurons were projected under ×1,000 magnification on a computer screen and the somal area (μm^2) of neuron was determined by tracing the outline of the neuronal cell body cut through the level of the nucleolus, using OpenLab software.

Nocturnal activity measurements. We measured total spontaneous nocturnal activities using an infrared beam activated movement-monitoring chamber (Opto-Varimax-Mini-A, Columbus Instruments). For each measurement, a mouse was placed at least 3 h before the starting time into a standard laboratory mouse cage with fresh bedding. Recording started at the time when the lights were normally turned off (19:00 Eastern Standard Time) and stopped at 8:00 the next morning, 1 h after the lights normally came back on.

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