

Up-regulation of glucocorticoid-regulated genes in a mouse model of Rett syndrome

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Rett syndrome (RTT) is a severe form of mental retardation, which is caused by spontaneous mutations in the X-linked gene *MECP2*. How the loss of MeCP2 function leads to RTT is currently unknown. Mice lacking the *Mecp2* gene initially show normal postnatal development but later acquire neurological phenotypes, including heightened anxiety, that resemble RTT. The *MECP2* gene encodes a methyl-CpG-binding protein that can act as a transcriptional repressor. Using cDNA microarrays, we found that *Mecp2*-null animals differentially express several genes that are induced during the stress response by glucocorticoids. Increased levels of mRNAs for serum glucocorticoid-inducible kinase 1 (*Sgk*) and FK506-binding protein 51 (*Fkbp5*) were observed before and after onset of neurological symptoms, but plasma glucocorticoid was not significantly elevated in *Mecp2*-null mice. MeCP2 is bound to the *Fkbp5* and *Sgk* genes in brain and may function as a modulator of glucocorticoid-inducible gene expression. Given the known deleterious effect of glucocorticoid exposure on brain development, our data raise the possibility that disruption of MeCP2-dependent regulation of stress-responsive genes contributes to the symptoms of RTT.

INTRODUCTION

Rett syndrome (RTT) affects predominantly females who are heterozygous for new mutations in the X-linked *MECP2* gene (1–3). Owing to random X chromosome inactivation, the patients are mosaic for expression of either the mutant or the wild-type (*wt*) gene. Affected girls develop apparently normally for 6–18 months, at which time they enter a crisis that leaves them with greatly impaired motor skills, repetitive hand movements, abnormal breathing, microcephaly and other symptoms. Males, who are hemizygous for comparable mutations, do not survive. *Mecp2*-null mice develop normally for several weeks after birth, but they acquire a variety of neurological symptoms at ~6 weeks of age leading to death at ~10 weeks. Several features of this delayed onset phenotype, which is fully penetrant, recall human RTT (4–6). Conditional deletion of the *Mecp2* gene only in mouse brain

causes the same symptoms as *Mecp2* deletion in the whole mouse (4,5). Therefore, although MeCP2 is ubiquitously expressed in cells of the mouse, the *Mecp2*-null phenotype appears to be entirely due to its absence in the brain. Consistent with this finding, biochemical and immunocytochemical studies established that MeCP2 expression levels are highest in the brain, specifically in neurons (7,8). Significantly, expression of MeCP2 in neurons alone prevents onset of the mouse phenotype (9).

Molecular approaches have demonstrated that MeCP2 is a methyl-CpG binding protein, which can inhibit transcription by recruitment of co-repressors that modify chromatin (10–13). Given the likely role of MeCP2 as a transcriptional repressor, an obvious hypothesis to explain the disease is that genes in the brain that should be silenced by MeCP2 escape repression in its absence, leading to aberrant neuronal function. An initial attempt to test this hypothesis

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using microarrays detected slight alterations in gene expression, which only became significant when groups of genes were considered together (14). A candidate approach, however, successfully identified the first mammalian MeCP2 target gene, encoding brain-derived neurotrophic factor (*Bdnf*) (15,16). *Bdnf* belongs to a set of proteins synthesized in response to neuronal activity and is thought to be essential for converting transient stimuli into long-term changes in brain activity. Its misregulation may therefore be implicated in the pathology of RTT, although studies in the *Mecp2*-null mouse have not so far revealed obvious changes in *Bdnf* gene expression. More recently, altered expression of the imprinted *UBE3A* and *GABRB3* genes (17) and *Dlx-5* (18) genes in mice and patient DNAs have been reported. The magnitude of the effects is small, but it is possible that these alterations contribute to the RTT phenotype.

We independently sought misexpression of brain genes in *Mecp2*-null mice and report here novel potential target genes that are over-expressed in the absence of MeCP2. Microarray experiments revealed that certain genes that are induced by glucocorticoids are over-expressed in the absence of MeCP2. Over-expression occurs both before and after the onset of neurological symptoms, and MeCP2 is found bound near the promoters of these genes in brain. We verified that both *Sgk1* and *Fkbp5* are normally up-regulated in the brain in response to glucocorticoid signalling. *Mecp2*-null mice do not, however, have elevated levels of circulating glucocorticoids, suggesting that MeCP2 is normally required to inhibit their basal expression. As long-term activation of the glucocorticoid signalling cascade affects severely the human and rodent brain, our results raise the possibility that misregulation of these genes contributes to the neurological phenotypes associated with MeCP2 deficiency.

RESULTS

Differential gene expression in the brain of *Mecp2*-null mice

To search for differential gene expression in *Mecp2*-null mice, total RNA from symptomatic *Mecp2*^{-y} brain was compared with that of wild-type littermate control brain using microarray analysis. RNAs were fluorescently labelled with Cy3 and Cy5 in a reverse transcription reaction and co-hybridized to a microarray of 13 627 cDNA clones. The results of ANOVA analysis showed 17 clones representing 11 different UniGene clusters that met the criteria of differentially expressed genes (Table 1; Materials and Methods, GEO accession No. GSE2805). Intensity ratios for the 17 clones ranged from 2.00 to 3.44, eight being increased and three decreased in the mutant RNA sample. Northern blots and quantitative real-time PCR analyses confirmed that three of the genes (*Sgk*, *Fkbp5* and *Cirp*) are consistently up-regulated in brain RNA from symptomatic *Mecp2*-null animals (Fig. 1).

At least five of the 11 differentially expressed genes (*Fkbp5*, *Sgk*, *Pomc1*, *Sult1A1* and *Hsp105*) are regulated by the glucocorticoid hormones, which are secreted in response to stress. Stress provokes release of corticotropin-releasing hormone by the hypothalamus, which stimulates synthesis of adrenocorticotrophic hormone (ACTH). ACTH in turn causes the adrenal

cortex to produce circulating glucocorticoids (cortisol in humans, corticosterone in rodents), which bind to glucocorticoid receptors and coordinate the transcriptional response (19). Negative feedback by glucocorticoids on the hypothalamus and pituitary ensures that the stress response is normally transient. RNAs-encoding pro-opiomelanocortin (POMC—the polypeptide precursor of ACTH) and the glucocorticoid-inducible genes, *Sgk*, *Fkbp5* and *Sult1A1* (19–21), were all increased in the *Mecp2*-null brain, whereas *Hsp105* mRNA, which is down-regulated by glucocorticoids (22), was decreased (Fig. 1A). Increased transcription of *Cirp* (the cold-inducible RNA binding protein gene) is induced by low temperature or low oxygen tension (23) but has not been shown to depend on glucocorticoids.

Up-regulation of *Fkbp5* and *Sgk* in pre-symptomatic and early symptomatic *Mecp2*-null animals

Mecp2-null mice acquire neurological symptoms at ~6 weeks of age and die at ~10 weeks. The postnatal onset of symptoms resembles RTT, as affected girls develop normally until the age of 6–18 months. We asked whether the de-regulated expression of the *Sgk* and *Fkbp5* genes seen in late symptomatic mice was also present in animals that had yet to develop symptoms. As there is considerable heterogeneity in the timing of symptom onset and progression, we classified *Mecp2*-null animals according to symptoms rather than age using three groupings: (1) pre-symptomatic; (2) early symptomatic; and (3) late symptomatic as shown in Table 2. Brain cDNA from nine mice at each stage (three pools each comprising three individual brain cDNA preparations) was compared by real-time PCR analysis, using *Gapd* mRNA as an internal control. We chose real-time PCR of pooled samples in preference to northern blotting to correct for inter-individual variation among mutants in expression of these genes (see for example Fig. 1A). The data showed that *Sgk* and *Fkbp5* mRNAs are both up-regulated in pre-symptomatic, early symptomatic and late symptomatic mice when compared with wild-type controls (Fig. 1B). Western blot analysis confirmed increased levels of these proteins in the *Mecp2*-null brain (Fig. 1C). Further analysis of the same cDNA samples established that many other genes show indistinguishable expression levels in mutant and wild-type mice at all three stages (data not shown), in agreement with previous findings (14). The de-regulation of *Sgk* and *Fkbp5* genes in mice with no obvious symptoms raises the possibility that these genes are not misregulated as a secondary consequence of MeCP2 deficiency, but might be primary targets of MeCP2 regulation.

Up-regulation of glucocorticoid-inducible *Fkbp5* and *Sgk* in *Mecp2*-null animals without elevation of glucocorticoid levels

A simple hypothesis to explain the up-regulation of these stress-induced genes is that MeCP2 deficiency causes secretion of glucocorticoids. As hyper-secretion of glucocorticoids has not been previously shown to induce expression of *Fkbp5* and *Sgk* in the brain, we initially implanted cortisol pumps into wild-type mice and measured the effect on their

Table 1. Gene expression changes in the brain of an *Mecp2*^{-/-} animal compared with a wild-type littermate control detected by microarray hybridizations

Fold change	Up (+)/down (-) regulated	Gene description	UniGene cluster	Accession no.
3.4412	+	<i>Sgk</i> , serum/glucocorticoid-regulated kinase	Mm.28405	AA273540
3.2992	+	<i>Sgk</i> , serum/glucocorticoid-regulated kinase	Mm.28405	AI527833
2.6313	+	<i>Fkbp5</i> , FK506-binding protein 5 (51 kDa)	Mm.276405	BC015260
2.5815	+	<i>Fkbp5</i> , FK506-binding protein 5 (51 kDa)	Mm.276405	BC015260
2.3051	+	<i>Fkbp5</i> , FK506-binding protein 5 (51 kDa)	Mm.276405	BC015260
2.4131	+	<i>Cirp</i> , cold-inducible RNA binding protein	Mm.17898	NM_007705
2.254	+	<i>Cirp</i> , cold-inducible RNA binding protein	Mm.17898	NM_007705
2.1947	+	<i>Cirp</i> , cold-inducible RNA binding protein	Mm.17898	NM_007705
2.2748	+	<i>Sult1a1</i> , sulfotransferase family 1A, phenol-preferring, member 1	Mm.17339	AB029487
2.2651	-	Sorcin	Mm.96211	AK008970
2.2386	-	<i>Hsp105</i> , heat-shock protein, 105 kDa	Mm.270681	AA105012
2.0727	-	<i>Hsp105</i> , heat shock protein, 105 kDa	Mm.270681	BC018378
2.2012	+	<i>Pomc1</i> , pro-opiomelanocortin-alpha	Mm.277996	BC061215
2.1584	+	<i>Scya17</i> , small-inducible cytokine subfamily A17	Mm.41988	NM_011332
2.1477	-	Gja12-pending, Gap junction membrane channel protein alpha 12	Mm.40016	AW742272
2.1375	+	S3-12-pending, Plasma membrane associated protein, S3-12	Mm.347924	NM_020568
2.0019	+	<i>RIKEN cDNA 4930546H06 gene</i>	Mm.227456	AK016052

Transcripts with >2-fold expression difference, as defined in the Materials and Methods section, are shown. Some genes are represented by more than one differential clone on the array and, therefore, listed several times. The identity of all 17 clones was confirmed by sequencing. Up-regulation (+) and down-regulation (-) in the *Mecp2*^{-/-} sample are indicated.

transcription. The results showed that 2 days of hormone exposure induced expression of both *Sgk* and *Fkbp5* compared with animals that received the solvent vehicle alone (Fig. 2A). These genes are therefore induced in the brain by hormone exposure. To determine whether the similar levels of induction of *Sgk* and *Fkbp5* in *Mecp2*-null mouse brain is due to increased circulating glucocorticoids, we measured basal and stressed hormone levels. There was no significant difference between the basal plasma glucocorticoid levels of *wt* and *Mecp2*-null mice (Fig. 2B), suggesting that elevated hormone levels are not responsible.

MeCP2 binds to genomic regions of *Fkbp5* and *Sgk*

As an alternative explanation for the observed gene induction, we considered the possibility that MeCP2 represses expression in the presence of normal, low levels of hormone. This scenario predicts that MeCP2 is bound to regulatory regions of the *Sgk* and *Fkbp5* genes. Chromatin immunoprecipitation (ChIP) was carried out to test this possibility and showed that upstream regions of both genes are associated with MeCP2 in the brain nuclei (Fig. 3). No immunoprecipitation of these DNA fragments was evident in the *Mecp2*-null brain, thereby ruling out that the results are due to antibody cross-reaction with another protein. As MeCP2 requires methylated CpGs for binding, we tested for CpG methylation using bisulphite sequencing of genomic DNA derived from brain tissue. The results showed multiple methylated CpG sites in the region of *Fkbp5*, which was immunoprecipitated by MeCP2

(Fig. 3D). These results are compatible with the hypothesis that MeCP2 associates with the *Sgk* and *Fkbp5* promoters and has a repressive effect that is over-ridden by elevated glucocorticoids in response to stress.

Studies of cultured primary neurons have demonstrated that MeCP2 vacates the *Bdnf* gene promoter III upon membrane depolarization (15,16). We asked whether the association between MeCP2 and the *Sgk* and *Fkbp5* genes was similarly lost when the genes are induced by hormone. Artificial administration of glucocorticoids by corticosterone implants did not show obvious changes in promoter occupancy by MeCP2 as measured by ChIP on whole brain (Fig. 3E). This suggests that MeCP2 and the GR/ligand complex bind to independent sites on the promoter region.

Localization of MeCP2, FK5 and SGK1 in mouse brain

We next examined the immunolocalization of FK5, SGK1 and MeCP2 proteins. The results revealed a significant co-localization of FK5 with cells that also synthesize MeCP2 (Fig. 4A). A few MeCP2-positive cells expressed low or undetectable levels of FK5 (e.g. cortical cells; Fig. 4A). Many cells co-expressing both SGK1 and MeCP2 were found, though fewer than co-expressed FK5 and MeCP2 (Fig. 4B). As these animals had not been stressed or glucocorticoid treated, the data suggest that presence of MeCP2 in a neuronal cell is compatible with *Fkbp5* and *Sgk* expression. Therefore, the presence of MeCP2 in a cell does not prevent expression of either gene. It is possible that the role of

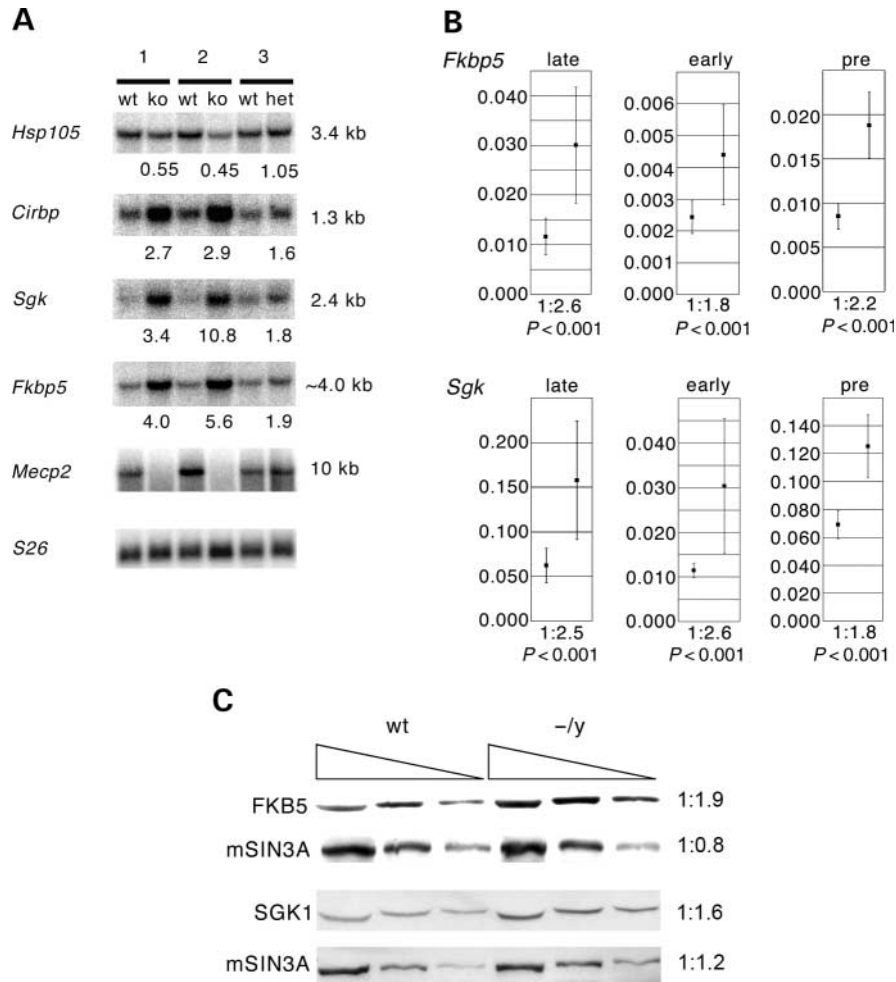


Figure 1. Altered expression of genes in the brain of *Mecp2*-null mice. (A) Northern blot analyses of *Hsp105*, *Cirbp*, *Sgk* and *Fkbp5* confirm the microarray results for these genes. Total brain RNA isolated from *Mecp2*^{-/-} (ko), heterozygote *Mecp2*^{+/-} (het) and wild-type (*wt*) control animals was used. Three littermate pairs (1, 2, 3) were investigated. The *Mecp2*^{-/-} animals showed advanced neurological symptoms. Intensities of the *Hsp105*, *Cirp*, *Sgk* and *Fkbp5* bands were normalized against a loading control (*S26 ribosomal protein* cDNA probe). Expression levels of wild-type animals were arbitrarily set to 1.0 and relative expression levels of the respective ko and het littermate samples are indicated below the lanes. (B) Real-time PCR analyses of *Fkbp5* and *Sgk* in pre-symptomatic (pre) animals and animals with early or late symptoms as defined in Table 2. Nine wild-type and nine *Mecp2*-null animals were investigated per stage. A significant up-regulation of both genes was detected in all three investigated stages (see *P*-values). The mean is shown as a dot; error bars indicate \pm SD. The *y*-axes represent arbitrary threshold cycle number converted into expression levels relative to *Gapd* expression. (C) Western blot analysis shows up-regulation of *Sgk* and *Fkbp5* proteins in the *Mecp2*-null brain. Three 2-fold dilutions of total brain protein from *wt* and symptomatic *Mecp2*-null (ko) littermates were probed with antibodies as described in the Materials and Methods. The control was mSin3a. Densitometric ratios between *wt* and mutant levels of each protein are shown adjacent to each dataset.

MeCP2 at these promoters is not to silence the genes, but to modulate levels of expression, as may be the case at promoter III of the *Bdnf* gene.

DISCUSSION

The search for genes that are misregulated in *Mecp2*-null mice has so far identified a small number of low magnitude effects. Initial expression microarray studies detected perturbed gene expression, but the effect only became statistically significant by pooling multiple different transcripts together for analysis (14). Transcription of the MeCP2-target gene *Bdnf* was also altered subtly in *Mecp2*-null neurons. Levels of *Bdnf* transcription after neuronal stimulation were not affected by the absence of MeCP2, but basal expression increased from 1 to

2% of the induced expression level (15). A 2-fold increase in transcription was reported at the *Dlx5* locus (18), whereas statistically significant reductions in *UBE3A* and *GABRB3* expressions were <2 -fold (17). The novel MeCP2-regulated genes identified in the present study, *Fkbp5* and *Sgk1*, exhibited 2–3-fold elevation of expression in the *Mecp2*-null brain. Expression of these two glucocorticoid-regulated genes could be increased by ~ 1.5 -fold in *wt* brain through chronic administration of cortisol. Therefore, the level of *Fkbp5* and *Sgk1* expressions in the mutant mice is at least as great as that induced by circulating hormone. We ruled out the possibility that over-expression is mediated by increased levels of circulating hormone in the mutant mice. Therefore, MeCP2 indirectly or directly influences expression of *Fkbp5* and *Sgk1*. The presence of MeCP2 in promoter-proximal

Table 2. Classification of *Mecp2*-null mice according to the manifestation of phenotype. The last column shows the average age of mice displaying the symptoms

Phenotype	Clasping	Inertia	Tremour	Weight loss	LOC ^a	Average age
Pre-symptomatic	–	–	–	–	–	~30 days
Early symptomatic	–/+	+	+	–	–	~55 days
Late symptomatic	+	+	+	+	+	~70 days

^aLOC—loss of coat condition.

regions of both genes in brain implies a direct role in their regulation.

The functions of both *Sgk1* and *Fkbp5* have been the subject of several studies. *Sgk1* plays a role in activating certain potassium, sodium and chloride channels, suggesting an involvement in the regulation of a variety of processes including cell survival and neuronal excitability (21). *Fkbp5* is a glucocorticoid receptor-regulating co-chaperone of Hsp90 that has been implicated as a modulator of steroid receptor function through association with Hsp90, which is itself a molecular chaperone with a central role in steroid hormone signalling. *Fkbp5* possesses peptidyl–prolyl isomerase activity, which is involved in modulation of glucocorticoid receptor activity. Glucocorticoid resistance in New World primates has been attributed to FKBP51 over-expression, suggesting that this co-chaperone can modulate hormone-binding affinity (24). In addition, an association has been reported between single-nucleotide polymorphisms in FKBP5 and clinical depression in humans (25). Although it is not possible to predict phenotypic outcomes due to moderate over-expression of *Sgk1* and *Fkbp5*, the fact that both genes have been linked with neuronal function makes their possible involvement in RTT a priority for further investigation.

It is noteworthy that absence of *Mecp2* leads to elevated levels of *Fkbp5* and *Sgk1* transcripts before the onset of overt symptoms in these mice. Misregulation of *Fkbp5* and *Sgk1* is therefore an early phenotype that is manifest during important stages of postnatal brain development. Numerous publications describe deleterious effects of glucocorticoid treatment on the developing brain (26–29). These include dendritic changes and osteopenia in both rodents and humans. Related changes occur in brains of RTT patients (30–34). Furthermore, seizures and heightened anxiety of mice with mutations in the *Mecp2* gene (5,6) and the over-alertness and agitation of RTT patients recall the known physiological consequences of glucocorticoid exposure.

As MeCP2 is a transcriptional repressor, the predominant current hypothesis to explain RTT is that critical genes are aberrantly expressed in its absence. It is conceivable that misregulation of a few genes is responsible. For example, aberrant expression of one or more of the MeCP2-target genes, *Bdnf*, *Dlx5*, *Ube3a*, *Gabrb3*, *Fkbp5* and *Sgk1*, might be key contributors to the phenotype. For each of these genes, a hypothetical case can be made that misregulation would have neurological consequences that could give rise to disease, although there is currently no experimental evidence that strongly supports these conjectures. An alternative

hypothesis is that RTT is due to an aggregate of many slight defects in gene expression, only a few of which are currently known. These two broad scenarios have therapeutic implications. If a small number of molecular pathways were to be involved, pharmacological intervention might be targeted to the affected processes. In contrast, a complex set of downstream effects resulting from MeCP2-deficiency may prove more difficult to address pharmacologically, and this could favour a strategy of re-introducing (or re-activating) a functional *MECP2* gene. To resolve these issues, it will be important to connect gene expression changes in mouse and human with aspects of the phenotype in each system.

MATERIALS AND METHODS

Animals

Adult mice were maintained under conditions of controlled lighting (lights on 07:00 to 19:00 h) and temperature (22°C) and allowed *ad libitum* access to food and water. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Stress testing

Basal blood samples were obtained from MeCP2^{-/-} and *wt* mice by tail venesection and collection of blood in EDTA-coated microvette tubes (Sarstedt), between 9 and 10 a.m. Mice were then placed in Perspex restraint tubes for 10 min and a further blood sample was taken, to reflect peak stress response of corticosterone. Blood tubes were centrifuged, plasma removed and stored at –20°C until corticosterone levels were measured by radioimmunoassay (29).

Corticosterone treatment

Adult, male C57BL/6J mice were used. In one experiment, corticosterone (Sigma) solution was delivered at a rate of 15 µg/h from mini osmotic pumps (Alzet, Charles River, Margate, UK) inserted subcutaneously under halothane anaesthesia. Control animals had a pump inserted, which contained the vehicle solution (1:1 ratio of DMSO and polypropylene glycol). The experiment was terminated 48 h later. Efficacy of the treatment was confirmed by plasma corticosterone measurement as described (35) (control: 56.5 ± 11.2 nM; treated: 417 ± 61 nM) and altered thymus weights (decrease 33%). In the second experiment, corticosterone implants containing 75 mg corticosterone in silastic elastomer (Dow Corning, MI, USA) or control implants (silastic elastomer alone) were inserted subcutaneously into the animals under halothane anaesthesia. Animals were sacrificed 3 days later. Plasma corticosterone levels were raised from 74 ± 18.2 nM in controls to 448 ± 28.1 nM in the corticosterone-treated group and thymus weights were decreased by 65%.

Microarray hybridization

Microarrays were generated as described previously (36). Total RNA from the brain of a 74-day-old MeCP2^{-/-} animal with late symptoms (gait ataxia, hind limb clasping, breathing

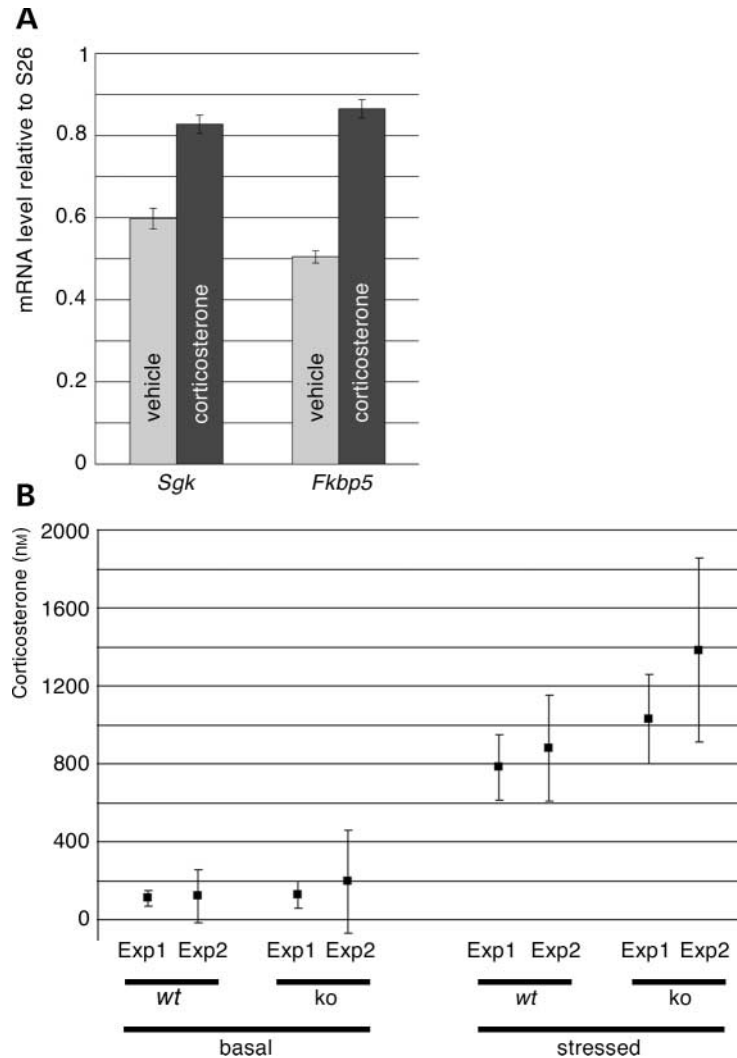


Figure 2. Hormone-dependent and hormone-independent induction of *Fkbp5* and *Sgk* in mouse brain. (A) Corticosterone administration induces *Fkbp5* and *Sgk* expression in *wt* mouse brain. Hormone ($n = 6$) or hormone-free vehicle ($n = 7$) was delivered continuously to *wt* mice for 2 days, after which *Fkbp5* and *Sgk* mRNA levels in mouse brain were measured by northern blotting. Results were normalized against S26 ribosomal protein mRNA levels on the same blots. (B) Corticosterone levels are not elevated significantly in *Mecp2*-null mice, although *Fkbp5* and *Sgk* are induced. Resting and stressed levels of plasma glucocorticoid were measured in symptomatic *Mecp2*-null mice (ko) and wild-type (*wt*) littermates. Experiment 1 ($n = 4$) and experiment 2 ($n = 5$) are shown separately. Boxes depict mean plasma glucocorticoid values and error bars show \pm SD. Statistical analysis using Student's *t*-test gave *P*-values for the comparison *wt* versus ko under basal conditions as 0.6673 (experiment 1) and 0.5891 (experiment 2). Under stressed conditions, *P*-values were 0.141 (experiment 1) and 0.1037 (experiment 2). We conclude that there are no significant differences between *wt* and ko hormone levels.

irregularities, uneven teeth, small stature) and a wild-type male littermate was isolated using TriReagent (Sigma) according to the manufacturer's protocol. Labelled target cDNA was generated by direct incorporation of fluorescent nucleotide analogues in a reverse transcription reaction. Total RNA (75 μ g of each sample) was used in an oligo dT primed reaction in the presence of 100 μ M Cy3- or Cy5-dUTP (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany), 200 μ M dTTP and 500 μ M dATP, dCTP and dGTP (Roche Molecular Biochemicals, Mannheim, Germany). The labelled cDNA was purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany). Labelled cDNA targets were resuspended in hybridization solution (50% formamide, 6 \times SSC, 0.5% SDS, 5 \times Denhardt's solution) to which 1 μ l

poly(dA) (10 μ g/ μ l) and 1 μ l mouse Cot-1 DNA (20 μ g/ μ l) were added. The resulting reaction solution was concentrated to 35 μ l, denatured at 95°C for 3 min and snap-cooled on ice. Hybridization took place under a coverslip at 42°C for 16 h. The slides were washed at room temperature in 0.2 \times SSC and 0.01% SDS for 5 min, followed by two washing steps in 0.2 \times SSC for 5 min each. In total, two co-hybridizations (*Mecp2*^{-/-} versus wild-type brain cDNAs) were performed with dyes swapped in the repeat experiment.

Data analysis

Image analysis was performed as described previously (36). The data sets of two co-hybridization experiments with

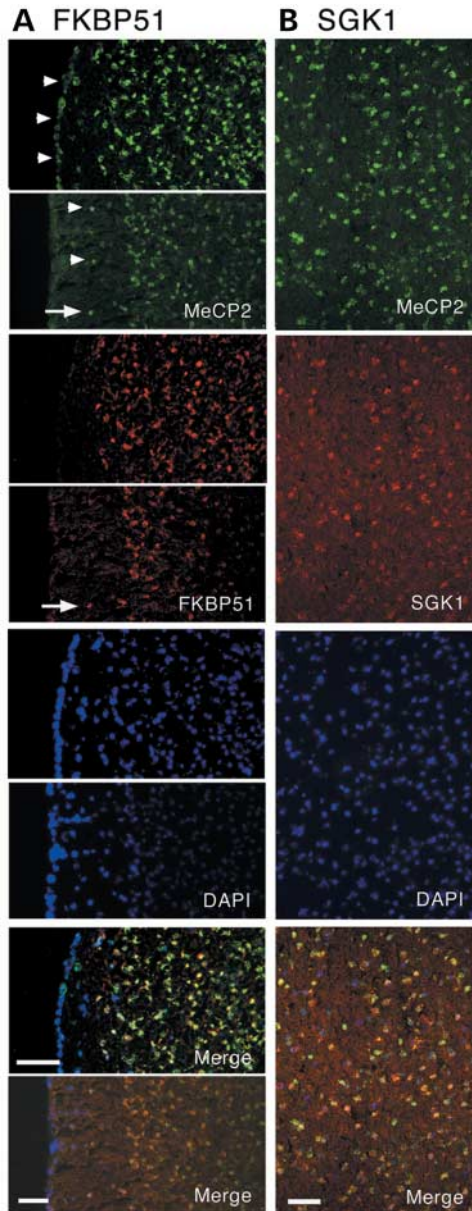


Figure 4. Expression of MeCP2, FKBP51 and SGK1 in the brain of adult female mice. Immunolocalization of MeCP2 (green signals, FITC-coupled secondary antibody), FKBP51 (red signals in A, Cy3-coupled secondary antibody) and SGK1 (red signals in B, Cy3-coupled secondary antibody) on cryostat sections of mouse brain. Panels show the temporal cortex (A) and a region immediately above the corpus callosum (B). Blue signals indicate DAPI-stained nuclei. A clear cellular overlap of the staining with anti-MeCP2 and anti-FKBP51 antibodies can be seen for the majority of cells in all brain regions. There are few MeCP2-positive cells with an absent or weak FKBP51 expression (arrow heads in A). Arrow in A points to a cortical cell with strong MeCP2 and FKBP51 signals. Many cells co-express MeCP2 and SGK1 in the brain, for example, cells above the corpus callosum (B). Scale bars in merged images equal 100 μm , (upper panels) and 50 μm , (lower panels).

dye swap were first quality checked for pin- and PCR plate-specific effects by local regression according to each pin or PCR plate. Furthermore, background intensities across the slide were checked for homogeneity. Spots with local contaminations were excluded from further analysis. Local

background-subtracted raw intensities on a logarithmic scale were normalized by ANOVA analysis using the MATLAB code, with slight modifications. The underlying ANOVA model we applied was

$$\log(y_{ijk}) = \mu + A_i + D_j + V_k + G_g + (AG)_{ig} + (VG)_{kg} + \varepsilon_{ijk}$$

$$i = 1, 2; j = 1, 2; k = 1, 2; g = 1, \dots, 13627;$$

(where y is the log intensity of each channel, μ the overall mean, A the overall array effect, D the overall dye effect, V the overall variety effect and G the overall gene effect; AG describes array-gene interactions and VG the variety-gene interactions). Genes were then sorted according to their variety-gene differences $[(VG)_{k=\text{sample},g} - (VG)_{k=\text{control},g}]$, which is an estimate of the log ratio of the sample versus control normalized-intensities. Clones showing >2 -fold changes were considered relevant. All data analyses were performed using MATLAB, Version 6.0.0.88, Release 12, MathWorks Inc., MA, USA.

Northern and western blot analyses

RNA from total brains of *Mecp2*^{-/-} male animals, *Mecp2*^{+/-} females and wild-type animals was isolated using TriReagent (Sigma) according to the manufacturer's protocol. Northern blots were prepared by standard procedures. We generated probes by PCR amplification of respective microarray cDNA clones (*Hsp105*, *Cirp*, *Sgk* and *Fkbp5*, see generation of cDNA microarrays). For the amplification of *Mecp2*, *S26 ribosomal protein* and β -*actin* cDNA probes, specific primers and cDNA templates were used. All blots were rehybridized to an S26 ribosomal protein cDNA probe as a loading control. Radioactive signals were detected with a PhosphorImager (Molecular Dynamics) and quantitated using ImageQuant software. Brains of symptomatic mutant and wild-type littermates were homogenized in 500 μl of PBS supplemented with Complete (Roche) protease inhibitors. The protein suspension (1.25–10 μl) was fractionated on an 8% SDS-PAGE gel. Western blots were carried out using standard protocols. Anti-Sgk antibodies were purchased from Upstate (07-315), anti-Fkbp51 antibodies from Affinity Bioreagents (PA1-020) and anti-mSin3a antibodies from Santa Cruz Biotechnology (sc-994).

Reverse transcription and real-time PCR analysis

RNA (20 μg) isolated from total mouse brain using the Trizol reagent (Invitrogen) was subjected to a reverse transcriptase reaction in the presence of 25 ng/ml oligo (dT) and 2.5 mM dA/C/G/TTP (Roche Molecular Biochemicals) with 10 U/ml SSII reverse transcriptase (Invitrogen) and without enzyme in negative control reactions. The resulting cDNA (2 μl) and negative control reaction were used in 50 μl PCR amplifications with 10 pmol of primers: mFkb5_exon1_exp_for (5'-gctggcagcaggtcttcta-3')/mFkb5_exon1_exp_rev (5'-gctttgataa cctggccttg-3'). Amplifications consisted of 35 cycles (94°C for 45 s, 56°C for 60 s and 72°C for 60 s).

Real-time PCR analysis was performed using an iCycler (Bio-Rad) real-time PCR machine. For each genotype and disease stage, three pools of RNA from nine mice were

analysed (three whole brain RNA preparations per pool). For *wt* controls, the same number of littermates were used. Four parallel reactions were carried out for each cDNA pool with IQ SYBR Green supermix (Bio-Rad) or home-made mix: 0.5× SYBR Green (Molecular Probes); 10 nm fluorescein (Sigma); 1× PCR buffer with MgCl₂ (Roche); 200 μM dNTP (ABgene) with 1 U FastStart *Taq* polymerase (Roche). Results were always displayed relative to *Gapdh* cDNA amounts and statistically compared using Student's *t*-test. Primers used for this analysis are as follows: *Gapdh* (5'-taccceaatgtgtcctcg-3' and 5'-cctgcttcaccaccttcttg-3'); *Sgk* (5'-cgccaagtccctcaacaa-3' and 5'-tgcccttcgatcacttcc-3') and *Fkbp5* (5'-gctggcaacaacacagag-3' and 5'-gaggaggccga gttcatt-3').

Immunofluorescence microscopy

Brains from female wild-type C57BL/6 animals were snap-frozen in isopentane pre-cooled in dry ice. Cryostat sections (6 μm thick) were fixed with acetone at -20°C for 10 min, dried at room temperature and stored at -80°C until use. Primary antibodies against FKB5 (1:100, sc-11518, Santa Cruz Biotechnology), SGK1 (1:100, sc-15885, Santa Cruz Biotechnology) and MeCP2 C-terminus (1:100, 07-013, Upstate/Biomol, Hamburg, Germany) were used. Sections were incubated in 0.1% Triton/PBS for 5 min, followed by three washes in PBS. After a blocking step with 5% donkey serum in PBS for 30 min, sections were incubated with primary antibodies for 1 h, followed by three washes in PBS. For negative control reactions, PBS instead of the primary antibody was used. Secondary antibodies coupled to Cy3 or FITC (Dianova, Hamburg, Germany) were applied for 30 min, and after three PBS washes, samples were rinsed with distilled water, dipped in ethanol and mounted with a DAPI-containing medium. Fluorescence images were taken with a Zeiss Axioskop epifluorescence microscope or with a Leica DM IRE2.

Chromatin immunoprecipitation

ChIP was performed using frozen total brain tissue pulverized with a mortar according to the Upstate Biotechnology ChIP kit protocol (Upstate/Biomol) with the following modifications. Prior to sonication, nuclei were isolated from lysed cells by the use of a sucrose gradient. Cross-linked chromatin was sheared by sonication in a 0.5% Triton solution to fragment sizes ranging from 500 to 1000 bp. The chromatin was then subjected to immunoprecipitation using antibodies specific to acetylated histone H3 (06-599, Upstate/Biomol), mSin3A (sc-994, Santa Cruz Biotechnology), the MeCP2 C-terminus (07-013, Upstate/Biomol) or a polyclonal MeCP2 antibody generated as described (13). Alternatively, brains were ground in liquid nitrogen and fixed for 15 min in 1% formaldehyde in PBS. Cells were lysed and insoluble material was pelleted by centrifugation. Crude nuclei were lysed, sonicated and subjected to immunoprecipitation. Precipitated fragments were immediately subjected to PCR amplification or after a two-step DOP-PCR using primers SR1 and SR2 (31). *Fkbp5* primers: m1_0_for (5'-tgctcccttagatcaccacac-3')/m1_0_rev (5'-ccactgctccgatacattctc-3'), m1_4_for (5'-agta

cccaacagaggtcaga-3')/m1_4_rev (5'-tgtggatacaaatattcca-3'), m2_1_for (5'-tcttgcccttacctttaa-3')/m2_1_rev (5'-agttctcaggg acttttcag-3'), m2_2_for (5'-acacagaacaatacaaaaag c-3')/m2_2_rev (5'-tcaacaatatggctgtagga-3'), m2_4_for (5'-g cacaatgctgactagata-3')/m2_4_rev (5'-ttaaattgtctcagtgctc-3'), Fkpd (5'-agccacggctctagatgagagc-3') and Fkpr (5'-gtgtgtg aaggagagtgccagaac-3'), Fkp1d (5'-tgctcccttagatcaccacac-3') and Fkp1r (5'-ccactgctccgatacattctc-3'). Amplifications consisted of 35 cycles, (94°C for 45 s, 49°C for 60 s and 72°C for 45 s; different annealing temperatures were used for primers m1_0_for/m1_0_rev (55°C), Fkpd/Fkpr (60°C), and Fkp1d/Fkp1r (60°C). *Sgk* primers: sgkp1d (5'-acggacggg ttaaggcagt-3') and sgkp1r (5'-cgtgaggaggtggcgagtagag-3').

Bisulphite sequencing

Genomic DNA from brain was isolated using the DNAeasy tissue kit (Qiagen). Conversion of genomic DNA by sodium bisulphite was performed as described in the CpGenome DNA Modification Kit manual (Chemicon, Temecula, CA, USA). Regions of interest were amplified with specific primers: Fkbp5_prom_1_4bis_for (5'-aataccaacaaaa tcaaaaaaa-3')/Fkbp5_prom_1_4bis_rev (5'-tgtggtataaaatattt atagtaaat-3'), Fkbp5_prom_2_1bis_for (5'-cacacttctataacctt cctttaa-3')/Fkbp5_prom_2_1bis_rev (5'-gttttagggatttttaggtt gtt-3'), Fkbp5_prom_2_2bis_for (5'-aaattaaaacaaaaacataa caaaaac-3')/Fkbp5_prom_2_2bis_rev (5'-ttgttttttaataatggtt gtagga-3'). Amplifications consisted of 35 cycles (94°C for 45 s, 49°C for 60 s and 72°C for 45 s). Cloning of the result ing PCR products into pCR 2.1-TOPO vectors (Invitrogen) was followed by sequencing with primers M13 for (5'-ct ggccctgctttac-3')/M13 rev (5'-caggaacagctatgac-3').

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Conflict of Interest statement. None declared.

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