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Cell-specific expression of wild-type MeCP2 in mouse models of Rett syndrome yields insight about pathogenesis

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Rett syndrome (RTT), a leading cause of mental retardation with autistic features in females, is caused by mutations in the gene encoding methyl-CpG-binding protein 2 (MeCP2). RTT is characterized by a diverse set of neurological features that includes cognitive, motor, behavioral and autonomic disturbances. The diverse features suggest that specific neurons contribute to particular phenotypes and raise the question whether restoring MeCP2 function in a cell-specific manner will rescue some of the phenotypes seen in RTT. To address this, we generated transgenic mice expressing inducible MeCP2 under the control of the brain-specific promoters calcium/calmodulin-dependent protein kinase II (CamKII) or neuron-specific enolase (Eno2) and bred them onto mouse models lacking functional MeCP2. Expression of normal MeCP2 in either CamKII or Eno2 distribution was unable to prevent the appearance of most of the phenotypes of the RTT mouse models. These results suggest that most RTT phenotypes are caused either by disruption of complex neural networks involving neurons throughout the brain or by disruption of the function of specific neurons outside of the broad CamKII or Eno2 distribution.

INTRODUCTION

Rett syndrome (RTT) is a neurodevelopmental disorder that affects the patient's ability to communicate and perform simple motor tasks. RTT affects mainly females, occurring with an incidence of up to 1:10 000 (1,2). Although the RTT phenotype is variable, most Rett patients have a distinctive disease course. They appear to develop normally from birth to about 6–18 months. After this initial apparently normal period, patients undergo a period of regression characterized by the loss of acquired speech, deceleration of head growth and the development of ataxia, seizures, scoliosis, breathing dysfunction and autistic behavior (3). A stereotyped repetitive hand-washing or hand-wringing gesture replaces purposeful use of the hands. After this regressive phase, patients stabilize but fail to acquire new skills and may also develop additional neurological abnormalities (3).

The vast majority of Rett cases are caused by mutations in the *MECP2* gene (4). *MECP2*, which maps to Xq28 and not expressed from the inactive X chromosome, encodes for methyl-CpG-binding protein 2 (MeCP2). MeCP2 is a methylation-dependent transcriptional repressor (5) and a splicing regulator (6) most abundant in brain, lung and spleen (7). In the mature brain, MeCP2 is present in virtually every neuron. The spectrum of *MECP2* mutations causing RTT includes missense, frameshift and nonsense mutations and intragenic deletions (2).

Mouse models of the disease have been generated using gene-targeting approaches. Mice null for *Mecp2* exhibit clinical manifestations of Rett: a period of apparently normal development followed by a severe progressive neurological dysfunction that includes abnormal gait and movements, irregular breathing, tremors and hypoactivity. They exhibit alterations in synaptic plasticity, and males die at ~8–12 weeks of

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age (8–10). In addition, we have generated a mouse model of Rett by truncating the *Mecp2* gene after amino acid 308 (full length is 486 amino acid). Male mutant mice survive to adulthood and show features of Rett in a progression that mimics the human disease. *Mecp2*$^{308/y}$ mice show stereotypic forelimb motions, tremor, motor and social behavioral abnormalities, seizures, hypoactivity, anxiety-like behavior and learning and memory deficits (11–13).

The initial period of apparent normal development displayed by RTT patients suggests that MeCP2 expression might be unessential in the developing brain. This is supported by the observation that the timing of MeCP2 expression in mouse and human correlates with the maturation of the central nervous system (CNS) (7,14). For example, MeCP2 levels in olfactory neurons become maximal after synaptogenesis (15), and deletion of *Mecp2* in postnatal CNS neurons reproduces the phenotypes of MeCP2 null mice (8,9). Furthermore, expression of wild-type MeCP2 in postmitotic neurons under the control of a neuron-specific promoter rescued the phenotype of the *Mecp2* null mice (16), strengthening the hypothesis of the dispensability of MeCP2 for normal development and indicating that the phenotype is caused by MeCP2 deficiency in the CNS. These data also suggest that the progression of Rett can be halted or reversed by restoring MeCP2 function or the function of its downstream effectors. In fact, it has been recently reported that most of the Rett-like phenotypes exhibited by *Mecp2* null mice are reversible, indicating that the dysfunctional neuronal network could be rescued by the sole expression of MeCP2 (17).

We set out to evaluate whether the restoration of functional MeCP2 in a subset of neurons was sufficient to rescue the phenotypic manifestations of MeCP2 mutant mice, with the reasoning that restoring the function of the protein in specific neurons might prevent phenotypes resulting from dysfunction of such neurons.

Mice overexpressing MeCP2 in most neurons exhibit a neurological disorder (16,18), revealing a particular CNS sensitivity to MeCP2 expression levels. Thus, we also set out to determine if different expression levels could affect the rescuing capabilities of MeCP2. For this purpose, we generated transgenic mice carrying an inducible MeCP2 (I-MeCP2) transgene under the control of promoters active in areas supposed to be relevant for the development of RTT phenotypes. We chose to use either the rat neuron-specific enolase (NSE) promoter (Eno2) or the mouse calcium/calmodulin-dependent protein kinase IIα (CamKIIα) promoter. The NSE is a glycolytic enzyme expressed in most terminally differentiated neurons and neuroendocrine cells (19). CaMKIIα is a protein kinase expressed in the neurons of the neocortex, hippocampus, amygdala and basal ganglia (20). To address the question of whether different mutant alleles of MeCP2 are susceptible to being rescued by expression of a MeCP2 transgene, we used two different mutants: *Mecp2*$^{-/y}$ and *Mecp2*$^{308/y}$.

RESULTS

Generation of a mouse with an inducible wild-type *MECP2* allele

This work was initiated as an effort to determine whether the Rett-like disease in the *Mecp2* mutant mouse models was reversible and the period of development during which therapeutic intervention could be successful. To answer these questions, we generated mice transgenic for an inducible wild-type *MECP2* allele that when introduced into a *Mecp2* mutant background would allow us to evaluate the phenotypic rescuing effects of wild-type MeCP2 expression at different stages of disease progression. Cell-specific transgenic MeCP2 expression, also able to be temporally regulated, was achieved with a system based on the tetracycline transactivator (tTA), a fusion protein between the tetracycline repressor TetR and a minimal transactivation domain of the viral VP16 protein. In the absence of tetracycline, tTA (encoded in one transgene) binds to *tetO* recognition sequences in the tetracycline-responsive element (TRE) adjacent of a CMV minimal promoter and activates RNA polymerase II-dependent transcription of MeCP2 and EGFP simultaneously (all contained in a second transgene). If tetracycline is present, tTA dissociates from the TRE and transcription ceases. We constructed *MECP2*-pBI-EGPF, a bi-directional construct designed to allow the simultaneous regulation of both human MeCP2 (wild-type) and EGFP by a central TRE (Fig. 1A). From 10 transgenic founders, with transgene copy number ranging from 5 to 15 copies, only six transmitted the transgene to their F1 progeny. MeCP2 should not be expressed from the transgene in these mice unless binding of the tetracycline-inhibitable transcription factor (tTA) is provided. Therefore, the six transgenic lines were crossed with mice expressing the tetracycline-inhibitable transcription factor (tTA) under the control of brain-specific promoters (CamKII–tTA or Eno2–tTA). The particular CamKII–tTA and Eno2–tTA transgenic lines that we used in this study have been shown to direct tTA-dependent transgene expression specifically in neurons of the forebrain and neurons of the striatum and cerebellum, respectively (21,22). The resulting double transgenics would express wild-type MeCP2 only in the subset of neurons in which the promoter that drives expression of the tTA is active. The advantage of using this system over simple transgenics with MeCP2 under the control of the same cell-specific promoters lies in the fact that the expression of transgenic MeCP2 is inhibitable through the administration of tetracycline, which abolishes transgene expression. Thus, this inducible system would allow us to let the disease progress (by the continuous supply of tetracycline) and then test the reversibility of the Rett-like phenotypes by inducing the expression of transgenic MeCP2 at variable stages of the disease.

The doubly transgenic *MECP2*-pBI-EGPF;promoter-tTA mice are referred to as inducible-MeCP2 (I-MeCP2), and when relevant, the specific promoter is indicated in brackets. Western blot analysis indicated that two of the transgenic lines do not express the transgenic protein. Southern analysis shows that one harbors an incomplete transgene, thus explaining the lack of transgene expression (data not shown). We hypothesize that transgene expression in the other line is 'off' probably due to a silencing effect arising from the integration site of the transgene. The transgenes from three expressing lines are solely expressed in the brain, as would be expected when using brain-specific drivers (Fig. 1B and data not shown). The other transgenic line also expresses the transgenic proteins in the brain, but in addition, the eyes also show EGFP fluorescence and expression of transgenic

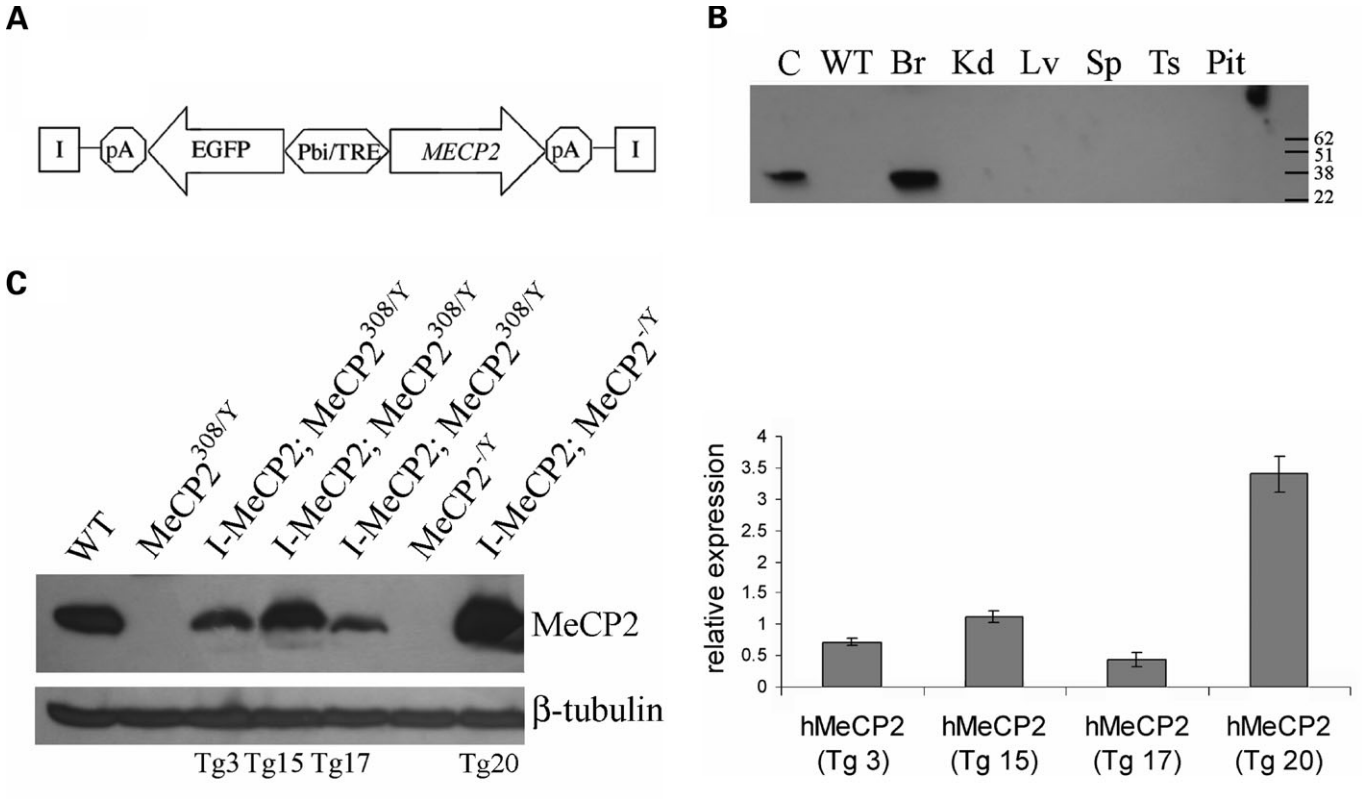

Figure 1. Brain-specific expression in I-MeCP2 and tTA bi-transgenic mice. (**A**) The tet-regulated system. A transgene consisting of reversely positioned *MECP2* and EGFP genes, regulated by a bi-directional tetracycline operator-based promoter (pbi/TRE). Cross-breeding of transgenic lines for this construct with mice bearing another transgene consisting of a tTA under the control of a brain-specific promoter generates a double-transgenic mouse capable of brain-specific expression of the MeCP2/EGFP transgene. tTA is constitutively expressed, binds specifically and with high affinity to *tetO* and activates transcription of both target genes. I, insulator; pA, poly-A; pbi, CMV-bidirectional promoter; TRE, tetracycline responsive element. (**B**) Tissue-specific transgene expression. Whole-brain protein extracts prepared from 6–10-week-old male mice doubly transgenic for *MECP2*-pBi-EGPF; CamKII–tTA (I-MeCP2) were subjected to immunodetection of EGFP. C, positive control for the detection of EGFP (extracts from transiently transfected cells); WT, extract from a 12-week-old non-transgenic wild-type mouse as a negative control; Br, brain; Kd, kidney; Lv, liver; Sp, spleen; Ts, testicle; Pit, pituitary. (**C**) Determination of levels of transgene expression. Top: Western blot for the detection of MeCP2, using a C-terminal MeCP2 antibody, to whole-brain protein extracts of 6–8-week-old I-MeCP2(CamKII) mice that also harbor either the *Mecp2*^{308} mutation (I-MeCP2;*Mecp2*$^{308/Y}$) or the *Mecp2* null mutation (I-MeCP2; *Mecp2*$^{-/Y}$). The transgenic or endogenous MeCP2 can be observed at the apparent molecular weight of 75 kDa. To control for equal loading, the blot was probed with an anti-β-tubulin antibody. Bottom: densitometry analyses of the MeCP2 bands (normalized to β-tubulin levels, $n = 3$) indicate that transgenic MeCP2 in line Tg15 is expressed at similar levels to those of endogenous MeCP2.

MeCP2 (Supplementary Material, Fig. S1; data not shown). One line (MeCP2tg15) expresses the transgenic protein at levels similar to the endogenous MeCP2 protein, as assayed by western blot of brain protein extracts. The expression in the lines MeCP2tg3 and MeCP2tg17 is lower (0.5–0.7-fold) than the expression of the endogenous Mecp2, whereas the expression levels in the MeCP2tg20 are higher (3–4-fold) (Fig. 1C and D). Thus, unless otherwise indicated, the experiments in this paper have been conducted with the MeCP2tg15 line.

Cell-specific expression of transgenic MeCP2

Examination of the tissue specificity for transgene expression indicated that the pattern of expression is dictated by the corresponding brain-specific promoter (CamKII or Eno2). When crossed with CamKII–tTA transgenics, all the lines express transgenic MeCP2 and EGFP specifically in neurons of the forebrain (Fig. 2A). On the other hand, when tTA is under the control of the NSE promoter (Eno2–tTA transgenics), the expression was specific to the striatum and cerebellum (Fig. 2B), with the exception of MeCP2tg20, in which the expression extends into the eyes. Transgene expression was abolished in the presence of doxycycline (Supplementary Material, Fig. S2).

We bred the transgenic mice onto a MeCP2 mutant background to determine the preventability (by constitutively expressing transgenic MeCP2) and reversibility (by post-symptomatic induction of transgenic MeCP2 expression) of the phenotypes. Through multiple matings, we generated mice carrying a *MECP2*-pBI-EGPF transgene, a promoter-tTA transgene and a mutation in the endogenous *Mecp2*. We first determined whether the tissue specificity of transgene expression was preserved in the presence of a truncated Mecp2 protein or in the absence of endogenous Mecp2. Expression analysis of the I-Mecp2(Eno2); *Mecp2*$^{308/Y}$ indicates that this is indeed the case (data not shown). Thus, the expression of the transgene from either promoter is not affected by the endogenous MeCP2 status.

MeCP2 exhibits a distinctive immunostaining pattern in mouse neurons, localizing to densely methylated heterochromatic foci

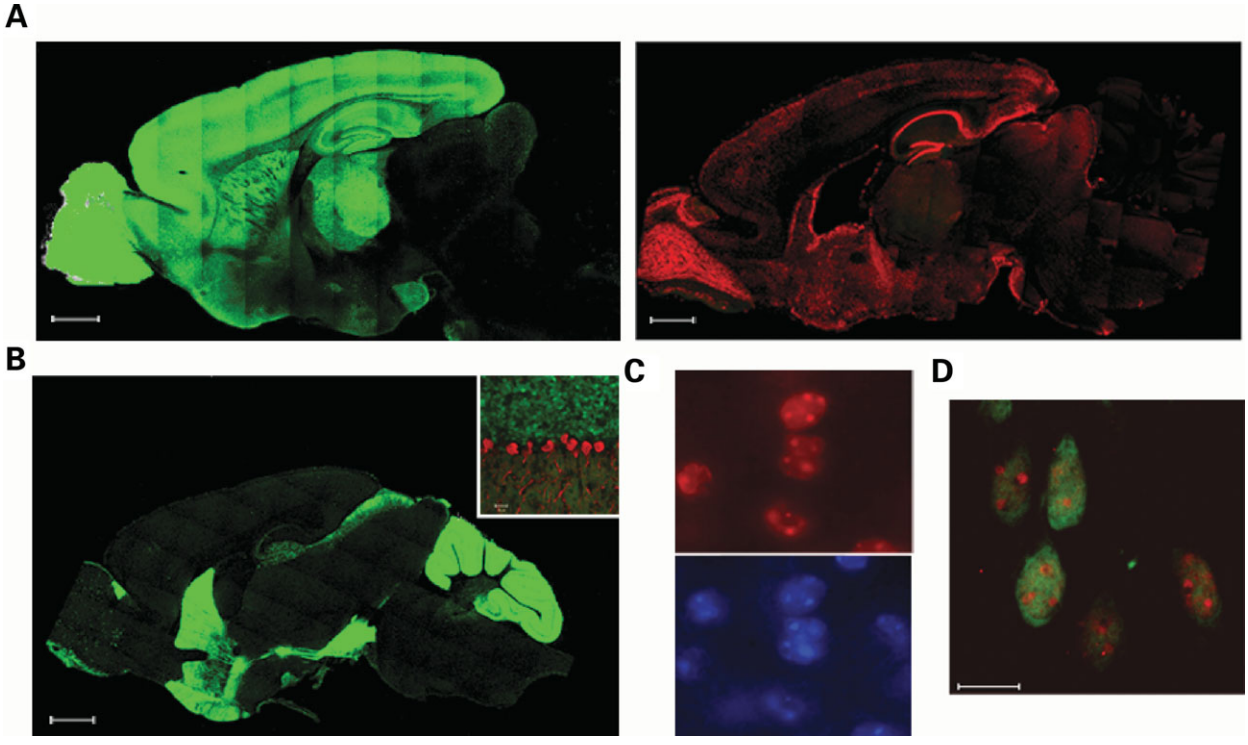

Figure 2. Tissue-specific expression of EGFP and MeCP2 from I-MeCP2 transgenic mice. EGFP expression was determined in sagittal brain sections of I-MeCP2 by EGFP immunoreactivity. (**A**) Eight-week-old I-MeCP2(CamKII) mice demonstrated EGFP (left) and MeCP2 (right) positive staining in the olfactory bulb, striatum, cortex, hippocampus, nucleus accumbens, thalamus and substantia nigra. (**B**) Expression of EGFP in the I-MeCP2 (Eno2) mice at 9 weeks was evident in the striatum, hippocampus, superior colliculus and cerebellum. The inset shows the EGFP expression in the granule cell layer in the cerebellum. Most Purkinje cells, stained with anti-calbindin (red), do not express the transgenic proteins (inset). (**C**) Heterochromatic localization of MeCP2 in the cells of the hippocampus. The intranuclear distribution of transgenic MeCP2 in cortical neurons was revealed by immunostaining of brain sections from 7-week-old I-MeCP2(CamKII); *Mecp2*$^{-/Y}$ mice, using an antibody directed to the C-terminus of MeCP2 (top) and Dapi staining of heterochromatin (bottom). (**D**) High magnification of caudate/putamen neurons from I-MeCP2(Eno2); *Mecp2*$^{-/Y}$ brain sections immunostained with an antibody that specifically recognizes the S421 phosphorylated form of MeCP2 indicates that the transgenic protein gets phosphorylated.

(23). Staining of brain sections from I-MeCP2 mice at 5–8 weeks of age revealed that the transgenic MeCP2 protein was also nuclear and localized to heterochromatic bodies (Fig. 2C). The recent demonstration that MeCP2 gets phosphorylated at S421 in response to neuronal activity prompted us to examine the functionality of the transgenic protein by analyzing whether it acquired this post-translational modification. Immunostaining with an antibody that specifically recognizes the S421 phosphorylated form of MeCP2 indicated that the transgenic MeCP2 is indeed phosphorylated. Interestingly, transgenic MeCP2 phosphorylated at S421 also exhibits heterochromatic localization (Fig. 2D). The specificity of the antibody was confirmed by testing its capacity of recognizing MeCP2 in extracts of neurons depolarized *in vitro* but not from unstimulated neurons (Supplementary Material, Fig. S3).

Since neurons normally exhibit variable MeCP2 expression levels (14), we compared the immunoreactivity of transgenic MeCP2 with the expression of the endogenous protein. Thus, we stained serial sections of *Mecp2*$^{+/Y}$ and I-MeCP2; *Mecp2*$^{-/Y}$ brains with either anti-NeuN or anti-MeCP2 antibodies. We observed that although the western blot results indicated that the transgenic protein was expressed approximately at wild-type levels, at the cellular level, the expression of transgenic MeCP2 did not correctly recapitulate endogenous levels of Mecp2 expression in all neurons. Although most transgenic neurons displayed levels of expression similar to the endogenous Mecp2, some exhibited higher while others displayed lower MeCP2 immunoreactivity than wild-type neurons (Supplementary Material, Fig. S4).

Timing of transgene expression

To determine the timing of expression of transgenic MeCP2, we carried out RT–PCR for the detection of MeCP2. We amplified a MeCP2 fragment that included parts of exons 3 and 4, absent in RNA obtained from *Mecp2*$^{308/Y}$ and *Mecp2*$^{-/Y}$ mice. When analyzing RNA derived from I-MeCP2(CamKII); *MECP2*$^{308/Y}$ mice, we detected the expression of transgenic *MECP2* starting at embryonic day 10 (E10, Fig. 3A). The expression of the transgene, when under the control of the Eno2 promoter, was first detectable at day P1 (Fig. 3D). We then studied the timing of the appearance of fluorescence of the co-expressed EGFP transgene. EGFP fluorescence started at E12.5 in the I-MeCP2(CamKII) transgenics (Fig. 3B and C), whereas in the I-MeCP2(Eno2) transgenics, EGFP fluorescence was detectable at P1 (Fig. 3E). Immunostaining for MeCP2 indicates that transgenic MeCP2 is co-expressed with EGFP even during

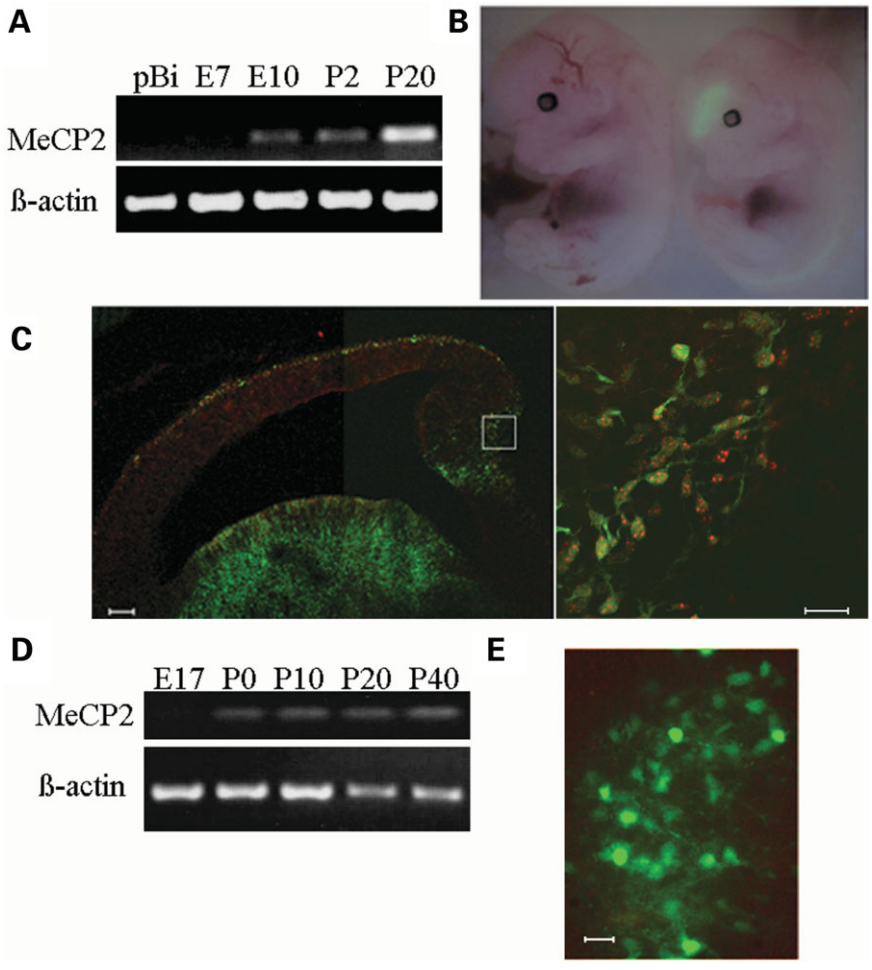

Figure 3. Timing of transgene expression. **(A)** Expression of the MeCP2 transgene was analyzed in I-MeCP2(CamKII); *Mecp2*$^{-/Y}$ mice by RT–PCR from whole brain obtained at indicated ages (top left). **(B)** EGFP expression was easily observed under a dissecting scope illuminated with a hand held UV lamp in I-MeCP2(CamKII) E12.5 embryos (embryo at right) but absent in I-MeCP2(Eno2) embryos of the same age (embryo at left). **(C)** A brain section of a I-MeCP2(CamKII) E12.5 embryo showing expression of EGFP (green) and MeCP2 (red) in the forebrain. Scale bar: 100 um. The framed region of the neocortex/hippocampus is magnified in the right panel to show MeCP2 staining (red). Scale bar: 20 μm. **(D)** RT–PCR for the detection of transgenic MeCP2 in whole brain of I-MeCP2(Eno2); *Mecp2*$^{-/Y}$ mice. **(E)** Direct fluorescence microscopic visualization of EGFP-positive cells from the striatum of an I-MeCP2(Eno2) mouse at P1. Scale bar: 20 μm.

development (E12.5), a stage when wild-type mice show only a weak signal for the expression of endogenous Mecp2 (Fig. 3C).

Effect of the *MECP2* transgene on the phenotype of *Mecp2*$^{308/y}$ mice

The phenotypes in the *Mecp2*$^{308/Y}$ mice are progressive and take months to develop (7,12). To determine whether the tissue-specific expression of transgenic MeCP2 resulted in the prevention of the development of Rett-like phenotypes, we followed the course of the I-MeCP2; *Mecp2*$^{308/Y}$ mice for a full year.

We and others have reported that mild overexpression of MeCP2 causes a neurologic phenotype (16,18). However, only the transgenic line with the highest expression levels (MeCP2tg20) has an overt phenotype before 32 weeks of age when the transgene is in a wild-type background (J.I.Y., unpublished data). Thus, the use of the transgenic lines without overt phenotypic manifestations allows us to study whether localized expression of MeCP2 could prevent the appearance of Rett-like phenotypes in *Mecp2* mutant mice.

Regardless of the promoter driving the expression of transgenic MeCP2, gross appearance of I-MeCP2; *Mecp2*$^{308/Y}$ mice was indistinguishable from *Mecp2*$^{308/Y}$ mice or from *Mecp2*$^{308/Y}$ mice expressing tTA. I-MeCP2; *Mecp2*$^{308/Y}$ mice exhibited forepaw stereotypies, tremors and kyphosis, in spite of the presence of transgenic MeCP2. These symptoms also developed at comparable times (Supplementary Material, Table S1).

The motor performance of I-MeCP2; *Mecp2*$^{308/Y}$ mice was analyzed by three behavioral tests: dowel, suspended wire and accelerating rotarod. At 20 weeks of age, I-MeCP2; *Mecp2*$^{308/Y}$ mice exhibited the same deficit as the *Mecp2*$^{308/Y}$ mice on the dowel test, which measures the ability of the mice to walk on a thin stationary elevated rod (Fig. 4A). The use of CamKII or Eno2 promoters made no difference in their performance on this test. When their ability to hang upside-down

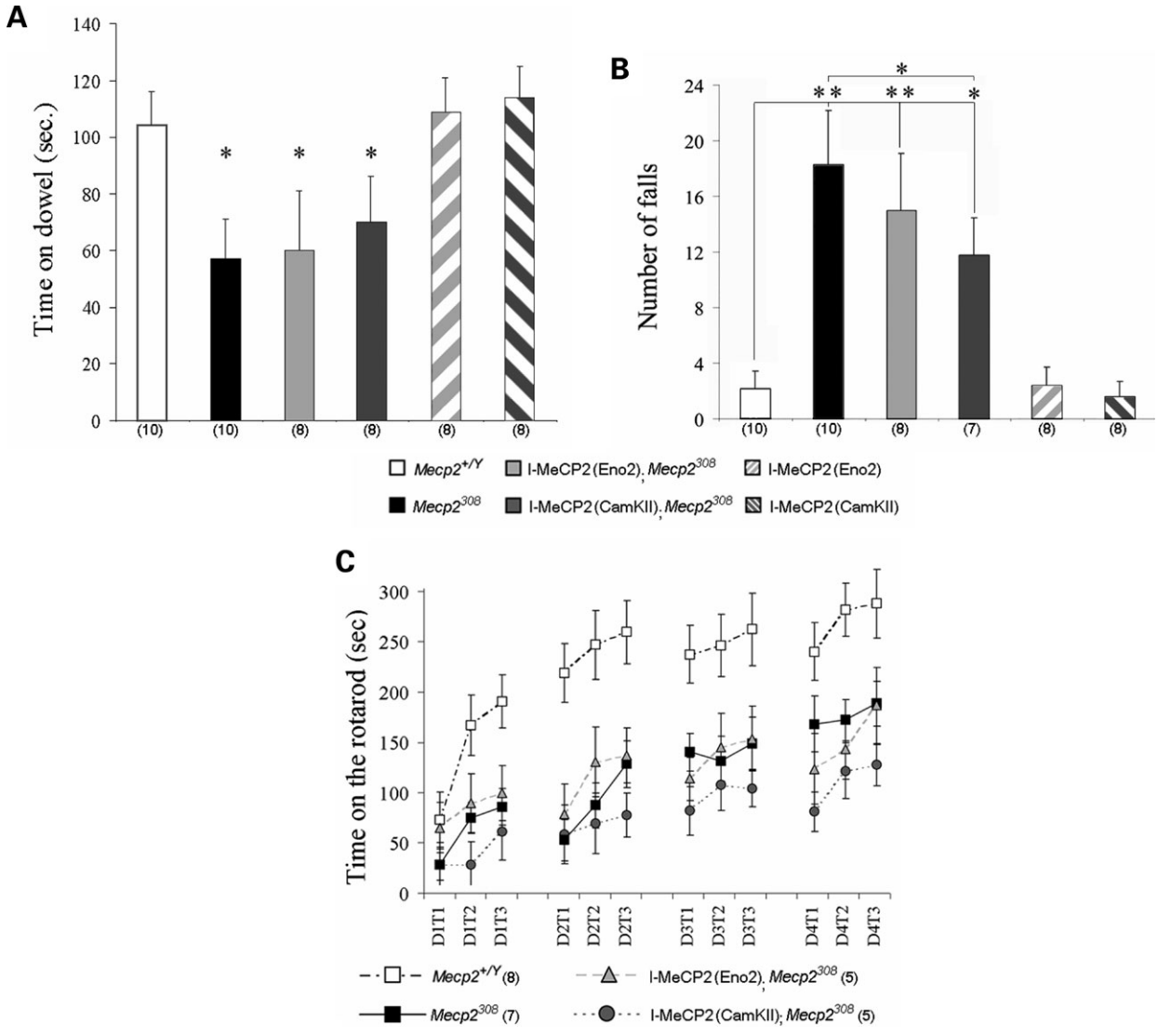

Figure 4. Evaluation of the extent of rescue by transgenic MeCP2 of the *Mecp2*$^{308/Y}$ phenotype. (**A**) The ability of the mice to stay on a thin wooden dowel (0.7 cm diameter) was determined. The test lasts for 2 min or until the mice fall for the 10th time. *Mecp2*$^{308/Y}$ mice fall from the dowel more often than their wild-type littermates. Expression of the transgene does not rescue this phenotype. $^{*}P < 0.05$. (**B**) We measured the ability of the mice to hold on to a wire (2 mm diameter) with their forepaws. The test lasts for 1 min and the number of falls is recorded. $^{*}P < 0.05$, $^{**}P < 0.01$. (**C**) Analysis of rotarod performance. Animals performed three trials a day for 4 days. For each trial, the mouse was placed on the rotating rod, its head directed against the direction of rotation. The amount of time the animal spent walking on the accelerating rod was recorded. The trial was stopped either when the mouse fell, held passively on to the rod for two consecutive cycles, or after 10 min. Rotarod performance improved in consecutive trials in all genotypes but was impaired in the mutant mice compared with the control mice, regardless of transgene expression (rm-ANOVA, genotype effect $P < 0.05$, trial effect $P < 0.001$). The number of animals for each assay is within brackets, either below each genotype bar (A and B) or beside genotype legends (C). All data are expressed as means ± SEM.

from a wire was tested (suspended wire test), a trend for a partial, but not statistically significant, rescuing effect of the *MECP2* transgene was noticeable when expressed under the CamKII promoter. The statistical comparison of the performance in this task of the I-MeCP2(CamKII); *Mecp2*$^{308/Y}$ mice with their wild-type counterparts indicates that the occurrence of the associated phenotype is not completely prevented. However, the different response of the I-MeCP2(CamKII); *Mecp2*$^{308/Y}$ and the *Mecp2*$^{308/Y}$ mice reaches statistical significance (Fig. 4B), uncovering a partial effect over this motor phenotype. Expression of the same transgene under the control of the Eno2 promoter, however, did not result in any detectable improvement in the *Mecp2*$^{308/Y}$ mice. These data suggest that part of the motor phenotypes may originate in the forebrain.

The rotarod test is used to screen locomotor performance, measuring motor coordination and balance control, but also provides a measure of motor learning. The performance of the *Mecp2*$^{308/Y}$ mice on the rotarod test is age-dependent. We chose to test mice at 28 weeks of age, as *Mecp2*$^{308/Y}$ mice at this age exhibit noticeable deficits in the accelerating rotarod test (Fig. 4C). Expression of MeCP2 from either the CamKII or the Eno2 promoter did not result in a significant improvement on this task (Fig. 4C).

We hypothesized that the lack of prevention by constitutive, cell-specific expression of the wild-type transgenic MeCP2

could be due to a dominant negative interference from the *Mecp2*^{308} allele, as *Mecp2*$^{308/Y}$ mice continue to make a mutant protein that binds DNA (11). To test this possibility, we analyzed the phenotype of the MeCP2 transgene on an *Mecp2*$^{-/Y}$ background, in which no Mecp2 mRNA or protein was detected (9).

Effect of the *MECP2* transgene on the phenotype of *Mecp2*$^{-/y}$ mice

Mecp2$^{-/Y}$ mice display tremor, irregular breathing and are less active and alert than wild-type littermates at 5–12 weeks. They exhibit hind limb clasping and forelimb immobility when suspended by their tails and die prematurely at 8–12 weeks. We investigated whether the transgenic MeCP2 was able to restore the function of MeCP2 in *Mecp2*-deficient mice. Notably, I-MeCP2; *Mecp2*$^{-/Y}$ mice also exhibited breathing abnormalities, tremor and helplessness when suspended. Further, the life span of the I-MeCP2; *Mecp2*$^{-/Y}$ mice did not differ from the *Mecp2*$^{-/Y}$ mice regardless of which promoter drove the expression of the transgene (Fig. 5A). However, we noticed that the I-MeCP2(CamKII); *Mecp2*$^{-/Y}$ mice exhibited more activity in their home cage than their *Mecp2*$^{-/Y}$ littermates (Supplementary Material, video). Therefore, we compared the level of activity of these mice at 8 weeks of age by placing them in a new open arena. The data indicate that the decrease in locomotor activity of the *Mecp2*$^{-/Y}$ mice was less pronounced in the I-MeCP2(CamKII); *Mecp2*$^{-/Y}$ mice (Fig. 5B). The I-MeCP2(eno2); *Mecp2*$^{-/Y}$ mice were indistinguishable from *Mecp2*$^{-/y}$ mice in all aspects studied.

Effect of the level of *MECP2* transgene expression on the longevity of *Mecp2*$^{-/y}$ mice

The lack of rescue by the MeCP2 transgene could be due to inappropriate levels of expression. To test this possibility, we determined the life span of 'transgenic rescue' lines with different expression levels of transgenic MeCP2. No modification of the shortened life span of the *Mecp2*$^{-/Y}$ mutants was observed for any of the four studied transgenic lines (data not shown).

Alternatively, brain areas in which the transgenes are not expressed might be important for the development of the observed phenotypes. For instance, transgenic MeCP2 is not expressed in the hypothalamus in either of our transgenic lines, as revealed by co-localization studies with hypothalamic markers such as ACTH (arcuate nucleus), neurophysin (supraoptic and paraventricular nuclei) or tyrosine hydroxylase (dopaminergic neurons in the hypothalamus) (Fig. 6). For these analysis, we chose to study the co-localization of EGFP with markers of neuronal identity as EGFP diffuses through the whole neuron (MeCP2 remains in the nucleus) and is therefore more comparable with the localization of the marker proteins (see co-localization with GAD1 in Fig. 6). Importantly, close to 90% of the neurons co-express EGFP and transgenic MeCP2 (Supplementary Material, Fig. S5). Other areas that do not express transgenic MeCP2, as are outside of the realm of the CamKII and Eno2 promoters, include the midbrain tegmentum and the medulla, with the exception of isolated neurons (Fig. 7).

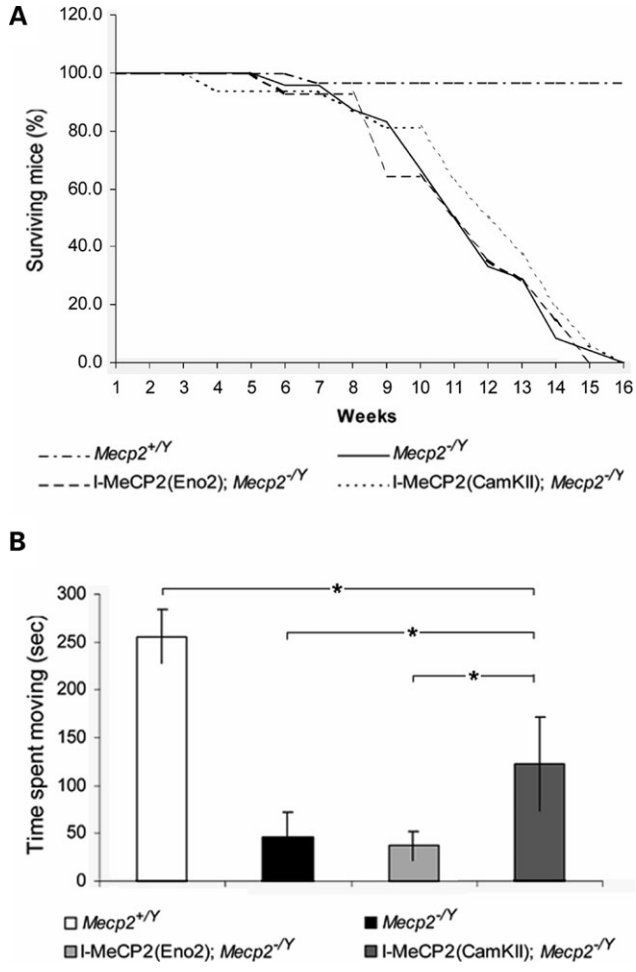

Figure 5. Survival and activity of I-MeCP2; *Mecp2*$^{-/Y}$ mice. (**A**) Survival curves of *Mecp2*$^{+/Y}$($n = 30$), *Mecp2*$^{-/Y}$($n = 24$), I-MeCP2(Eno2); *Mecp2*$^{-/Y}$ ($n = 14$) and I-MeCP2(CamKII); *Mecp2*$^{-/Y}$ ($n = 10$) mice are shown. (**B**) Spontaneous locomotor activity was measured in 8-week-old mice in an open arena. Partial rescue of this phenotype was observed with forebrain-specific transgene expression [I-MeCP2(CamKII); *Mecp2*$^{-/Y}$] [$F(3,23) = 59.42$, $P < 0.0001$]. The asterisk denotes significant differences, $P < 0.01$. Error bars indicate SEM.

DISCUSSION

Our rationale to pursue cell-specific rescue was inspired by the need to determine whether any of the RTT phenotypes originate from specific neurons. Defining such neurons might help in the future design of effective therapies.

As a first step towards this goal and based on previous reports indicating that MeCP2 expression in all neurons was capable of preventing the appearance of phenotypic manifestations in mice with mutant endogenous *Mecp2* (16,18), we bred transgenic mice expressing MeCP2 conditionally in specific areas of the brain to mouse models of Rett. These transgenic lines express different levels of MeCP2 in different regions of the brain in a regulated manner and lack overt phenotypic manifestations. Hence, they represent a great tool to test the hypothesis that RTT phenotypes originating in different neurons may be reversible.

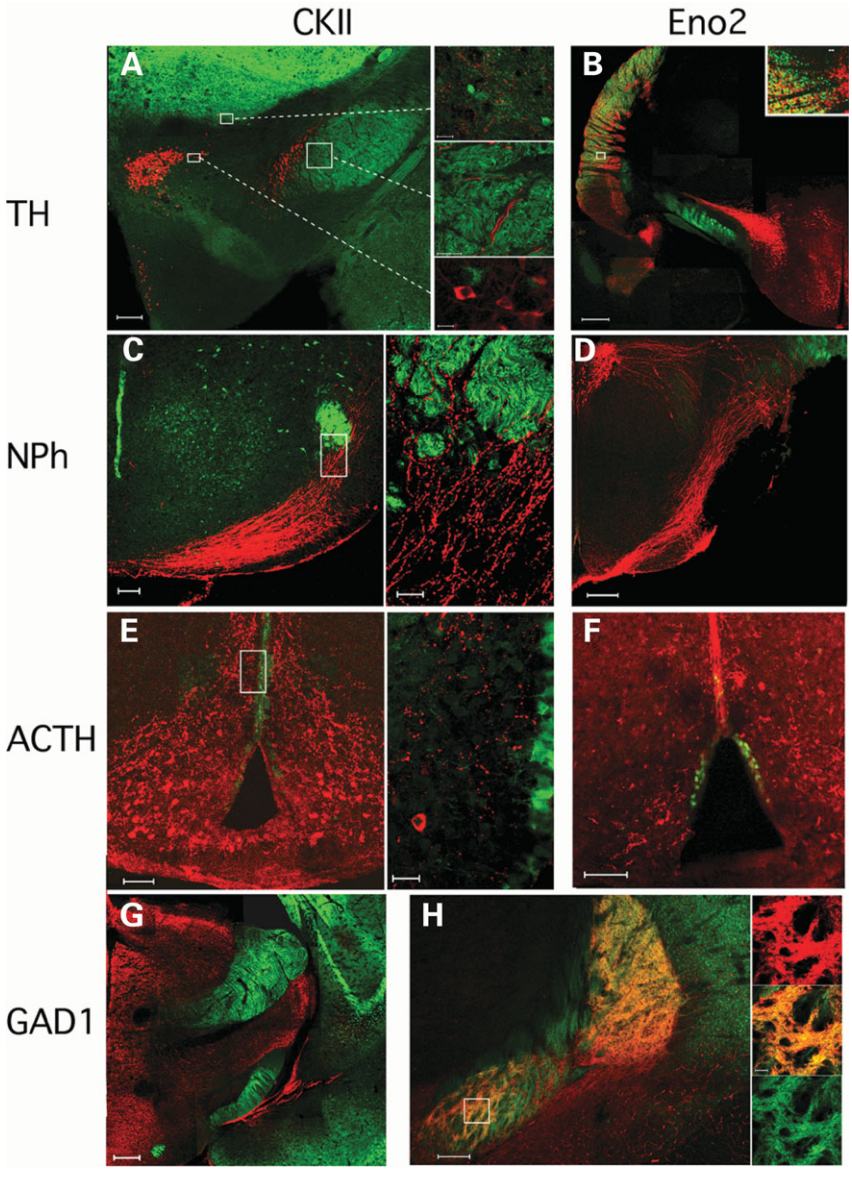

Figure 6. Transgenic MeCP2 expression patterns in a variety of neurons. Simultaneous detection of EGFP fluorescence (green) and immunostaining for tyrosine hydroxylase (TH, red), neurophysin (NPh, red), adrenocorticotrophin (ACTH, red) or glutamic acid decarboxylase (GAD1) in diencephalic midcoronal sections of brains from I-MeCP2(CamKII) (**A**, **C**, **E** and **G**) and I-MeCP2(Eno2) (**B**, **D**, **F** and **H**) adult mice. Scale bar: 100 μm in (C, E and F); 200 μm in (A, D and H); 400 μm in (B) and 500 μm in (G). Insets show magnified areas. Scale bar: 20 μm.

To our surprise, however, region-specific expression of transgenic MeCP2 was not able to prevent the development of most of the assessed phenotypes notwithstanding the specific pattern of transgene expression. Thus, the inability of constitutive, cell-specific MeCP2 transgene expression to prevent the development of Rett-like features precludes the study of phenotypic reversibility in these mice. Notably, widespread expression of transgenic MeCP2 in the forebrain, including areas believed to be relevant for the Rett phenotype such as the cortex and hippocampus (10,13,16,24–31), was unable to produce a major impact on the observed phenotypes of our mice. Although I-MeCP2(CamKII); *Mecp2*$^{-/Y}$ mice were less hypoactive than *Mecp2*$^{-/Y}$ mice, all other aspects studied remained unchanged. It is important to note that CamK-Cre-induced deletion of MeCP2 results in a delayed Rett-like phenotype that includes behavioral, motor and structural manifestations.

Expression of the transgene in the striatum and cerebellum, whose dysfunction may contribute to the deterioration of the motor system seen in patients with RTT, did not prevent appearance of any phenotype either.

In our transgenics, only one of the two splice variants identified is expressed (MeCP2-e2) (32,33). This should not be a problem, however, since a pan-neuronal postmitotically expressed *Mecp2-e2* transgene, encoding solely the splice variant expressed by our transgene, was able to prevent the

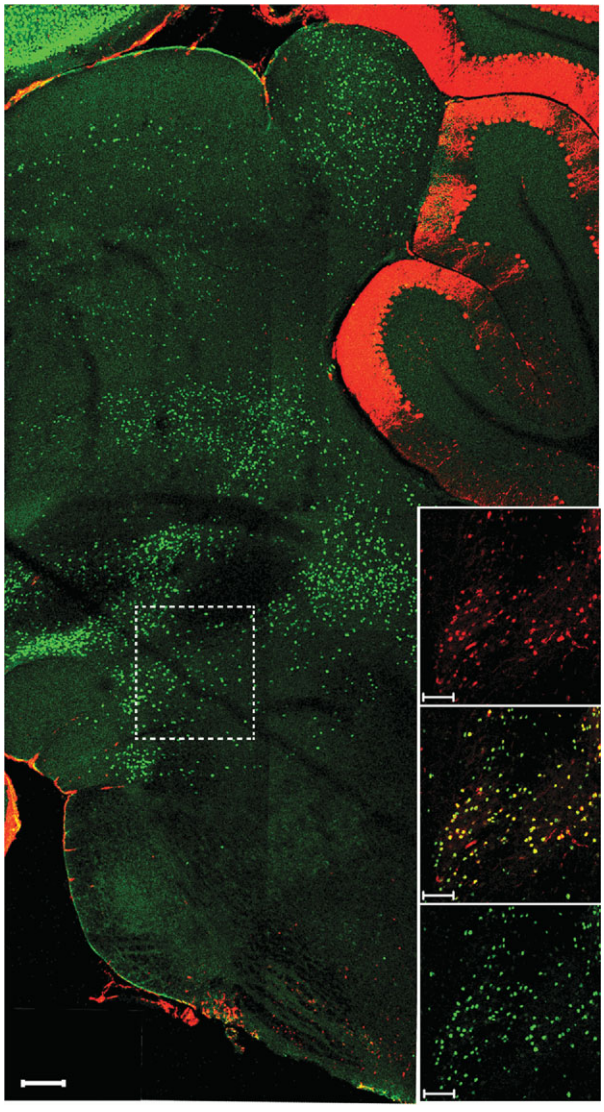

Figure 7. Transgene expression in the brainstem. Simultaneous detection of EGFP fluorescence (green) and immunofluorescence for calbindin (red) in midsagittal cryosections from I-MeCP2(CamKII) mice. Scale bar: 200 μm. The insets show magnification of calbindin-positive neurons (red, top panel) in the raphe nucleus expressing the transgene (green, bottom panel). The middle panel shows the merged image. Scale bar: 100 μm.

development of phenotypes of *Mecp2* null mice (18). Also, the lack of phenotype prevention is probably not due to species-specific differences in the human and mouse proteins, since we have previously shown that the expression of transgenic human MeCP2 was able to prevent lethality in *Mecp2* null mice (16).

An explanation for the inability of the CamKII- or Eno2-driven transgene to prevent the phenotypes of the *Mecp2* mutants might be that the levels of transgene expression are not suitable to achieve full MeCP2 function. Although the level of expression of one of the transgenic lines analyzed is similar to the endogenous expression of MeCP2 as measured by western blot, the amount of endogenous MeCP2 is variable depending on the neurons considered, and the expression of the transgenic MeCP2 does not recapitulate this variable pattern, generating cell-specific deficits and excesses in transgene expression levels. Alternatively, brain areas such as the midbrain, or the brain stem, in which the transgenes are not expressed in our mice, might be 'the' relevant areas for RTT clinical manifestations (34,35). The latter possibility is less likely given that CamKII-Cre deletion of MeCP2 suffices to cause a Rett-like phenotype. Therefore, the data suggest that Rett is caused by disruption of a neuronal network that includes the CamKII 'domain' but also extends outside of it. Thus, restoration of MeCP2 in only a portion of the network is not enough to rescue the complete function of the network, whereas perturbation of particular components of the network suffices to alter its function. Hence, a more widespread expression of MeCP2 in the CNS might be necessary to obtain full rescue.

Our data differ markedly with a recent report of CamKII promoter-directed reactivation of MeCP2 at postnatal day 15 that resulted in partial amelioration of Rett-like symptoms and a small extension of life span (36). The discrepant results could stem from the use of different mutant alleles; the *Mecp2* null mice that we used in this study lacked exons 3 and 4, whereas Giacometti *et al*. (36) used mice with deleted exon 3. In addition, the activity and cellular specificity of the CamKII transgenic promoters used in the two studies could differ due to different sites of transgene integration, and dissimilar transgene composition and copy number, factors known to affect transgene expression (37). Also, strain background differences could account for the dissimilar results.

Our results indicate that phenotypes in patients with RTT result either from dysfunction of neurons in the brain stem and hypothalamus or from dysfunction of a neuronal network that involves neurons located throughout the brain.

MATERIALS AND METHODS

Transgenic mice

The *MECP2*-pBi-EGFP transgene was generated by subcloning a full-length human MECP2 cDNA into the pBi-EGFP vector (Clontech). Subsequently, *MECP2*-pBi-EGPF was inserted between insulators into pJ13-1 and linearized to release vector sequences. The linear fragment was introduced into pronuclei of FVB mice by microinjection. Genotyping of the progeny was performed by PCR and Southern blot. To generate conditional MeCP2-expressing mice, *MECP2*-pBi-EGFP transgenic mice were crossed with transgenic mice that express tTA under the control of either the promoter for the α subunit of calcium/calmodulin-dependent protein kinase II [B6;CBA-TgN(CamK2tTA)1Mmay, from Jackson Laboratories] or the promoter for the NSE [B6.Cg-TgN(Eno2tTA)5021Nes, from Jackson Laboratories].

PCR and Southern blot analysis

To isolate genomic DNA for PCR and Southern blot analyses, mouse-tail biopsies were subjected to proteinase K digestion, followed by ethanol precipitation. After preliminary screening with PCR amplification, genotypes were further confirmed by

Southern blot analysis. Briefly, 20 μg of purified genomic DNA was digested with restriction enzymes, fractionated on 0.7% agarose gels, and transferred onto nylon membrane (Hybond *N*+; Amersham). DNA was hybridized with a ^{32}P-labeled probe at 65°C in hybridization buffer. Non-specific signals were removed by stringency washes in 2 × 1× and 0.1× SSC buffer. Hybridization signals were detected by X-ray autoradiography.

Western blot

Protein was extracted from dissected tissue by Dounce homogenization in TSTE buffer [150 mM NaCl, 50 mM Tris, pH 7.5, 0.5% Triton X-100, 2 mM EDTA and 1× protease inhibitor cocktail (Sigma P-8340)], incubated on ice for 4–6 h, centrifuged at 14 000*g* and supernatant pulled to a fresh tube, stored at −80°C till use. Equal protein was loaded and analyzed by standard western blot protocols. An affinity-purified polyclonal antibody against EGFP (Novus Biologicals) was used at a 1:1000 dilution. A rabbit polyclonal C-terminal antibody against MeCP2 (UPSTATE) was used at a 1:1000 dilution. Western blots were developed using Pierce Super-Signal West Dura Extended Duration Substrate, according to the manufacturer's directions.

Immunofluorescence

Mouse brains were fixed by transcardial perfusion followed by overnight immersion in PBS-buffered 4% formaldehyde. The brains were embedded in OCT (Tissue Tek) and cryosectioned (50 μm). Sections were either analyzed directly for EGFP fluorescence or blocked for 1 h in 2% normal goat serum and 0.3% Triton X-100 in PBS. After blocking, the sections were incubated for 48 h at 4°C in blocking solution containing primary anti-MeCP2 (Upstate) (1:100), anti-tyrosine hydroxylase (Chemicon) (1:250), anti-ACTH (1:200), anti-neurophysin (1:100), or anti-GFP (Novus Biologicals) (1:150) antibodies. The sections were washed four times in PBS and were incubated for 48 h in blocking solution containing anti-rabbit-conjugated Alexa Fluor 488 (Molecular Probes), or anti-rabbit-conjugated Cy3 (Jackson Immunoresearch) used at a dilution of 1:500. The expression levels of transgenic versus endogenous MeCP2 were compared by normalizing the immunolabeling with the use of the NeuN antibody. Serial brain sections from I-MeCP2 (CamKII); Mecp2$^{-/y}$ and from Mecp2$^{+/y}$ mice containing only the *MECP2*-pBi-EGFP transgene were labeled with either anti-NeuN (1:200) or anti-MeCP2 antibodies. The same neuronal groups were identified and the intensity of NeuN immunolabeling was compared with the intensity of MeCP2 immunolabeling in brains from both genotypes (two mice of each genotype). The antibody concentrations used in this experiment turned the intensities of NeuN and endogenous MeCP2 immunolabeling similar, which facilitated the determination (we counted approximately 40 neurons for each brain region).

Reverse transcriptase–polymerase chain reaction

Total RNA was extracted with Trizol according to the method provided by the manufacturer. RNA was treated with DNase I for 30 min at 37°C and reverse-transcribed at 42°C for 50 min by using random hexamers and ThermoScript (Invitrogen) to synthesize single-stranded cDNA. PCR reactions were carried out to detect *MECP2, EGFP* and β-actin mRNAs. Primers used for PCR were 5′- CACGGAAGCTTAAG-CAAAGG -3′ and 5′-AGTCCTTTCCCGCTCTTCTC-3′ for *MECP2* and 5′-TGGATAGCAACGTACATGGC-3′ and 5′-ATTCCTATGTGGGCGACGAG-3′ for β-actin. Control RT reactions were carried out without reverse transcriptase.

Behavioral testing

Stereotypic forepaw movements Mice were suspended by the tail for 10 s and the presence or absence of stereotypic forepaw movements was scored.

Body tremor Mice were held on the hand of the examiner for 10 s and the presence or absence of body tremor was recorded.

Suspended wire test Mice were suspended by their forepaws on a 2 mm wire, and the amount of time they remained on the wire was recorded. Statistical significance was determined using Student's *t*-test.

Rotating rod test Mice were placed on the accelerating rotarod apparatus (Ugo Basile) for 12 trials (three trials on four consecutive days) with a 30–60 min rest interval between trials. Each trial lasted for a maximum of 10 min, during which the rod accelerated linearly from 3 to 30 r.p.m. The amount of time for each mouse to fall from the rod was recorded for each trial. If the mouse held on to the rod and passively rotated 360°, the time of the second rotation was reported as the time of falling off the rod. Analysis was performed using a two-way ANOVA (genotype × trial) with repeated measures (VassarStats).

Dowel test Mice were placed in the center of a horizontal dowel (0.7 cm diameter), and the time they remained on the dowel was recorded. If mice walked across and off the dowel, they were placed back onto the dowel. Trials lasted for a maximum of 2 min. Data were analyzed using Student's *t*-test.

Novel arena test Mice were placed in the center of an elevated (22 cm) platform (28 × 28 cm^2). Activity was quantified by measuring the amount of time the mice displaced themselves on the field, with the use of a stopwatch. Each test session was 5 min long. Analysis of data was performed using one-way (genotype) ANOVA and Tukey's HSD test for pairwise comparisons of sample means (VassarStats).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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