Mild Overexpression of Mecp2 in Mice Causes a Higher Susceptibility toward Seizures

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An intriguing finding about the gene encoding methyl-CpG binding protein 2 (MeCP2) is that the loss-of-function mutations cause Rett syndrome and duplication (gain-of-function) of MECP2 leads to another neurological disorder termed MECP2 duplication syndrome. To ensure proper neurodevelopment, a precise regulation of MeCP2 expression is critical, and any gain or loss of MeCP2 over a narrow threshold level may lead to postnatal neurological impairment. To evaluate MeCP2 dosage effects, we generated Mecp2WT_EGFP transgenic (TG) mouse in which MeCP2 (endogenous plus TG) is mildly overexpressed (approximately 1.5×). The TG MeCP2WT_EGFP fusion protein is functionally active, as cross breeding of these mice with Mecp2 knockout mice led to alleviation of major phenotypes in the null mutant mice, including premature lethality. To characterize the Mecp2WT_EGFP mouse model, we performed an extensive battery of behavioral tests, which revealed that these mice manifest increased aggressiveness and higher pentylenetetrazole (PTZ)-induced seizure propensity. Evaluation of neuronal parameters revealed a reduction in the number of tertiary branching sites and increased spine density in Mecp2WT_EGFP transgenic (TG) neurons. Treatment of TG neurons with epileptogenic compound-PTZ led to a marked increase in amplitude and frequency of calcium spikes. Based on our ex vivo and in vivo data, we conclude that epileptic seizures are manifested as the first symptom when MeCP2 is mildly overexpressed in mice. (Am J Pathol 2013, 183: 195e210; http://dx.doi.org/10.1016/j.ajpath.2013.03.019)

The MECP2 gene encodes for methyl-CpG-binding protein 2 (MeCP2). MeCP2 binds to methylated CpG DNA sequences and modulates transcription of genes in the vicinity of the methylated sequence.1 The ability of MeCP2 to bind to the methylated CpGs, and its predominant localization to the highly methylated heterochromatin regions of the nucleus,2 led researchers to propose that MeCP2 may act as a global transcriptional repressor.3 Subsequently, the majority of studies substantiated the role of MeCP2 in repression of transcription (reviewed in Chahrour and Zoghbi4 and Guy et al5). Furthermore, MeCP2 was shown to interact with Sin3A and HDAC (histone deacetylases).6 By recruiting HDAC at the methylation loci, MeCP2 further represses transcription at such sites. However, recent studies have also highlighted the role of MeCP2 in transcriptional activation. It was shown that MeCP2 associates with actively transcribed genes.6,7 MeCP2 promotes activation of transcription by associating with the transcriptional activator CREB1 (cAMP responsive element binding protein 1) on an active but not repressed form of the same gene.6 A multifaceted role of MeCP2 was further corroborated by the discoveries of its involvement in RNA splicing and chromatin compactions.8,9

Mutations in MECP2 gene have been shown to cause a neurodevelopmental disorder known as Rett syndrome (RTT).10 Classically, RTT patients develop normally until the age of 6 to 18 months. After this normal phase of development, RTT patients fall into a stage of developmental stagnation, followed by rapid regression. The patients start...
showing symptoms, such as progressive loss of motor skills, stereotypic hand movements, breathing abnormalities, microcephaly, epileptic seizures, and social interaction impairments reminiscent of autism.11 Due to X-chromosome inactivation, females are mosaics for MECP2 mutations, which can have a mitigating effect, with phenotypes ranging from classic RTT to asymptomatic individuals.12 However, male patients carrying MECP2 mutations usually show a very severe phenotype. They typically suffer from neonatal encephalopathy and die in early childhood.13 Until now, more than 700 MECP2 mutations have been reported for RTT (https://portal.biobase-international.com/hgmd/pro/all.php, HGMD Professional 2012.4, last accessed December 14, 2012). All types of mutations have been reported, such as missense, nonsense, splice site, deletion, and insertion. This wide spectrum of MECP2 mutations suggests loss-of-function as the likely common pathomechanism for RTT.

In addition to these loss-of-function mutations in the MECP2 gene, which cause RTT, until now more than 100 cases of copy number gains of the MECP2 gene have been reported.14,15 In the majority of cases, duplication of MECP2 locus was observed, however, in several instances, triplication was also detected.16,17 The gain of MECP2 lead to a neurological disorder termed MECP2 duplication syndrome, which primarily affects males who display variable phenotypes. Common symptoms include infantile hypotonia, severe mental retardation, autism or autistic features, poor speech development, recurrent infections, progressive spasticity, ataxia, and epilepsy.14,15,16 MECP2 duplication is 100% penetrant in males.14 The triplication of the MECP2 locus causes a more severe phenotype.17,18 Most of the reported MECP2 duplications are inherited, however, de novo cases have also been reported.18,20,21

Previously, a TG mouse (MECP2-Tg1) was generated that overexpresses MeCP2 at twice the level of endogenous MeCP2.22 The phenotypes of these mice are reminiscent of the human MECP2 duplication syndrome. Until the age of 10 to 12 weeks, these TG mice appear normal. Afterward, they develop progressive neurological symptoms that include stereotyped and repetitive movements, motor dysfunction, epilepsy, spasticity, ataxia, hypoactivity, kyphosis, and premature death.23 Increasing the copy numbers of MeCP2 in the TG mice, resulting in higher MeCP2 levels, causes even more severe phenotypes.22 Interestingly, in another study, evaluation of a MeCP2fox allele revealed that due to the modification of the 3’UTR of MeCP2 by insertion of a neomycin cassette, the expression of MeCP2 was reduced by 50%.23 These MeCP2fox/Y mice manifest a spectrum of disabilities, such as learning and motor deficits, decreased anxiety, and breathing dysfunction.

Overall, it is evident that the central nervous system is highly sensitive to the functional level of MeCP2 and any gain or loss of MeCP2 over a narrow threshold level can lead to postnatal neurological impairment. This precise regulation of MeCP2 expression to ensure proper neurodevelopment has important ramifications regarding devising potential therapeutic strategies for correcting the expression level of MeCP2 in RTT patients. To study the pathomechanism underlying the neurological impairment in MECP2 duplication syndrome, a mouse model with only mild overexpression of MeCP2 may help to identify and characterize the earliest pathologies elicited due to gain-of-function properties of MECP2. Therefore, we generated MeCP2WT-EGFP TG mice, which mildly overexpress MeCP2 (approximately 1.5×).

Materials and Methods

Generation of MeCP2WT-EGFP Transgenic Mouse Line

A bacterial artificial chromosome (BAC) clone, plasmid (p)BAC_B22804 containing approximately 120 Kb of murine genomic fragment with intact MeCP2 gene and the flanking Opsin1 and Irak1 genes was used for generating the TG construct.24 To generate pBAC_Mecp2WT-EGFP construct, the enhanced green fluorescent protein/kanamycin-resistant gene (EGFP/Kan) cassette was PCR-amplified using pEGFP1 vector as the template with primers containing 50 bp flanking sequence from either side of the MeCP2 stop codon. Furthermore, the endogenous stop codon was replaced by two glycine residues in frame between the MeCP2 protein and the EGFP protein to facilitate the two proteins to fold and function independently. The amplified EGFP/Kan cassette was electroporated into Escherichia coli harboring the BAC clone and pGET recombination system25 to facilitate the homologous recombination of EGFP/Kan cassette at the site of stop codon of MeCP2. The correct insertion of EGFP/Kan cassette after the recombination event into the BAC clone was confirmed by sequencing. The Mecp2 flanking genes, Opsin1 and Irak1 were deleted from the modified BAC clone using additional BAC recombineering with the Zeo selection cassette (containing the BAC homology arms and Zeocin (InvivoGen, Toulouse, France) antibiotic marker gene driven by EM7 promoter from pSELECT vector) to avoid any additional phenotype arising from the overexpression of these genes. During the process of Opsin1 deletion, a Mlu I restriction site was introduced into the BAC clone.

The final BAC construct pBAC_Mecp2WT-EGFP was linearized with the Mlu I restriction enzyme and micro-injected into the male pronuclei of the fertilized mouse oocytes derived from the FVB/N strain. Next, the injected oocytes were transplanted into the uteri of the foster mothers. The genomic DNA isolated from tail biopsies of newborn pups were analyzed for the presence of the transgene by PCR analysis, as previously described.24

Western Blot Analysis

For Western blot experiments, total protein extracts were isolated from brain biopsies of fresh male adult mice. In brief, the brain biopsies were collected in ice cold PBS and transferred to cold lysis buffer (20 mmol/L PIPES [piperazine-N,N’-bis(2-ethanesulfonic acid)], 2 mmol/L...
EGTA, 1 mmol/L EDTA, 1 mmol/L DTT, and 0.3 mmol/L phenylmethylsulfonyl fluoride) containing protease inhibitors (Roche, Mannheim, Germany) followed by homogenization using tissue micromotor. The lysates were sonicated on ice and the supernatant was collected after centrifugation at 12,000 g for 10 minutes at 4°C. An aliquot of 50 μg protein lysates were denatured by boiling in a buffer containing SDS and dithiothreitol, which were resolved by SDS-PAGE using a NuPAGE 4 to 12% Bis-Tris precast gel in 2-(N-morpholino)ethanesulfonic acid buffer (Invitrogen, Karlsruhe, Germany). The resolved proteins were electrotransferred onto a nitrocellulose membrane (Amersham Biosciences, Braunschweig, Germany). Afterward, the membrane was blocked in 5% lyophilized milk powder in Tris-buffered saline-Tween (100 mmol/L Tris-Cl, pH 7.5; 150 mmol/L NaCl; 0.1% Tween 20) and then incubated with either anti-MeCP2 antibody (dilution 1:8000) raised in rabbit using MeCP2-strep tag fusion protein (InMeubl Laccolith, Luxembourg) at room temperature (Karlsruhe, Germany) with subsequent incubation with anti-MeCP2 antibody (dilution 1:5000) at 4°C overnight. The following day after washing, the immunoblot was incubated with secondary antibody conjugated with alkaline phosphatase (dilution 1: 10,000) (Sigma-Aldrich, Munich, Germany) for 1 hour at room temperature. Finally, Western blot analysis was developed by using nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Carl-Roth, Karlsruhe, Germany). The intensity of the protein bands in the Western blot analysis were quantified using ImageJ software version 1.46 (NIH, Bethesda, MD).

RNA Preparation and RT-qPCR Analysis

Total RNA was extracted from the cerebellum and hippocampus of 6-week-old male mice using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. The cDNA was synthesized from 2 μg of RNA using the SuperScript II (Invitrogen, Karlsruhe, Germany) and oligo-dT primer in a total volume of 20 μL. For real-time quantitative RT-PCR (RT-qPCR) analysis, the cDNA was further diluted (1:8) and was used as a template in Platinum SYBR Green qPCR SuperMix-UDG with ROX system (Invitrogen, Karlsruhe, Germany) with MeCP2 gene specific primers (Mecp2_F; 5’-TGTTAGCTGGGATGTTAGGG-3’ and Mecp2_R; 5’-CCTTCTTCTTCTTCTCGCC-3’). The RT-qPCR reactions were run in 7900 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). The housekeeping gene, β-actin ampolcon was used for data normalization. The RT-qPCR experiments were repeated four times with three technical replicate. The data were analyzed by SDS software version 2.2 (Applied Biosystems, Darmstadt, Germany) using standard curve method and Microsoft Excel version 2010 (Immeuble Laccolith, Luxembourg).

Immunohistochemistry

The adult male mice were transcardially perfused with 4% paraformaldehyde in PBS for 3 hours at 4°C. The whole brain was dissected and submerged in 15% sucrose for 1 hour at 4°C followed by incubation in 30% sucrose solution for 3 days, and then cryosectioned to 40 μm thickness. The brain sections were washed three times with PBS for 5 minutes each and then permeabilized with 0.2% Triton-X for 30 minutes at 4°C. Thereafter, the slides were washed again three times with PBS for 5 minutes and incubated in 4% bovine serum albumin and 0.1% Tween-20 (Promega, Mannheim, Germany) for 1 hour at room temperature. The brain sections were then incubated with anti-MeCP2 antibody (1:200; Eurogentec, Cologne, Germany) at room temperature for 1 hour in 2% bovine serum albumin solution. After washing steps, the sections were incubated with secondary antibody conjugated with Cy3 (Sigma-Aldrich) for 1 hour at room temperature in 2% bovine serum albumin solution. After final washing, the slides were air-dried and VectaShield solution (Linaris, Wertheim, Germany) was applied before mounting the slides with coverslips. The sections were observed under a BX60 fluorescence microscope (Olympus, Hamburg, Germany).

Animal Management and Behavioral Analysis

All experiments have been approved by the local Animal Care and Use Committee. Mice used for the experiments were kept in our environmental unit under a temperature-controlled environment (21 ± 2°C) on a 12-hour light/dark cycle with food and water ad libitum, unless stated otherwise. A battery of behavioral tests were performed on preweaning and postweaning stages of mice. In all experiments, the experimenter (M.T.) was unaware (blinded) of the genotype.

Drug Used in Animal Experiments

Pentylenetetrazole (PTZ; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), a noncompetitive GABA antagonist with epileptogenic properties was dissolved in saline for subcutaneous injection.

Preweaning (Neonatal) Assessments

Male TG mice were bred with FVB/N wild-type (WT) female mice to obtain WT and MeCP2 TG animals. A daily inspection for the presence of new litters in the cages was carried out twice a day, and the day a litter was first observed was scored as day 0 for that litter. After birth, animals were kept untouched in the home cage with their mothers until postnatal day (P) 3, and at P3 animals feet were tagged. Neurodevelopmental evaluation tests were performed daily starting from P4 until P21. The battery of tests provides an assessment of development throughout the neonatal period and the behaviors measured are expressed at different periods throughout the first 21 days of life. These tests have been extensively described elsewhere26–28 and consist of observations covering the maturation of physical landmarks, as well as developmental milestones (Supplemental Figure S1). Weaning was performed at 22 days of age. For the assessment of preweaning behavior, 14 male WT and 18 male TG litters were used.
Postweaning (Adult) Behavior
Breeding pairs of Mecp2 TG male and WT female FVB/N mice were used to obtain WT and Mecp2 TG mice. Shortly after weaning, mice were transferred to the behavioral unit for testing. On arrival and during the whole period of behavioral testing, mice were housed individually in standard plastic cages (26.5 × 20 × 14 cm). Adult behavioral testing was performed starting at the age of 5 weeks. The animal numbers of individual experiments are given in the legend of each corresponding figure.

Experimental Design

Neonatal Assessments
Neonatal assessments were comprised of three domains: i) maturation measures targeting physical development, ii) neurodevelopmental measures targeting the development of neurological reflexes, and iii) the development of neuro-motor coordination.

Maturation Measures (Physical Development)

Body weight. The body weight of each pup was registered daily, starting from P4 through P21.

Ear opening. The day when an opening in the ear was visualized, the mouse was registered.

Eyes opened. The day when the eyes were opened, the mouse was registered. An eye was considered open when any visible break in the membrane was noticed.

Neurodevelopmental Measures

Surface righting reflex. Animals were restrained on their back on a table and then released. The time needed for each pup to right itself was recorded and the performance was monitored for 3 consecutive days, starting from P4. Score 0 was assigned when pup did not show surface righting reflex and score 1 when surface righting reflex was present.

Postural reflex. Starting on P7 onward, animals were put in a small box and shaken up and down, left and right. Animals were tested until an appropriate response (animals splaying their four feet) was observed and scored as 1.

Cliff avoidance. Observed daily from P6 until each test pup, when placed on an edge with forepaws and nose just over the edge, showed retraction within 10 seconds. Then score 1 was assigned.

Negative geotaxis reflex. Observed daily from P7 onward, animals were put in a head-down position on a 30° inclined surface. The response of each pup was observed for 30 seconds and registered as shown in Supplemental Figure S1.

Neuromotor Coordination Measures

Wire suspension. The animals were forced to grasp a 3 mm wire and hang from it on their forepaws. Mice were tested starting on P10 onward until all test pups were able to hold the wire for 30 seconds.

Swimming ability. Mice were tested on P8, P12, and P14. On each observation day, one at a time, test pups were placed in a tank of water (30°C) for a period of 5 to 15 seconds and observed for three aspects of swimming: i) direction, ii) head position relative to water surface, and iii) use of limbs. Scoring for direction was: sank, 0; floated, 1; swam in circle or arch, 2; straight, 3. Scoring for angle: submerged, 0; nose at the surface, 1; nose and top head at or above the surface but ears still below the surface, 2; similar to the last except for waterline is now at mid-ear level, 3; nose and top of head and ears above the surface, 4. Scoring for limb usage: no paddling, 0; paddling with all four limbs, 1; paddling with hind limbs only and forelimbs held stationary, 2.

Adult Behavior

Elevated Plus Maze
The mouse was placed in the central platform, facing an open arm of the plus maze inside a room with light intensity set to 130 lx. Behavior was recorded by an overhead video camera and a computer equipped with Viewer software version 2 (Biobserve GmbH, Sankt Augustin, Germany) to calculate the time each animal spends in open or closed arms. The proportion of time spent in open arms was used for estimation of open arm aversion (fear equivalent).

Open Field
Spontaneous activity in the open field was tested in a gray Perspex arena (120 cm in diameter, 25 cm high) (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) placed inside a room with light intensity set to 130 lx. The mouse was positioned in the center and allowed to explore the open field for 7 minutes. The behavior was recorded by a computer-linked overhead video camera. Viewer software was used to calculate velocity, distance traveled, and time spent in central, intermediate, or peripheral zones of the open field.

Hole Board
The hole board test measures exploratory activity. The apparatus consisted of a transparent Perspex chamber (21 × 21 × 36 cm) with a nontransparent floor raised 5 cm above the bottom of the chamber with 16 equally spaced holes (2 cm in diameter). Mice were allowed to explore the chamber for 5 minutes and the number of explored holes (head dips) was monitored by 2 layers of infrared photo beams connected to a computer with ActiMot software version 2 (TSE Systems GmbH, Bad Homburg, Germany). The light intensity was set to 5 lx during the test phase.

Pre-Pulse Inhibition Test
In this test of sensorimotor gating, individual mice were placed in small metal cages (90 × 40 × 40 mm) to restrict major movements and exploratory behavior. The cages were equipped with a movable platform floor attached to a sensor that records vertical movements of the floor. The cages were placed in four sound-attenuating isolation cabinets (TSE Systems GmbH). Startle reflexes were evoked by acoustic stimuli delivered from a loudspeaker that was suspended above the
cage and connected to an acoustic generator. The startle reaction to an acoustic stimulus, which evokes a movement of the platform and a transient force resulting from this movement of the platform, was recorded with a computer during a recording window of 260 msec (beginning with the onset of prepulse) and stored for further evaluation. The recording window was defined from the onset of the acoustic stimulus.

An experimental session consisted of a 2-minute habituation to 65 dB background white noise (continuous throughout the session), followed by a baseline recording for 1 minute at background noise. After baseline recording, 6 pulse-alone trials using startle stimuli of 120 dB intensity and 40 msec duration were applied to decrease influence of within-session habituation. These data were not included in the analysis of the prepulse inhibition. For tests of prepulse inhibition, the 120 dB/40 msec startle pulse was applied either alone or preceded by a prepulse stimulus of 70 dB, 75 dB, or 80 dB intensity at 20 msec duration. An interval of 100 ms with background white noise was used between each prepulse and pulse stimulus. The trials were presented in a pseudorandom order with an interval ranging from 8 to 22 seconds. The amplitude of the startle response (expressed in arbitrary units) was defined as the difference between the maximum force detected during a recording window and the force measured immediately before the stimulus onset. Amplitudes were averaged for each individual animal, separately for both types of trials (ie, stimulus alone or stimulus preceded by a prepulse). Prepulse inhibition was calculated as the percentage of the startle response using the following formula:

\[
\text{Prepulse inhibition} \% = 100 - \left( \frac{\text{[startle amplitude after prepulse and pulse]}}{\text{[startle amplitude after pulse only]}} \right) \times 100. \tag{1}
\]

**Rota-Rod**
Rota-rod is a test for motor function, balance, and coordination, and comprises a rotating drum (Ugo Basile Srl, Comerio, Italy) that is accelerated from 4 to 40 revolutions per minute over the course of 5 minutes. Each mouse was placed individually on a drum and the latency of falling from the drum was recorded using a stopwatch. To assess motor learning, the rota-rod test was repeated 24 hours later.

**Grip Strength**
A grip strength meter (TSE Systems GmbH) was used to assess forelimb grip strength. Mice were lifted and held by their tail so that their forepaws could grasp a wire grid. The mice were then gently pulled backward by the tail with their posture parallel to the surface of the table until they released the grid. The peak force applied by the forelimbs of the mouse was recorded in gram-force. Each mouse performed the test three times, and the average of the three trials was used for statistical analysis.

**Assessment of Hearing**
Individual mice were placed in small metal cages (90 × 40 × 40 mm) to restrict major movements and exploratory behavior. The cages were equipped with a movable platform floor attached to a sensor that records vertical movements of the floor. The cages were placed in four sound-attenuating isolation cabinets (TSE Systems GmbH). Startle reflexes were evoked by acoustic stimuli delivered from a loudspeaker that was suspended above the cage and connected to an acoustic generator. The startle reaction to an acoustic stimulus (pulse), which evokes a movement of the platform and a transient force resulting from this movement of the platform, was recorded with a computer during a recording window of 100 msec and stored for further evaluation. The recording window was defined from the onset of the acoustic stimulus. An experimental session consisted of a 2-minute habituation to 65 dB background white noise (continuous throughout the session), followed by a baseline recording for 1 minute at background noise. After baseline recording, stimuli of different intensity and fixed 40 msec duration were presented. Stimulus intensity was varied between 65 dB and 120 dB, such that 19 intensities from this range were used with 3 dB step. Stimuli of the each intensity were presented 10 times in a pseudorandom order with an interval ranging from 8 to 22 seconds. The amplitude of the startle response (expressed in arbitrary units) was defined as the difference between the maximum force detected during a recording window and the force measured immediately before the stimulus onset. Amplitudes of responses for each stimulus intensity were averaged for individual animals. Mean values for each experimental group were plotted on the graph to provide the stimulus-response curves.

**Visual Cliff Test**
This test was used to assess more profound visual deficiencies (eg, in contrast perception). The test apparatus consisted of an open-topped box (70 × 35 cm floor, 30 cm high). The walls of the box were made from white Perspex, and the base was made from clear Perspex. The box was positioned on the edge of a laboratory bench so that half of the base was placed on the bench (ground side), and the other half over the edge of the bench, suspended 1 m above the floor (air side). Mice were placed in the middle of the base at the edge of the cliff, and their activity was monitored for 5 minutes by a computer and the video-tracking system Viewer 2 (Biobase GmbH). The percentage of time each mouse spent on the ground and the air side of the box was calculated. The light intensity inside the room was set to 150 lx.

**Olfaction Test**
In the first 3 days mice were habituated to the test cages (29.5 × 18.5 × 13 cm); mice were placed in the cages for 2 × 20 minutes per day. On day 3, mice were deprived of food for 24 hours before testing, with water *ad libitum* and a chocolate cookie was placed within the home cage. From days 4 to 6, mice were again placed into test cages with...
a chocolate cookie inside (2 × 20 minutes). After habituation was finished on each day, mice received food for 1 hour per day within their home cages. On day 7, the test took place, and individual mice were placed into test cages, in which a piece of a chocolate cookie was hidden under 1.5 cm standard bedding at the end of the cage. The mouse was positioned in the right corner at the opposite end of the cage, and the food-finding time (ie, the time from the moment the mouse was placed into the cage to the time it located the cookie and initiated burrowing) was recorded. As soon as the cookie was uncovered, the mouse was not allowed to consume it and was removed from the cage. To control for other sensory, motor, or motivational deficiencies that might influence the food-finding latency, the test was repeated as previously described with a piece of chocolate cookie clearly visibly placed on the bedding. A fresh cage and bedding was used for each trial, and all mice were subjected to identical testing procedures. Cut off time was set to 3 minutes.

**Sucrose Preference Test**

The evaluation of anhedonia-like behavior was assessed using the sucrose preference test, which was performed using a two-bottle procedure. During the sucrose preference test, mice have free access to both water and a sucrose solution. Animals were first habituated for 48 hours to consume water from the two small (100 mL) bottles. After habituation, mice were deprived of water and the sucrose preference was measured during the next 3 days. The first two days served as a habituation to sucrose solution. The results of day 3 were used for the evaluation of sucrose preference. Each day, single-housed mice were presented for 60 minutes with two bottles (one with tap water and one with 2% sucrose solution) in the home cage. Consumption of water or sucrose solution was measured by weighing the bottles before and after the session. Bottles were counterbalanced across the left and the right sides of the cage, and their position was alternated from test to test. Sucrose preference (percentage) was calculated as follows:

\[
\text{preference} = \frac{\text{sucrose solution intake (mL)}}{\text{total fluid intake (mL)}} \times 100
\]  

**Resident Intruder**

Intermale aggression was studied in the resident-intruder paradigm. As standard opponent males, we used group-caged males of the same age, also from the same FVB/N strain. A standard opponent was introduced into the home cage of the tested resident male and observation started when a tested resident male sniffed the opponent for the first time. The observation was stopped immediately after the first attack (an attack being defined as a bite) to prevent wounding, but lasted 5 minutes if no attack occurred. The latency of attack was recorded by a stop watch.

**PTZ-Induced Seizure**

The method used here has been previously described by Ferraro et al.\(^3\)\(^0\) Seizure activity was induced in wakeful mouse using a single subcutaneous injection of 50 mg/kg body weight PTZ. After injection of the compound, the mouse was placed in a small, clear home cage and was closely observed for 30 minutes. Latencies to focal [partial clonic (PC)], generalized [generalized clonic (GC)], and maximal [tonic-clonic (TC)] behavioral seizures were recorded. Furthermore, four phases in the continuum of behavioral response to subcutaneous PTZ injection were defined as follows: i) hypoactivity (a progressive decrease in motor activity until the animal came to rest in a crouched or prone position with the abdomen in full contact with cage bottom); ii) partial clonus (PC) (clonus seizure activity affecting face, head, and/or forelimb or forelimbs); iii) generalized clonus (GC) (sudden loss of upright posture, whole body clonus involving all four limbs and tail, rearing and autonomic signs); and iv) tonic-clonic (TC) (maximal) seizure (generalized seizure characterized by tonic hind-limb extension, also associated with death).

Finally, latencies to PC, GC, and TC seizures were summed to assign each mouse a seizure score that was used as a quantitative trait measure for mapping according to the following equation:

\[
\text{Seizure score} = \left[ (0.2)\left( \frac{1}{\text{PC latency}} \right) + (0.3)\left( \frac{1}{\text{GC latency}} \right) + (0.5)\left( \frac{1}{\text{TC latency}} \right) \right] \times 1000
\]  

The weighing factors (0.2, 0.3, and 0.5) in the equation were included as means of incorporating a measure of the progressive nature of the PTZ-induced seizure phenotype into the severity rating because GC is regarded as a more significant event than PC, and TC (hind-limb extension) is regarded as the most severe component of the phenotype. Therefore, the seizure score reflects the degree of progression of the seizure phenotype in each mouse.\(^3\)\(^0\)

**Hind Limb Clasping Test**

To quantify hind-limb claspings,\(^3\)\(^1\) P40 stage mice were picked up from the cage by tail and suspended 10 cm above the cage for 30 seconds to allow the clasping of the hind limbs. During this time mice were videotaped. The video was analyzed, and the duration of hind-limb clasping was calculated for each mouse.

**Primary Neuronal Culture and Immunocytochemical Analysis**

For neuronal cell preparation, brains from three P7 stage male mice were collected and put into dissection solution (0.72 g bovine albumin, 0.347 g MgSO\(_4\) in 240 mL Hanks
medium) on ice. The hippocampus was dissected from the whole brain, the meninges were removed, and the tissue samples were cut into small pieces and incubated in Hanks solution for 5 to 10 minutes on ice.

The tissue samples were then digested at 37°C for 10 minutes with 6 mg of trypsin and 60 μg of DNAsse (2000 U/µL) in 2 mL of digestion solution (0.8 g NaCl, 35 mg NaHCO3, 37 mg KCl, 99 mg Na2HPO4 in 100 mL of H2O, pH approximately 7.3 to 7.4). The reaction was stopped by addition of 2.4 mg of trypsin inhibitor (Sigma-Aldrich) in 4 mL of dissection solution and incubated at room temperature for 10 minutes. The samples were then incubated in a solution of 0.5 mL of horse serum and 2.5 mL of dissection solution for 10 minutes at room temperature. The digested hippocampal cells were triturated with three different sizes of pipettes coated with Sigmacote (Sigma-Aldrich).

The homogenate was centrifuged at 100 × g at 4°C for 10 minutes. The cells obtained were resuspended in 1 mL of culture medium A [0.5 g D-glucose, 0.2 mL (12.5 mg/mL) insulin (Sigma-Aldrich), 1 mL (200 mmol/L) GLUTAMAX (Gibco, Paisley, UK), 50 μL (10 mg/mL), gentamicin, 10 mg transferring, and 10 mL horse serum up to 100 mL with minimal essential medium Eagle (Sigma-Aldrich). The cell suspension was seeded (4000 to 8000 cells per well) in preincubated culture plates with coverslips coated with poly-D-lysine and laminin (BD Bioscience, Heidelberg, Germany) in culture medium A and incubated at 37°C in 5% CO2. The medium was replaced after overnight incubation. After 4 days, culture media was changed to medium B [50 μL gentamicin (Sigma-Aldrich), 125 μL bFGF (Invitrogen, Karlsruhe, Germany), 250 μL L-Glutamine, 2 mL B27 supplement (Gibco, Darmstadt, Germany), 125 μL cytosine arabinoside (4 mmol/L) up to 100 mL with Neurobasal medium (Gibco, Darmstadt, Germany)]. The medium was changed every 2 days until the day of neuronal cell analysis, when cells were fixed and immunostained with anti-microtubule-associated protein 2 (MAP2) antibody (Cell Signaling, Frankfurt, Germany) (dilution 1: 500) (AbaCam, Cambridge, UK) and visualized by the BX60 fluorescence microscope (Olympus, Hamburg, Germany).

The evaluation of neuronal parameters, such as cell soma area and number of primary, secondary, and tertiary branch sites, was performed by ImageJ software and the total neurite lengths were measured by NeuronJ software version 1.4.2 (http://www.imagescience.org/meijering/software/neuronj, last accessed March 2012). The analysis was performed with the experimenter (C.B.) being unaware (or blinded) of the genotype. The average data were derived from WT and TG mice (9 to 10 each).

Sholl Analysis

To evaluate the complexity of a neuron, such as dendrite distribution and arborization, we performed Sholl analysis on captured images of primary neurons. In brief, concentric circles (Scholl lines) were drawn at 5 μm increments up to a radius of 250 μm from the centroid of the neuron soma until the tip of the longest neurite. The numbers of crossings made by neurites originating from soma through each Scholl line were calculated by ImageJ software. The crossings of neurites at each Scholl line were summed and the means and SD were calculated.

Measurements of Calcium Influx in Primary Neurons

Primary culture of hippocampal neurons was prepared as previously described. The coverslips with neurons were placed on the microscope stage (at room temperature) in a chamber continuously superfused at 34°C at 1 mL per minute with HEPES-buffered salt solution (136 mmol/L NaCl, 5 mmol/L KCl, 1.25 mmol/L CaCl2, 0.8 mmol/L MgCl2, 6 mmol/L glucose, 10 HEPES, pH 7.4). The experimental solutions were produced by adding aliquots of corresponding stock solutions directly to the chamber solution. The neurons were treated with 1 μmol/L fura-2-AM (calcium indicator dye, Invitrogen, Karlsruhe, Germany) for 20 minutes. The cells were viewed through a 40× objective in an upright Axioskop2 microscope (Zeiss, Goettingen, Germany). Excitation (380 nm) was generated by light-emitting diode and the fluorescence (520 ± 19 nm) was recorded with a cooled CCD camera. Images were captured at intervals of 0.5 seconds, and analyzed using MetaMorph software (Roper Scientific, Planegg/Martinsried, Germany). Free calcium values (C) were obtained according to Grynkiewicz et al as:

\[
C = K \frac{1 - f}{f - R} \tag{4}
\]

where the dissociation constant for calcium binding to fura-2 is \( K = 0.22 \) μmol/L. \( f = F/F_{max} \) The maximal fluorescence \( F_{max} \) was measured after application of 1 μmol/L ionomycin in calcium-free solution and \( F_{min} \) was obtained after introduction of 5 mmol/L calcium into the chamber. The calibration gave \( R = F_{min}/F_{max} = 0.21 \pm 0.02 \).

To examine slow calcium buffering in neurons, we induced calcium loads by depolarization-activated calcium influx with 50 mmol/L KCl and followed calcium recovery to the basal levels. The time-constants of calcium recovery, together with depolarization-induced calcium increases and resting calcium levels allow to assess the contribution of mechanisms involved in calcium regulation. The mean values of time-constants, resting, and elevation of calcium were determined in the WT and TG cells. PTZ tests were applied to examine responses of neurons to epileptogenic stimuli. Calcium changes were recorded for 12 to 16 neurons in the image field. We determined the amplitude and frequency of calcium spikes, counted the number of active neurons, and averaged the data for six biological replicates of WT and Mecp2WTEGFP TG neuron.
Golgi-Cox Staining

The whole mouse brains were dissected from 3 weeks old mice and Golgi staining was performed using the FD Rapid GolgiStain Kit (FD Neurotechnologies, Columbia, MD). The dissected brains was rinsed with double distilled water and then immersed in a Solution A/B mixture (impregnation) for 6 to 7 days at room temperature. Afterward, the brains were incubated in Solution C (cryoprotection) for 2 days and thereafter frozen in anisopentane/dry ice bath. The brains were immediately sectioned into 100 to 150 μm slices using a CM 1900 cryostat (Leica, Wetzlar, Germany) and the slices were mounted on slide pre-coated with 0.4% gelatin (Sigma-Aldrich). After the samples were dried, they were dehydrated by immersing them in solution with incremental proportion of ethanol. The staining procedure for development of the black precipitate was performed using kit reagents according to the manufacturer instruction. Afterward, coverslips were mounted using Eukitt quick for development of the black precipitate was performed using kit reagents according to the manufacturer instruction. Afterward, coverslips were mounted using Eukitt quick hardening mounting medium (Fluka, Neu Ulm, Germany) and the slides were visualized using the BX60 microscope (Olympus, Hamburg, Germany).

The morphology of pyramidal neurons was traced from the captured images through a graph pad by using NeuronJ software and neuronal parameters were evaluated as described above. The spine density was calculated as average number of spines sprouting from a block unit of 10 μm of neurite. The average data were derived from WT and TG mice, 5 to 6 each.

Statistical Analysis

For neuronal parameters evaluation, the differences between WT and Mecp2WT_EGFP transgenic neurons were calculated by applying Wilcoxon’s Rank sum statistics or, if appropriate, by using the Student’s t-test. We set the overall level of significance to 5%. For the number of branching sites as the mainly focused phenotypes were corrected for multiple testing according to Bonferroni assuming four independent tests (area, length, number of spines per 10 μm, and branching points). Data are represented as means ± SD in both the figures and text.

In the preweaning behavior assessment, regression analyses were used to compare the performance between TG and WT littermate control mice: variables scored 0 or 1 were analyzed by logistic regression. Additionally, Fisher’s exact test for categorical data on each observation day and two-way repeated measures analysis of variance including Bonferroni post hoc test were used where applicable.

Statistical significance for adult behavioral phenotyping was evaluated using the U-test and two-way repeated measures analysis of variance including the Bonferroni post hoc test where applicable. A critical value for significance of $P < 0.05$ was used throughout the study. Data are represented as means ± SEM in both the figures and text.

For RT-qPCR data, the statistical significance between WT and Mecp2WT_EGFP TG mice were calculated by Student’s t-test.

All statistical analyses were performed with either GraphPad Prism 4.0 (La Jolla, CA) or SAS Version 9.2, SAS Institute Inc. (Cary, NC) or SPSS for Windows, version 17.0 (Chicago, IL).

Results

Reducing Expression of MeCP2 from Mecp2WT_EGFP Allele

The Mecp2WT_EGFP TG mouse was generated by micro-injection of linearized pBAC_Mecp2WT_EGFP construct (Figure 1A) into fertilized oocytes of FVB/N mice. For the generation of the pBAC_Mecp2WT_EGFP construct, we used a BAC clone (B22804). This BAC contains approximately 120 kb of a mouse genomic fragment, which consists of the complete Mecp2 gene including all four exons, as well as flanking/regulatory sequences. The BAC clone also contains the complete transcriptional unit of Opsin1 gene, which is 5’ upstream of Mecp2 and part of the Irak1 gene, which is located downstream 3’ end of the Mecp2 gene. The sequences of Opsin1 and Irak1 genes were deleted from the BAC clone by GET recombination using the Zeocin selection cassette to exclude any toxic effect in the TG animals due to duplication of these genes (Figure 1A).

The pups obtained after microinjection were tested for the integration of the transgene into the genomic sequence by PCR genotyping using a primer pair specific for the transgene. Through PCR genotyping, several founders were identified, however, subsequent breeding and transmission analysis of Mecp2WT_EGFP allele among the founders led to the establishment of one successful TG line. From the breeding of this TG line with FVB/N mice, we obtained equal numbers of male and female mice suggesting that the transgene was integrated into an autosomal chromosome. The Mecp2WT_EGFP TG mouse line was maintained in a heterozygote state (T WT/−) by backcrossing with FVB/N mice. From such breeding, we obtained 50% TG mice (T WT/−) and 50% WT littermates.

To determine whether the MeCP2WT_EGFP is expressed in the TG mice, we performed Western blot analysis with total protein extracted from the brain biopsies from the adult Mecp2WT_EGFP TG male mice. The immunoblot with antibody against MeCP2 revealed expression of two MeCP2 proteins. In addition to the endogenous MeCP2 (approximately 70 kDa) band, a TG MeCP2WT_EGFP band of approximately 100 kDa in size was detected in the brain of TG mice. In contrast, only endogenous MeCP2 was detectable in WT mice (Figure 1B). Moreover, immunoblot analysis with green fluorescent protein antibody detected the approximately 100 kDa MeCP2WT_EGFP protein, only in TG mice (Figure 1B). From Western blot analysis, it is obvious that the intensity of MeCP2WT_EGFP
TG protein is remarkably lower as compared to endogenous MeCP2 band. This observation prompted us to quantify the relative intensity of the TG protein to endogenous protein by ImageJ software. Densitometrical quantification of the protein bands intensities from five independent immunoblots revealed that relative expression of MeCP2WT_EGFP was approximately 40\% to that of endogenous MeCP2 (Figure 1C). To determine whether the reduced expression MeCP2WT_EGFP protein from the transgene was due to lower amount of mRNA, we performed quantitative real-time PCR analysis. Indeed, we also detected a reduced level of TG mRNA (Figure 1D).

To determine the cellular expression pattern of the MeCP2WT_EGFP protein, we performed immunohistochemical analysis of brain sections. MeCP2WT_EGFP protein was

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**Figure 1** Expression analysis of MeCP2 from the BAC transgene. A: Schematic diagram illustrating the generation of the pBAC_Mecp2WT_EGFP construct using GET recombination in the BAC clone. An EGFP/Kan cassette was inserted in frame at stop codon of Mecp2 in exon 4 and was replaced with two glycine residues in the BAC clone. The sequences of Opsin1 and Irak1 genes were deleted from the BAC clone by GET recombination using the Zeocin selection cassette. B: Western blot analysis using MeCP2 antibody on the total protein extract isolated from brain samples revealed in addition to the endogenous MeCP2 (70 kDa) an additional MeCP2 protein corresponding to MeCP2WT_EGFP of size approximately 100 kDa in the TG adult mice. Additionally, immunoblots with anti-GFP antibody confirmed that the approximately 100 kDa size protein was indeed MeCP2WT_EGFP. Furthermore, Western blot analysis with brain extract of rescued mice (IWT/-: -/y), generated by breeding Mecp2WT_EGFP TG mice with Mecp2 knockout mice revealed only expression of MeCP2WT_EGFP of size of approximately 100 kDa. C: Quantification of the protein bands intensities from at least five immunoblots revealed that relative expression of MeCP2WT_EGFP was approximately 40 ± 4.1\% to that of endogenous MeCP2. D: Quantitative real-time PCR analysis suggests that reduced expression of TG MeCP2WT_EGFP is due to the diminished level of mRNA. E: Immunohistochemical analysis of hippocampus showed a similar expression pattern for the TG MeCP2WT_EGFP protein as compared to endogenous MeCP2. KO, knockout mice adult mice. Scale bars: approximately 10 kb (A); 50 μm (E).
visualized by green fluorescent protein fluorescence and MeCP2 antibody staining, which also detected endogenous MeCP2 protein. The expression pattern of MeCP2\textsubscript{WT,EGFP} protein was similar to that of endogenous MeCP2 (Figure 1E and Supplemental Figure S2).

**Prolonging Lifespan of \textit{Mecp2} Null Mutant Mice on Expression of \textit{Mecp2}\textsuperscript{WT,EGFP} Allele**

We bred \textit{Mecp2}\textsuperscript{WT,EGFP} TG mice with \textit{Mecp2} knockout mice\textsuperscript{31} to express the TG allele in \textit{Mecp2} null background (T\textsuperscript{WT,; };y). Western blot analysis with MeCP2 antibody using total protein extracted from the brain revealed expression of MeCP2\textsubscript{WT,EGFP} protein only (approximately 100 kDa) (Figure 1B). Remarkably, the premature lethality at the age of approximately 10 weeks, reported for null mutants, was rescued by the expression of the TG allele. These mice have a prolonged life span, as our observation over a period of approximately 9 months did not detect any early deaths. Hindlimb clumping, a typical feature of \textit{Mecp2} null mutant mice, was also absent in these mice (Supplemental Figure S3).

\textit{Mecp2}\textsuperscript{WT,EGFP} Transgenic Mice Exhibit Increasing Aggressiveness and Seizure Propensity

The TG mice appeared normal on cursory observation. To characterize the behavioral phenotype of \textit{Mecp2}\textsuperscript{WT,EGFP} TG mice, we performed a series of behavioral tests at both preweaning (0 to 3 weeks) and postweaning (after 5 weeks onward) stages.

**Pre-Weaning Behavior Analysis**

The number of animals used in the analysis of maturation markers and neurodevelopmental measures was: \textit{Mecp2} TG (TG) male \textit{(n = 18)}, WT littermate male \textit{(n = 14)}.

**Maturation Measures**

**Body Weight**

Body weight of animals was taken daily from P4 through P21 and the data were analyzed with two-way repeated measurement analysis of variance. We did not find any significant differences between genotypes in the body weight measured (Supplemental Table S1). As expected, all mice progressively increased their body weight as we observed from P4 through P21 (data not shown), indicating a comparable growth rate between TG and WT littermates.

**Ear and Eye Opening**

The day when at least one eye was open and the day an aperture was seen in the ear was registered daily starting from P4. No differences existed between genotypes regarding the day of the ear and the eye opening (Supplemental Table S1). All test mice have their ears and eyes fully opened on P14 (data not shown).

**Neurodevelopmental Measures (Neurological Reflexes and Neuromotor Coordination)**

There were no significant differences observed between groups in terms of the neurological reflexes tested in surface righting, postural, cliff avoidance, and negative geotaxis reflex (Supplemental Table S1). We also observed a comparable performance between groups during the acquisition of neuromotor coordination assessed from wire suspension and neonatal swimming activity (Supplemental Table S1).

**Postweaning (Adult) Behavior Analysis**

Adult behavioral phenotyping of male TG mice with mild \textit{Mecp2} overexpression, as well as their WT littermates, was performed beginning at the age of 5 weeks (Table 1). Behavioral analysis was performed with less stressful tests at the beginning and more stressful tests toward the end in the following order: elevated plus maze, open field, hole board, prepulse inhibition, rota-rod, hearing, visual cliff, sucrose preference, grip strength, olfaction, resident intruder, and finally, PTZ-seizure induction. The number of animals used in the adult behavioral analysis is given in the corresponding figure legends of each read out. For behavioral analyses, we used 10 to 27 WT and 6 to 22 TG mice (dependent on the complexity of the test, lower or higher numbers of mice are needed).

**Table 1** Summary of Phenotypes in Adult \textit{Mecp2}\textsuperscript{WT,EGFP} Transgenic Mice

<table>
<thead>
<tr>
<th>Domain</th>
<th>Behavioral tests</th>
<th>WT versus TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic behavioral functions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity (locomotion)</td>
<td>Open filed</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Anxiety</td>
<td>Elevated plus maze</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Exploratory</td>
<td>Hole board</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Anhedonia</td>
<td>Sucrose preference</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Seizure susceptibility</td>
<td>PTZ-induced seizure</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Motor functions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motor learning and coordination</td>
<td>Rota-rod</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Muscle strength</td>
<td>Grip strength</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Sensory functions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olfaction</td>
<td>Food finding</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Vision</td>
<td>Visual cliff</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Hearing</td>
<td>Hearing (PPI set up)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Social behavior</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggression</td>
<td>Resident intruder</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Sensorimotor gating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensorimotor gating</td>
<td>Prepulse inhibition</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>

Mild MeCP2 overexpression does not alter basic behavior, motor, sensory, or sensorimotor gating readouts in mice, but leads to alterations in social behavior (aggression) and seizure activity. Wild-type (WT) mice = 10 to 27; transgenic (TG) mice = 6 to 22. Data are presented as means ± SEM. PPI, prepulse inhibition; PTZ, pentylenetetrazole.

The American Journal of Pathology
Mild Overexpression of Mecp2 Selectively Influences Behavioral Alterations

Although basic behavior, sensory, and motor functions, as well as sensorimotor gating remain unchanged by mild overexpression of Mecp2, our results revealed that social behavior and seizure propensity of TG animals overexpressing Mecp2 were altered (Table 1, Figure 2, and Supplemental Figure S4). During daily handling of the animals, we observed that mice displayed a higher tendency to bite when handled. We performed the resident-intruder paradigm to investigate whether mild Mecp2 overexpression was associated with an increased aggressive behavior. Indeed, tested at the age of 8 weeks, we found that TG mice were more aggressive compared to the WT littermates (Figure 2A). The latency of the first attack of TG mice toward the intruder was significantly reduced, compared to their WT littermates (P = 0.048).

We also repeatedly observed spontaneous seizures in the TG mice. Thus, to assess seizure susceptibility, we induced seizures by administering PTZ (50 mg/kg s.c. of body weight).30 We detected increased PTZ-induced seizure activity in 30-week-old TG mice (P = 0.042) compared to their WT littermates (Figure 2B).

Mild Mecp2 Overexpression Causes Increase in Soma Size and Fewer Branching Sites in Cultured Neurons

The ex vivo cultured hippocampal neurons are considered a well-characterized model for the evaluation of neuronal development.37 We cultured hippocampal neurons from P7 Mecp2WT_EGFP TG and WT male mice to determine the effect of mild overexpression of Mecp2 during the process of neuronal development and differentiation. The primary neurons were cultured for 14 days, then immunostained with anti-MAP2 antibody (Figure 3, A and B). MAP2 is a neuron-specific cytoskeletal protein that is enriched in dendrites.38 The stained neurons were traced and analyzed by ImageJ software for calculating the soma size. The neuronal parameters, such as total length of neurites, number of neuritis, and number of primary, secondary, and tertiary branch sites, were evaluated by NeuronJ software.

The mean soma size of 91 TG neurons was slightly larger compared to those of 83 WT neurons (P = 0.0008) (Figure 3C). However, no significant difference could be seen for the total length of neurites projecting from TG neurons (P = 0.1536) (Figure 3D). Analysis of branching sites of neurites revealed an average lower number of branching sites in TG neurons, although this was clearly significant only for tertiary sites (primary sites: P = 0.6000, secondary sites: P = 0.0496, and tertiary sites: P < 0.0001) (Figure 3, E and G). To further evaluate the complexity of neurons, we performed Sholl analysis on the traced images.34 Sholl analysis is used to evaluate the morphological complexity of imaged neurons by counting the number of neurite intersections for concentric circles of increasing radius arising from the cell soma (Supplemental Figure S5). This quantitative analysis revealed diminished apical neurite branching in TG neurons.

Pyramidal Neurons of Mecp2WT_EGFP Transgenic Mice Show Higher Spine Density

To evaluate the effect of mild Mecp2 overexpression on neurons in vivo, we visualized the hippocampal neurons by Golgi-Cox staining of tissue sections. The Golgi-Cox staining allows high resolution visualization of dendrites and spines in the histological section, thus enabling us to calculate the qualitative and quantitative parameters of neurons.39–41 The stained pyramidal neurons (Figure 4A) were reconstructed by semiautomatic tracing using NeuronJ software (Figure 4B) and were analyzed similarly, as previously described for cultured neurons. We did not observe significant difference in soma size between TG and WT neurons (Figure 4C), although the total length of TG neurites was increased (Figure 4D). To determine the spine density of neurites, we used higher magnification images of basal dendrites from CA1 region (Figure 4E). Spine density for neurite unit length of 10 µm revealed, on average, a slightly higher density of spines in TG neurites (mean, 14.4 µm) than WT neurites (mean, 13.6 µm; P = 0.0324) (Figure 4F).

Calcium Homeostasis in Mecp2WT_EGFP Transgenic Neurons

Calcium responses of cultured primary hippocampal neurons were evaluated by applying depolarization-induced calcium influx (50 mmol/L KCl) and IP3-mediated calcium release from endoplasmic reticulum after activation of P2Y receptors with 1 mmol/L ATP.42 Our measurements revealed that TG neurons in comparison to WT neurons, had higher levels of resting calcium (0.075 ± 0.05 µmol/L versus 0.052 ± 0.03 µmol/L), calcium elevations due to depolarization were also higher (0.34 ± 0.08 µmol/L versus 0.25 ± 0.06 µmol/L), and the amplitudes of ATP-driven calcium release were smaller.
(0.21 ± 0.04 μmol/L versus 0.31 ± 0.05 μmol/L) (Figure 5, C and D). The time course of calcium recovery in TG neurons was distinctly slower, both after depolarization and calcium release (Figure 5). The mean time constant of calcium recovery in WT was 6.1 ± 0.4 seconds (Figure 5A), and in TG cells it was approximately three times longer (18.6 ± 1.2 seconds) (Figure 5B). We further observed that TG neurons were more prone to spontaneous calcium oscillations that may indicate intrinsic differences in the vulnerability toward external stimuli. Spontaneous calcium spiking was seldom observed in WT neurons (6 of 97 cells [6%]), whereas in TG neurons, spontaneous activity was encountered more frequently (12 of 98 cells [12%]).

In the behavioral test, the TG mice manifested a higher propensity toward PTZ-induced seizure activity (Figure 2C), we evaluated the response of cultured hippocampal neurons to PTZ. Spontaneous and elicited calcium spikes (indicators of spontaneous discharges of the neurons) are usually considered as a correlate of epileptic seizures.33,44 The WT neurons rarely showed spontaneous calcium transients and 10 mmol/L PTZ-induced only a weak effect (Figure 5E). The TG neurons frequently demonstrated spontaneous calcium spikes whose amplitude and frequency were markedly increased after PTZ treatment (Figure 5F).

Discussion

Evaluation of the quantitative effect of MeCP2 expression level on phenotypes is highly relevant, as duplication of
MECP2 causes severe neurological impairment,\textsuperscript{14,18,22} and on the contrary, loss of function mutations causes Rett syndrome.\textsuperscript{10} It is evident that precise regulation of MeCP2 expression is essential for proper neurodevelopment. To determine the consequence of MeCP2 dosage, we generated a TG Mecp2\textsuperscript{WT_EGFP} mouse model mildly overexpressing MeCP2. The BAC construct used for the generation of Mecp2\textsuperscript{WT_EGFP} TG mice consist of complete promoter and regulatory elements necessary for the spatiotemporal expression pattern of MeCP2.\textsuperscript{22,24} In addition, to exclude the possibility of interference in the phenotype of Mecp2\textsuperscript{WT_EGFP} mice due to overexpression of Opsin1 and Irak1, we deleted both genes in the BAC construct. When expression pattern of MeCP2\textsuperscript{WT_EGFP} from the transgene was compared with endogenous MeCP2, we observed a similar expression pattern, thus confirming that the BAC transgene mimics the endogenous MeCP2 expression. Additionally, in a previous study, we showed that expression pattern of a TG MeCp2\textsuperscript{270_EGFP} (generated from the same BAC clone) was comparable to the endogenous protein.\textsuperscript{24} In the pBAC_Mecp2\textsuperscript{270_EGFP} construct, the EGFP/Kan cassette was inserted after 270 amino acids into exon 4 of MeCP2. Overall, the MeCP2\textsuperscript{270_EGFP} TG protein is equivalent to human p.R270X mutation.\textsuperscript{24}

Interestingly, using Western blot analysis, the intensity of TG MeCP2\textsuperscript{WT_EGFP} protein band was much weaker than endogenous MeCP2. Quantification of protein bands intensity revealed that the expression level of TG protein was nearly half (40 ± 4.1\%) as compared to the endogenous MeCP2 protein. To determine whether the reduced expression from the transgene was due to lower level of mRNA or post-transcriptional regulation, we performed RT-qPCR analysis. Our data indicates that the diminished level of mRNA may be the underlying reason for lower level of TG MeCP2\textsuperscript{WT_EGFP} protein. A previous study has shown that modification of 3' UTR of MeCP2 by insertion of a neomycin cassette and polyadenylation sequence in MeCp2\textsuperscript{floX} allele causes 50\% reduction of expression as compared to the endogenous MeCP2 protein.\textsuperscript{23} During the generation of Mecp2\textsuperscript{WT_EGFP} TG mice, we also modified the 3' untranslated region of MeCP2 by insertion of an EGFP/Kan cassette, which could be the underlying cause for reduced expression. However, a regulatory inhibition (at least partially) at the site of TG integration cannot be completely excluded.
and further investigation will be necessary to exclude any late stage symptoms.

*Mecp2*<sup>WT_EGFP</sup> TG mice appear phenotypically normal and were negative for hind limb claspings (observation over a 9-month period). To identify any relevant behavioral symptoms in these mice, we performed a battery of tests involving basic and social behavior, motor performance, sensory functions, and sensorimotor gating. Our investigation revealed that in most behavioral parameters the performance of TG mice were comparable to that of WT siblings, and with the progression of age, there was no deterioration in the symptoms. However, the animals showed increased aggression and propensity toward PTZ-induced seizures. The phenotype of *Mecp2*<sup>WT_EGFP</sup> TG mice (approximately 1.5×) was in stark contrast to the previously reported *MECP2*-Tg1 mice (2×) that manifested severe neurological phenotypes. In *MECP2*-Tg1 mice display stereotyped and repetitive movements, motor dysfunction, epilepsy, spasticity, ataxia, hypoactivity, kyphosis, and premature death.22 In *MECP2*-Tg1 mice, the phenotypes appear at an early stage (10 to 12 weeks) and deteriorate progressively. Overall, by means of our generated mouse model expressing approximately 1.5× the level of MeCP2, we could reinforce the notion that the effect of MeCP2 on neurodevelopment is quantitative. Furthermore, unlike doubling of MeCP2 dosage, which causes severe neurological impairment, only approximately 1.5× the level of overexpression of MeCP2 elicits a milder phenotype.

The expression level of MeCP2 is critical for early neuronal development and MeCP2 function is vital even at a single-neuron level.15 To assess the effect of mild overexpression (approximately 1.5×) of MeCP2 on neurons, we evaluated neuronal parameters of cultured neurons. We observed a slight but statistically significant increase in soma size. The reduced number of tertiary branching sites in *Mecp2*<sup>WT_EGFP</sup> TG as compared to WT neurons was also statistically significant. When we analyzed the neuronal parameters of Golgi-Cox stained, we observed no difference in soma size. This discrepancy could be due to sensitivity of two different experimental systems used for neuronal parameters. The morphological features of neurons are explicitly visible in primary culture; therefore, even subtle changes can be calculated. However, exogenous conditions in the primary culture can also create distortion, which might not be representative to an *in vivo* form. Remarkably, in Golgi-Cox stained neurons, we detected a slight increase (statistically significant) in spine density in the TG neurons. Previous studies have shown that loss of MeCP2 leads to substantial reduction in spine density,46–48 thus, it is reasonable to expect enhanced spine density during MeCP2 overexpression. Overall, it seems that mild overexpression of MeCP2 subtly deters the maturation of neurons.

Furthermore, we observed an impaired calcium homeostasis with a slower time course of calcium decay in *Mecp2*<sup>WT_EGFP</sup> TG neurons, which was manifested by an increase in basal calcium levels, prolongation of calcium
recovery to basal levels after brief depolarization, and a smaller amount of calcium released from intracellular stores in TG neurons. The calcium imbalance could be the underlying cause for the observed exaggerated response of the TG neurons to epileptogenic compound-PTZ.\(^{30,44}\) Improper calcium handling can make these neurons more vulnerable to external stimuli encountered during early development. A similar disturbance in calcium homeostasis was also observed in Mecp2 null mutant neurons,\(^{49}\) which suggest that both lack and overexpression of MeCP2 may lead to deleterious effect.

It is interesting to note that epileptic seizures are a major phenotype reported both in RTT and MECP2 duplication syndrome. Remarkably, MECP2 disruptions also lead to neuropsychiatric disorder, such as Angelman-like syndrome, and in many instances these patients also manifest epileptic seizures.\(^{50,51}\) In this context, it is noteworthy that the primary phenotype observed in the Mecp2\(^{30,44}\) TG mice was an increased propensity to epileptic seizures, as observed in our behavioral tests, as well as at the resolution of single neurons (ex vivo). In both in vivo and ex vivo settings, the seizure activity was induced by epileptogenic compound-PTZ, and in the TG condition, the induction was remarkably stronger.

In conclusion, it is evident from our study that epileptic seizure propensity is highly sensitive to the functional level of MeCP2 and it appears as a primary phenotype in the mouse due to mild overexpression of MeCP2. In future gene therapy approaches to correct the MeCP2 expression either in RTT or MECP2 duplication syndrome, the optimum dosage of the treatment may have to be determined so that epilepsy does not occur as a consequence.

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Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2013.03.019.

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