A partial loss of function allele of Methyl-CpG-binding protein 2 predicts a human neurodevelopmental syndrome

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Rett Syndrome, an X-linked dominant neurodevelopmental disorder characterized by regression of language and hand use, is primarily caused by mutations in methyl-CpG-binding protein 2 (MECP2). Loss of function mutations in MECP2 are also found in other neurodevelopmental disorders such as autism, Angelman-like syndrome and non-specific mental retardation. Furthermore, duplication of the MECP2 genomic region results in mental retardation with speech and social problems. The common features of human neurodevelopmental disorders caused by the loss or increase of MeCP2 function suggest that even modest alterations of MeCP2 protein levels result in neurodevelopmental problems. To determine whether a small reduction in MeCP2 level has phenotypic consequences, we characterized a conditional mouse allele of Mecp2 that expresses 50% of the wild-type level of MeCP2. Upon careful behavioral analysis, mice that harbor this allele display a spectrum of abnormalities such as learning and motor deficits, decreased anxiety, altered social behavior and nest building, decreased pain recognition and disrupted breathing patterns. These results indicate that precise control of MeCP2 is critical for normal behavior and predict that human neurodevelopmental disorders will result from a subtle reduction in MeCP2 expression.

INTRODUCTION

Rett Syndrome (RTT, OMIM #312750) is an X-linked neurodevelopmental disorder characterized by regression of language and hand use after a period of normal initial cognitive development (1). During this regression, autistic features can manifest, sometimes leading to the misdiagnosis of autism. After regression, characteristic clinical features such as distinctive hand stereotypes, movement abnormalities, breathing irregularities, autonomic dysfunction, seizures and sleep disruption become prominent. The disorder primarily affects girls at a frequency of 1:10 000–20 000 live female births (2).

Mutations in Methyl-CpG-Binding Protein 2 (MECP2) are found in over 95% of typical RTT (3), with ~70% of these cases caused by point mutations. MeCP2 primarily functions as a transcriptional repressor (4,5) by recruiting histone deacetylases to DNA that contains the epigenetic mark of methylated cytosines (6). Point mutations in MECP2 behave similarly to a complete deletion of the coding sequence of MECP2, indicating that they are complete or partial loss of function alleles (3). In females, these point mutations cause features characteristic of RTT; in contrast, the same point mutations may result in severe infantile encephalopathy and early death in males (7). Additionally, a number of point mutations in MECP2 not associated with RTT have been identified in males with moderate mental retardation, movement abnormalities and psychiatric features (8–16). These mutations are often found in X-linked mental...
retardation (XLMR) families with the female carriers displaying mild mental retardation or learning disabilities.

The discovery that mutations in MECP2 cause the neurodevelopmental disorder RTT led to the analysis of MECP2 in other neurodevelopmental disorders with similar clinical features. For example, MECP2 mutations have subsequently been discovered in girls with a number of disorders such as idiopathic autism (17), Angelman-like syndrome (15) and mental retardation (18). In addition, MeCP2 expression is decreased in the brains of individuals with neurodevelopmental disorders such as autism, Down syndrome, Angelman syndrome and Prader–Willi syndrome (19,20). Interestingly, duplications of the genomic region spanning MECP2 have been discovered in humans who have neurological abnormalities (21–26). Features of this disorder are typically found in males and are characterized by hypotonia, cognitive impairment, autistic features and language deficits. Of note, the human disorder was initially predicted by abnormalities observed in transgenic mice that over-express Mcp2 2-fold (27). These results point to the importance of MeCP2 expression levels and suggest that neurodevelopmental disorders may result from either increases or decreases in Mcp2 levels.

To determine whether a slight reduction in MeCP2 level resulted in phenotypic abnormalities, we made use of a conditional allele of Mecp2 (‘floxed’) that retains a neomycin selection cassette and polyadenylation sequence within the 3’-UTR (28) which disrupts the Mecp2 3’-UTR isoform that predominates in the brain (29). In many instances, retention of selection cassettes has resulted in the generation of hypomorphic alleles of the engineered locus (30). When tested, we found that the ‘floxed’ allele of Mecp2 caused reduced expression of both Mecp2 mRNA and MeCP2 protein by ~50% and resulted in a broad spectrum of phenotypic abnormalities. This demonstrates that precise Mcp2 levels are critical for neuronal function and that slight reduction in Mcp2 levels in humans is likely to result in clinical abnormalities.

RESULTS

The Mecp2Flox allele decreases mRNA levels of both Mecp2 isoforms and reduces MeCP2 protein level in the brain

To investigate the molecular consequences of the ‘floxed’ Mecp2 allele, we used quantitative real-time PCR (qRT-PCR) to measure the expression level of Mecp2 mRNA in F1 129S6.B6 Mecp2Flox/y animals and wild-type (WT) littermate controls at 26–27 weeks of life. Mecp2 is expressed as two isoforms that differ in the choice of start sites (31,32). In the e1 isoform, exon 1 provides the start site and is spliced to the common exon 3. The e2 isoform is formed by utilizing the start site in exon 2, which is then spliced to exon 3. We designed qRT-PCR primers and probes that either span the exon 1–3 boundary (e1–3) or span the exon 2–3 boundary (e2–3) to quantify the expression of these Mecp2 isoforms. Additionally, we designed a qRT-PCR primer/probe set that is entirely contained within the common exon 3, allowing us to quantify the total Mecp2 transcript level. We find that the Mecp2Flox allele results in an ~50% decrease in the brain in isoform e1, isoform e2 and exon 3 (Fig. 1A).

To assess whether the reduction in Mecp2 mRNA also resulted in a comparable decrease in protein expression, we analyzed whole brain extracts by western immunoblotting (Fig. 1B) from F1 129S6.B6 Mecp2Flox/y animals and WT littermate controls at 26–27 weeks of life. We used an antibody that recognizes the common carboxy-terminus (C-term) of MeCP2. MeCP2 protein levels are decreased in Mecp2Flox/y animals compared with WT littermate controls. Quantification of band intensity normalized to Gapdh revealed that MeCP2 levels in Mecp2Flox/y animals are decreased by 42% compared with WT controls (P < 0.001).

Mecp2Flox/y animals have normal survival and slight weight alterations in certain genetic strain backgrounds

As a complementary approach to examine MeCP2 protein levels and to determine whether the reduction in Mcp2 expression was uniform throughout the whole brain, we analyzed sagittal sections of brain tissue obtained from Mecp2Flox/y animals. Indeed, when identical settings were used to capture confocal images of a WT littermate control animal (Fig. 1C) and an F1 129S6.B6 Mecp2Flox/y animal (Fig. 1D), the overall MeCP2 expression is markedly attenuated in the mutant animals. Higher power magnification (inset in Fig. 1C and D) reveals that MeCP2 retains the same overall punctuate nuclear staining pattern in the mutant animals as the WT animals, but the expression intensity is reduced. We observed similar attenuated Mcp2 levels in animals in the F1 129S6.FVB strain (data not shown).

Mecp2Flox/y animals have decreased motor performance

Despite the lack of lethality and absence of overt hindlimb clasping, we assessed the performance of the mutant mice on a variety of motor tasks. In the accelerating rotating rod task, a test for motor coordination and learning, the animal is subjected to four trials a day for 4 days. The mean latency to fall for each day is recorded. F1 129S6.B6 Mecp2Flox/y animals spend less time on the rod on Day 1, indicating a
baseline deficit in motor coordination (Fig. 2B). Although the mutant animals continue to fall off the rod sooner than WT littermate controls on all 4 days, they exhibit the expected increase in the time spent on the rod over the successive days, demonstrating that they are capable of motor learning. This problem with motor coordination was not apparent in F1 129S6.FVB animals (not shown).

The coordination deficit is also observed when the mutant animals are subjected to two additional motor tasks, the dowel walking task and the wire hanging task. F1 129S6.B6 Mecp2<sup>Flox/y</sup> animals fell off the wire sooner than WT littermate controls (Fig. 2C). The mutant animals also have fewer number of side touches during the wire hanging task (not shown) and for the dowel walking task (Fig. 2D). These differences in motor coordination were not observed in F1 129S6.FVB animals (not shown).

**Mecp2<sup>Flox/y</sup>** animals have decreased pain recognition but intact pain sensitivity

Individuals with RTT have altered pain thresholds (D. Glaze, personal communication); therefore, we tested F1 129S6.B6 Mecp2<sup>Flox/y</sup> animals to determine whether they have alterations in these neurological systems. In the tail flick assay, which assesses the pain sensitivity and is based on a nociceptive response (34), a reflex is generated within the spinal column that does not depend on the pain recognition systems in the brain. Mecp2<sup>Flox/y</sup> animals showed a normal response to the tail flick assay at 40, 50 and 60°C, indicating that the nociceptive reflex is intact (Fig. 3A). However, they show an increased latency to respond when exposed to the hot plate assay, a measurement of pain recognition (Fig. 3A). This requires the transmission of pain sensation between the spinal cord and the brain to generate a muscle response leading to paw withdrawal. Also, 5 out of 15 WT mice (33%) licked their hindlimbs in response to the heated plate. In contrast, mutant mice only displayed hindlimb paw withdrawal. The dissociation between the tail flick and the hot plate seen in these animals suggests a primary deficit in pain recognition rather than a problem with the peripheral sensation of pain. When these assays were performed on F1 129S6.FVB animals, similar results were observed with no difference between mutant WT animals in pain sensitivity, but decreased pain recognition in the mutant animals (not shown).

**Mecp2<sup>Flox/y</sup>** animals have decreased acoustic startle and prepulse inhibition

Sensorimotor gating involves the inhibition of a startle response to a stimulus when that stimulus is shortly preceded by a less intense stimulus (the prepulse). Alterations in this gating system have been characterized in neuropsychiatric conditions such as schizophrenia (35,36). To assess any abnormalities in sensorimotor gating in Mecp2<sup>Flox/y</sup> animals, we used the prepulse inhibition assay. In this assay the animals are exposed to a stimulus (120 dB white noise) and their startle response is recorded. The animals are also...
exposed to increasing levels of prepulse (74, 78 and 82 dB) shortly before the stimulus and their startle response is recorded. The amount of startle to the stimulus after hearing the prepulse is then expressed as a percentage of the startle without the prepulse. F1 129S6.B6 animals have a diminished startle response compared with WT littermate controls (P = 0.001, not shown). However, Mecp2Flox/y animals show increased inhibitory gating at 74 and 82 dB (Fig. 3B). Similar results were observed in the F1 129S6.FVB animals (not shown).

**Mecp2Flox/y animals have altered social behavior**

To assess social behavior, we used the partition test, a test of social interaction without physical contact (37). In this test, the test animal is singly housed for 4 days in a standard mouse cage divided into two equal halves by a partition. The partition is clear and has multiple holes that allow the mouse to see and smell the adjacent chamber. On the fifth day of single housing, an adult conspecific male mouse is placed in the adjacent chamber. The pair is co-housed for at least 18 h. The once novel mouse partner is now considered ‘familiar’ by the test subject. The following day, the time the test mouse spends at the partition engaged in social interest directed at the partner mouse is recorded in three sequential 5 min test encounters: test animal versus the ‘familiar’ mouse, test animal versus a novel ‘unfamiliar’ mouse and test animal versus the same ‘familiar’ mouse. The first two test encounters assess social interest. The last test encounter is a measurement of social recognition and tests the ability of the test animal to recognize their original co-house partner. Using this assay, F1 129S6.B6 Mecp2Flox/y animals spend a larger fraction of the time at the partition interacting with both the unfamiliar mouse and the second exposure to the familiar mouse (Fig. 3C). We have also observed this behavior in Mecp2Flox/y animals in the F1 129.FVB strain background (not shown).

**Mecp2Flox/y animals have hippocampal and amygdala-dependent learning problems**

To characterize the effect of Mecp2Flox allele on learning, we tested F1 129S6.FVB Mecp2Flox/y animals and WT littermate controls on the fear conditioning task. In this task, the animal is placed in a chamber (the context) exposed to a 30 s sound pulse (the cue) before receiving a mild electrical shock. During the training day, the animal is exposed to a 30 s sound pulse (the cue) before receiving a mild electrical shock. During the training day, the animal is re-introduced into the training chamber (context) and the percent time spent freezing (a fear behavior in mice which indicates memory of the context) is recorded. The animal is then exposed to a
chamber with altered visual and odor stimuli. The cue is presented and the percent time freezing is recorded. Mecp2<sup>Flox/y</sup> animals have decreased freezing (Fig. 4A) both when re-exposed to the context (hippocampal dependent) and to the cue (amygdala- and hippocampal-dependent). The learning deficits were not apparent in F1 129S6.B6 animals (not shown).

**Mecp2<sup>Flox/y</sup> animals have altered respiratory patterns**

Because girls with RTT (38) and RTT mouse models (39–41) have disrupted breathing patterns, we analyzed breathing in F1 129S6.FVB Mecp2<sup>Flox/y</sup> animals and WT littermate controls at 4 months of life. The respiratory pattern shows qualitative differences between WT and mutant animals (Fig. 5A and B). There was a marked increase in the incidence of apneas (defined as ~1 s duration with at least two missed breaths) in Mecp2<sup>Flox/y</sup> animals (39.5 ± 3.7 per hour) relative to WT (5.8 ± 0.9 per hour) mice (Fig. 5C). Additionally, the coefficient of variability of the respiratory rhythm was significantly higher in Mecp2<sup>Flox/y</sup> animals (1.6 ± 0.1) relative to WT (0.71 ± 0.07) mice (Fig. 5D).

**DISCUSSION**

Detailed characterization of the Mecp2<sup>Flox/Y</sup> animals revealed a surprising array of phenotypes associated with subtle alterations of MeCP2 levels. The ‘floxed’ allele of MeCP2 results in an ~50% reduction of MeCP2 levels, which is likely secondary to the presence of the neomycin cassette within the 3’-UTR (28,30). This reduction creates a hypomorphic allele of Mecp2, which results in a variety of behavioral and physiological changes such as learning problems, movement abnormalities and breathing dysfunction. These results further underscore the critical importance of the tight regulation of MeCP2 levels. The phenotypic abnormalities of transgenic animals with a mild over-expression of MeCP2 (27) and...
levels by as little as 50%. This decrease in expression may impact the overall conclusion of the paper that a 50% reduction of MeCP2 results in behavioral abnormalities, rather this suggests that such a reduction in MeCP2 is sensitive to modifier effects. This observation is likely to extend to humans and suggests that the array of phenotypes associated with subtle reduction in MeCP2 levels is likely to be broad and that inter-individual clinical variability will be common.

This work also has important ramifications concerning potential therapeutic strategies for RTT. Recently, novel compounds that allow translational read-through of premature stop-codons have been used to treat animal models of muscular dystrophy (42). Because many common RTT causing MECP2 mutations create such premature stop codons (3), interest has developed in utilizing this approach for treatment of RTT. The difference is that whereas the restoration of 40–50% of the expression of a structural protein such as dystrophin might be sufficient to improve the function of muscles, functional restoration in RTT might require expression much closer to WT endogenous levels.

Importantly, this study predicts that human neurodevelopmental disorders will result from a decrease of MeCP2 levels by as little as 50%. This decrease in expression may be the result of sequence changes in the MECP2 locus that occur either in cis (enhancers, promoter or within the 3'-UTR), or possibly via the trans-factors that regulate MeCP2 expression. This has been suggested by studies that found reduced MeCP2 levels in the brain of a number of neurodevelopmental disorders (19,20), but the concern has been that the decreased MeCP2 levels in these post-mortem samples of neurodevelopmental disorders reflects a non-specific decrease in neuronal function rather than a specific finding of the disorders. Other work has identified sequence polymorphisms in the 3'-UTR or MECP2 in individuals with autism (43–45). The challenge with that work has been establishing the functional significance of the sequence polymorphisms. The work described here demonstrates that a 50% decrease in MeCP2 levels might indeed cause disease, and that misregulation of MeCP2 may be a common feature of many neurodevelopmental disorders.

**MATERIALS AND METHODS**

**Animal husbandry**

Mice were maintained on a 12 h light:12 h dark cycle with standard mouse chow and water *ad libitum*. All of the mice used in these experiments were generated by crossing heterozygous female *Mecp2*+/− mice that had been backcrossed to 129S6/SvEvTac for at least five generations to male mice on either a pure FVB/N background or male mice on a pure...
C57BL/6J background to generate isogenic F1 animals. Only male animals were used for all the experiments listed with WT littermate males serving as the controls. Mecp2\(^{\text{Flox/Y}}\) and WT littermate controls were co-housed immediately after weaning. Behavioral experiments were performed with 15–20 mice per genotype with the exception of the open-field assay, which was performed with 10–12 mice per genotype. All research and animal care procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

**Behavior assays**

*Accelerating rotating rod.* Mice were placed on an accelerating rotating rod apparatus (Ugo Basile North America, Inc., Schwenksville, PA, USA) for 16 trials (four trials per day for four consecutive days). The rod accelerated linearly from 3.6 to 36 rpm for the first 4.5 min. Each trial lasted a maximum of 5 min with a 30–60 min inter-trial interval. The latency for each mouse to fall from the rod was recorded for each trial. The mean fall time for each day was calculated.

Data were analyzed using a one-way ANOVA (genotype).

*Dowel walking.* Mice at 11–12 weeks of life were placed in the center of a 0.9 cm wooden dowel balanced between the two wooden poles. The latency to fall, the latency to reach either end of the wooden dowel defined as a ‘side touch’ and the number of side touches were recorded. In the event of a side touch, the timer was stopped, the side touch recorded and the mouse was returned to the center of the dowel. Trials lasted for a maximum of 2 min. Data were analyzed using the Mann–Whitney U-test.

*Wire hanging.* Mice at 11–12 weeks of life were suspended by their forepaws on a 2 mm wire and the time remaining on the wire, the time to first side touch and the number of side touches, as described above, was recorded. Maximum time for each trial was 2 min, and similarly to the dowel, whenever a mouse touched the side wall it was replaced to the center of the wire. Data were analyzed using the Mann–Whitney U-test for fall time and number of side touches.

*Tail flick.* Mice at 16–17 weeks of life were tested for pain sensitivity using the Tail-Flick Analgesia Meter (Columbus Instruments, Columbus, OH, USA). Animals were allowed to acclimate to a plexiglass restraint for 2 min and tails were placed over the sensing groove. Testing commenced upon activation of an intense light beam directed at the tails 4 cm from their base. The latency to observe a tail flick in response to the light beam was recorded. Each animal was tested using...
three different light beam temperature settings (40, 50 and 60°C) presented in random order.

*Hot plate.* Mice at 16–17 weeks of life were tested for pain recognition using the Hot-Plate Analgesia Meter (Columbus Instruments). Animals were placed on a 55°C heated surface. A response to the discomfort served as a functional readout of pain recognition. Responses included hindlimb licking, shaking or twitching. The latency to respond to the heated surface was recorded and the data were analyzed using ANOVA (genotype).

*Prepulse inhibition.* Mice at 15–16 weeks of life were subjected to acoustic prepulse inhibition. The acoustic prepulse inhibition task consists of presenting the animal with two closely paired sound pulses: a prepulse at +0 dB, +4 dB (74 dB), +8 dB (78 dB), +12 dB (82 dB) and over background followed 100 ms later by a pulse of 120 dB. The amount of startle the pulse induces in the animal is recorded using a startle chamber for mice (SR-Lab, San Diego Instruments, San Diego, CA, USA) which records activity for 65 ms after the pulse. The maximum amplitude recorded over the 65 ms is recorded and compared using an ANOVA (genotype) at each prepulse level.

*Partition test.* Test mice at 20 weeks of age were individually housed in standard housing cages for 4 days. Each cage was separated into two compartments by a perforated barrier which allows social interaction without direct physical contact. On Day 5 of individual housing, age- and gender-matched C57BL/6J partner mice were placed into the compartment opposite the test mice. Paired mice were co-housed in the separate halves of the partitioned cage for at least 18 h. Following this period of induced familiarity, the time that test mice displayed directed interest in their partner was recorded during three different paradigms: test subject versus familiar partner, test subject versus unfamiliar partner and repeated test subject versus familiar partner. Each behavioral paradigm was assessed during three 5-min intervals and was performed in sequential order. Data were analyzed using a one-way ANOVA (genotype).

*Nest-building assay.* Singly-housed mice at 19 weeks of life were tested for their ability to build nests. Nest material (Kimwipes, Kimberly Clark, Dallas, TX, USA) was placed in each cage 1 h prior to the onset of the dark cycle and were left undisturbed for 14 h. Nest-building was assessed based on a three-point scale (1 = 0–25% of material shredded, 2 = less than 50% shredded with material gathered in a nest, 3 = fully shredded) with material gathered in a nest. Data were analyzed using a one-way ANOVA (genotype).

*Fear conditioning.* Mice were tested at 22 weeks of life in a chamber that contains a grid floor that can deliver an electric shock (Actimetrics chamber system, Med Associates, St. Albans, VT, USA). On Day 1 of the test, mice were placed within the chamber and left undisturbed for 2 min after which a 30 s white noise sound pulse (‘cue’) was delivered. At the end of the cue, the mouse was shocked (2 s, 0.4 mA). Two minutes later, a second pairing of sound cue followed by shock was delivered. Thirty seconds after the final shock, the animal was removed and replaced in the home cage. The following day, the animals were replaced to the same chamber (‘context test’) and freezing behavior was recorded for 6 min. Freezing behavior was recorded automatically by the instrument. One hour after the context test, the animals were placed into a chamber which had been cleaned with an unfamiliar agent (ethanol) and the wall color, the chamber shape and the odor (artificial vanilla) had been changed to remove the contextual cues of the chamber. The animals were then monitored for 3 min. After 3 min, the white noise cue was started and lasted 3 min. The amount of freezing was recorded separately for the first 3 min and for the last 3 min (cue test). The number of freezing intervals was converted to a percentage of freezing for both the context test and the cue test, and the data were analyzed using a one-way ANOVA (genotype).

*Open-field analysis.* Mice at 12 weeks of life were placed in the center of chamber (40 x 40 x 30 cm) and the activity was measured by photobeams connected to a computer-operated Digiscan optical animal activity system (AccuScan, Columbus, OH, USA). This system measures both X/Y position as well as z-activity (rearing). The test was performed with 60 dB white noise and 150 lux illumination. The activity was measured for 30 min and data were analyzed as three 10 min intervals. The analysis of data was performed using a one-way ANOVA (genotype).

*Quantitative real-time polymerase chain reaction*  
Freshly dissected whole brains (n = 4 per genotype) from 26–27-week-old mice were placed in 2 ml Trizol (Invitrogen, Carlsbad, CA, USA) on ice and immediately homogenized using a Polytron homogenizer at half maximal. The resultant homogenates were processed per the manufacturer’s instructions. RNA was DNase digested and cleaned using the Qiagen RNeasy Mini kit per manufacturer’s instructions (Qiagen Inc., Valencia, CA, USA). First-strand cDNA was synthesized from 5 µg of the purified RNA using SuperScript III (Invitrogen). Quantitative PCR was performed using Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) according to manufactures instructions using Taqman gene expression assays. Primers and Taqman probes were designed to assess *Mecp2* transcript levels with the Primer Express v2.0 software program (Applied Biosystems). The primers and probe sequences are as follows:

*Mecp2e1–3* (forward primer 5’-AGGAGGAGAGACTGGAGGAAAG-3’, reverse primer 5’-CTTCTTCTTGGCCCTTCCTTAAACTTCAG-3’; probe 5’-FAM-AAGACCAGGATCTCAGGGCCCTGTA-GA-TAMRA-3’)

*Mecp2e2–3* (forward primer 5’-GATCCATGTCACTGGGATGATG-3’, reverse primer 5’-TCTAGGGCCCTGGGATCCTTT-3’; probe 5’-FAM-AGGGCTCAGGGAGGAAAGTGAA-3’)

*Mecp2e3* forward primer 5’-TACAACCTTACGGCGAACC-3’; reverse primer 5’-CTGAGCTTCTTGATGTTC
were Dounce homogenized in ice-cold RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton-X-100, 0.1% SDS) with Roche complete protease inhibitors and 1 mM PMSF. Samples were rotated for 10 min at 4°C and then spun at maximum speed in a microcentrifuge for 10 min at 4°C to pellet insoluble material. Forty micrograms of each sample were boiled in a sample buffer, loaded onto a NuPAGE Bis-Tris 4–12% gel (Invitrogen) and transferred to nitrocellulose. Samples were rotated for 10 min at 4°C in 1× phosphate-buffered saline (PBS), brains were cryoprotected in 30% sucrose and then embedded in O.C.T. and stored at −23°C. Fifty micrograms of mid-sagittal sections were cut and suspended in 24-well tissue culture plates containing 1× PBS and then incubated for 48 h at 4°C in 1:500 Alexa 488 labeled goat-anti rabbit (Molecular Probes). Sections were washed four times for 20 min in 1× PBS and then mounted with ProLong Gold antifade mounting medium (Invitrogen cat# P36930). Images were collected from optical sections using a Zeiss 510 (Carl Zeiss, Thornwood, NY, USA) confocal microscope and processed using ImageJ software (http://rsb.info.nih.gov/ij/).

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