BEHAVIORAL AND ANATOMICAL ABNORMALITIES IN MECP2 MUTANT MICE: A MODEL FOR RETT SYNDROME

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Abstract—Over 90% of Rett syndrome (RTT) cases have a mutation in the X-linked gene encoding methyl CpG binding-protein 2 (MeCP2). A mouse model that reprises clinical manifestations of the disease would be valuable for examining disease mechanisms. Here, we characterize physical and behavioral measures, as well as brain region volumes in young adult mice that have mutations in mouse methyl CpG binding-protein 2 gene (Mecp2) to serve as a baseline for other studies. Hemizygous males, which produce no functional protein, exhibit hypoactivity and abnormalities in locomotion, stereotypies, and anxiety reminiscent of the clinical condition. The mutant males also exhibit cognitive deficits in fear conditioning and object recognition relative to wildtypes. Volumetric analyses of male brains revealed a 25% reduction in whole brain volume in mutants relative to wildtypes; regional differences were also apparent. Mutants had decreased volumes in three specific brain regions: the amygdala (39%), hippocampus (21%), and striatum (29%). Heterozygous females, which produce varying amounts of functional protein, displayed a less severe behavioral phenotype. The mutant females exhibit abnormalities in locomotion, anxiety measures, and cognitive deficits in object recognition in an open field. This study provides the first evidence that the abnormal motor and cognitive behavioral phenotype in Mecp2 mice is consistent with specific volume reductions in brain regions associated with these behaviors, and shows how these data parallel the human condition. The Mecp2 mutant mice provide a very good model in which to examine molecular and behavioral mechanisms, as well as potential therapeutic interventions in RTT. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: zero maze, swim maze, fear conditioning, AMIRA, volumetric analysis, object recognition.

Rett syndrome (RTT), a major cause of mental retardation in females, is a disorder with a broad array of clinical manifestations (Hagberg, 1985; Hagberg et al., 1999). Four stages of classic RTT include 1) relatively normal development until 6–18 months of age, when head growth slows down, 2) rapid regression at 1–3 years, when acquired skills are lost and stereotypic movements, mental retardation, and emotional disturbances become apparent, 3) pseudo-stabilization at 5–10 years, when autistic symptoms lessen but mental retardation and stereotypies persist, and breathing abnormalities develop. After stabilization, which can last until adulthood, 4) osteoporosis, scoliosis, and dystonia may develop. The median age of death in one study was 24 years; however, when nutritional requirements are met and physical therapies applied, most RTT females survive longer (Schneider and Glaze, 2002).

Magnetic resonance imaging (MRI) studies and post-mortem analysis of RTT individuals suggest that these clinical manifestations are correlated with underlying anatomical changes. Volumetric MRI studies of RTT brains and age-matched controls show that on average, there is an overall reduction in whole brain size; however, this reduction does not appear to be uniform (Casanova et al., 1991; Reiss et al., 1993; Armstrong, 2005). The brain regions most significantly affected in RTT individuals appear to be associated with the two most prominent clinical features of RTT: impaired motor function and impaired higher cognitive processes. Voluntary motor function is regulated through the basal ganglia circuit connecting cortex to striatum and thalamus. Both the cortex and caudate nucleus of the striatum are reduced in RTT individuals (Reiss et al., 1993; Takakusaki et al., 2004). Coordination of movement and fine motor control are putatively regulated by the cerebellum, which is also reduced in RTT individuals (Murakami et al., 1992; Mauk et al., 2000). Brain regions involved in higher cognitive functions are also affected in RTT individuals; the prefrontal cortex, in addition to the striatum, is reduced (Reiss et al., 1993). Additionally, the white matter tract bridging the two cerebral hemispheres, the corpus callosum, has also been shown to be reduced in some cases (Gotoh et al., 2001). However, in general it appears that gray matter is affected more than white matter (Casanova et al., 1991; Reiss et al., 1993; Naidu et al., 2001).

Mutations of human methyl CpG binding-protein 2 gene (MECP2), an X-linked gene encoding methyl CpG binding-protein 2 (MeCP2), are documented in over 90% of sporadic RTT cases (Williamson and Christodoulou, 2006). MeCP2 is a ubiquitously located transcription repressor that affects gene transcription rates by binding methylated DNA, changing chromatin structure and rendering it transcriptionally inactive (Wan et al., 2001).
thought that only females with the mutation survive, it appears that males with mutations in their single MECP2 gene often present with a more severe phenotype (reviewed in Bienvenu and Chelly, 2006). It is believed that the wide range of symptom severities in both males and females is due both to the large number of documented mutations in MECP2 (over 2000), as well as random X chromosome inactivation in females. Random X inactivation would result in relatively more or fewer cells expressing normal MeCP2 protein (Webb and Latif, 2001; Naidu et al., 2003; Bienvenu and Chelly, 2006).

The precise role of MeCP2 in producing the clinical RTT phenotypes is still unclear. Experiments examining the spatial and temporal expression pattern of MeCP2 protein suggest that MeCP2 may play an important role in controlling neuronal maturation through the regulation of synapse formation and maintenance (Mullaney et al., 2004; Kaufmann et al., 2005). MeCP2 expression parallels the timing of post-migration differentiation in neurons and appears to play a prominent role in maturation of cortex, hippocampus, cerebellum, thalamus, and striatum, many of the same regions, which show significant volumetric reductions in RTT (Shahbazian et al., 2002; Mullaney et al., 2004; Kaufmann et al., 2005). In these regions, the absence of the appropriate levels of MeCP2, as seen in RTT, result in shorter, underdeveloped dendritic arborizations and reduced neuronal size as a consequence of inappropriate synaptic maturation. Reduced dendritic arborization and neuronal size are believed to account for decreases in brain volume (Bauman et al., 1995). Recent evidence suggests that MeCP2 may play a role in synapse maturation and maintenance of appropriate dendritic arborization by regulating activity-associated expression of BDNF in neuronal cells (Chen et al., 2003). Interestingly, the timing of the onset of symptoms in humans and the regression of learned behaviors suggest that disruption of normal synaptic maturation and stabilization of dendritic arborization underlie the abnormal motor and cognitive phenotype in this disorder (Johnston et al., 2001; Zoghbi, 2003).

The potential benefit of animal models that reprise symptoms of RTT is enormous, and has led to the creation of several mouse models expressing alterations in mouse methyl CpG binding-protein 2 (Mecp2). Four mouse models currently exist. Three models result in the loss of functional Mecp2 protein either through the deletion of exon 3 of the Mecp2 gene (Mecp2lox, Chen et al., 2001) or by the deletion of exons 3 and 4 (Mecp2floxed, Guy et al., 2001; Mecp2tm1Tam, Pelka et al., 2006). In the fourth model, the Mecp2 protein is truncated after codon 308 (Mecp2308), retaining several key functional domains (Shahbazian et al., 2002). In all four models, mutants exhibit motor abnormalities; however, only a small subset of cognitive tasks has been performed in the Mecp2tm1Tam and Mecp2308 mouse models (Moretti et al., 2006; Pelka et al., 2006). To our knowledge a battery of cognitive tasks has not been performed on a single animal model of RTT, nor have researchers attempted to associate motor, anxiety, and cognitive impairments with the anatomical changes in the brain which are also hallmarks of RTT. The Mecp21lox mouse model of RTT, utilized for these studies, allowed us to determine the effects of the loss of Mecp2 function on both behavior and anatomy without the added complication of determining the functional consequence of a truncated Mecp2 protein. Additionally, the severity of symptoms previously described in the Mecp21lox model, both onset of symptoms and lifespan, appeared more reminiscent of RTT in humans than other available mouse models.

Here we describe the phenotype of Mecp21lox mice of both sexes between 4 and 12 weeks of age including motoric, cognitive and anxiety-related abnormalities. Both male hemizygous and female heterozygous (Fhetero) mice were used for all behavioral studies in contrast to most other studies which examine only male mice. We used male hemizygous animals for reasons similar to those cited by other researchers: male hemizygous mice represent completely null animals (no Mecp2 protein) and random X-inactivation leaves an unpredictable proportion of X-chromosomes carrying the Mecp2-null allele in the female making statistical comparisons potentially difficult. Despite this last drawback, we decided to use Fhetero animals also because most research in humans studies female patients with RTT, and we found that the motor impairment in male mutants made some physically demanding cognitive tasks difficult to interpret. In addition in the current study, we examine brain volumes in regions associated with the specific behavioral tasks. Because the most pronounced and reliable motoric and behavioral changes were in the mutant males, we examined brain volumes, only in males, at 5 weeks of age. We measured the volumes of several regions, focusing on regions associated with the RTT abnormal behavioral phenotype such as amygdala, cerebellum, hippocampus, somatomotor cortex, striatum, and thalamus as well as the white matter tracts the anterior commissure and corpus callosum.

**EXPERIMENTAL PROCEDURES**

**Subjects**

Mecp2lox mice were generated as described previously (Chen et al., 2001). Two female founder mice heterozygous for the Mecp2lox null allele were obtained from Dr. R. Jaenisch (Massachusetts Institute of Technology, Cambridge, MA, USA) and used to establish a colony of Mecp2lox animals in the Department of Biological Sciences, Wellesley College. These heterozygous females of mixed genetic background (primarily BALB/C with some 129 and C57BL/6) were mated to wildtype C57BL/6J males; the Fhetero offspring were mated to wildtype C57BL/6J males. All experiments were conducted on Mecp2lox mice, back-crossed for more than eight generations, and their wild-type littermates. Mice were maintained on a 12-h light/dark cycle with lights on at 07:00 h, and food and water available *ad libitum* except during behavioral testing. All procedures were approved by the Wellesley College Institutional Animal Care and Use Committee (IACUC) and conformed to the standards set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All attempts were made to reduce the number of animals used and to prevent their suffering.
Genotyping

DNA was prepared from a small tail-tip biopsy taken at 21–28 days of age. The Mecp2 alleles were identified by PCR using two sets of primers. Primer set 1 (5'-primer: 5'-CAC CAC AGA AGT ACT ATG ATC-3' and 3'-primer: 5'-CTA GGT AAG AGC TCT TG1 TGA-3') yields a product of 180 bp identifying the wildtype allele. Primer set 2 (5'-primer same as for primer set 1 and 3'-primer: 5'-ATG CTG ACA AGC TTT CTA-3') yields a product of apparent size 260 bp identifying the null allele. PCR products were electrophoresed through a 2% NuSieve 3:1 agarose gel (Cambrex Bio Science, Rockland, ME, USA) containing 0.5 μg/mL ethidium bromide, and examined under UV light.

Experimental design

The effect of the Mecp2 mutation was characterized in young mice, 4–12 weeks of age. The physical evaluation and behavioral testing compared male wildtype (Mwt) to male null (Mnull) mice and female wildtype (Fwt) to Fhetero mice. A battery of tasks was conducted including physical and sensory measures: weight, neurological battery, grip strength, visual acuity, hearing evaluation (Preyer’s reflex); motoric measures: dark cycle locomotor activity and motor coordination (rotorod); anxiety measures: elevated plus and zero mazes; and cognitive measures: 1-day and multi-day water maze tasks, pavlovian fear conditioning, and object recognition in an open field test. Because so many different behavioral tests were employed, different groups of mice were used for different behavioral tasks; the ages and numbers of mice used in each task are specified in the results. The effect of the Mecp2 mutation on brain volumes was measured at 5 weeks of age in male mice. For all experiments, the genotypes of the mice were concealed from the researcher until after the analysis was complete. The approximate timing of behavioral and anatomical testing is shown in Fig. 1.

Behavioral testing

Physical and sensory measures. To assess general health, mice were weighed (g) and observed in their home cages for stereotypic behaviors and autonomic irregularities including body tremors, labored breathing, and piloerection two or three times/week beginning at weaning. A neurological battery was performed at the same time as the general health assessment to evaluate the basic motor skills of the mice. In the neurological battery we measured reaching and righting reflexes, and grip strength (g). Reaching reflex was measured four times by suspending the mouse by its tail and bringing it close to a flat surface. A score of ‘normal’ was given when the mouse reached for the surface with its front paws. Righting was used to measure postural reflex. The mouse was turned on its back and the ability to right itself was recorded. Grip strength was measured as the average of three consecutive trials, recorded by a digitized meter (San Diego Instruments). Each apparatus consisted of a triangular pull attached to a 2 kg Chatillon strain gauge, mounted in-line with a mouse support table. The mouse was held by the tail and allowed to grasp the triangular pull with its forepaws, then pulled back until its grip was released, yielding a value of the strain on the gauge (g). Visual acuity was assessed in an apparatus similar to that described previously (Crawley, 1999; Lione et al., 1998). A sheet of transparent Plexiglas was placed over a two-level staircase painted with a black and white checkerboard pattern. Mice were placed on a 5-cm wide, 1-cm high riser on the Plexiglas located at the junction between upper and lower levels. Mice with normal vision were expected to perceive and avoid the area over the cliff (lower level), and spend most of their time in the safe areas (on the riser or above the upper level). Mice were videotaped for 2 min, and subsequently scored for time spent over the cliff or in the safe areas. Shock reactivity was tested as previously described (Arters et al., 1998). Mice were given a series of 1 s foot shocks of increasing intensities (0.05 mA, 0.1 mA, 0.15 mA, and 0.2 mA). Vocalizations were scored on a 0–3 scale (0 indicated no response and 3 indicated an extreme response). Jumping, another measure of sensitivity to foot shock, was not scored because the Mnull mice displayed motor deficits, which limited their ability to jump. Preyer’s reflex, a measure of gross hearing loss, was evaluated. Reaction to a sudden handclap with the presence or absence of a rapid movement of the whole body of the mouse was recorded (Jero et al., 2001).

Motoric measures. Dark cycle locomotor activity was measured for 12 h as described previously (Arters et al., 1998), using a Photobeam Activity System (San Diego Instruments). Each

![Fig. 1. The timing of behavioral and anatomical experiments. A representation of the approximate timing of the behavioral and anatomical experiments is shown.](image-url)

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<tr>
<th>Age (weeks)</th>
<th>Behavioral Measure</th>
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<td>4</td>
<td>home cage observations</td>
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<td>5</td>
<td>anatomy</td>
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<td>neurological battery grip strength</td>
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<td>9</td>
<td>zero maze plus maze 1-day swim task</td>
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<td>10</td>
<td>Mnull time of death</td>
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<td>neurological battery females</td>
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mouse was placed individually into a cage (47×25×21 cm) that was placed into a rectangular arena equipped with three photobeams along its length. The number of photobeam breaks was monitored for 12 h starting at 18:00 h. The total number of photobeam breaks/1 h interval and the number of successive breaks of two different photobeams (ambulatory breaks) were recorded. Motor coordination/rotorod. Performance on an accelerating rotorod (San Diego Instruments) was used to measure the mouse’s balance and motor coordination. Each mouse was given three trials on the apparatus with the rate of rotation increasing from 4–40 r.p.m. over 4 min. The time spent on the rotorod was recorded by an automated unit, which stopped when the mouse fell and broke a photobeam. The mouse was placed back in its home cage for a minimum of 10 min between each trial.

Cognitive measures. Water maze. Spatial learning and memory were tested using 1-day or multi-day water maze paradigms. The 1-day water maze was conducted as described previously (Frick et al., 2000b). Mice were pretrained to escape from a pool of water by climbing onto a visible platform. Spatial trials. During spatial trials, the mice learned to locate a hidden platform using extra maze visual cues, and to escape from the water by climbing onto it. Spatial trials were arranged in three blocks of four trials. A maximum of 60 s/trial was allowed, with 30 min rests between blocks. Probe trials. Thirty minutes after the third spatial trials, mice received one probe trial in which the platform initially was lowered to make it unavailable for escape. The mice were allowed 30 s to attempt to locate the platform, then the platform was raised and another 30 s allowed for the mice to locate and climb onto it. Cued trials. The extra-maze cues were removed. The location of a visible platform was changed to a different quadrant for each of four cued trials. During all trials, data were recorded for subsequent analysis (HVS Image, Hampton, UK). Parameters measured for spatial trials were path length, swim time (time to platform), corridor (a measure of the amount of time the mouse spends in a path, one platform wide, between the place of entry into the pool and the platform), and swim speed. For cued trials, the same measures, except for the corridor measure, were recorded. For the probe trial, proximity, a measure of distance from the former platform location, quadrant time (time spent in the target quadrant), and platform crossings were measured for the first 30 s. The multi-day water maze task for mice was run essentially as previously described (Frick et al., 2000a). This task is similar to the 1-day water maze, but has a different temporal arrangement. Spatial trials. Mice received four spatial trials each day for 5 days, with a rest period of about 20 min between each trial. Probe trials. On each of the 5 days of the spatial trials, after the fourth spatial trial, mice received one probe trial, conducted as for the 1-day water maze. Cued trials. Following the last day of spatial/probe trials, mice received four cued trials/day for each of three consecutive days. Data were recorded and analyzed as described for the 1-day water maze task.

Contextual and cued fear conditioning

Associative learning was assessed at 6–7 weeks using an automated fear conditioning system for mice (Coulbourn Instruments, Allentown, PA, USA) with the following parameters: day 1, acquisition: 3 min in context A, followed by a 30 s tone (60 dB), the last 2 s paired with a 0.5 mA shock, then a further 1.5 min in context A, followed by a second identical tone-shock pairing, then a further block of 30 s in context A (total time 5.5 min). Then the mouse was returned to its home-cage. Context A was cleaned thoroughly with 70% ethanol between mice. Day 2, contextual retention: 5.5 min in context A with no tone or shock. Freezing, defined as the lack of movement except for respiration and heartbeat (Fanselow and Bolles, 1979) was recorded by an automated system (Graphic State 3.0 software; Coulbourn Instruments). Then the mouse was returned to its home-cage, and context A was cleaned. At least 1 h following contextual retention testing, the mice were tested for cued retention: 3 min in context B (having different visual, tactile and olfactory cues to that of context A), followed by 3 min tone (80 dB), during which time freezing was recorded (total time 6 min). The mouse was returned to its home-cage and context B cleaned and resented with the context B scent cue (pure vanilla essence) between mice. The number of freezing intervals during contextual and cued retention testing was converted to a percentage freezing value, prior to statistical analysis.

Open field test with objects

Reactions to spatial and object novelty were assessed by the open field test procedure described previously (Ricceri et al., 2000). Open field testing was performed between 09:30 and 15:30 h under red light. A mouse was placed in a 180 cm diameter black acrylic pool. Mice were submitted individually to seven successive 6 min sessions, separated by 3 min delays during which the subjects were returned to their home cage. Between sessions, the objects and tank were cleaned. Data were collected using a video recorder, placed above the open field arena, and the software program “The Observer” (Noldus, Wageningen 6700 AG, The Netherlands). During session 1, the frequencies of the following responses were measured: rearing (standing on hind legs), wall rearing (standing on hind legs and placing forelimbs on the wall of the arena) and crossings (crossing one of the annuli with which the floor of the arena was subdivided on the monitor). From sessions 2–7, object exploration was measured as time spent by the mouse in contact with an object, defined as the subject’s snout or forelimbs physically touching the object. Habituation to the objects was assessed by averaging the duration of contacts with the objects during sessions 2–4 in each group. In session 5, the spatial arrangement of the objects was modified and response to spatial change was assessed by comparing the mean time spent in contact with displaced objects (DO) and non-displaced objects (NDO) in session 5 minus the mean time spent in contact with the same object category in session 4. Finally, in session 7, the response to object novelty was assessed by comparing mean time spent in contact with the substituted object (SO) and familiar, non-substituted objects (NSO) in session 7 minus the mean time spent with objects located in the corresponding position in session 6.

Anxiety measures. Anxiety-related behavior in the mice was examined on elevated plus and zero mazes. On both mazes, a mouse was placed on the maze individually and was videotaped for 5 min for later scoring. The elevated plus maze apparatus, with open and closed arms radiating from the center, was constructed as previously described (Frick et al., 2000a). Five measures were recorded: latency to arm entry, percentage of time in open arms, closed arm entries, open arm entries, and total entries. The zero maze apparatus, with open and closed arms formed in a circle, was constructed as described elsewhere (Cook et al., 2001). Seven measures were recorded: percentage of time in open areas, number of transitions to open arms, number of double transitions between open and closed arms, number of head dips, number of rearings, time spent freezing, and time spent grooming.

Volumetric measures: perfusion

Mwt (n=6) and Mnull (n=6) mice were anesthetized with 200 μl 1:10 Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI, USA), then perfused with 15 mL of phosphate-buffered saline (PBS) (Dulbecco’s 1× Phosphate Buffered Saline, Invitrogen, Carlsbad, CA, USA) and 15 mL 4% paraformaldehyde (PFA) in PBS. Brains were post-fixed in 4% PFA for 2 h then cryoprotected in 20% sucrose in PBS overnight and frozen at −70 °C. Brains were sectioned (50 μm slices) with a Leica cryostat. Every fifth slice was collected and mounted on a 1% gelatin-coated slide. Slices were air dried overnight at room temperature. To visualize the
sections for tracing, the mounted brain sections were stained with Cresyl Violet.

**Tracing protocol.** Slides were scanned into Adobe Photoshop (San Jose, CA, USA). Each section was cropped to an identical canvas size (374x503 pixels/inch) and saved. Individual files were loaded in successive series into AMIRA™ (Berlin, Germany). Brain regions were traced according to pre-determined boundaries described below, and volumes (mm³) were automatically generated by AMIRA based on the area included in the traced regions. These traced regions were re-assembled three-dimensionally to verify the accuracy of the tracing. Each section was traced twice in two independent sessions with a test-retest reliability coefficient of over 0.99.

**Regions traced.** A stereotaxic atlas was used to determine regional borders of structures (Paxinos and Franklin, 2001).

**Whole Brain:** Tracing began at Bregma +2.22 mm (plate 12) and ended at Bregma −6.36 mm (plate 84; Fig. 2A–C). Anatomical landmarks such as the staining patterns of gray matter around the anterior commissure were used to assess the starting and ending points. **Anterior commissure:** Tracing was performed on both the left and right hemispheres from Bregma +2.22 mm (plate 12) to Bregma −0.46 mm (plate 35). Tracing started when the area around the anterior commissure was uniform in color and ended when the anterior commissure was no longer visible. **Amygdala:** The amygdala was defined as the space between the fork in the corpus callosum between Bregma −0.58 mm (plate 36) and Bregma −2.18 mm (plate 49) on both the left and right sides of the brain. Tracing began when the corpus callosum forked and ended when the corpus callosum ran along the edge of the hippocampus. **Cerebellum:** The cerebellum was defined by Bregma −5.40 mm (plate 76) to Bregma −6.36 mm (plate 84). The starting point was the beginning of the cerebellum and the end point was the declive of the structure. Only the anterior portion of the cerebellum was traced because this is the area of the cerebellum associated with motor control, and the posterior part of the cerebellum had been lost in blocking. **Corpus callosum:** The corpus callosum was measured from Bregma +1.54 mm (plate 18) to Bregma −2.46 mm (plate 51). Tracing commenced with the first appearance of the corpus callosum and ended when the corpus callosum became indiscernible from the hippocampus. **Somatomotor cortex:** The somatomotor cortex was measured from Bregma +1.10 mm (plate 22) to Bregma −0.58 mm (plate 36). The starting section was defined as where the corpus callosum joined the two hemispheres together, and the ending point was when the corpus callosum began to fork. The cortical area between the cingulated and the rhinal fissures was traced. The right side of the cortex was traced and the volume multiplied by two to determine the total somatomotor cortical volume. **Hippocampus:** The hippocampus was measured from Bregma −1.34 mm (plate 42) to −3.52 mm (plate 60). **Striatum:** The striatum was defined as the area between the ventricles and the corpus callosum; the ventral boundary was the top of the anterior commissure. The right striatum was traced from Bregma +1.10 mm (plate 22) to Bregma −0.94 mm (plate 39). The complete volumetric measurement of the striatum was determined by multiplying the right volume by two (a sample reconstruction shown in Fig. 2D). **Thalamus:** The thalamus was traced on the left and right side of the brain between Bregma −2.18 mm (plate 49) and Bregma −0.46 mm (plate 35). The start point was classified by the first appearance of the bowl-like structure of the thalamus, and end point was located at the beginning of the hypothalamus.

**Data analysis**

In all cases, behavioral data for the two sexes were analyzed separately. Data were analyzed using Student’s t-test, one- or two-factor analysis of variance (ANOVA) or repeated measures ANOVA using SuperANOVA (Abacus Concepts, Inc., Cary, NC, USA). Post hoc Newman-Keuls tests were used to examine dif-
frequencies between specific groups following ANOVAs. Shock reactivity scores were analyzed using the Mann-Whitney test for non-parametric data using SPSS data analysis software (SPSS, Inc., Chicago, IL, USA). Brain volume data were analyzed using two-tailed Student’s t-test.

RESULTS

Time course of the onset of the Mecp2 phenotype

Males. Mnull mice appeared normal at birth and relatively normal when very young but could be identified occasionally by an altered gait as early as 4 weeks of age and by a significantly reduced body weight by 5 weeks of age. The body weight of Mnulls (n=21) was about half that of Mwts (n=19) (13.4±0.82 and 21.0±1.00 g respectively) [t(38)=46.9, P<0.001]. In all males, body tremors and shaking paws were noted by 5 weeks of age, and piloerection and periods of labored breathing as early as 6 weeks. Reaching and righting reflexes were normal, but reduced forepaw grip strength was noted in some Mnulls as early as 5 weeks, and in most Mnulls by 6 weeks (Mwt, 82.7±6.4 g; Mnull, 45.2±2.5 g, [t(38)=31.99, P<0.001]). Qualitative measure of grip strength of hind paws was also noticeably decreased, and was quite pronounced after hind leg clamping (holding one or both hind legs against the body when lifted by the tail) developed. At 6–8 weeks of age the Mnulls were able to walk normally, but had difficulties swimming; they would use either one or the other hind leg for swimming but were unable to move the hind legs in a normal alternating fashion. Assessment of motor coordination by rotarod testing, confirmed that the motor coordination of Mnulls (n=21) was significantly worse than that of Mwts (n=19). Mean time to fall was 55.3±5.3 s and 90.6±5.2 s, respectively [t(38)=22.05, P<0.001]. A rapid, repetitive forepaw face/head grooming movement, sometimes with rearing, was noted in about two-thirds of the null mice. Age of Mnulls at euthanizing (when death was imminent) was 70±8 days (range 46–121). Females. The time course for the development of the motor and autonomic symptoms appeared slower in the mutant females than in the mutant males. Fheteros appeared normal at birth. At 5 weeks of age, the body weight of Fheteros (n=14) was only slightly reduced compared with Fwt mice (n=15) (15.6±0.6 g and 17.5±0.3 g respectively; [t(27)=6.4, P<0.01]). Stereotypic movements and light forepaw grip were noted occasionally (about 5%) in individual Fheteros as young as 5 weeks of age. Most Fheteros took 6 months or longer to develop hind leg clamping, as well as those of piloerection and heavy breathing. Reaching and righting reflexes were normal between 4 and 12 weeks of age. However, assessment of motor coordination by rotarod testing at 6 weeks revealed that Fheteros (n=14) were less coordinated than Fwts (n=15). Mean time to fall was 60±4.0 and 77.7±5.6, respectively [t(27)=32.5, P<0.001].

Mecp2 mice were hypoactive relative to their sex-matched littermate controls

Locomotor activity decreased throughout the 12 h dark cycle in all mice. Males. At 6 weeks of age, Mwts (n=19) were more active than Mnulls (n=21) over the dark cycle [F(1,30)=6.46, P=0.016] (Fig. 3A) and made more ambulatory movements [F(1,30)=10.83, P=0.003], data not shown. In addition to an overall reduction in activity by the Mnulls, there was a dissimilar pattern of activity between the two groups as revealed in significant interactions between genotype and time interval for activity [F(11,330)=1.9, P=0.04] and for ambulatory movements [F(11,330)=2.2, P=0.014]. Mwts appeared to spend more time exploring their cage before activity levels decreased (about 7 h) whereas activity levels in Mnulls began to decrease immediately. These data suggest that the Mnulls were both hypoactive when compared with Mwts, and showed a different pattern of motor habitation to the cage environment. Females. Similar to the males, Fwts (n=15) were more active than Fheteros (n=21) over the 12 h dark cycle [F(1,34)=5.41, P=0.026] (Fig. 3B) and made more ambulatory movements [F(1,34)=4.28, P=0.046], data not shown. Unlike the males, while the Fheteros were hypoactive relative to Fwts, there were no significant interactions between genotype and time interval suggesting that the pattern of habitation to the cage was similar for Fwts and Fheteros.

![Fig. 3. Dark cycle locomotor activity in wildtype and mutant mice. Total photobeam breaks per 1-h interval for males (A) and females (B) are shown. Mwts were significantly more active over the dark cycle than Mnulls, and Fwts were significantly more active than Fheteros (P<0.05). There was also a significant interaction between genotype and interval for the male mice (P<0.05).](image-url)
Mecp2 mutant males were impaired motorically on both water maze tasks making cognitive performance difficult to interpret

Mice performed a 1-day spatial navigation task at about 8 weeks of age. This is a strenuous series of 17 trials that assesses hippocampal-dependent (spatial and probe trials) and hippocampal-independent (cued trials) learning and memory over a 3 h period (Frick and Berger-Sweeney, 2001; Frick et al., 2001). Males. Mwts (n=20) performed normally for all measures, and similarly to our previously published reports in C57BL/6 mice (Frick et al., 2001); path lengths and swim times decreased, and corridor measures increased across the spatial trials. Although Mnulls (n=10) could walk relatively normally at the time of 1-day water maze testing, they had difficulty swimming and steering themselves in the water. In all three phases of the water maze task, spatial, probe, and cued trials, Mnulls had significantly shorter path lengths [F(1,9)=17.2, P<0.002] and longer swim times [F(1,9)=62.9, P<0.001] than Mwts due to their much slower swim speeds [F(1,9)=39.78, P<0.001]. While we saw significant differences on many of the spatial, probe, and cued measures, we could not separate out motoric difficulties from potential cognitive impairments.

Because the Mnulls had difficulty performing the strenuous 1-day water maze task at 8 weeks, we assessed the mutants’ performance using a multi-day swim task that is less physically taxing (five trials for each of 5 days), at an earlier age (6 weeks). Mnulls exhibited similar swimming and navigational difficulties to those described in the 1-day water maze task resulting in similar differences in measures of cognitive performance. The obvious motor difficulties in both the 1-day and multi-day water maze task, noted as slower swim speeds in the mutants, made it difficult to assess whether cognitive deficits existed in addition to the motor deficits.

Females performed the 1-day and multi-day water maze task similarly

Both Fwt (n=9) and Fheters (n=12) mice were able to learn the 1-day hippocampal-dependent spatial navigation task and the multi-day water maze task (Fwt [n=14], Fheters [n=10]); there was an expected decrease in path lengths and latencies over the three blocks of spatial trials. There were no significant differences between the two groups in path length and swim speed, or on the probe measures suggesting similar hippocampal-dependent retention of the spatial information. In addition, there were no significant differences between the groups on any cued measure, nor any significant interactions between any cued measure and genotype suggesting no hippocampal-independent cognitive impairment.

Mutant males, but not females, exhibited impaired cued fear conditioning in the absence of obvious somatosensory or auditory deficits

Pavlovian fear conditioning was used to assess a mouse’s ability to associate an environment (context) and a cue (an audible tone) with an aversive stimulus (a foot shock). The former association is considered a hippocampal-dependent task, and the latter an amygdala-dependent task (Fanselow and LeDoux, 1999; Rudy et al., 2002). Males. Both Mwts (n=19) and Mnuls (n=17) exhibited increased freezing responses (59.3±6.1% and 48.9±6.1%, respectively) to the context in which they received a foot shock 24 h earlier; there was no statistically significant difference between the groups (Fig. 4A).

Freezing in response to the cue, however, was significantly higher in Mwts (49.3±5.1%) than in Mnulls (13.9±5.7%) ([t(34)=21.7 P<0.001), indicating impaired cued conditioning in the Mnulls. Significant deficits in conditioning to a cue in the absence of a deficit in conditioning to a context suggest abnormalities in the amygdala but unaffected hippocampal function. Females. The Fheters (n=14) exhibited increased baseline freezing, before presentation of the tone, when compared with Fwts (n=15) ([t(27)=10.8, P<0.01]. However, after the pairing of the tone and shock, there was no statistical difference between the performances of the two groups; in other words, neither contextual nor cued conditioning differed between Fwts and Fheters (Fig. 4B).

To determine if fear conditioning responses in Mnulls were confounded by sensory deficits, we assessed the tactile and auditory acuity of both groups in response to the foot shock and to the audible tone. To evaluate shock sensitivity, vocalizations and jumping were measured in response to
increasing foot shock; however, only vocalizations were analyzed statistically because of the obvious motor deficits in the Mnulls. Vocalizations were not significantly different between Mnulls (n/11005/6) and Mwts (n/11005/7) or between Fheteros (n/11005/7) and Fwts (n/11005/7). These data suggest that the deficits in fear conditioning performance in the Mnulls could not be explained by altered sensitivity to pain.

To determine whether the mice could hear the tone, the videotapes of the acquisition phase of contextual fear conditioning were reviewed to see if the mice responded by orienting to the onset of the tone. All mice (Mwt, n/11005/19; Mnull, n/11005/17; Fwt, n/11005/15; Fhetero, n/11005/14) reacted by orienting to the tone, jumping, running to a different location in the chamber, and/or freezing. In addition, a subset of mice was tested for Preyer’s reflex (gross hearing loss). All mice (Mwt, n/11005/6; Mnull, n/11005/6; Fwt, n/11005/6; Fhetero, n/11005/6) tested responded to the auditory stimulus by orienting to it and then freezing, jumping or running. Therefore, deficits in amygdala-dependent fear conditioning performance in the Mnulls were not likely explained by a gross hearing loss.

Mecp2 mice of both sexes exhibited an impaired ability to recognize spatial changes in an open field

The open field with object test is a task used to examine basic motoric and exploratory activity and to quantify how a mouse reacts to spatial novelty and object novelty (Ricceri et al., 2000). Males. In session 1, Mwts (n=8) have significantly more crossings and wall rearings than Mnulls (n=7), [ts(13)>4.8, Ps<0.04], suggesting higher motility and exploration in the wildtype mice than mutant males. In session 2, five small objects were placed in the open field. The Mwts and Mnulls spent similar amounts of time with the objects. Additionally, across sessions 2–4, the two groups habituated to the presence of objects in a similar fashion. In session 5 when two of the five small objects were spatially rearranged, the Mwts spent markedly more time than Mnulls with the two DOs suggesting that only wildtypes appeared to recognize the object rearrangement [F(1,13)=9.1, P=0.01] (Fig. 5A). The two groups spent similar amounts of time with the three NDOs. In session 7 when a novel/SO replaced one of the five objects, Mwts and Mnulls spent similar amounts of time with the SO and familiar objects (Fig. 5B).

Females. In session 1, Fwts (n=11) have significantly more crossings and wall rearings than Fheteros (n=14), [ts(23)>6.8, P<0.03], suggesting higher motility and exploration in the wildtype than heterozygous females. In session 2, Fwts and Fheteros spent similar amounts of time with the objects and habituated to the presence of the objects in a similar fashion across sessions 2–4. In session 5, the Fwts spent more time than Fheteros with the two DOs [F(1,23)=14.3, P<0.001] (Fig. 5C), but similar amounts of
time with the three NDOs. In session 7, Fwts spent significantly more time than Fheteros with the SO \( [F(1,23)=2.6, P<0.03] \) (Fig. 5D), but a similar amount of time with the NSO.

In total, both mutant males and females had difficulty recognizing the spatially rearranged object, which is considered a hippocampal-dependent task (Lenck-Santini et al., 2005). Additionally, the mutant females were impaired in recognizing the novel object. These results suggest hippocampal-dependent cognitive deficits in the mutant mice of both sexes.

**Mecp2 mice of both sexes exhibited altered anxiety**

The elevated plus maze, a test used commonly to assess anxiety levels in mice (Crawley, 2000), revealed differences among the groups for both males and females (Table 1). **Males.** Mwts \((n=10)\) spent significantly less time in the open arms than did Mnulls \((n=10)\) \([t(18)=2.3, P<0.04]\). There were no significant differences between the groups in entries into the closed or open arms, or total entries. **Females.** Fwts \((n=12)\) also spent significantly less time in the open arms than Fheteros \((n=20)\), \([t(30)=2.9, P=0.02]\), and made significantly fewer open arm entries \([t(30)=4.6, P=0.01]\).

The zero maze, another task commonly used to measure anxiety in mice (Cook et al., 2001), revealed similar differences among the groups (Table 1). **Males.** Mwts \((n=10)\) spent significantly less time in open arms than Mnulls \((n=8)\), \([t(16)=10.2, P=0.005]\). Mwts also spent significantly less time freezing and less time grooming than Mnulls \([t(16)=4.2, P<0.05]\). **Females.** Fwts \((n=9)\) spent a smaller percentage of time in open arms, had more transitions, fewer double transitions, and more rearings than Fhetero mice \((n=10)\), \([t(17)>4.8, all PS<0.04]\).

To ensure that altered performance on these maze tasks was not due to impaired visual acuity in the mutants, mice (Mwts, \(n=21\); Mnulls \(n=19\); Fwts \(n=15\); Fheteros \(n=21\)) were tested on a visual cliff task. All mice spent similar amounts of time, between 73% and 83%, on the safe side of the arena. These data suggest that visual acuity and motivation to stay safe were similar for the control and mutant mice and should not account for the increased amount of time spent by both Mnull and Fhetero in the open maze arms.

In addition to the standard maze tests for anxiety, thigmotaxis (wall hugging) on swim maze trials and freezing in a new context in the absence of the cue (tone) are also indicators of anxiety in mice (Simon et al., 1994). **Males.** The Mnulls were swimming so poorly on the navigation tasks that it was not appropriate to measure thigmotaxis. However, Mwts spent a significantly larger percentage of time freezing \((20.5\pm4.5)\) than Mwts \((4.1\pm0.8)\) during the first 2 min of the acquisition phase of Pavlovian fear conditioning, before the shock was administered \([t(34)=14.26, P<0.001]\). **Females.** Fheteros exhibited significantly more thigmotaxis than Fwts during the first block of trials of the 1-day water maze, spending more time in the outer one-third zone of the pool than Fwts \((91.4\pm3.7)\) s versus \(77.8\pm2.9\) s, \([t(13)=3.1, P=0.01]\). During the fear conditioning trials, the Fheteros also exhibited more freezing in a new context without a cue \((36.9\pm5.2)\%\) as compared with Fwts \((21.9\pm4.8)\%\), a statistically significant difference between the groups \([t(27)=2.8, P=0.04]\). Together, these four tests of anxiety suggest that mutants of both sexes displayed altered anxiety when compared with their wildtype littermates.

**Table 2** summarizes all the phenotypic and behavioral results described above.

**Region-specific decreases in brain volumes were noted in the mutant males**

To determine whether motoric, anxiety, and cognitive impairments described in these Mecp2 mice were associated with changes in brain anatomy similar to changes described in RTT patients, whole brain volumes as well as regional volumes were calculated in AMIRA for Mwts.
Table 2. Summary of Mecp2 mutant phenotype at 5–12 weeks of age

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mnull</th>
<th>Phetero</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical and Sensory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurological reflexes (righting, reaching)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hind leg claspning</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Stereotypies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grip strength</td>
<td>Reduced</td>
<td>Normal</td>
</tr>
<tr>
<td>Visual acuity</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Shock sensitivity</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Hearing</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Motor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark cycle locomotor activity</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>Rotorod</td>
<td>Impaired</td>
<td>Impaired</td>
</tr>
<tr>
<td>Anxiety</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plus maze</td>
<td>Altered</td>
<td>Altered</td>
</tr>
<tr>
<td>Zero maze</td>
<td>Altered</td>
<td>Altered</td>
</tr>
<tr>
<td>Cognitive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water maze</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pavlovian fear conditioning</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Object Recognition (spatial and novelty reactions)</td>
<td>Altered</td>
<td>Normal</td>
</tr>
<tr>
<td>Brain volume</td>
<td>Reduced</td>
<td>Normal</td>
</tr>
<tr>
<td>Early death</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

(n=6) and Mnulls (n=6) at postnatal day (PD) 35. The specific regional volumes were calculated for the following brain areas: amygdala, cerebellum, cortex, hippocampus, thalamus and striatum and the following two white matter tracks: anterior commissure and corpus callosum.

Significant reductions were found in the whole brain volume as well as in three specific brain regions; amygdala, hippocampus and striatum and the following two white matter tracks: anterior commissure and corpus callosum.

DISCUSSION

Motor deficits are associated with reductions in striatal volume

We have characterized mice with a deletion of Mecp2. Mnulls, with a mutated copy of the Mecp2 gene on their single X chromosome and no functional Mecp2 protein (Chen et al., 2001), exhibit severe motor deficits compared with wildtype males based on observations of stereotypies, abnormalities in gait and grip strength, reduced motor coordination, reduced dark cycle locomotor activity, and severely impaired swim performance. Many of these motor abnormalities are observable by 5 weeks of age. Phetero mice with one normal allele and one mutated Mecp2 allele also have several motor abnormalities; however, their symptoms are less severe than those in the Mnulls. Overt abnormal motor phenotypes are seen only rarely in Pheteros in the first 5 weeks. At 6 weeks of age, the Pheteros are less active and less coordinated than Fwts; however, gait and grip strength appear normal through 12 weeks of age. Despite apparently normal appearance in the first 4 weeks for both male and female mutants, we have reported elsewhere that the mutant mice of both sexes exhibit subtle abnormalities in sensory-motor reflexes and responses to social isolation detectable as early as PD5 (Picker et al., 2006).

These motor deficits are consistent with the human pathology of RTT (see Table 4), which includes stereotypic hand wringing, loss of fine motor control, and gait apraxia. We found that these behavioral changes are associated with a significant volumetric reduction in the striatum (29%), a brain region also affected in the human pathology (Reiss et al., 1993). This reduction in striatal volume is significant in light of volumetric MRI and pharmacological studies which link reduced striatal volume and defects in dopamine neurotransmission to stereotyped movements in other disorders (Backman et al., 1997). From these human studies as well as studies in animal models, it has been hypothesized that the basal ganglia circuit and in particular the striatum is associated with the production of stereotypies (Mason et al., 1978).

Surprisingly, we did not observe reduced volumes in two other motor regions frequently noted in the human pathology of RTT, the frontal cortex or cerebellum. Decreases in cortical volume may have been missed because we measured somatomotor cortex instead of a region containing only motor cortex. However, the data in our study are consistent with data from the Mecp2<sup>tm1-1Bird</sup> null, which also does not exhibit cortical volume reductions (Metcalf et al., 2001).

Table 3. Mean whole brain and regional volumes for Mwt and Mnull mice

<table>
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<th>Region</th>
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<th>Mnull</th>
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<tr>
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<td>320.4±4.3*</td>
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<td>28</td>
</tr>
<tr>
<td>Grey matter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amygdala</td>
<td>0.64±0.08*</td>
<td>0.39±0.01</td>
<td>39</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>7.4±1.2</td>
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<td>—</td>
</tr>
<tr>
<td>Somatomotor cortex</td>
<td>13.8±2.6</td>
<td>11.2±3.5</td>
<td>—</td>
</tr>
<tr>
<td>Hippocampus</td>
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<td>6.1±0.4</td>
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</tr>
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</tr>
<tr>
<td>White matter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior commissure</td>
<td>0.48±0.11</td>
<td>0.44±0.07</td>
<td>—</td>
</tr>
<tr>
<td>Corpus collicus</td>
<td>4.0±0.6</td>
<td>3.4±0.4</td>
<td>—</td>
</tr>
</tbody>
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* Values indicate volume (mm<sup>3</sup>, mean±S.E.M.) unless noted.  
P<0.05.

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P<0.05.
Additionally, we did not note changes in cerebellar volumes most likely because the variability of the volumes measured in this study was higher than in any other region and this large variability may have obscured significant difference between the groups. While we did not observe significant differences in the current study, recent studies in our laboratory using MRI to measure total cerebellar volumes show that Mnulls have significantly reduced cerebellar volumes on PD 17, but by PD 35 (the timing of the current study), cerebellar volumes appear similar to those of Mwts (N. H. Kolodny, J. Berger-Sweeney, unpublished observations). In the Mecp2<sup>tm1-1Bird</sup> null mouse model of RTT, cerebellar volumes are reported to be reduced (Saywell et al., 2006).

### Cognitive deficits in hippocampal and amygdala-dependent tasks are associated with reduced regional volumes

We tested Mecp2 mice on a variety of cognitive tasks evaluating the function of several brain regions including the hippocampus (spatial water maze, contextual fear conditioning, and spatial recognition in an open field), the striatum (cued water maze task), and the amygdala (cued fear conditioning) to more precisely determine which brain regions appear to be affected in mutant animals. In general, mice of both sexes showed impaired performance on the least stressful hippocampal-dependent task (spatial recognition in an open field) and Mnulls showed impaired performance on the amygdala-dependent cued fear conditioning task. The Mnulls have severely impaired swim navigation performance when compared with Mwts. Their navigation abilities were so impaired that it is not possible to separate motoric from cognitive deficits with this task. The Mnulls exhibited impaired cued, but normal contextual, fear conditioning. The mutants had normal shock reactivity, hearing and visual acuity, adding support to interpreting their performance in fear conditioning as a cognitive deficit. Importantly, impaired cued fear conditioning resulted because the mutants froze less than wildtypes in response to the tone. If the performance of the mutants

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Table 4. Comparison between mouse models and human RTT

<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
<td>Mutation</td>
<td>Deletion of exon 3</td>
<td>Deletion of exon 3 and part of exon 4</td>
<td>Truncated after amino acid 308</td>
<td>Deletion of exon 3 and part of exon 4</td>
<td>Mutations in the MECP2 in about 80% of cases</td>
</tr>
<tr>
<td>Lifespan</td>
<td>M: ~75 d F: &gt;1 y</td>
<td>M: ~10 wk</td>
<td>M: Most &gt;1 y, but some die by 10 mo</td>
<td>M: ~5 wk</td>
<td>Shorter than normal</td>
</tr>
<tr>
<td>Motor deficits/gait ataxia</td>
<td>M: Hypoactive,* swimming difficulties,* hind limb clasping*</td>
<td>M: Stiff, uncoordinated gait at 3–8 wk</td>
<td>M: Difficulties with wire suspension; rotorod deficits at ~8 wk</td>
<td>M: Hypoactive; rotorod deficits; unusual gait; hind limb clasping</td>
<td>Gait ataxia; loss of fine motor coordination</td>
</tr>
<tr>
<td>Stereotypic behaviors</td>
<td>Yes*</td>
<td>Yes</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Hand wringing or flapping</td>
</tr>
<tr>
<td>Autonomic irregularities</td>
<td>Yes*</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Seizures/abnormal EEG</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Anxious behavior</td>
<td>M: Altered plus/zero maze behavior*</td>
<td>Not reported</td>
<td>M: Less exploratory activity</td>
<td>M: More time in open arms</td>
<td>Yes</td>
</tr>
<tr>
<td>Cognitive performance</td>
<td>M: Impaired fear conditioning,* impaired object recognition*</td>
<td>Not reported</td>
<td>Not observed</td>
<td>M: Impaired cerebellar learning; both contextual and cued fear conditioning deficits</td>
<td>Mental retardation; loss of speech</td>
</tr>
<tr>
<td>Social interactions</td>
<td>Not reported</td>
<td>Not reported</td>
<td>HM: abnormal on resident intruder tests</td>
<td>Not reported</td>
<td>Some autistic traits; reduced social interactions</td>
</tr>
</tbody>
</table>

F, heterozygous females; M, hemizygous males.

* Reported in the current study.
was confounded by hypoactivity, we would predict that they would freeze more than wildtype mice, which was not the case. The Mnulls also exhibited impairments in spatial recognition on an open field with objects. The mutants spent similar contact time to wildtypes with objects that had not been displaced, but were impaired in recognizing that several of the objects had been moved. Generally, a spatial rearrangement of objects in a familiar open field induces a re-exploration of the DO; intact hippocampal and parahippocampal functioning is considered critical for this task (Broadbent et al., 2004; Lenck-Santini et al., 2005).

The MeCP2 Fheteros did not exhibit swim maze deficits and performed a fear conditioning task normally, but were impaired on the object recognition task. They had difficulty in recognizing spatial rearrangement of objects as well as object novelty. In humans, recognizing object novelty is associated with perirhinal cortex in the medial temporal lobe and with the hippocampus (Kohler et al., 2005); the data in rodents appear similar, however the fine anatomical distinctions are not possible.

Together these data suggest that the Mnulls have cognitive deficits on two behavioral tasks in which the motoric skills required to perform the task are relatively minimal. Our data suggest an interesting feature about this model of RTT: the cognitive deficits in Mnulls are severe, but not global. Hemizygous males exhibit a severe cued fear conditioning deficit, in the absence of a contextual deficit. The males are also impaired on the object recognition task but are able to react to object novelty. Fheteros have a more subtle cognitive deficit than the males that is apparent only on the object recognition task. We have noted previously that subtle cognitive deficits are sometimes obscured on tasks that are highly stressful such as swim maze and fear conditioning (Ricceri et al., 1999). It is therefore, not surprising that the females’ subtle deficit is apparent only on this latter behavioral task.

Normal amygdala functioning appears critical for cued conditioning, and normal hippocampal functioning appears critical for spatial recognition in open fields (Fanselow and LeDoux, 1999; Rudy et al., 2002; Broadbent et al., 2004). These data are supported by the anatomical data that reveal a significant decrease in amygdala and hippocampal volume in MeCP2 Mnulls. Considering the cognitive deficits that we have noted in the mutants of both sexes and the rather severe deficits in the mutant males, it is perhaps somewhat surprising that somatomotor cortical volumes were not reduced. This is inconsistent with human data which suggest reduced prefrontal and frontal cortical volumes in RTT individuals (Casanova et al., 1991; Reiss et al., 1993; Naidu et al., 2001). However, data in the current study are consistent with the data from another MeCP2 null mutant, which also does not exhibit cortical volume reductions (Metcalf et al., 2006). There are numerous possible explanations for these disparities between RTT and the MeCP2 null mouse. It is possible that the human condition, which encompasses many different types of MECP2 mutations and not solely the null mutation, has many different anatomical variants. It is also possible, and very likely, that the regional subdivisions in larger and more complex human brain make it possible to observe cortical regional differences that are not observable in the simpler mouse cortex. Nevertheless, the overall correlation between the human and mouse anatomical data, and the consistency between the anatomical and the behavioral data in the MeCP2 mutants are impressive.

### Anxiety abnormalities are associated with reductions in amygdala volume

The four anxiety measures we examined suggest that the mutant mice of both sexes have altered anxiety relative to their wildtype littermates, but the exact nature is unclear. On both the plus and zero mazes, mutants spent an increased amount of time in open arms relative to controls (the MeCP2<sup>tm1Tam</sup> mouse model performed similarly, Pelka et al., 2006). While more time in open arms is generally considered a sign of reduced anxiety, this finding contradicts results obtained from other anxiety measures. The preponderance of results showing increased baseline freezing on zero maze and in fear conditioning, reduced exploratory activity on both the zero maze and in the open field, and particularly thigmotaxis in the water maze and double transitions (from open to closed to open arms or vice versa within a short time) on zero maze, all suggest that the mutants are more anxious than their wildtype littermates. Although the other anxiety measures (increased freezing and reduced exploratory activities) may be confounded by general reductions in activity in the mutants, these last two measures of anxiety (thigmotaxis and double transitions, which are associated with either normal or increased levels of activity) are particularly convincing in light of the mutants’ motor deficits.

Overall, the data suggest that this MeCP2 mouse model exhibits abnormalities in anxious behaviors reminiscent of RTT (Sansom et al., 1993). Anxious behaviors in humans generally comprise reports of apparent panic attacks or fear/anxiety in unfamiliar situations (Mount et al., 2002). The variability in anxiety measures we report in the MeCP2 mutants may be due to the types of anxiety measures tested. We do describe several instances of increased anxiety, including a general reduction in exploratory activity and an increase in freezing in unfamiliar situations, which is similar to RTT patients. In addition, a few reports in the literature suggest that anxiety levels in RTT patients are widely variable based both on the age of the patient (anxiety decreases as patients age; Sansom et al., 1993) and on the specific mutation (Robertson et al., 2006).

Numerous studies in rodents and humans, link circuits in the amygdala to social anxious behaviors (Adolphs, 1999). In the current study, we note a 39% decrease in amygdala volume in the Mnulls. We infer that the reduction in volume in the amygdala alters circuitry and affects amygdala-dependent tasks (discussed above) and emotions associated with the amygdala, such as anxiety. Anxious behaviors in humans, the abnormal anxiety measures in MeCP2 mutant mice, and the physical changes in the amygdala we report here are consistent with the hypothesis that reduced amygdala functioning may be a neurobiological substrate for the abnormal anxious behaviors in
RTT (Mount et al., 2002, 2003). To our knowledge, volumetric MRI studies of the amygdala have not been performed in human RTT patients.

**How does this model of RTT compare with other published models, and more importantly to characteristics of RTT in humans?**

To date, behavioral characterization of four mouse models of RTT have been reported (Table 4). In all four models, severe motor deficits and breathing irregularities are noted. Notably, gait ataxia and stereotypic hand wringing motions and abnormalities in breathing are prominent features of RTT (Hagberg, 1985). Mental retardation is also a prominent feature of RTT, although the degree of severity varies (Hagberg and Witt-Engerstrom, 1986; Mount et al., 2002). The data presented here suggest that Mecp2 Mnulls present with cognitive deficits as revealed by fear conditioning and spatial and object recognition deficits. In contrast, in the mice developed by Zoghbi, there were no deficits in water maze or fear conditioning (Shahbazian et al., 2002). In this aspect, the mutants described here exhibit cognitive deficits and reprise an important characteristic of RTT that is not seen in this other model. In addition to motoric and cognitive deficits, the Mnulls exhibit changes such as piloerection, breathing irregularities, and body tremors that may reflect impaired autonomic functioning, another feature of this model that links it to RTT (see Table 4). These autonomic abnormalities probably lead to premature death in the Mnulls at about 70 days.

A recent article suggests that Mecp2\textsuperscript{2floxed-1Bird} null mice express mitochondrial dysfunction, and that mitochondrial dysfunction may contribute to the Rett phenotype, and account for similarities between Rett individuals and those with other mitochondrial disorders (Kriaucionis et al., 2006). The findings in the current study in Mecp2\textsuperscript{2floxed} mice of motor ataxia, reduced general activity, reduced body weight, and early death support this idea, and are also consistent with mitochondrial dysfunction phenotypes (Schapira, 2006).

**Insights from developmental studies**

The loss of MeCP2, in both humans and in the mouse model of RTT characterized here, clearly leads to selective volumetric reductions in a very specific subset of brain regions whereas MeCP2 is present ubiquitously throughout the brain. We considered the developmental timeline of each brain region examined in the current study. In rodents, the thalamus and the amygdala develop from embryonic day 14 to embryonic day 17, the hippocampus, striatum and cerebral cortex develop from embryonic day 15 to embryonic day 20 (Bayer et al., 1993). While some changes occur in these regions postnatally, when MeCP2 expression is increasing, the amygdala, hippocampus, and striatum develop at the same time as structures that are not significantly reduced (the cerebral cortex and the thalamus) (Bayer et al., 1993). These data suggest that the regional reductions observed in the current study do not occur during neurogenesis of these brain regions.

Small dendritic arborizations are a hallmark of RTT, and volumetric reductions could result from shorter dendritic arborization leading to a higher packing density (Kaufmann et al., 2005). It is likely that reduced cellular volumes and/or dendritic arborizations are responsible for the volume reductions observed in the Mnulls in the current study. These results are consistent with impaired neuronal maturation in the null mice. Because white matter structures anterior commissure and corpus callosum did not exhibit reduced volume in the Mnulls, we would hypothesize that reduced glial volume does not likely underlie the volumetric changes that we observe.

**Insights from memory studies**

We were curious as to why the amygdala, hippocampus, and striatum might specifically exhibit greater volume reductions than the other brain regions measured in this study. Converging evidence from studies of memory processes suggests that memory is organized into multiple systems, including the striatum and hippocampus, which are functionally linked. The striatum and hippocampus have long been associated with cued response and spatial memory respectively. In rodents, when an animal is given a choice between using striatal- or hippocampal-based learning to solve a cognitive task, animals with striatum lesions express a preference for using spatial memory systems to solve the task (Devan and White, 1999). Conversely, when lidocaine is administered in rat hippocampi to retard hippocampal functioning, the rats express a preference for using cued memory systems (Packard and McGaugh, 1996). These data and others (reviewed in Poldrack and Packard, 2003) suggest that the striatum and the hippocampus may interact competitively during memory processing.

It is hypothesized that this “competition” is mediated either through direct projections between hippocampus and striatum or through indirect modulation from another structure. While some evidence exists for direct connections, an increasing amount of evidence suggests that the amygdala may modulate the relative influence of the hippocampus and striatum in memory processes (Packard and Knowlton, 2002). These data together with recent evidence that mature MeCP2 expression is dependent on synaptic activity suggest an interesting hypothesis that reductions of MeCP2 in one or two brain regions, such as the amygdala, could lead to abnormal synaptic maturation in all areas of this memory circuit.

**CONCLUSIONS**

The Mecp2 mutant mice described, both here and in other studies, exhibit a phenotype that is less severe than RTT. For example, Mnull mice survive to early adulthood, and Fheteros survive to middle-age, in contrast to human RTT reports in which males only survive a year or two past birth and females survive to early adulthood (Schneider and Glaze, 2002). These differences in the effects of Mecp2 deletions in mice versus humans could result from differences in MeCP2 function in the two species, or from the
fact that in humans, many cases of RTT result from nonsense, frameshift mutations to MeCP2, and from null mutations (Shahbazian and Zoghbi, 2001). Nevertheless, our data suggest that the mutant mice described here provide an excellent model for RTT in that they exhibit 1) motor deficits, including stereotypic movements and loss of coordination, that are noted in RTT, 2) cognitive deficits that are reminiscent of mental retardation in RTT, and 3) alterations in regional brain volumes that support the abnormal behavioral phenotype and are generally consistent with volumetric MRI studies of RTT. As such, these MeCP2 mutant mice provide an excellent opportunity to examine the relationship between specific molecular and anatomical changes and their associated behavioral abnormalities, as well as provide an extraordinary vehicle for testing therapeutic interventions that may lead to amelioration and an eventual cure of this devastating disorder.

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REFERENCES


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