Mecp2 deficiency is associated with learning and cognitive deficits and altered gene activity in the hippocampal region of mice

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Rett syndrome (RTT) is a debilitating neurological condition associated with mutations in the X-linked MECP2 gene, where apparently normal development is seen prior to the onset of cognitive and motor deterioration at 6–18 months of life. A targeted deletion of the methyl-CpG-binding domain (MBD) coding region and disruption of mRNA splicing was introduced in the mouse, resulting in a complete loss of Mecp2 transcripts and protein. Postnatal comparison of XO and XY mutant Mecp2 allele-containing null mice revealed similar effects on mouse growth and viability, suggesting that phenotypic manifestations are not modulated by the Y-chromosome. Further assessment of Mecp2-null XY mice highlighted cerebellar and hippocampal/amygdala-based learning deficits in addition to reduced motor dexterity and decreased anxiety levels. Brain tissues containing the hippocampal formation of XY Mecp2-null mice also displayed significant changes in genetic activity, which are related to the severity of the mutant phenotype.

Keywords: Rett syndrome; methyl CpG-binding protein 2; gene expression; behaviour; hippocampal formation

Abbreviations: ES = embryonic stem; MBD = methyl-CpG-binding domain; MeCP2 = methyl-CpG-binding protein 2; QRT–PCR = quantitative real time reverse transcription–polymerase chain reaction; RTT = Rett syndrome; TRD = transcriptional repression domain


Introduction

Rett syndrome, RTT (MIM 312750), is a neurodevelopmental disorder associated with onset of cognitive and motor deterioration at 6–18 months of life (Hagberg et al., 1983). Up to 80% of classical RTT patients harbour mutations in the X-linked MECP2 gene encoding the methyl-CpG-binding protein 2 (MeCP2) (Amir et al., 1999). MeCP2 binds selectively to methylated CpG dinucleotides in mammalian genomic DNA (Lewis et al., 1992) via a methyl-CpG-binding domain (MBD) (Nan et al., 1993) and represses transcription of target genes by transcriptional repression domain (TRD) initiated interactions with histone deacetylases (HDAC1 and HDAC2) and the transcriptional co-repressor SIN3A (Nan et al., 1998). Mecp2 mRNA, in particular the 10 kb isoform, is highly expressed in the mouse brain (Pelka et al., 2005) and the protein is abundantly expressed in the CNS of both human and mouse (LaSalle et al., 2001), with most prominent expression being in the hippocampus, cerebellum and the cortex (Mullaney et al., 2004).

Initial attempts to produce Mecp2-null animals resulted in embryonic lethality (Tate et al., 1996), leading to conditional knockout approaches with Cre recombinase-mediated deletion of exon 3 (Chen et al., 2001) or exon 3 and part of exon 4 (Guy et al., 2001) of the Mecp2 gene. The male mutant mice develop an uncoordinated gait and a decline in spontaneous movement commencing at 3–8 weeks of age and subsequently exhibit body trembling, laboured breathing, hind-limb clasping and signs of physical deterioration prior to death at ~10 weeks (Chen et al., 2001; Guy et al., 2001). Onset of symptoms was variable in heterozygous mutant females between 3 and 9 months, with 50% developing breathing
irregularities and hind-limb claspng motions and showing a reduction in activity (Guy et al., 2001).

Mutant mice with a truncated MECP2 protein, similar to that seen in RTT patients, were generated by introducing a stop codon after the TRD (Shahbazian et al., 2002). These mice, termed MeCP2<sup>308</sup>, presented a milder phenotype. The hemizygous males developed motor abnormalities and tremors from 2 months of age, kyphosis at 4 months, EEG abnormalities, reduced activity and stereotypic limb movements. Ninety per cent of males survived to at least 1 year and maintained a normal body and brain weight, whereas heterozygous females showed minimal symptoms even at 1 year of age. The MeCP2<sup>308</sup> hemizygous males were studied as a model of the female RTT condition, circumventing the difficulties caused by the early lethality of MeCP2-null animals and allowed a detailed analysis of symptoms without the confounding effect of X-inactivation (reviewed by Warby, 2002).

Despite the emphasis on the assessment of male hemizygous mice it still remains untested whether Y-linked gene activity in the male may influence any downstream genetic activity that will affect the manifestations of the null–mutant phenotype. RTT is associated predominantly with females yet activity that will affect the manifestations of the null–mutant activity in the male may influence any downstream genetic features. The extended mouse longevity allowed investigation of behavioural deficits associated with motor function, anxiety level and learning capacity. Importantly, we show that in MeCP2-null male mice, cerebellar learning and hippocampal-/amygdala-based cognition are impaired, which has not been reported in published accounts of the truncated protein mutants (Shahbazian et al., 2002; Moretti et al., 2005). We also show that increases in disease severity are accompanied by a progressive increase in dysregulated expression of a set of genes.

**Materials and methods**

**Gene targeting and mouse crosses**

The BAC clone B22804 (Reichwald et al., 2000) containing the entire MeCP2 gene was digested with BamHI and a 10.8 kb fragment containing exon 3, and the first 3 kb of exon 4 was cloned into a pBluescript (Stratagene) vector. An Acyl digest released an 800 bp fragment to which the splice acceptor region from exon 3 and NotI and Acyl restriction sites were added to the 5′ end. This modified fragment was then cloned back into the construct. An Hpal and NotI digest subsequently removed 2 kb of sequence containing exon 3 and the first ~170 bp of exon 4 that code for the MBD. A loxP flanked Pgk-neo cassette was inserted into the Hpal/NotI ends of the targeting vector. Electroporations were performed using 800 V, 3 µF and 200 ohms (BioRad Gene Pulser), 1 × 10<sup>7</sup> R1 129 embryonic stem (ES) cells (Nagy et al., 1993) and 80 µg construct DNA. Following selection with G418, colonies were screened by polymerase chain reaction (PCR) and integration confirmed by Southern analysis (see Fig. 1 and Supplementary Figure 1). Correctly targeted ES cells were injected into blastocysts, which were subsequently transferred into

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**Fig. 1** Targeting of the MeCP2 gene in ES cells. Targeting strategy resulting in removal of MBD coding regions. Initial ES cell clone screening was performed with primers flanking the neomycin cassette, resulting in a 2.1 kb PCR product compared with a 1.8 kb wild-type product. Confirmation of correct integration was performed with primer sets external to the arms of homology and in the neomycin cassette giving 3.3 and 3.4 kb bands for primer sets spanning the 5′ and 3′ homologous arms, respectively. Southern analysis on NotI/EcoRV-digested DNA using a probe complementary to sequence downstream of the 3′ arm of homology showed an 8.7 kb band in the correctly targeted clone owing to insertion of a NotI site, in contrast to a 20.3 kb wild-type band.
pseudo-pregnant females. Resulting chimeric males were bred with 129 females to generate heterozygous females, which were then mated with 129 males. Heterozygous females from this line, termed Mecp2\(^{tm1neoTam}\), were mated with Paf males on a C3H/HeSnJ background (Lane and Davison, 1990) and the resulting 129/C3H/HeSnJ mixed background F1 offspring that contained 39 X\(^{Mecp2-O}\) and 40 X\(^{Mecp2-Y}\) genotypes was assessed. Heterozygous Mecp2\(^{tm1neoTam}\) females were also bred with Tnap-Cre males of a 129 background (Lomeli et al., 2000), resulting in Cre-mediated excision of the Neomycin cassette. The resulting heterozygous females were then mated with 129 males or C57BL6 males to generate the Mecp2\(^{tm11Tam}\) line on a pure 129 or mixed 129/C57BL6 background, respectively.

**Mouse phenotype analysis**

**Growth measurement**

Mouse weights for mutant mice and wild-type littermates were measured every 7 days commencing from postnatal day (P) 28.

**Freewheel activity**

Mouse activity was measured by placing freewheels in cages of solitary confined mice. Magnetic switches triggered with freewheel use and custom-designed software recorded the number of revolutions in 10 s increments. Up to 12 mice at a time were monitored over 9 days, with the first and last days excluded from analysis.

**Elevated plus maze**

The experimental animal was placed at the centre of a maze with a plus sign configuration, measuring 1.5 m across, which was raised 1 m above the ground. Two arms of the maze consisted of only a black Perspex floor, whereas the other two were enclosed with black Perspex. The amount of time spent in each type of arm and in the centre region was recorded over a 5 min test period.

**Rotating rod test (rotarod)**

The rotarod apparatus consisted of a 3-cm-diameter rotating cylinder with 24-cm-diameter end walls, where the speed of rotation could be increased. A mouse was placed on the rotating cylinder, which was set at the lowest speed, and the rotational speed was increased every 15 s. The time taken for the mouse to fall off the rod or be rotated twice along with the rod was recorded. Four successive trials on the rod were performed for each mouse with a 5 min interval between each trial.

**Cued and contextual conditioning task**

The stimulus unit of the economical exercise treadmill (model EXER-04, Columbus Instruments, Columbus, Ohio, USA) consists of stainless steel rods and was used to deliver a peak current of 1.6 mA. A cage was modified and the base removed, allowing the steel rods to form the floor of the cage. The test procedure (Wehner et al., 1997) was spread across two days. On Day 1 the mouse was placed in the test chamber and allowed to explore the cage for 2 min, after which a 30 s tone of white noise (static) was played, immediately followed by an electrical shock lasting 2 s, delivered through the steel coils. The tone and foot shock procedure was repeated 30 s later and the mouse was then placed back into its home cage. Twenty-four hours later the animal was placed back into the testing cage (context) and was observed for 5 min, with this time broken down into 10 s intervals, which were scored if the mouse froze in that interval. The mouse was then returned to its home cage. One hour later the animal was placed in a different test chamber. This chamber was larger and contained bedding material and an almond scent was applied. The number of freezing bouts was measured as before during a 3 min period, broken into 10 s intervals, and then the auditory cue (static) was played during a further 3 min and the number of freezing episodes was noted.

**Investigation of gene expression in mutant mice**

Quantitative real time reverse transcription–polymerase chain reaction (QRT–PCR) analysis was performed on 10 mg brain tissue samples from the region of the hippocampal formation that were conservatively dissected from 11 to 12-week-old Mecp2\(^{tm1Tam}\) male mice and wild-type littermates of a mixed 129/C57BL6 background. RNA was isolated using the Nucleospin RNA II Kit (Macherey Nagel) and quantified by spectrophotometry (Cary Bio300). cDNA was generated from 500 ng RNA using the Advantage RT kit (BD Biosciences) with oligo-dT primers. QRT–PCR was performed using the Rotorgene 2000 thermal cycler (Corbett Research) with SYBR green I (Molecular Probes) using heat-activated Taq polymerase (Amplitaq Gold, PE Applied Biosystems). Reaction conditions and primer sequences are shown in Supplementary Table 1. The PCR products were run in 2% gel to confirm correct band size. The levels of Gapdh and β-actin were used for the normalization of sample results. All samples were measured in duplicate. Melt curve analysis confirmed presence of a single amplified product following each run.

**Statistical analysis**

Means, standard deviations and standard errors of the mean (SEM) were calculated using Microsoft Office Excel 2003. Analysis of mouse behavioural data was performed using KyPlot version 2.0 (http://www.kahest.co.jp/Download/KyPlot/kyplot_e.htm), with one way analysis of variance (ANOVA), Wilcoxon–Mann–Whitney rank sum tests and unpaired t-tests being applied using a significance level of 0.05. QRT–PCR data tests of significance (0.05) were performed by a Wilcoxon–Mann–Whitney rank sum test (KyPlot).

**Results**

**Generation of Mecp2-deficient mice**

Mecp2-deficient mice were produced by replacing the coding sequence for the MBD with a floxed Pgk-neo cassette. A non-functional splicing site was introduced at the 5’ end of the gene sequence encoding the TRD, preventing splicing and transcription of downstream Mecp2 sequence for the TRD, the C-terminal domain and the 3’ UTR. The correctly targeted ES cells (Fig. 1 and Supplementary Figure 1) were used to produce mutant mice by germline transmission. Recombinase-mediated deletion of the floxed Pgk-neo (Supplementary Figure 2) in the Mecp2\(^{tm1neoTam}\) mutant mice was achieved by mating with Tnap-Cre mice (Lomeli et al., 2000) to generate the Mecp2\(^{tm11Tam}\) mice. No detectable Mecp2
transcripts were found in hemizygous mutant male brain tissue. Western analyses using mice with antibodies to both the N- and C-terminal ends of MECP2 also revealed an absence of detectable protein (Supplementary Figure 2). Mecp<sup>2m<sub>Tam</sub></sup> mice are therefore completely null.

**Y-chromosome function does not have any significant impact on the manifestation of Mecp2-null phenotype**

In our C3H/HeSn-Paf/J breeding stock (JAX no. 001529), the loss of the Y chromosome, due to non-disjunction during male meiosis (Lane and Davison, 1990), results in an overall 62.1 : 38.9 ratio of females : males. Among the females, 12.1% were 39 XO mice (Jameson et al., 1998). Mecp2-null 39 XO mice were produced by crossing Mecp2<sup>2m<sub>Tam</sub></sup> female mice with Paf male mice. In total, 153 females (56%) and 121 (44%) males were produced, which is slightly less, but not significantly, than the expected ratio. In the Mecp2<sup>+/Y</sup> × Paf/Y cross, ~49% (59 out of 121) of all male pups were mutant, which is consistent with the expected 50% of males being mutant for Mecp2. About 6.2% (17 out of 274) of mice were Mecp2− 39 XO R1 129 × C3H/HeSn, which is consistent with the fact that half of the expected 12.1% of XO females will inherit the mutant allele from the mother. Overall, 11% (17/153) of female mice were Mecp2− 39 XO. Since not all wild-type female mice were successfully karyotyped to distinguish between 40 XX and 39 XO, it is not known if the Mecp2− 39 XO has indeed made up 50% of all the XO females as expected. Nevertheless, the data suggest that there was no significant loss of any mutant mice before birth, irrespective of the genotype (i.e. Mecp2−/Y, Mecp2+/− × 40 XX or Mecp2+/− × 39 XO).

Both the Mecp2− 39 XO and Mecp2− 40 XY mice displayed a high mortality rate during postnatal development. The age by which 50% of the mice died was 7 weeks for Mecp2− 39 XO (n = 16) mice and 8 weeks for Mecp2− 40 XY (n = 48) (Fig. 2A). Only two male and one XO female survived to the 18th week. Mecp2− 39 XO mutant females grew like wild-type, heterozygote Mecp2+/− 40 XX and wild-type females (either 40 XX or 39 XO) during the first 6–7 weeks. The growth curve of Mecp2− 39 XO mutants deflected downwards at 7–8th week owing to the onset of growth retardation in some mice (Fig. 2B). The surviving XO mutants as a group showed progressive loss of weight after 11 weeks (Fig. 2B). Most XO mutant mice followed the trend of the population. However, some showed growth arrest at as early as the 4–5th week. Mecp2− 40 XY males displayed a growth profile similar to that of the wild-type males, until the age of 5 weeks, after which there was a progressive decline in weight gain (Fig. 2C). Malocclusion was relatively common in both mutant males and females, which could have underpinned the failure to thrive due to feeding problems and malnourishment. Death of the mice 39 XO and 40 XY mutants was preceded by cessation of weight gain, then weight loss and onset of lethargy. In summary, our data show that the mutant 40 XY and 39 XO mice exhibit similar profiles of postnatal viability and growth (Fig. 2C), suggesting that Y-linked gene activity has no significant impact on the manifestation of these two features of the Mecp2-null phenotype.

Mecp2<sup>2m<sub>Tam</sub></sup> mice generated by removal of the neomycin cassette were maintained on a 129 and a 129 × C57BL6 background after two backcrosses to C57BL6 mice. Mutant males on the 129 background displayed an earlier onset of postnatal lethality with half of the population succumbing by 5 weeks (Fig. 2D). Mutant males on the 129 × C57BL6 background showed a similar rate of lethality as the XY mutant of 129 × C3H/He background in the Paf crosses, with half of the animals dying at the 8th week (Fig. 2D). The mutant mice began to show failure to thrive from 5 weeks of age (Fig. 2E) and attained a mean weight of only 15.2 ± 1.3 g compared with 21.0 ± 0.7 g for wild-type littermates at 8 weeks (t-test \( P = 0.009 \)). No difference in growth and viability between heterozygous and wild-type littermate females over the first 13 weeks of life was observed (Fig. 2F). Mecp2<sup>2m<sub>Tam</sub></sup> males exhibited a marked decline in weight ~1 week prior to death and shed as much as 50% of their body weight. Prior to their demise mutant mice displayed the typical features of other Mecp2 mutants (Chen et al., 2001; Guy et al., 2001). They showed an unusual gait with sprawling hind limbs, claspings of hind limb when suspended by the tail, dishevelled fur and erected whiskers, laboured breathing, tremors and episodes of seizures. The brains of mutant mice were significantly lighter (360 ± 6 mg, \( n = 9 \)) than those of wild-type male littermates (450 ± 8 mg, \( n = 8 \), t-test \( P = 3.19 \times 10^{-7} \)). The reduction in brain weight (by 30% for wild-type), however, was not in proportion to that of the body weight, at 50% reduction (12.8 ± 1.4 g versus 23.2 ± 0.6 g, t-test \( P = 9.86 \times 10^{-6} \)).

**Mecp2<sup>2m<sub>Tam</sub></sup> mice display reduced locomotory activity and cerebellar learning**

To test if apraxia and ataxia exhibited by RTT patients (reviewed by Jellinger, 2003) is related to loss of MECPT2 function, we have investigated the motor abilities of the Mecp2<sup>2m<sub>Tam</sub></sup> XY mice. Mutant mice were selected while they were still generally active and still gaining weight, whereas those displaying seizures, laboured breathing or discernible tardiness were excluded from the analysis. The locomotory activities of mice were assessed by their activity on freewheels, which recorded the number of revolutions over a 7 day period. Mutant mice showed significantly lower levels of activity over the 7 days (t-test \( P = 1.04 \times 10^{-7} \)), with a mean daily revolution count of 1251 ± 121, compared with 8711 ± 335 of the wild-type littermates (Fig. 3A).

The rotarod test, consisting of a rotating rod that progressively accelerates, measures motor coordination and balancing ability (Crawley, 2000). Mutant mice remained on the rod for a much shorter mean time of 88 ± 5 s, compared with 264 ± 25 s for wild-type counterparts (Fig. 3B), representing...
a significant difference in motor ability (t-test $P = 0.0029$). To test the cerebellar learning ability (Crawley, 2000) the performance over successive trials was monitored. Successive trials on the rotarod for the MeCP2-null mice revealed significant deficits in cerebellar learning, with mutant mice failing to show significant improvements in their ability to stay on the rotarod (ANOVA $P = 0.7135$) compared with wild-type littermates (ANOVA $P = 0.0028$).
Reduced levels of fear/anxiety in \textit{Mecp2}\textsuperscript{-null} mice

To test if \textit{Mecp2} deficiency produces altered behaviour to their surroundings, we assessed the fear/anxiety of the mice on an elevated plus maze (Crawley, 2000). Over a 5 min period the total time spent in enclosed and open arms of the maze and in the semi-sheltered centre region was recorded (Fig. 3C). The natural tendency for wild-type mice is to prefer dark enclosed spaces when anxious. Mutant animals spent more time (56 ± 8 s) in the open arms and the centre region (68 ± 10 s) compared with 10 ± 3 and 41 ± 5 s, respectively, for the wild-type mice. The significant differences in the duration spent in different areas of the maze (t-test, open \( P = 0.00003 \), centre \( P = 0.0279 \)) suggested that mutants had apparently lower levels of anxiety. In addition, wild-type mice very rarely explored the open arms and none ventured to the ends of these arms, whereas mutant mice generally explored all areas of the maze.

\textit{Mecp2}\textsuperscript{-null} mice show impaired fear conditioning and contextual association

The cued and contextual conditioning task was undertaken to test if \textit{Mecp2-null} mice have any cognitive and learning deficits. Mice were trained to associate a foot shock with an audible tone (cue) in a new environment (context). Mice that previously experienced the combination of shock, cue and context were assessed for their ‘freezing’ response to the testing environment and to associate and relate the context and cue with prior foot shocks. As a control, to measure baseline freezing, the mice were also placed in a different environment (altered context). The results showed that mutant mice were less capable in remembering the context and associating this with prior foot shocks (Fig. 3D). Only 7 ± 1.2 freezing episodes were recorded for the mutant mice \(( n = 10 )\) compared with 14.8 ± 0.8 freezing events for wild-type animals \(( n = 12 , \text{ } t\text{-test } P = 1.80 \times 10^{-6})\). The ability of mutants to associate the auditory cue with shock was also significantly impaired—1.4 ± 0.4 freezing episodes compared with 5.75 ± 0.25 \(( t\text{-test } P = 6.86 \times 10^{-8})\). Both groups showed minimal freezing in the altered context with 1.25 ± 0.25 for wild-types and 1.2 ± 0.25 for mutants \(( t\text{-test } P = 0.89)\).
shown) and the frontal cortex of human RTT brain (J. Gibson and J. Christodoulou, unpublished results) and an association with a RTT-like phenotype (Cdkl5) (Tao et al., 2004; Weaving et al., 2004). The amygdala is believed to be involved in fear conditioning (Fanselow and Kim, 1994), whereas the hippocampus, cingulate cortex, prefrontal cortex, perirhinal cortex and sensory cortex are associated with contextual association (Eichenbaum et al., 1996; Squire and Zola, 1996; Logue et al., 1997), all areas of which show increased Mecp2 expression (Pelka et al., 2005). Given the cognitive deficits in Mecp2<sup>tm1Tam</sup> mice, we decided to focus the assessment of gene expression to the hippocampal region of these mice.

QRT–PCR on hippocampal brain samples from six male wild-type mice, six mutants with minimal phenotypic features (termed active mutants) and six mutants displaying significant disease features (sick mutants) were performed. Gapdh and β-actin housekeeping genes were used to normalize samples, and all 18 brain samples were analysed in duplicate in the same QRT–PCR run for each gene to minimize inter-run variability. Following amplification, melt curve analysis confirmed the presence of a single band (Fig. 4).

Of the 17 non-X-linked genes analysed, 11 were found to be significantly dysregulated between the sick mutant animals and wild-type littermates, whereas 6 showed non-significant changes (Fig. 4A). The direction and magnitude of significant gene expression changes in active and sick mutants is indicated in Fig. 5. Genes that showed significantly increased expression between sick mutants and their wild-type littermates included Sgk, Sup30 and Pdk4. Sgk displayed the greatest fold-change increase, reaching levels 4.5 times that seen in wild-type animals, and an ~2-fold increase was seen in Sup30 and Pdk4 expression. Significantly decreased expression levels were observed for Fabp7, His2b, Gap43, Kif1b, Lin7b and Zfp40, with fold-change reductions ranging from 1.5× for Zfp40 to 3.5× for Fabp7. No pattern of chromosomal localization was identified, and Chromosomes 8 and 10 each coded for two of these genes, whereas a single gene was located from Chromosomes 2, 4, 6, 7, 13, 16 and 17. For the X-linked genes, two had significant changes (Fig. 4B) and their expression change for active and sick mutants is indicated in Fig. 5C and I. Irak1 expression was increased 2.5-fold, whereas Xlr3a was decreased 4.5-fold in sick mutants relative to wild-type controls. Both of these genes reside on band A7.2, which incidentally also contains Mecp2. Active and sick mutant results were tested separately to reveal any differences in genes expression that might be present between these groups. No significant changes were observed when active and wild-types were compared with each other except for Sgk (Fig. 5A), indicating that most changes are exacerbated in the more severe disease state. The direction of change was consistent for each gene, with a progressive increase between gene dysregulation in active and sick mutant sample groups.

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**Fig. 4** Autosomal (A) and X-linked (B) genes assayed by QRT–PCR. A single gene specific product is produced for each gene analysed. Chromosomal origins of genes are indicated, mt indicates a mitochondrial encoded gene and band positions are given in the case of X-linked genes. A plus (+) signifies that a significant increase in transcript level was detected in sick mutants compared with wild-type littermates, whereas a minus (−) indicates a significant reduction (data in Fig. 5). Genes for which non-significant changes were found are labelled ns. Significance was assessed using the non-parametric Wilcoxon rank sum test.

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Effects of Mecp2 deficiency in mice

Brain (2006), 129, 887–898 893

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Fig. 5 Genes assayed by QRT–PCR whose expression was significantly affected in Mecp2<sup>tm1Tam</sup> male mice. Genes found to be significantly increased included (A) serum glucocorticoid-regulated kinase, (B) Sin3-associated polyepptide 30 kDa, (C) the X-linked interleukin 1 receptor-associated kinase and (D) pyruvate dehydrogenase kinase 4. Significantly downregulated genes included (E) fatty acid binding protein 7, (F) H2B histone family member S, (G) notch-regulated ankyrin repeat protein, (H) growth-associated protein 43, (I) X-linked lymphocyte-regulated 3a, (J) kinesin family member 1B, (K) lin 7 homolog b and (L) zinc finger protein 40. WT, wild-type XY littermates; M active, Mecp2<sup>tm1Tam</sup> XY mice with few disease features; M sick, Mecp2<sup>tm1Tam</sup> XY mice with advanced disease features. Each sample was measured in duplicate; n = 6 for each group type and inter-sample variability normalized using Gapdh and Actin, with Mecp2 deficiency having no significant impact on these housekeeping genes. Error bars indicate the SEM.
The increased magnitude of dysregulation observed in sick mutants may be responsible for the progression of the disease phenotype. It should be noted that animals used in the assessment of motor and learning deficits in our mutant mice would be classified as active mutants, demonstrating that the level of gene regulation disruption among this mutant subgroup if indeed responsible for the ‘sick’ mutant phenotype was sufficient to cause behavioural deficits. The level of Cdkl5 expression was found to remain stable in both active and sick mutants with means of 1.28 ± 0.22 and 1.00 ± 0.16, respectively compared with 1.38 ± 0.24 in the wild-type group (P = 0.87; P = 0.34). This is consistent with the previous finding that Cdkl5 and Mecp2 activity is regulated independently (Weaving et al., 2004; Mari et al., 2005).

**Discussion**

Mecp2<sup>tm1Tam</sup> mice display motor deficiencies, altered levels of anxiety and reduced cognitive function

Detailed behavioural analysis of the impact of Mecp2 deficiency has only previously been conducted on Mecp<sup>2<sub>308</sub></sup> XY mice, which have a considerably milder phenotype (Shahbazian et al., 2002; Moretti et al., 2005). Our Mecp2<sup>2<sub>308</sub>/Y</sup> mice aged 8–11 weeks exhibited significant reductions in activity and motor coordination with significant decreases in freewheel use and time spent on the rotarod, respectively. This is in contrast to the 10-week-old Mecp2<sup>2<sub>308</sub>/Y</sup> mice, which did not show any motor deficits (Moretti et al., 2005), at least not until 5 months of age, where a slight but statistically significant reduction in time spent on the rotarod was observed (Shahbazian et al., 2002). Our data demonstrate for the first time that the complete absence of Mecp2 leads to motor deficits as early as 8–11 weeks. Motor behaviour is dependant on many factors, with even small irregularities in axonal signal transduction or neurotransmitter levels at synapses being able to be manifested as motor impairments. Presumably, both granular and Purkinje cells are responsible for the coordination deficits observed in Mecp2-deficient animals, given that mice with deficits in cerebellar neuroanatomy such as hot-foot (defective innervation of Purkinje cells) and staggerer (progressive loss of granule and Purkinje cells) all perform poorly on the rotarod and fall off between 0–100 s compared with the 300–400 s seen in wild-types (Lalonde et al., 1996).

Our finding of reduced anxiety levels in 8- to 11-week-old Mecp2<sup>2<sub>308</sub>/Y</sup> mutant males conducted using the elevated plus maze contrasts with observations on the Mecp2<sup>2<sub>308</sub></sup> mouse. At a similar age, Mecp2<sup>2<sub>308</sub>/Y</sup> mice displayed normal anxiety responses (Moretti et al., 2005); however, when 8-month-old Mecp2<sup>2<sub>308</sub>/Y</sup> mice were tested by the open field test, a reduced inclination to venture into the centre of the open field became apparent (Shahbazian et al., 2002), suggesting increased levels of anxiety.

The cued and contextual conditioning task on the Mecp2<sup>2<sub>308</sub>/Y</sup> mice revealed that there are cognitive deficits associated with memory and learning, highlighting for the first time that the complete absence of Mecp2 affects cognitive processes. Conversely, published studies on Mecp2<sup>2<sub>308</sub>/Y</sup> mutant mice did not show such a decrease, with normal cognitive function in these animals being reported (Shahbazian et al., 2002). Aside from our behavioural assessment implicating Mecp2 deficiency with learning impairments, recent work overexpressing human MECP2 in mice at twice the level of the endogenous protein found enhanced cerebellar motor learning at 10 weeks and hippocampal- and amygdala-dependent learning at 20 weeks (Collins et al., 2004). These animals, however, subsequently became hypoactive and developed seizures and kyphosis at 30–52 weeks.

Repeated trials on the rotarod with our Mecp2<sup>2<sub>308</sub>/Y</sup> mice revealed that they failed to improve significantly, suggesting further learning deficiencies in mastering this task. Both C57BL6 and 129/SvJ mice have previously been shown to be good learners and consequently perform well on the cued and contextual task (Crawley, 2000). Both strains also show improvement in successive trials on the rotarod (Homanics et al., 1999), further highlighting the poor performance of our Mecp2<sup>2<sub>308</sub>/Y</sup> animals. Our findings suggest that in addition to hippocampal and amygdala-based learning deficiencies, Mecp2<sup>2<sub>308</sub>/Y</sup> mice also exhibit deficits in cerebral function, not only in terms of motor ability but also in the capacity to learn the task, and as a result they do not show improved performance over successive trials on the rotarod.

Our findings showed a more severe impairment of mouse ability compared with the Mecp2<sup>2<sub>308</sub></sup> mouse model for which extensive behavioural studies have been conducted (Shahbazian et al., 2002; Moretti et al., 2005). However, cells of Mecp2<sup>308</sup> mice express a truncated protein with the MBD and TRD elements of the protein still intact, suggesting residual protein function, as thought to be the case in RTT patients presenting with milder disease features. The disparity in anxiety and cognitive responses between these two mouse lines may be a consequence of having a truncated Mecp2 protein compared with no protein at all. The difference in disease severity between the Mecp2<sup>2<sub>308</sub></sup> mouse and Mecp2-null mice (this study and Chen et al., 2001; Guy et al., 2001) are clearly evident and reflect the situation that among RTT patients early truncating mutations or those affecting the MBD may produce more severe disease features than mutations at the 3’ end of the gene (Weaving et al., 2003; Smeets et al., 2005). Importantly, our study for the first time demonstrates that a specific behavioural response and thereby the underlying mechanistic process may be differentially affected between different introduced mutations.

**Mecp2 deficiency and gene expression in hippocampal tissue**

A current focus of the research on RTT is centred on the identification of direct MECP2 targets and downstream
genes affected by Mecp2 mutation in an effort to elucidate the pathophysiology of RTT. Putative target genes of MECP2 include the following: Hairy2a, a transcriptional repressor protein responsible for silencing neuronal genes in non-neuronal cells (Stancheva et al., 2003); Bdnf, believed to be involved in the maintenance of neurons (Chen et al., 2003; Martinowich et al., 2003) and the maternally imprinted gene Dlk5, which is implicated in forebrain and craniofacial development (Horike et al., 2005). The QRT–PCR assays undertaken using our mice have identified a number of genes whose expression is dysregulated and could potentially contribute to the RTT phenotype. Not analysing whole-brain samples has the advantage of avoiding brain regions where cells are potentially less sensitive to a lack of MECP2, which could mask the true extent of gene dysregulation in regions responsible for eliciting RTT features. We focused our analysis on hippocampal tissue because of the association of learning/memory memory process with this brain region and the impaired performance of our mice in the cued and contextual conditioning task. Functional aspects of genes found to be significantly dysregulated are shown in Supplementary Table 2 along with effects on mouse behaviour. We found that the extent of dysregulation was progressive and increased in animals manifesting a more severe phenotype. The changes identified in gene expression may, however, be a consequence of secondary effects induced by Mecp2 deficiency, rather than the primary, direct interaction between Mecp2 and its target gene.

Having discounted the Y chromosome in modulating the severity of phenotype in mice, it can be inferred that other genes on the X-chromosome or genes on the autosomes are likely to be influencing the RTT phenotype. Given the high concentration of loci involved in cognition on the X-chromosome (Zechner et al., 2001) we hypothesized a greater level of X-linked gene dysregulation; however, only two genes, Xlr3a and Irak1, were found to exhibit significantly altered levels of expression in our Mecp2-null mice. Recent microarray investigations comparing the expression patterns of X-linked genes and parent of origin effects present in XO mice revealed that Xlr3b, Xlr4b and Xlr4c are imprinted (Davies et al., 2005; Raefski and O’Neill, 2005). The ~3-fold increase in levels of Xlr3b present in mice with a maternally derived X have been attributed to cognitive deficits associated with use of a Y-maze-based reversal learning task, which involves the pre- and orbital–frontal cortex and hippocampal regions (Davies et al., 2005) and parallel behavioural deficits in Turner syndrome patients with a maternal X. The Xlr3a gene was found not to be imprinted (Davies et al., 2005). The impact of the 4.5-fold decrease in Xlr3a levels in our Mecp2<sup>2<sup>null</sup></sup> mice in terms of possible phenotype contribution is unknown, as the role of Xlr3 genes in the context of cognition remains enigmatic; they are, however, part of a larger family of chromatin-associated proteins (Bergsagel et al., 1994). Similarly, the impact of a 2.5-fold increase in Irak1 expression on the brain remains unknown.

Two genes, Gap43 and Kif1b, were downregulated in the hippocampus of the Mecp2<sup>2<sup>null</sup></sup> mice. Growth-associated protein 43 (Gap43) is highly expressed during axonal growth, but expression persists in the adult in highly plastic regions and in pre-synaptic terminals (Benowitz et al., 1998; Udvardia et al., 2001; Feig, 2004). The protein is also upregulated during long-term potentiation, indicative of an involvement in learning and memory processes (Cammarota et al., 1997; Pascale et al., 2004). Gap43-null mice displayed significant impairments in muscle strength, balance and limb coordination, hyperactivity and reduced anxiety, with heterozygous mice showing milder impairments (Metz and Schwab, 2004). The performance deficits in Gap43-deficient mice are similar to that observed in Mecp2<sup>2<sup>null</sup></sup> mice. The kinesin family member 1B (Kif1b) gene is associated with axonal-based transport, involved in supplying essential organelles and materials to nerve endings using microtubules (Hirokawa, 1998). Disruption of the Kif1b gene leads to mice that develop neurological disorders reminiscent of Charcot–Marie–Tooth (CMT) disease (Zhao et al., 2001). The homozygous mice die within 30 min of birth and exhibit respiratory distress, have reduced brain size with a reduction in the number of neurons and have abnormal synapses. Heterozygous mice displayed progressively reduced motor capabilities associated with muscle weakness and motor dis-coordination with impaired performance on the rotarod and balance beam test. Analysis of the sciatic nerves of 2-month-old Kif1B<sup>+/−</sup> mice revealed a reduction in synaptic vesicle proteins in nerve axons but not in nerve cell bodies (Zhao et al., 2001). It is possible that reduced levels of this motor protein, as a consequence of Mecp2 deficiency, may not sustain the chemical or energy requirements of neurons, leading to impairment of nerve function and the features of RTT.

In summary, behavioural analysis of our Mecp2-null mice revealed that Mecp2 deficit not only impacts motor function and affects anxiety levels but also cognitive function involving the amygdala and hippocampus, which has not been shown before. Our mouse model along with models of functionally null mutations may provide a novel perspective on the impact of Mecp2 deficiency on animal behaviour.

**Supplementary material**

Supplementary data are available at Brain Online.

**Acknowledgements**

We thank Mehtap Baserdem, Melissa Jones and Irma Villaflor for care of mice and Peter Rowe for comments on the manuscript. We also extend our gratitude to Visalini Nair-Shalliker, Kata Popovic and Nanthakumar Subramaniam for technical assistance pertaining to western blots. The animal experiments were approved by the CMRI/CHW Animal Ethics Committee (ACEC project 156). Our work was supported by the National Health and Medical Research Council (NHMRC) of Australia, the Rett Syndrome Association of New South Wales, the Rett Syndrome Australian Research Fund, the Country Women’s Association of New South
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Wales, the Clive and Vera Ramaciotti Foundation and Mr James Fairfax. G.J.P. was a NHMRC Dora Lush scholar and a Western Sydney Genetic Program Rett Syndrome Scholar. P.P.L.T. is a NHMRC Senior Principal Research Fellow.

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