Minireview

Experimental models of Rett syndrome based on Mecp2 dysfunction

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Abstract

Rett syndrome (RTT) is a neurodevelopmental disorder predominantly occurring in females with an incidence of 1:10,000 births and caused by sporadic mutations in the *MECP2* gene, which encodes methyl-CpG-binding protein-2, an epigenetic transcription factor that binds methylated DNA. The clinical hallmarks include a period of apparently normal early development followed by a plateau and then subsequent frank regression. Impaired visual and aural contact often lead to an initial diagnosis of autism. The characterization of experimental models based on the loss-of-function of the mouse *Mecp2* gene revealed that subtle changes in the morphology and function of brain cells and synapses have profound consequences on network activities that underlie critical brain functions. Furthermore, these experimental models have been used for successful reversals of RTT-like symptoms by genetic, pharmacological and environmental manipulations, raising hope for novel therapeutic strategies to improve the quality of life of RTT individuals.

Keywords: Rett syndrome, MeCP2, neurodevelopmental disorder, synapse, dendritic spine, hippocampus, BDNF

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Introduction

Rett syndrome (RTT; Online Mendelian Inheritance in Man #312750; http://www.ncbi.nlm.nih.gov/omim/), first recognized and described in German by Andreas Rett,[1] is a neurodevelopmental disorder predominantly occurring in females. Almost 20 years later, Hagberg *et al.*[2] presented the first description of RTT in the English language, leading to worldwide diagnosis in all ethnic and racial groups. Currently, the incidence of RTT is estimated to be approximately 1:10,000 female births.[3,4] Early studies proposing a genetic basis for RTT were later confirmed by Zoghbi and co-workers[5] with the identification of mutations in the *MECP2* gene located at chromosome Xq28. *MECP2* encodes methyl-CpG-binding protein-2, a transcription factor that binds methylated DNA and is ubiquitously expressed in mammalian tissues.[6] Following the identification of loss-of-function mutations in the *MECP2* gene in RTT individuals, research efforts expanded rapidly on an international scale with comprehensive clinical investigations into the complex array of medical and behavioral issues. In addition, intensive laboratory-based studies have been spurred by the availability of human autopsy tissue and experimental animal models, such as deletions of the endogenous *Mecp2* gene (knockout), insertions of premature STOP codons or RTT-associated mutations (knock-in) common in the human *MECP2* gene and overexpression of Mecp2 to model the newly identified *MECP2* duplication syndrome.[7–9]

RTT is a sporadic condition in >99% of the cases, with the risk of familial recurrence being extremely low. Indeed, *MECP2* mutations appear to be spontaneous transitions occurring in the paternal germline,[10,11] explaining in part the paucity of males with RTT or carrying *MECP2* mutations. The clinical hallmarks of RTT include a period of apparently normal early development followed by a plateau or stagnation in development and a subsequent frank regression. It is during this period that both visual and aural contact is impaired, leading to an initial diagnosis of autism. The convergence of clinical presentations in RTT and autism in association with *MECP2* mutations represents an intriguing link between these disorders and other neurodevelopmental conditions with an established genetic basis and clinical features consistent with autism spectrum disorders, such as fragile X syndrome and Down's syndrome.[12] Here, we briefly review the features of RTT and its genetic bases. We then provide a detailed description of the existing mouse models of RTT based on MeCP2 dysfunction, and their experimental use to test novel therapeutic approaches for the reversal of established neurological deficits.

RTT neuropathology

Gross anatomy and cellular morphology in autopsy brains from RTT individuals reveal very consistent and distinct features.[13] First and foremost, the absence of any recognizable pattern of neuronal or glial cell atrophy, degeneration or death, gliosis, demyelination or neuronal migration defects, as well as the lack of disease progression is critical to differentiate RTT from a neurodegenerative disorder.[14,15]

Reduced brain and neuronal size with increased cell density is consistently observed in several brain regions, including the cerebral cortex, hypothalamus and the hippocampal formation.[16,17] In addition, biopsies of the nasal epithelium revealed far fewer terminally differentiated olfactory receptor neurons and significantly greater numbers of immature neurons in RTT individuals compared with unaffected controls.[18] Furthermore, the size and complexity of dendritic trees was reduced in pyramidal cells of the frontal and motor cortices and of the subiculum,[19,20] while levels of microtubule-associated protein-2 (MAP-2), a protein involved in microtubule stabilization, were lower throughout the neocortex of RTT autopsy material.[21,22] In addition, autoradiography in the frontal cortex and basal ganglia of autopsy RTT brains revealed complex, age-related abnormalities in the density of neurotransmitter receptors, such as excitatory NMDA-(*N*-methyl-D-aspartate), AMPA-(α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid), kainate- and metabotropic-type glutamate receptors (GluRs) as well as inhibitory GABA receptors.[23,24] Furthermore, 1H spectroscopy at 4.1 T revealed that the ratio of glutamate to *N*-acetylaspartate was elevated in the gray matter of RTT individuals compared with their unaffected siblings, while unchanged in white matter.[25] Finally, the density of dendritic spines is reduced in pyramidal neurons of the frontal cortex[14,26] and of the CA1 region of the hippocampal formation (Figure 1),[27] which is consistent with a reduced expression of cyclooxygenase, a protein enriched in dendritic spines.[28] This so-called spine dysgenesis phenotype[29] is common to other neurodevelopmental disorders, including Down's syndrome, autism, Angelman syndrome and fragile X syndrome.[12,20,30] Such striking commonality across a spectrum of disorders, most with distinct molecular mechanisms, suggests a fundamental linkage through common pathways of neurobiological development responsible for cognitive performance.[31]

Gene function: *MECP2*

MeCP2 is a member of a family of proteins that bind regions of DNA enriched with methylated CpG regions, i.e. cytosine and guanine nucleosides separated by a phosphate group.[32] Two major functional domains characterize this family of proteins: the methyl-CpG binding domain (MBD) and the transcriptional repression domain (TRD).[33,34] The best and first characterized function of Mecp2 is to repress gene transcription by recruiting co-repressor and the mSin3a/histone deacetylase complex and altering the structure of genomic DNA.[35] This classical view of Mecp2 as a global transcriptional repressor exclusively has been questioned by the recent realization that out of all the genes misregulated in the hypothalamus of both *MECP2* overexpressing and *Mecp2* knockout mice (see below), the majority (~85%) were activated in the overexpressing mice and down-regulated in the knockout mice, which indicates that Mecp2 has a broader gene transcription role than originally thought.[36] In addition, Mecp2 interacts with the RNA-binding protein Y box-binding protein 1 and regulates splicing of reporter minigenes, which may explain the aberrant alternative splicing patterns observed in *Mecp2*$^{308/Y}$ mice,[37] which carry a premature STOP codon and express a truncated non-functional Mecp2 protein (see below).[38]

The human *MECP2* gene has four exons,[39] from which two protein isoforms differing in their N-termini are expressed by alternative splicing: MECP2-e1 (previously identified as MECP2B/MECP2α), the most abundant isoform; and MECP2-e2 (previously identified as MECP2A/MECP2β).[40,41] Total protein and mRNA expression from the mouse *Mecp2* gene (without differentiating between isoforms) is widely distributed throughout the developing and adult brain.[38,42,43] More recently, brain-region-specific splicing of the *Mecp2* gene was observed during mouse brain development: *Mecp2-e2* mRNA was enriched in the dorsal thalamus and layer V of the cerebral cortex, while more *Mecp2-e1* transcript was detected in the hypothalamus than in the thalamus between postnatal days 1 and 21.[44]

So far, more than 250 different *MECP2* mutations have been identified in individuals with RTT. However, eight common point mutations (R106W, R133C, T158M, R168X, R255X, R270X, R294X and R306C) account for about 65% of those with RTT, while large deletions involving one or more exons, and 3′ deletions account for another 15–18% of RTT individuals (RettBASE: IRSF *MECP2* Variation Database; http://mecp2.chw.edu.au/). While the vast majority of individuals expressing a *MECP2* mutation do fulfill the diagnostic criteria for typical RTT, significant phenotypic variation is associated with such mutations.[45,46] These include individuals meeting variant atypical RTT criteria, female carriers who may be normal or have mild learning or cognitive disabilities, and more significantly include individuals with prominent behavioral phenotypes, such as autism and obsessive–compulsive and aggressive behaviors in associations with moderate to severe cognitive delay. Individuals meeting the RTT variant criteria, such as preserved speech but with a significant delayed onset (up to 10 years of age or more) are noted in association with milder involvement, whereas early onset seizures and congenital onset are noted with more severe clinical features.[45,47] Inasmuch as RTT is a sporadic condition with extremely low risk of familial recurrence, female carriers represent less than 3% of the total participants in the RTT natural history study.[48] However, this group is likely to be underrepresented because they would not be recognized if it not were for an affected child or sibling.

A possible explanation for the wide phenotypic variability in clinical presentations is skewing of the normally expected random X chromosome inactivation (XCI), whereby the inactivation of one of the X chromosomes in

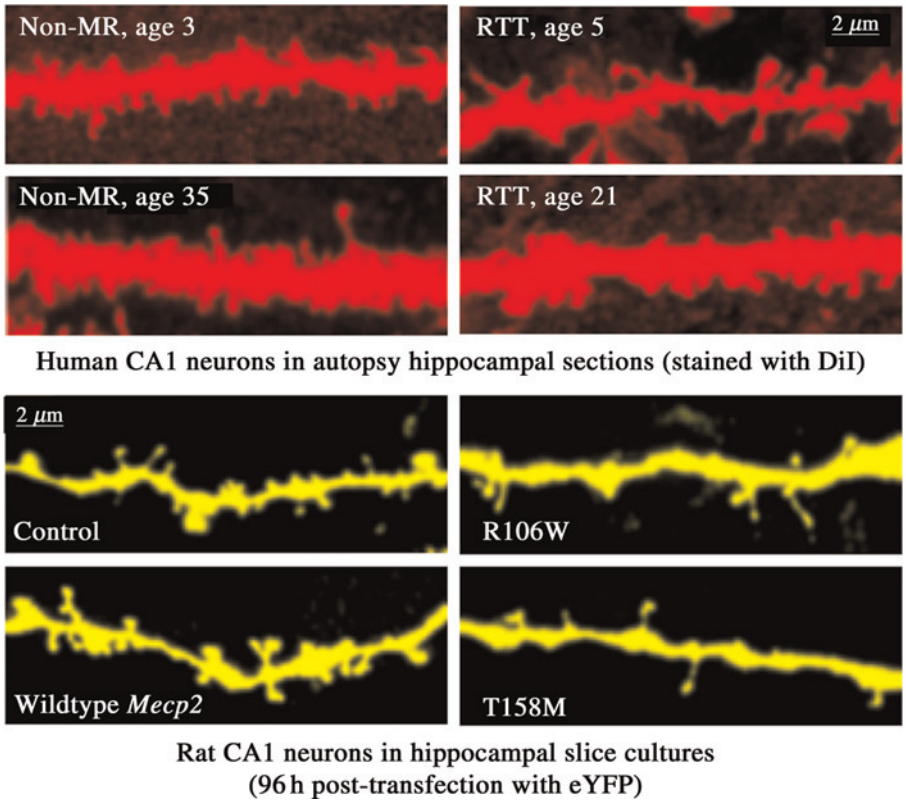

Figure 1 Dendritic spines of pyramidal neurons in rat hippocampal slice cultures and from human hippocampus. Upper panels: CA1 pyramidal neurons from human hippocampus stained with DiI by 'diolistics' showing marked reduction and aberrant morphology of dendritic spines in samples from a child (age 5) and an adult (age 21) with RTT, compared with non-MR controls, a child (age 3) and an adult (age 35), respectively. Lower panels: Rat CA1 neurons (96 h post-transfection with enhanced yellow fluorescent protein [eYFP]) in slice cultures showing an increase in spine density (wildtype MECP2 overexpression) and decrease in spine density (mutant R106W and T158M MECP2 overexpression). Modified with permission from Chapleau *et al.*[27] (A color version of this figure is available in the online journal)

every female cell typically permits the expression of genes from the active X chromosome in the adult. As such, individuals with normal function or only mild involvement would represent cases of wildtype MECP2 expression in the majority of cells. However, it is important to mention that this explanation may account for only ~20% of RTT cases related to variances in severity.[49]

Among males, the most common *MECP2* dysfunction is associated with duplications of the Xq28 region, which include variable numbers of other genes. However, the principal clinical features in these male individuals appear to relate to the overexpression of *MECP2*, because overexpressing the human *MECP2* gene in mice caused significant neurological deficits (see below).[50] Thus, a *MECP2* duplication syndrome has been defined.[7–9] Male individuals carrying *MECP2* mutations generally have non-specific cognitive delay with or without progressive motor problems or a severe early infantile encephalopathy,[51] as well as clear autistic features.[52] As with carrier females, these males are likely under-represented, unless a sibling has been identified with RTT or a progressive encephalopathy. In addition, typical RTT features have been described in a small number of males with either somatic mosaicism or in combination with Klinefelter syndrome (47XXY), because in both instances, two cell populations of X chromosomes would exist, similar to females with RTT.

Model systems: *Mecp2* knockout, *Mecp2* mutant and *MECP2* knock-in mice

To advance the basic knowledge of the role of Mecp2 on brain development and function, as well as to understand the molecular and cellular bases of RTT pathophysiology, several different mouse models were generated based on targeted manipulations of the endogenous *Mecp2* gene or targeted introduction of the human *MECP2* gene, either wildtype or carrying RTT-associated mutations. The following mouse models have been generated, and their similarities to the clinical presentation in RTT individuals will be discussed in the next section.

(1) Mecp2 knockout mice. The first report of constitutive *Mecp2* deletion in mice described embryonic lethality,[53] which led to the generation of mice using the Cre recombinase-loxP system of conditional deletion in selected tissues at desired times.[54] Embryonic lethality was prevented by breeding mice that have *Mecp2* exons 3 and 4 flanked by loxP sites (i.e. 'floxed' or *Mecp2*^{2lox}) with 'Cre deleter' mice that ubiquitously express a Cre transgene in

the X chromosome.[55] The resulting progeny (*Mecp2*$^{tm1.1Bird}$, 'Bird strain') carry deletions spanning *Mecp2* exons 3 and 4 starting in early embryonic development.[56] These mice showed no signs of Mecp2 expression using antibodies directed against either N- or C-termini of the protein.[56,57] In addition, brain-specific targeting to the neuron and glial lineage was achieved by breeding floxed *Mecp2* mice with mice expressing a *Nestin-Cre* transgene, which is highly expressed in neural precursor cells beginning at E12.[58] However, it should be noted that the mice with *Mecp2* exons flanked by loxP sites used in the generation of these knockouts (Bird *Mecp2*$^{2lox/Y}$) already show ~50% lower levels of Mecp2 protein than wildtype controls (without Cre-mediated recombination), and thus should be considered '*Mecp2* hypomorphs' (see below).[59]

Another strain of *Mecp2* knockout mice was generated by removal of the coding MBD sequence (entire exon 3 and part of exon 4) and introduction of a non-functional splicing site at the 5′ end of the gene sequence encoding the TRD, preventing splicing and transcription of the downstream *Mecp2* sequence for the TRD, the C-terminal domain and the 3′ UTR (*Mecp2*^{tm1Tam}).[60] These mice showed no signs of Mecp2 protein expression using antibodies directed against either N- or C-termini, and developed a postnatal behavioral phenotype that resembles RTT.

(2) Mecp2 mutant mice expressing a truncated protein: Brain-specific deletions of *Mecp2* exon 3, which encodes 116 amino acids including most of the MBD, led to the expression of a truncated protein in the neuron and astrocyte lineage beginning at E12 using a *Nestin-Cre* transgene[61] (*Mecp2*$^{2lox/X}$, *Nestin-Cre*). Because these mice express a truncated Mecp2 protein lacking the MBD but with an intact C-terminus – which includes the nuclear localization signal and potentially the TRD and other downstream domains[57,61,62] – they should be considered mutant mice rather than 'knockout' or 'null' mice. Therefore, the consequences of the expression of a mutant protein of unknown function should be taken into account. In addition, it is unknown whether the introduction of the loxP sites flanking *Mecp2* exon 3 in Jaenisch *Mecp2*^{2lox} mice (before Cre-mediated loxP deletion) had consequences on Mecp2 protein expression similar to that detected in Bird *Mecp2*$^{2lox/Y}$ mice.[59] It should be noted that most studies of this so-called 'Jaenisch strain' use mice derived by germline recombination (*Mecp2*^{1lox}) where the mutations in *Mecp2* are not longer brain-specific.[61]

The introduction of a premature STOP codon in the mouse *Mecp2* gene led to the expression of a protein truncated at amino acid 308 (*Mecp2*^{308}).[38] These mice express a truncated protein with the MBD and a portion of the TRD elements still intact, suggesting residual protein function, as thought to be the case in RTT patients presenting with milder disease features.

(3) Cell-type-specific Mecp2 deletions or mutations: The Cre/loxP recombination system has been used to generate the following conditional *Mecp2* knockout or mutant mice:

(i) Breeding Jaenisch *Mecp2*^{2lox} mice with mice expressing *Cre* under control of the *CamkII* promoter (*CamkII-Cre*^{93} line)[63] allowed the *Mecp2* mutations to be selectively expressed in forebrain excitatory neurons after approximately postnatal-day 20.[61] It should be noted that these mice express a milder behavioral phenotype with a delayed onset compared with that caused by more widespread deletion[56] or truncation[61] of Mecp2 using the *Nestin-Cre* transgene;

(ii) Breeding Bird *Mecp2*$^{2lox/Y}$ mice with mice expressing *Cre* under control of the *Sim1* promoter[64] yielded cell-type-specific *Mecp2* deletions in hypothalamic neurons;[65]

(iii) Breeding Bird *Mecp2*$^{2lox/Y}$ mice with mice expressing *Cre* in tyrosine hydroxylase (TH) neurons[66] generated another cell-type-specific *Mecp2* deletion;[67]

(iv) Breeding Bird *Mecp2*$^{2lox/Y}$ mice with mice expressing *Cre* in PC12 *ets* factor 1 (PET1)-expressing neurons[68] generated another cell-type specific *Mecp2* deletion;[67]

(v) Lastly, infusions of *Cre*-expressing lentiviruses into specific brain areas of Jaenisch *Mecp2*^{2lox} mice yielded useful mouse models of RTT amenable for behavioral studies without confounding issues of brain development.[69]

(4) Mice expressing reduced levels of Mecp2: The introduction of loxP sites flanking *Mecp2* exons 3 and 4 for the generation of conditional knockout mice of the Bird knockout mouse line[56] may be the reason for a ~50% reduction in the expression of Mecp2 protein in male *Mecp2*$^{2lox/Y}$ compared with wildtype littermates.[59] Interestingly, these mice express a delayed and milder behavioral phenotype similar to other *Mecp2*-deficient mice, which may originate from reduced expression levels of wildtype Mecp2 protein.

An alternative approach to reduce the expression of endogenous Mecp2 is by knock-down with small interference RNAs.[70,71] Intraventricular injections of lentiviruses that express a *Mecp2*-targeted short hairpin RNA (shRNA) in one-day-old rat pups reduced *Mecp2* mRNA levels and caused subtle but transient sensory-motor impairments.[72] So far, the *in utero* transfection of shRNA constructs to knock-down Mecp2 expression in sparsely distributed cortical pyramidal neurons in mice has been used to map intracortical connectivity by glutamate uncaging and laser scanning photostimulation in brain slices.[73]

(5) Mice overexpressing wildtype full-length MeCP2: The introduction of the human *MECP2* gene under control of its entire regulatory promoter using the P1-derived artificial chromosome led to an approximately two-fold increase in expression levels compared with the endogenous mouse *Mecp2* (MeCP2^{Tg1} mice).[50] Another mouse line overexpressed a Tau-MeCP2 fusion protein selectively in postmitotic neurons from the *Tau* locus in homozygous *Tau* knockout mice.[62]

(6) Knock-in mice carrying RTT-associated MECP2 mutations: So far, only two mouse strains carrying RTT-associated mutations have been generated: the R168X mouse (*Mecp2*^{R168X})[74] and the MeCP2 A140V mouse.[75] In theory, female heterozygous mice with mosaic expression of RTT-associated mutations in *MECP2* would represent the closest experimental animal model of the human disease. The next closest experimental model would be human neurons derived from induced pluripotent stem cells

obtained from individual RTT patients (e.g. ref.[76]), with the obvious limitation of being a dissociated culture system allowing only studies at the molecular and cellular level, and not network or behavioral studies.

The most important feature of all these genetically manipulated mice is that all present some behavioral features that correlate well with specific clinical symptoms observed in RTT individuals, although no single mouse line truly mimics the human disease (reviewed by Ricceri *et al.*[77] and Tao *et al.*[78]).

It should be stressed that most studies use male hemizygous mice (i.e. *Mecp2*$^{-/y}$) because they consistently develop a severe and characteristic behavioral phenotype much earlier than female heterozygous mice (i.e. *Mecp2*$^{-/+}$), which express a mosaic pattern of wildtype and mutant cells due to XCI. However, XCI is not uniform in female heterozygous mice from the *Mecp2*$^{tm1.1Jae}$ and *Mecp2*$^{308/X}$ mutant lines, or from the *Mecp2*$^{tm1.1Bird}$ knockout strains, being skewed towards the wildtype *Mecp2* allele.[57,79] Intriguingly, wildtype cells in *Mecp2*$^{tm1.1Jae}$ mutant and *Mecp2*$^{tm1.1Bird}$ knockout mice express lower levels of Mecp2 protein than in wildtype mice.[57] Therefore, the delayed appearance and variability of the behavioral phenotypes observed in *Mecp2*$^{-/+}$ heterozygous female mice could be caused by the combination of mosaic expression of mutant *Mecp2*, the degree of XCI unbalance, as well as reduced Mecp2 levels in wildtype cells. To simplify the analyses by reducing the contribution of these confounding factors, *Mecp2*$^{-/y}$ male hemizygous mice are used as a more homogenous population, which seem more amenable for experimental work in the laboratory. There are, however, a number of studies that compare RTT-like phenotypes between *Mecp2*$^{-/+}$ heterozygous female and *Mecp2*$^{-/y}$ hemizygous male mice and their wildtype littermates, and all agree that female heterozygous mice display a delayed onset, milder phenotype than male hemizygous mice (e.g. refs.[80–90]).

Another critical point is that only one study characterized the consequences of the complete deletion of the Mecp2 protein without potential deleterious effects of genetic engineering (e.g. *Neo* cassettes),[60] since at least one of the mouse lines commonly called '*Mecp2* null' or '*Mecp2* knockout' in fact expresses an internally deleted protein detectable by Western blotting and immunohistochemstry.[57,61,62] Despite these limitations, Mecp2-based mouse models represent useful experimental models of RTT in which to test novel therapeutic approaches before moving to the clinic.

General features of mouse models based on Mecp2 dysfunction

Male hemizygous mice of the *Mecp2* knockout strains (*Mecp2*$^{tm1.1Bird}$ and *Mecp2*^{tm1Tam}),[56,60] and of the *Mecp2* mutant line (*Mecp2*$^{tm1.1Jae}$)[61] appeared normal until the 4th–5th week of life when a consistent behavioral phenotype appears, which include unusual gait with splaying hind limbs, clasping of hind limb when suspended by the tail, dishevelled fur and erected whiskers, labored breathing, tremors and episodes of seizures. This phenotype progresses in severity, and also includes varied neurological features of sensory-motor or cognitive origin, such as increased anxiety-related behaviors and reduced social interactions with novel mice and learning and memory deficits. From the onset of these symptoms, marked weight loss, kyphosis and irregular breathing become more severe, until these mice die at ~10 weeks of life. Considering that a conditional deletion in neural precursor cells starting at ~E12 using *Nestin-Cre* mice[56,61] gave similar phenotypes to widespread deletions using X chromosome-Cre transgenic mice,[56] the complex phenotype seems to arise from impaired *Mecp2* expression in the central nervous sysytem (CNS). It should be noted that more restricted deletions in forebrain excitatory neurons starting at ~P20 using *CamkII*-Cre deleter mice cause a delayed and milder phenotype,[61] suggesting that proper *Mecp2* expression in both neurons and glial cells is critical for CNS development (see below). As mentioned above, this symptomatology is highly reminiscent of the complex clinical manifestations observed in RTT individuals used for inclusion criteria, with the exception of the progressive process.[2,48,91]

Similar to the *Mecp2* knockout and mutant strains, male hemizygous of the *Mecp2*$^{308/Y}$ strain appeared normal until the sixth week of life when they develop a similar but milder neurological phenotype. The features of that phenotype included tremors, motor impairments, hypoactivity, increased anxiety-related behavior, seizures, kyphosis and stereotypic forelimb motions,[38] as well as deficits in hippocampus-dependent spatial memory, contextual fear memory and social memory.[92]

Consistent with XCI of the mutant *Mecp2* allele in a fraction of cells, female heterozygous mice of the *Mecp2* knockout, mutant and truncated *Mecp2*^{308} strains develop a milder behavioral phenotype than the hemizygous male littermates, but with a delayed onset. In addition, the features of the phenotype in heterozygous females are much more variable than in hemizygous males.

Forebrain neuron-specific conditional *Mecp2* mutant mice generated by crossing *CamkII-Cre*[93] mice[63] with Jaenisch floxed mice (*Mecp2*^{2lox}) showed behavioral abnormalities similar to RTT phenotypes, including hind limb clasping, impaired motor coordination, increased anxiety and abnormal congener social behavior.[61] However, locomotor activity and context-dependent fear conditioning were unaffected in these mice.[93]

Hypothalamus-specific conditional *Mecp2* knockout mice generated by crossing *Sim1-Cre* BAC transgenic mice[64] with Bird floxed mice (*Mecp2*$^{2lox/Y}$) lacked Mecp2 protein during embryonic development and after birth in the paraventricular, supraoptic and posterior hypothalamic nuclei, as well as in the nucleus of the lateral olfactory tract of the amygdala.[65] These mice recapitulated the abnormal physiological stress response that is seen upon expression of a truncated non-functional Mecp2 protein in the entire brain.[38] Hypothalamus-specific *Mecp2* conditional knockout mice were also aggressive, hyperphagic and obese, consistent with a role for Mecp2 in the regulation of social and feeding behaviors. It should be noted that reduced Mecp2 protein levels in Bird *Mecp2*$^{2lox/Y}$ mice were taken into

consideration when comparing different genotypes in this study.[65]

Deletion of *Mecp2* in TH-positive dopaminergic and noradrenergic neurons by breeding TH-Cre transgenic mouse line[66] with Bird *Mecp2*$^{2lox/Y}$ mice caused a specific alteration in locomotor activity (i.e. decreased total distance and vertical activity in the open field, poor performance on the dowel walking task), without any impairments in motor learning, anxiety, social interaction, breathing patterns, and learning and memory.[67] In this study as well, reduced Mecp2 protein levels in Bird *Mecp2*$^{2lox/Y}$ mice were taken into consideration.

Deletion of *Mecp2* in serotonergic neurons of the dorsal and medial raphe nuclei by breeding PET1-Cre transgenic mouse line[68] with Bird *Mecp2*$^{2lox/Y}$ mice caused an increased aggressive behavior (i.e. congener resident intruder test), without affecting motor function, anxiety, self-grooming or repetitive behaviors, breathing patterns, motor learning or learning and memory. Also here, reduced Mecp2 protein levels in Bird *Mecp2*$^{2lox/Y}$ mice were taken into consideration.[67]

Bilateral injections of adeno-associated viruses that express GFP-tagged Cre recombinase in the basolateral amygdala of Jaenisch *Mecp2*^{2lox} mice caused elevated anxiety-like behavior with normal locomotion, motor coordination and social interaction behavior.[69] In addition, these mice have impaired cue-dependent fear conditioning. However, it is unknown whether Jaenisch *Mecp2*^{2lox} mice show a similar reduction in Mecp2 protein like that detected in Bird *Mecp2*$^{2lox/Y}$ mice.

Supporting the notion that proper and tightly controlled dosage of MeCP2 protein is critical for brain development and function, mice overexpressing human MeCP2 at approximately twice the endogenous levels (MeCP2^{Tg1}) exhibited a delayed (10th week) neurological syndrome that included enhanced motor and contextual learning and enhanced synaptic plasticity in the hippocampus. After 20 weeks of age, these mice developed seizures, became hypoactive and approximately 30% of them died by one year of age.[50] Without exception, higher levels of MeCP2 caused a more severe neurological syndrome,[50] even when expressed exclusively in postmitotic neurons.[62]

Consistent with this model of tightly controlled dosage of MeCP2 protein, a ~50% reduction in expression of the wildtype Mecp2 protein led to a neurological phenotype in male Bird *Mecp2*$^{2lox/Y}$ mice, before crossing with any Cre deleter line. These mice showed learning and motor deficits, decreased anxiety, altered social behavior and nest building, decreased pain recognition and disrupted breathing patterns.[59] Although no typical RTT-like symptoms were observed in rat pups injected with lentiviruses expressing *Mecp2*-targeted sh/RNAs, they displayed some transiently altered sensory-motor reflexes and neurobehavioral abnormalities during early development.[72]

The introduction of human *MECP2* carrying RTT-specific mutations also caused neurological symptoms related to the human disease. The R168X mice (*Mecp2*^{R168X}) carry a premature STOP codon in position 168, and thus do not express full-length wildtype Mecp2 protein. The truncated protein contains an intact MBD but lacks the TRD and C-terminal domains including the nuclear localization signal. As in the other *Mecp2* mouse lines, male hemizygotes are more severely affected than the female heterozygotes, with a shortened lifespan and neurological features starting by the 5th–6th week of life, which include forelimb stereotypies, hindlimb clasping and atrophy, hypoactivity and breathing irregularities; female heterozygotes showed similar signs only after six months of age.[74] It should be noted that, during the cloning of the mutant constructs, additional changes were introduced between codons 167 and 172. Because the STOP codon is at codon 168, these changes are not represented in the mutant Mecp2 protein; however, these mice are not useful to test the effectiveness of read-through compounds that skip premature STOP codon mutations.[94]

The A140V MeCP2 mutation is noted only in ~0.6% of individuals, the majority being males with X-linked mental retardation, and including some with manic-depressive or schizophrenic behaviors (RettBASE: IRSF *MECP2* Variation Database; http://mecp2.chw.edu.au/). Of the seven females carrying this *MECP2* mutation listed in the RettBASE, most were unaffected siblings and none had RTT. In addition, the A140V MeCP2 mutation has been described elsewhere in familial X-linked mental retardation, and in parkinsonism/pyramidal signs/macroorchidism/X-linked mental retardation.[95] The A140V mutant MeCP2 protein has been reported to preserve the methyl-CpG binding while compromising its ability to bind to the mental retardation associated protein ATRX.[96] In contrast to the other mouse models, male hemizygous mice carrying the A140V Mecp2 mutation have an apparently normal lifespan and normal weight gain patterns without obvious seizures, tremors, breathing difficulties or kyphosis. However, they show some typical features of autopsy brains from RTT individuals, such as increased cell packing density and reduced complexity of dendritic branching.[75]

Morphology of neurons and synapses in Mecp2-based mouse models of RTT

The most prototypical morphological features of autopsy brains from RTT individuals at the anatomical, histological and cytological levels are present in brains from Bird *Mecp2* knockout and Jaenisch *Mecp2* mutant mice, which are the most widely studied mouse models of RTT. For example, these mice have smaller cortical neurons packed at a higher density than their wildtype littermates.[43,61,81] In addition, pyramidal neurons in the cortex[43] and hippocampal CA3 region, as well as granule cells of the dentate gyrus show reduced dendritic complexity.[97] In addition, Jaenisch *Mecp2* mutant mice show a disorganized olfactory neuroepithelium indicative of delayed terminal differentiation, in addition to impaired turnover of olfactory neurons.[98] In MeCP2 A140V mice, pyramidal neurons in layer II/III of the somatosensory cortex also have decreased dendritic complexity compared with wildtype controls.[75] However, dendritic branching in layers III and V pyramidal neurons of the frontal cortex of male *Mecp2*$^{308/Y}$ mice was comparable to that in wildtype controls.[92]

In addition to smaller neurons with fewer dendritic branches, RTT mouse models exhibit the so-called spine dysgenesis phenotype,[29] which is common to other neurodevelopmental disorders, including Down's syndrome, autism, Angelman syndrome and fragile X syndrome.[12,20,30,31] Pyramidal neurons in the somatosensory cortex of six-week-old Bird *Mecp2* knockout mice have lower spine densities compared with wildtype controls.[99] Newly generated granule cells in the dentate gyrus of eight-week-old Jaenisch *Mecp2* mutant mice also show impaired dendritic spine density and distribution.[100] Dendritic spine density is also lower in pyramidal neurons of layers V–VI of the motor cortex from male hemizygous and female heterozygous Bird *Mecp2* knockout mice.[86] The onset of this dendritic spine phenotype is delayed in female heterozygous mice, and it seemed more severe in Mecp2-expressing neurons than in Mecp2-lacking neurons, suggesting both cell autonomous and non-cell autonomous consequences of *Mecp2* deletion.[86] In addition to similar lower spine densities, the intensity of immunolabeling for PSD-95 (a postsynaptic marker of mature excitatory synapses) was lower in layer V pyramidal neurons of the motor cortex of Jaenisch *Mecp2* mutant mice.[101] Furthermore, pyramidal neurons in the CA1 region of the hippocampus from Bird *Mecp2* knockout mice and Jaenisch *Mecp2* mutant mice also show lower spine densities, in addition to dendritic swelling, reduced diameter of dendritic spine heads and elongation of their necks.[102] Intriguingly, this study described significant differences between the two mouse strains examined, suggesting different consequences of a deletion[56] and expression of a truncated Mecp2 protein.[61]

It should be noted that the lower dendritic spine density observed in RTT mouse models is not a consistent phenotype; for example Kishi and Macklis[43] described comparable spine densities in cortical layers II/III of either Bird *Mecp2* knockout or Jaenisch *Mecp2* mutant mice and their wildtype controls at eight weeks of age. Similarly, neither dendritic spine density in layers III and V pyramidal neurons of the frontal cortex nor asymmetric spine synapse density in area CA1 were affected in male *Mecp2*$^{308/Y}$ mice, despite significant impairments in hippocampal-dependent learning and memory, as well as hippocampal synaptic transmission and plasticity (see below); however, the length of individual postsynaptic densities was shorter in the mutant mice.[92]

With regard to the morphology of axons and presynaptic terminals, the motor cortex of Bird *Mecp2* knockout mice show defects in axonal fasciculation.[102] Similarly, Jaenisch *Mecp2* mutant mice show impairments in olfactory axonal fasciculation, guidance and targeting before synaptogenesis.[103] In addition, the immunolabeling intensity of VGLUT1 (vesicular glutamate transporter) – a presynaptic marker of mature excitatory synapses – is lower in the dendritic region of the hippocampal CA1 region from Bird *Mecp2* knockout compared with wildtype controls, but only at two weeks of age.[104] Since the immunolabeling intensity of the postsynaptic dendritic marker MAP-2 was not different, the authors interpreted that *Mecp2* deletion caused a reduction in the number of mature synapses in area CA1, consistent with their results from autaptic neuronal cultures (see below).[104] On the other hand, the number of docked and total synaptic vesicles within presynaptic terminals was unaffected in area CA1 of *Mecp2*^{308}.[92]

The consequences of *Mecp2* deletion on synaptic circuits are different across the CNS, even for specific neuronal subtypes such as inhibitory GABAergic interneurons. The cerebellum of Jaenisch *Mecp2* mutant mice shows more GABA-positive presynaptic terminals from stellate and basket cells onto Purkinje cells than in wildtype controls, while the density of GABA-positive presynaptic terminals was not affected in layer III of the somatosensory cortex.[88] These differences may even occur within individual brain regions. In the thalamus of Bird *Mecp2* knockout mice for example, the ventral basal complex shows fewer puncta immunopositive for VGAT (vesicular GABA transporter, a presynaptic marker of mature inhibitory synapses), while the reticular thalamic nucleus shows more VGAT-positive puncta.[105]

Physiology of neurons and synapses in Mecp2-based mouse models of RTT

Intrinsic properties

Despite the consistent observations of smaller neuronal somata and dendritic arborizations, most electrophysiological studies in brain slices describe that intrinsic membrane properties are not affected in *Mecp2* knockout or *Mecp2* mutant mice. It should be noted that the majority of such studies were performed in brain slices from young mice before the appearance of RTT-like symptoms, and thus it is unknown whether these features deteriorate later in symptomatic mice. For example, layer V pyramidal neurons in slices of primary somatosensory cortex from Jaenisch *Mecp2* mutant mice have similar resting membrane potential (RMP), input resistance (Ri) and intrinsic excitability (i.e. spike rate versus current injection relationships) to their wildtype controls.[106] Pyramidal neurons layer II/III of the frontal cortex of Bird *Mecp2* knockout mice also show input resistances comparable to those in wildtype, although the effect of ouabain, a Na^+, K^+-ATPase inhibitor, was smaller in knockout neurons. The reduced basal activity of this electrogenic pump in *Mecp2* null neurons seems to originate from increased expression levels of FXYD, a small, single-spanning membrane protein that controls cell excitability by modulating Na^+, K^+-ATPase activity.[107]

Similarly, CA3 and CA1 pyramidal neurons in hippocampal slices from Bird *Mecp2* knockout mice show unaffected RMP, Ri and action potential amplitude and threshold.[108] Intrinsic membrane properties – such as RMP, Ri, membrane capacitance (Cm), action potential threshold and firing patterns – were also unaffected in inhibitory neurons of the reticular thalamic nucleus.[105] Relay neurons in the nucleus tractus solitarius of the brainstem from Jaenisch *Mecp2* mutant mice also showed RMP, Ri, action potential amplitude and threshold, after hyperpolarizations and intrinsic excitability (i.e. spike rate versus current injection relationship) comparable to those in wildtype controls.[109] In addition, the expression of a truncated non-functional protein did not affect Ri and Cm in principal neurons of the

lateral nucleus of the amygdala from *Mecp2*$^{308/Y}$ mice.[110] Finally, Mecp2 knockdown in cortical layer II/III pyramidal neurons by *in utero* electroporation did not affect RMP, Ri, membrane time constant, voltage–current relationship, rheobase (i.e. lowest-amplitude current step evoking at least one action potential), action potential half-width, spike frequency adaptation or firing–current relationships.[73]

On the other hand, TH-positive neurons in the locus ceruleus of Jaenisch *Mecp2* mutant mice have lower membrane conductance, Cm, slow afterhyperpolarizations and spike frequency adaptation than wildtype controls (all consistent with significantly smaller neuronal somata), all of which could contribute to their enhanced intrinsic excitability (i.e. spike rate versus current injection relationship).[111]

Synaptic properties

As with morphological features, the consequences of *Mecp2* dysfunction on the physiology of synapses vary across different brain regions and with neurotransmitter types. In slices of primary somatosensory cortex from Jaenisch *Mecp2* mutant mice, the cumulative charge (*Q*) of spontaneous excitatory postsynaptic currents (EPSCs) recorded in layer V pyramidal neurons was smaller, while the charge of spontaneous inhibitory postsynaptic currents (IPSCs) was larger than wildtype controls, which may contribute to the lower spontaneous firing rate of *Mecp2* mutant pyramidal neurons.[106] These differences seem to arise from impaired quantal transmitter release from excitatory synapses because action potential-independent miniature EPSCs (mEPSCs) were smaller in *Mecp2* mutant neurons (without changes in their frequency), whereas miniature IPSCs (mIPSCs) were unaffected.[106] In addition, studies of monosynaptic connections between layer V pyramidal neurons in cortical slices revealed reduced connectivity and weaker individual connections,[112] which likely contribute to reduced network activity that is reflected in the lower frequency of spontaneous action potentials recorded in *Mecp2* mutant neurons.[106,113] On the other hand, the input–output relationship of extracellularly recorded fEPSPs evoked in layer II/III of primary somatosensory cortex by stimulation in white matter/layer IV was comparable between Jaenisch *Mecp2* mutant mice and wildtype controls.[88] Similarly, input–output relationships of fEPSPs in layers II/III of slices from primary motor or primary sensory cortices were unaffected by expression of truncated Mecp2 in *Mecp2*$^{308/Y}$ mice.[92]

The consequences of embryonic Mecp2 knockdown on the degree of synaptic connectivity in postnatal cortical slices was also evaluated by comparing input maps generated by glutamate uncaging onto layer II/III pyramidal neurons using laser-scanning photostimulation. *In utero* electroporation of Mecp2-specific shRNA constructs caused a significant reduction in local excitatory input pathways in Mecp2-knockdown neurons compared with untransfected neighbors, without changes in inhibitory synaptic inputs.[73]

The brainstem of *Mecp2* knockout and *Mecp2* mutant mice also exhibits altered synaptic transmission. The amplitude and frequency of spontaneous EPSCs recorded from neurons in the rostroventrolateral medulla (which included the pre-Bötzinger complex) were higher, while the amplitude and frequency of spontaneous IPSCs were lower in brainstem slices from one-week-old *Mecp2* knockout mice compared with wildtype controls, well before any RTT-like symptoms are observed.[114] Such a consequence on GABAergic transmission reflects differences in presynaptic function because the frequency of both spontaneous mIPSCs and sucrose-evoked postsynaptic currents was lower in *Mecp2* mutant neurons; however, postsynaptic $GABA_A$ receptors were also impaired, as reflected by smaller agonist-induced currents in *Mecp2* knockout slices.[114] Similarly, evoked EPSCs and spontaneous mEPSCs were larger in relay neurons of the nucleus tractus solitarius in the brainstem of *Mecp2* mutant mice than in wildtype controls, which led to a higher probability of action potential firing in response to afferent stimulation.[109] These studies demonstrate an imbalance of excitatory and inhibitory synaptic input to brainstem circuits responsible for the respiratory rhythm, and thus strongly implicate this synaptic dysfunction in the breathing irregularities experienced by RTT individuals. Raising hopes for novel therapeutic approaches for RTT, breathing irregularities in *Mecp2* mutant mice were reverted by treatment with an AMPAkine drug that facilitates activation of glutamatergic AMPA receptors and increases the expression of brain-derived neurotrophic factor (BDNF),[115] consistent with the reversal of synaptic dysfunction by direct application of recombinant BDNF to brainstem slices.[109]

Altered synaptic properties were also found in the amygdala of *Mecp2*$^{308/Y}$ mice that express a truncated nonfunctional protein, albeit restricted to its cortical inputs. Intracellular whole-cell recordings from principal neurons of the lateral nucleus of the amygdala revealed that both synaptic maturation and elimination during development were enhanced in slices from *Mecp2*$^{308/Y}$ mice, but only on those synapses made by cortical afferents and not by projections from the thalamus.[110] Whether the probability of neurotransmitter release (Pr) at these cortico-amygdalar synapses is affected in *Mecp2*$^{308/Y}$ slices is unclear, because the paired-pulse ratio is smaller (consistent with higher Pr, see ref.[116]) but the maximal amplitude of evoked EPSCs, the amplitude of EPSCs evoked by minimal stimulation, and the frequency of spontaneous mEPSCs are all smaller than in wildtype slices,[110] which is consistent with lower Pr (see ref.[116]).

On the other hand, studies of the consequences of *Mecp2* dysfunction on synaptic transmission in the hippocampus have yielded more conflicting results. Extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) evoked in area CA1 by stimulation of Schaffer collaterals (the axons of CA3 pyramidal neurons) revealed that basal excitatory synaptic transmission is not affected by either *Mecp2* deletion in Bird knockout mice or expression of a mutant protein in Jaenisch mutant mice.[117] However, paired-pulse facilitation of fEPSPs at these synapses was smaller in *Mecp2*-deficient slices, which could be interpreted as a higher release probability (Pr) (see ref.[116]); it is unclear though, why this potential difference in Pr was not reflected in the amplitude of post-tetanic potentiation or in the slope of input–output relationships.[117] On the other hand, similar

recordings performed in area CA1 of hippocampal slices from *Mecp2*$^{308/Y}$ mice expressing a truncated Mecp2 protein showed that excitatory synaptic transmission is enhanced compared with wildtype controls, as reflected in steeper input–output relationships of fEPSPs and reduced paired-pulse facilitation (at the shortest interstimulus intervals, 10 and 20 ms).[92]

The CA3 region of the hippocampus seems to exhibit pronounced changes in *Mecp2* knockout mice. Intracellular whole-cell recordings from CA3 pyramidal neurons demonstrated that spontaneous IPSCs were larger in hippocampal slices from Bird knockout mice, suggesting that the activity of GABAergic interneurons is more synchronized than in wildtype slices.[108] In addition, the cumulative charge transfer and the amplitude of spontaneous EPSCs in CA3 pyramidal was smaller than in wildtype slices, which may contribute to the slower frequency of spontaneous rhythmic field potentials exhibited by the *Mecp2* knockout slices. Paradoxically, this combination leads to a hippocampal circuitry more prone to hyperexcitability, as reflected by the repetitive sharp wave-like discharges induced in area CA3 by a brief high-frequency stimulation to the alveus and CA3 *stratum oriens*, which contain recurrent collaterals of CA3 pyramidal neurons.[108]

Consistent with the notion that tightly controlled MeCP2 levels are critical for brain development and function, fEPSPs evoked in area CA1 of slices from *MECP2* overexpressing (MeCP2^{Tg1}) showed larger paired-pulse facilitation than in wildtype slices. Because the input–output relationships of fEPSPs was unaffected by *MECP2* overexpression, those results were interpreted as presynaptic differences in release probability.[50]

Long-term synaptic plasticity

Despite the various discrepancies mentioned above regarding the consequences of *Mecp2* dysfunction on synaptic transmission and short-term plasticity at excitatory synapses, more consistent results have been obtained with respect to long-term synaptic plasticity.[118] Several studies in *Mecp2*-deficient mice have documented impairments in long-term potentiation (LTP) and depression (LTD), two widely accepted forms of synaptic plasticity thought to underlie experience-dependent modifications of brain function, including learning and memory.[119]

When recorded with extracellular electrodes, the strength of excitatory synapses within layer II/III of slices from the primary somatosensory cortex of Jaenisch *Mecp2* mutant mice displayed smaller LTP after theta-burst stimulation (TBS) of white matter/layer VI inputs than that recorded in wildtype controls, an impairment reversed by environmental enrichment (see below).[88] Extracellular fEPSPs evoked in layer II/III of slices from primary motor or primary sensory cortices of *Mecp2*$^{308/Y}$ mice expressing truncated Mecp2 also showed smaller TBS-induced LTP than that shown by wildtype controls.[92] However, an intracellular study of monosynaptic connections between pyramidal neurons in layer V of the primary somatosensory cortex of Jaenisch *Mecp2* mutant mice demonstrated that LTP was intact at these synapses, provided that sufficient postsynaptic depolarization was achieved by step depolarization or by evoked action potentials during the induction of spike-timing-dependent plasticity.[112] Thus, the sparser connectivity with weaker synapses could be the reason of reduced LTP amplitude when induced without control of postsynaptic depolarization (as with extracellular recordings of fEPSPs). Alternatively, differences in GABAergic inhibition and the overall excitation/inhibition balance could also contribute to impaired LTP induction in *Mecp2*-deficient slices. Finally, a potential 'ceiling' effect on LTP induction has not been considered to explain its reduction or absence in *Mecp2*-deficient slices (i.e. saturation of LTP induction).

Similar to the studies in cortical slices, extracellular field EPSPs evoked in CA1 by CA3 afferent stimulation showed smaller LTP in hippocampal slices from either Bird *Mecp2* knockout or Jaenisch *Mecp2* mutant male mice compared with that induced in wildtype slices, a difference that occurred only after the appearance of RTT-like symptoms.[117] Such impaired LTP was observed using either high-frequency afferent stimulation or a more physiological pattern of afferent stimulation with shorter trains at theta frequency (5 Hz, i.e. TBS). Furthermore, high-frequency- and TBS-induced LTP was impaired in area CA1 of slices from mice where the endogenous *Mecp2* gene is silenced by insertion of a *lox-Stop* cassette (which can be conditionally activated under the control of its own promoter and regulatory elements by cassette deletion; see below).[120] In addition, LTP induced by either high-frequency or TBS stimulation to CA1 afferents was smaller in slices from *Mecp2*$^{308/Y}$ mice expressing a truncated Mecp2 protein than that in wildtype slices.[92]

Again consistent with the critical role of tightly regulated MeCP2 levels, LTP of field EPSPs in area CA1 was enhanced in MeCP2^{Tg1}-overexpressing mice.[50] The enhanced synaptic plasticity in young MeCP2^{Tg1} mice might have contributed to their better behavioral performance in motor and contextual learning paradigms, but it also could have caused their progressive neurological symptoms (e.g. forepaw clasping, aggressiveness, kyphosis and hypoactivity) and premature death.[50]

Regarding LTD, the other form of long-term synaptic plasticity, low-frequency afferent stimulation (15 min at 1 Hz) induced smaller LTD in area CA1 of slices from either Bird *Mecp2* knockout or Jaenisch *Mecp2* mutant mice, but only in symptomatic mice.[117] Similarly, LTD induced in area CA1 by paired-pulse low-frequency stimulation of its afferent fibers was impaired in slices from *Mecp2*$^{308/Y}$ mice.[92]

Morphological and physiological phenotypes are expressed *in vitro* by cultured neurons from Mecp2-deficient mice

A few studies evaluated whether neurons dissociated from either embryonic or early postnatal *Mecp2*-deficient mice, and cultured *in vitro* for several weeks display morphological and physiological characteristics resembling those described in more intact preparations from older presymptomatic and symptomatic animals (i.e. brain slices). As we summarize below, somewhat contradicting results have been obtained so far, which may be due to very different cell culture conditions.

On the one hand, dendritic length and branching were significantly affected in conventional high-density cultures of hippocampal neurons from *Mecp2* knockout or *Mecp2* mutant mice.[97,121] Consistently, rat hippocampal neurons transfected with a Mecp2-specific shRNA showed shorter dendrites than control cells in mass cultures.[122] On the contrary, the dendritic length and branch points of hippocampal neurons cultured at low density on glial micro-islands were not affected by *Mecp2* deletion or *MECP2* overexpression.[104] This type of neuronal cultures is often called 'autaptic' because individual neurons make synapses onto themselves, i.e. autapses.[123] It is unclear whether differences in the morphological consequences of *Mecp2* deletion between 'mass' high-density cultures and autaptic cultures are due to their widely different cell densities and the ensuing neuronal activity (see below).

In addition, conventional mass cultures of mouse hippocampal neurons transfected with full-length *Mecp2* displayed enhanced dendritic and axonal length and branching, a feature absent in cells expressing mutant *Mecp2* coding for a truncated protein that retains the MBD and a putative nuclear localization signal, but terminates within the TRD ($MeCP2^{293}$).[124] Similarly, rat hippocampal neurons transfected with wildtype human *MECP2* showed enhanced dendritic and axonal length and branching.[122] Consistent with the human neuropathology, two different *MECP2* mutations common in RTT individuals, T158M and R106W, caused dendritic and axonal shortening, an effect prevented by overexpression of *Bdnf*,[122] one of the gene targets of MeCP2 (see below).

This role of MeCP2 in dendritic complexity is also evident in more mature preparations, such as organotypic cultures of postnatal hippocampal slices, where pyramidal neurons have decreased dendritic branching after shRNA-mediated Mecp2 knockdown or overexpression of wildtype *Mecp2*, while expression of a phosphorylation mutant (S421A) had no effect on dendritic morphology.[125] In addition, dendritic spines of neurons overexpressing wildtype *Mecp2* were longer, thinner and more filopodia-like (i.e. resembling immature spines) than those in control neurons or neurons transfected with the S421A mutant MeCP2, while shRNA Mecp2 had no effect.[125] It should be noted that Mecp2 protein levels were up to four-fold higher than control values in these experiments, and that slice cultures were co-transfected with the antiapoptotic protein Bcl-XL. Incidentally, Bcl-XL promotes dendritic spine formation in cultured hippocampal neurons.[126]

On the other hand, similar experiments in hippocampal slice cultures but with lower MeCP2 expression levels (~2-fold endogenous levels) revealed that the RTT-associated T158M mutation caused only a small and transient increase in dendritic complexity in CA3 pyramidal neurons, while the wildtype protein only transiently reduced dendritic branch points.[27] In addition, shRNA-mediated Mecp2 knockdown caused a reduction of dendritic length and branch points. In regard to dendritic spines, the effects of the two different RTT-associated *MECP2* mutations were much more pronounced than the gain or loss of the intact protein. CA3 and CA1 pyramidal neurons expressing T158M and R106W showed fewer and more immature dendritic spines than control cells, while overexpression of wildtype *MECP2* only transiently reduced spine density (Figure 1). Similarly, shRNA-mediated Mecp2 knockdown only shifted the proportion of morphological spine types promoting the immature ones, but not their density.[27] Considering that the T158M and R106W mutations cause single amino acid substitutions in the MBD that alter MeCP2 binding to methylated DNA, and subsequent DNA transcription,[127–130] the consequences of their expression on dendritic spine density and morphology may reflect a 'toxic' gain-of-function of the mutant protein.

At the physiological level, hippocampal neurons from postnatal day-1 Bird *Mecp2* knockout mice maintained in conventional mass cultures for 11–14 days *in vitro* showed a lower frequency of spontaneous action potential-independent mEPSCs than that recorded in neurons from wildtype mice, without differences in their amplitude or kinetics.[131] Given that neither (1) the number of synapsin-positive presynaptic terminals in contact with MAP-2-positive dendrites; (2) the size and destaining kinetics of the total recycling pool of vesicles labeled with FM1-43 and high K^+ solutions; nor (3) the size of the readily releasable pool of vesicles estimated by the amplitude of membrane currents evoked by hypertonic sucrose were affected by *Mecp2* deletion, the lower mEPSC frequency was interpreted to reflect differences in the probability of vesicular neurotransmitter release (Pr). Indeed, the kinetics of depression of evoked EPSCs during 10 Hz stimulation (reflecting vesicle depletion) and the time-course of their recovery were both enhanced in *Mecp2*-deficient neurons, suggesting higher Pr. Consistently, the responses to paired pulses were reduced in *Mecp2* knockout neurons, but only at the shortest intervals (i.e. higher frequencies). A similar synaptic phenotype was observed after acute deletion in cultures from $Mecp2^{2lox}$ mice transfected with Cre-expressing lentiviruses. On the contrary, the frequency and amplitude of mIPSCs were not affected, suggesting a selective role of Mecp2 at excitatory synapses.[131]

Conversely, a study of autaptic connections between isolated hippocampal neurons grown in micro-island cultures concluded that MeCP2 controls the number of synapses, but not their individual properties. In this study, glutamatergic hippocampal neurons from *Mecp2*-deficient mice showed smaller membrane currents evoked by a single action potential fired by the cell under recording (autaptic EPSCs), while neurons from $MeCP2^{Tg1}$ mice had larger autaptic EPSCs. The authors concluded that those effects were likely due to differences in the number of glutamatergic synapses per neuron because (1) the frequency of mEPSCs was decreased in the *Mecp2*-deficient neurons and increased in overexpressing cells; (2) the size of the ready releasable pool (RRP) of synaptic vesicles (as estimated by integrating the membrane currents evoked by hypertonic sucrose applications[132]) was smaller in *Mecp2*-deficient neurons and larger in overexpressing cells; and (3) neither release probability per vesicle (estimated from the RRP); per terminal (estimated by the MK-801 block method[133]); nor short-term plasticity were affected

by loss or doubling of MeCP2.[104] Whether differences in these physiological consequences of MeCP2 manipulations are due to the widely different cell densities of micro-islands versus mass cultures or, more importantly, different patterns or magnitude of neuronal activity induced by autapses versus synapses is unclear at this time. For example, the consequences of synaptotagmin-I deletion on evoked synaptic transmission are different when evaluated at autapses in micro-island cultures than when tested at synapses between neurons maintained at higher density in mass cultures.[134]

The studies summarized so far strongly suggest that the pathology observed in RTT individuals and *Mecp2*-based mouse models results from Mecp2 protein dysfunction in neurons, partly because the initial studies of Mecp2 immunolocalization indicated an exclusive expression in neurons,[38,42,43] and also because transgenic *Mecp2* expression[62,83] or reactivation of endogenous *Mecp2*[120,135] in postmitotic neurons reverted some phenotypes of the *Mecp2*-deficient mice (see below). However, postnatal Cre-mediated *Mecp2* deletion in forebrain neurons using the *CamkII* promoter resulted in a delayed and milder phenotype than that caused by germline or *Nestin*-Cre deletions,[61] while transgenic *Mecp2* expression in *Mecp2* mutant mice using strictly neuronal promoters did not improve the RTT-like phenotype, unlike the widespread re-expression of endogenous *Mecp2* by removal of a conditional STOP codon (see below).[120] In addition, Mecp2 protein has been recently detected in all types of glial cells, including astrocytes, oligodendrocyte progenitor cells and mature oligodendrocytes,[97,121] as well as in microglia.[136] Moreover, astrocytes and microglia from either Bird *Mecp2* knockout or Jaenisch *Mecp2* mutant mice caused impaired dendritic branching in co-cultured neurons from either wildtype or mutant mice.[97,121,136] The pathogenic effect of *Mecp2*-deficient glia is reproduced by their conditioned media, suggesting that aberrant secretion of soluble factors (e.g. glutamate[136]) may cause non-cell autonomous effects on neuronal morphology (e.g. ref.[86]).

Reversal of behavioral and cellular impairments in MeCP2-based mouse models of RTT

The experimental reversal of behavioral and synaptic impairments in several models of neurodevelopmental disorders by pharmacological approaches in adult animals has raised hope for similar interventions in humans after the onset of neurological symptoms.[137] For example, the behavioral impairment in a mouse model of Down's syndrome (Ts65Dn) caused by an excitatory/inhibitory imbalance of synaptic function in the hippocampus can be reverted by $GABA_AR$ antagonists in symptomatic animals.[138,139] A mouse model of neurofibromatosis-1 ($Nf1^{+/-}$) also has higher levels of inhibition than their wildtype littermates (but comparable excitation), which can be reversed by decreasing Ras/MAPK signaling.[140,141] Also, mice that model tuberous sclerosis ($Tsc2^{+/-}$) improve after treatment with inhibitors of the mTOR/Akt signaling cascade.[142–144] In addition, a mouse model of Rubinstein-Taybi syndrome ($CBP^{+/-}$) improves after treatment with inhibitors of either phosphodiesterase 4[145] or histone deacetylases.[146] Using a genetic manipulation, most neurological deficits in a mouse model of Fragile X syndrome ($Fmr1\ -/-$) are prevented after breeding them with heterozygous *mGluR5* mice.[147] Likewise, reducing αCaMKII inhibitory phosphorylation in a mouse model of Angelman syndrome (*Ube3a* mutants) by crossing them with mice harboring a targeted αCaMKII mutation (T305V/T306A) prevents the development of Angelman syndrome-like behavioral deficits.[148] As we reviewed in the preceding sections, the neuropathology in RTT individuals and *Mecp2*-deficient mice is subtle, including reduced neuronal complexity and dendritic spine density rather than severe neuronal degeneration,[26,27,43,86,102,149,150] thus raising the possibility that some specific deficits in RTT individuals may be reversible.[151]

Several experimental approaches have been tested for the reversal of behavioral impairments in symptomatic *Mecp2*-deficient mice, four based on gene expression manipulations, two on pharmacological treatments, in addition to a dietary supplementation and behavioral interventions in the form of rearing in enriched environments.

(1) To selectively increase the expression of full-length wildtype Mecp2 in postmitotic neurons of *Mecp2* mutant mice, they were crossed with a mouse line overexpressing a Tau-MeCP2 fusion protein from the *Tau* locus in homozygous *Tau* knockout mice. The resulting offspring showed improved body and brain weights, as well as locomotor activity and fertility compared with *Mecp2* mutants, and seemed indistinguishable from their wildtype littermates.[62]

(2) Inducible and neuron-specific expression of human *MECP2* in either *Mecp2* knockout mice or $Mecp2^{308/Y}$ mice (which express a truncated non-functional protein) was achieved by using tetracycline-inducible *MECP2* under control of either the *CamkII* or the *Eno2* promoters. Despite the presence of specific patterns of transgene expression, most behavioral impairments in $Mecp2^{308/Y}$ mice (i.e. dowel test, suspended wire and accelerating rotarod) were not improved by neuron-specific *MECP2* transgene expression. Similarly, neuron-specific *MECP2* transgene expression failed to extend the longevity or prevent the tremors and breathing irregularities of *Mecp2* knockout mice, with a subtle effect on locomotor activity in their home cages.[152] The conclusion of these studies was that either the levels of MeCP2 achieved were insufficient or not in the relevant brain regions. Alternatively, these results may reflect the critical role of proper Mecp2 expression in glial cells and its non-cell autonomous consequences on neuronal structure and function (see the preceding section).

(3) *Mecp2* overexpression in postmitotic neurons of *Mecp2* knockout mice was achieved by using tetracycline-inducible *Mecp2-e2* cDNA under control of the *CamkII* promoter. Females of this rescue line showed improved rearing activity, overall mobility and rotarod performance compared with female $Mecp2^{-/+}$ mice, reaching levels of performance comparable to wildtype littermates.[83]

(4) The reactivation of the endogenous *Mecp2* gene under control of its own promoter and regulatory elements was achieved by silencing it with a *lox-Stop* cassette, which can be removed by transgene expression of a fusion protein between Cre recombinase and a modified estrogen receptor (*Cre-ER*). The Cre-ER protein remains in the cytoplasm unless exposed to the estrogen analog tamoxifen, which induces its nuclear translocation. Male mice of this strain ($Mecp2^{lox-Stop/y}$) developed RTT-like symptoms at 16 weeks of age and survived for ~11 weeks, being comparable to *Mecp2* knockout mice. Tamoxifen injections in $Mecp2^{lox-Stop/y}$ mice with advanced symptoms (between 7 and 17 weeks of age) led to milder symptoms and extended lifespan, demonstrating that reactivation of the endogenous *Mecp2* gene reverses established symptoms. Similar behavioral results were obtained in female $Mecp2^{lox-Stop/+}$ mice after treatment with tamoxifen. Furthermore, the impairment in high-frequency- and TBS-induced LTP in area CA1 of slices from symptomatic female $Mecp2^{lox-Stop/+}$ was completely prevented by tamoxifen treatment.[120]

(5) A conditional *Mecp2* transgene that can be activated by Cre-mediated deletion of a loxP-STOP-loxP cassette was used in *Mecp2* mutant mice to show that increasing Mecp2 levels as late as 2–4 weeks of age prevented the onset of RTT-like symptoms. The mouse *Mecp2e2* cDNA was placed downstream of a loxP-flanked STOP cassette, which in turn was downstream of the synthetic CAGGS promoter/enhancer/intron. Mice carrying this rescue transgene were crossed with *Mecp2* mutant mice, and the resulting offspring bred with *Cre* deleter mice. When Cre was driven by the *Nestin* promoter (E12 in neural precursors of neurons and glia) or by *Tau* promoter (postmitotic neurons), the lifespan of the rescue mice increased to more than eight months (compared with 10–12 weeks in *Mecp2* mutants). The activation of the *Mecp2* transgene by the *CamkII* promoter also extended the life span, but to a lesser extent. Nocturnal locomotor activity was also improved in all the lines of rescue mice, albeit more efficiently in those where Cre expression was driven earlier and in most neurons, i.e. Nest-Cre and Tau-Cre. Finally, rescue mice lacked the decrease in brain weight and neuronal soma size in the hippocampus and cerebral cortex characteristics of *Mecp2* mutant mice.[135]

(6) *Bdnf*, the gene coding for brain-derived neurotrophic factor, was one of the first *Mecp2* targets to be identified, binding to its promoter region.[153,154] The initial interpretation of Mecp2 as a transcriptional repressor of *Bdnf* was later confronted with the observations that *Mecp2* mutant mice express lower levels of BDNF mRNA and protein in the cerebral cortex and cerebellum than wildtype controls, and that conditional postnatal deletion of *Bdnf* in the forebrain, parts of midbrain and hindbrain of *Mecp2* mutant mice exacerbated the onset of their locomotor dysfunction and shortened their longevity, two consistent RTT-associated impairments in these mice.[113] Furthermore, a microarray study comparing hypothalamic samples from *Mecp2*-deficient and $MeCP2^{Tg1}$ overexpressing mice found that BDNF mRNA levels were lower in the absence of *Mecp2* and higher when MeCP2 levels were doubled.[36] Considering the well-established role of BDNF on synaptic transmission and plasticity,[31,155–158] restoring proper levels of BDNF in Mecp2-deficient brains is an attractive therapeutic strategy.

To selectively overexpress *BDNF* in postmitotic forebrain neurons of *Mecp2* mutant mice, they were first bred with a mouse line that expresses Cre recombinase under control of the *CamkII* promoter (cre93 transgenic line).[159] The resulting mice ($Mecp2^{+/-}$; cre93) were then crossed with mice carrying a human *BDNF* transgene under regulation of the synthetic CAGGS promoter/enhancer/intron followed by a loxP-STOP-loxP cassette. In the presence of Cre in postmitotic forebrain neurons, the STOP cassette is removed resulting in the activation of the *BDNF* transgene. The resulting overexpression of *BDNF* in postnatal forebrain neurons in a *Mecp2*-deficient background extended the lifespan, and prevented a locomotor defect (hypoactivity) as well as an electrophysiological deficit (low spike firing frequency in cortical layer V pyramidal neurons) consistently observed in *Mecp2* mutant mice.[113] In support of these observations, an *in vitro* study showed that overexpression of *Bdnf* in primary hippocampal cultures rescued the dendritic phenotype caused by either shRNA-mediated *Mecp2* knockdown or expression of RTT-associated *MECP2* mutations.[122] Altogether, these studies indicate that BDNF levels can be targeted for therapeutic interventions to alleviate RTT symptoms, and are the bases of two pharmacological approaches and the beneficial effect of environmental enrichment (see below).

(7) The inability of BDNF to cross the blood–brain barrier has hampered its use as a therapeutic agent in several neurological disorders. AMPAkines are a family of allosteric modulators of AMPA-type glutamate receptors known to enhance BDNF mRNA and protein levels.[160,161] In support of their use to ameliorate RTT symptoms by elevating BDNF levels, the breathing pattern irregularities in *Mecp2* mutant mice are alleviated by a 10-day treatment with the AMPAkine CX546.[115] Consistently, direct application of recombinant BDNF to brainstem slices from *Mecp2* mutant mice reversed their synaptic dysfunction phenotype.[109] Intriguingly, cultured neurons from *Mecp2* knockout mice are able to release more BDNF than wildtype cells, despite showing lower BDNF expression levels. Such hypersecretion phenotype was also observed for catecholamine release from chromaffin cells.[162] The parsimonious interpretation of these observations is that *Mecp2* null neurons may eventually exhaust their pool of releasable BDNF.

(8) Supporting the potential use of 'BDNF-mimetic' trophic factors to reverse the RTT-like impairments in Jaenisch *Mecp2* mutant mice, a two-week treatment with an active tri-peptide fragment of insulin-like growth factor 1 (IGF-1) extended the lifespan, improved locomotor function, ameliorated breathing patterns, reduced heart rate irregularity, and increased brain weight. Indeed, the IGF-1 receptor activates intracellular pathways common to those induced by BDNF signaling through its TrkB receptor (i.e. PI3K/Akt and MAPK).[163] Furthermore, IGF-1 partially restored dendritic spine density in pyramidal neurons of layer V in the motor cortex, the amplitude of spontaneous EPSCs in pyramidal neurons of sensorimotor cortex, the cortical expression of the synaptic scaffolding protein PSD-95

and stabilized cortical plasticity in *Mecp2* mutant mice to wildtype levels.[101]

(9) Daily injections of desipramine, a selective inhibitor of the norepinephrine transporter used to increase extracellular levels of this neurotransmitter, improved respiratory rhythm, the number of tyrosine hydroxylase-expressing neurons in the brainstem, as well as longevity in *Mecp2* knockout mice.[164]

(10) Dietary choline supplementation improved motor coordination and locomotor activity in male *Mecp2* mutant mice, and enhanced grip strength in female *Mecp2* mutant mice.[165] Increased NGF protein levels in the striatum[166] and of *N*-acetylaspartate content, as measured by NMR spectroscopy,[167] suggests improved neuronal proliferation and survival after choline supplementation in *Mecp2* mutant mice.

(11) In several neurological disorders, environmental enrichment has beneficial effects on various behavioral and cellular phenotypes, including increased levels of BDNF expression.[168,169] Potentially related to the ability of either *Bdnf* overexpression[113] or increased BDNF levels after AMPAkine treatment[115] to improve RTT-like symptoms in *Mecp2*-deficient mice, rearing them in enriched environments also ameliorated some of their behavioral and synaptic phenotypes. For example, the cerebellar and hippocampal/amygdala-based learning deficits, as well as the reduced motor dexterity and decreased anxiety levels characteristic of heterozygous *Mecp2*^{tm1Tam} females were prevented by their housing in larger-sized home cages with nesting material, a variety of objects with different textures, shapes and sizes, and running wheels starting at four weeks of age.[84] Similarly, housing male mice of the Jaenisch *Mecp2* mutant line in enriched environments starting at weaning (postnatal day 21) improved their locomotor activity, but not motor coordination or contextual or cued fear conditioning. Curiously, magnetic resonance imaging revealed a reduction in ventricular volume after environmental enrichment in both *Mecp2* mutant and wildtype littermates, without changes in total brain volume. Together with the known reduction in brain size in *Mecp2*-deficient mice, this observation suggests that environmental enrichment selectively increased grey and white matter.[170]

Intriguingly, more robust effects were obtained when environmental enrichment began earlier in the development of the pups and included their dams, which displayed enhanced maternal care behaviors. Rearing Jaenisch *Mecp2* mutant mice and their dams from postnatal day 10 in enriched environments led to improved motor coordination and motor learning compared with control *Mecp2* mutant mice kept in standard housing conditions. In addition, environmental enrichment prevented the deficit of TBS-induced LTP in layer II/III of slices from primary somatosensory cortex typical of *Mecp2* mutant mice kept in standard housing. Despite not being different between wildtype and *Mecp2* mutant mice, the density of spine synapses in layer III of the primary sensory cortex, as well as of parallel fiber-Purkinje cell synapses in the molecular layer of the cerebellum were higher in mice reared in enriched environments than in standard housing, suggesting that *Mecp2*-deficient neurons are still capable of structural plasticity. Consistent with previous reports in rats and mice, this protocol of early environmental intervention increased the levels of BDNF mRNA and protein in the cerebral cortex of both wildtype and *Mecp2* mutant mice.[88]

Altogether, these successful therapeutic approaches that reversed many RTT-like symptoms in both Jaenisch *Mecp2* mutant and Bird *Mecp2* knockout mouse lines provide further support to the potential pharmacological reversal of neurodevelopmental disorders in adults.[137]

Summary

The wide range of clinical symptoms in RTT individuals and of phenotypes in MeCP2-based mouse models was initially thought to originate from unbalanced X chromosome inactivation. Ample evidence in mouse models now indicate that the role of MeCP2 in neuronal development and function is very different across various brain regions, suggesting that dysfunction in specific neuronal populations due to differential distribution of mutant MeCP2 also contributes to phenotypic variability. In addition, the recently uncovered role of *Mecp2* dysfunction in glial cells leading to neuronal pathology cannot be overlooked, raising more questions regarding the primary deficits that initiate the cascade of events leading to clinical symptoms. Also, the realization that MeCP2 acts as both a repressor and activator of potentially thousands of genes has increased the complexity of this once thought simple monogenetic disorder. Finally, none of the experimental approaches tested in MeCP2-based mouse models fully reversed their RTT-like phenotypes, suggesting additional molecular and cellular deficits. In spite of these seemingly overwhelming limitations in our state of knowledge, we should remind ourselves that we have learned more in the last decade since the discovery that MeCP2 mutations cause RTT than in the preceding 30 years from the first description by Andreas Rett. Following this trajectory, it is likely that rational therapies grounded on basic scientific knowledge will be available for RTT individuals within the next decade.

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