Human Molecular Genetics, 2005, Vol. 14, Review Issue 1 **R19–R26**
doi:10.1093/hmg/ddi102

MeCP2 in neurons: closing in on the causes of Rett syndrome

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Received January 3, 2005; Revised January 25, 2005; Accepted February 23, 2005

The discovery in 1999 that Rett syndrome (RTT) is caused by mutations in a gene encoding the methyl-CpG-binding repressor protein MECP2 provided a significant breakthrough in the understanding of this devastating disease. The subsequent production of *Mecp2* knockout mice 2 years later provided an experimental resource to better understand how mutations in the *MECP2* gene result in RTT. This paper reviews the recent progress in understanding when and where MeCP2 function becomes important in the developing brain, why MeCP2 protein levels are crucial, which genes are normally silenced by MeCP2, and how misexpression of these targets might lead to the clinical manifestations of RTT.

INTRODUCTION

Rett syndrome (RTT; OMIM #321750) is a neurological disorder that affects primarily girls, with an incidence of 1/10 000 to 1/15 000 live births (1–4). The affected individuals are born asymptomatic and develop normally during the first few months of life, achieving the expected milestones in motor language and social areas. After 6–18 months of age, however, acquired speech and motor skills begin to be lost (3). Purposeful hand movements are replaced by characteristic hand-wringing motions and gait apraxia, followed by growth retardation and deceleration of head growth. In addition, half of affected individuals develop seizures and autistic behaviour, and by 4–7 years display gross cognitive and motor impairment together with profound hypoactivity which remains throughout their lives (3).

Histopathological studies on post-mortem RTT brains have revealed that the observed microcephaly correlates with reduced brain size, particularly of the prefrontal, posterior frontal and anterior temporal regions (5). This characteristic reduction in size roughly correlates with both a decrease in the size of individual neurons and increased neuronal packing densitiy in these regions (6,7). Additionally a reduction of dendritic arborization in cerebral cortical layers II and IV in the frontal, motor and inferior temporal regions has been observed (8).

MeCP2

RTT is caused by mutations in the *MECP2* gene (9). MeCP2 is a member of a family of proteins capable of binding to methylated DNA and recruiting chromatin modifying activities to silence transcription (10–14). *MECP2* is an X-linked gene (15), and girls with RTT are normally heterozygous for a mutation in *MECP2*. Random X-chromosome inactivation (XCI) in these girls means that, on average, half of their cells have inactivated the X chromosome bearing the mutant *MECP2* allele and are effectively normal. The other half of their cells have inactivated the X chromosome bearing the wild-type allele and are effectively null for *MECP2*. Thus, RTT patients are chimeras for wild-type cells and *MECP2*-null cells. Although favourable XCI ratios have been reported in some normal or mildly affected obligate mutation carriers (16–18), RTT is unusual among X-linked diseases in that profound skewing of XCI patterns does not normally occur (19,20). The vast majority of RTT-causing mutations are sporadic and inherited from the paternal germ line (21), hence males very rarely inherit mutations in *MECP2*. However, in the rare case when a boy does inherit a RTT-causing mutation the condition is very severe, usually presenting within days of birth and resulting in death within ~2 years (22,23).

A large body of evidence indicates that MeCP2 is predominantly expressed in differentiated neurons, with strongest expression in the more mature neurons (24–26). Studies in both rat (24) and mouse (27) identify the thalamus, medulla and basal ganglia as the first brain regions to express MeCP2 in neonatal animals, but both show reduced expression after 1–2 weeks of age. At birth, Cajal–Retzius neurons in the marginal zone and subplate cells, which are the first neurons to

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Figure 1. Alternate transcripts from the *MECP2* locus. The *MECP2* gene is represented on the middle line with exons indicated as boxes. The predominant transcript in somatic tissues, called *MECP2B* in humans or *Mecp2α* in mice, is indicated above the line and does not include exon 2. The minor form (which predominates in murine embryonic stem cells) is called *MECP2A* in humans or *Mecp2β* in mice, and is indicated below the gene. The ORFs in each transcript are shown in blue; the MBD is indicated in red and the TRD is shown in green. Note that the *MECP2B*/*Mecp2α* ORF initiates in exon 1, whereas the *MECP2A*/*Mecp2β* ORF initiates in exon 2.

be born in the cortex, are positive for MeCP2 staining. Within days cortical plate cells, which later generate neurons, begin expressing high levels of MeCP2. This pattern of expression correlates roughly with maturation of the cortex (25,26). Increased MeCP2 expression similarly parallels maturation in the hippocampus, initiating in the pyramidal cell layer and continuing in the granule cell layer of the dentate gyrus (24,28). Similarly, in the cerebellum MeCP2 is expressed only in the purkinje and golgi cells (neurons born during embryonic development) before cerebellar development is complete at ~3 weeks of age (29). It is not until after the third week when granule cells and stellate neurons, from the granule cell and molecular layers, respectively, start to show expression of MeCP2.

TWO ISOFORMS OF *MECP2*

It has recently been discovered that exon 2 of the four-exon *MECP2* gene is not included in all forms of the *MECP2* transcript (30,31) (Fig. 1). The result of this alternate splicing is that two different isoforms of MECP2 are produced using different initiator methionines, one in exon 1 and the other in exon 2. The two isoforms differ only at their very N-termini, whereas the remainder of the protein, including the methyl-CpG-binding domain (MBD) and transcriptional repression domain (TRD), are not affected by this alternate splicing (Fig. 1). This newly identified form of the *MECP2* transcript, called *MECP2B* in humans or *Mecp2α* in mice, is more abundant than the previously identified *MECP2A*/*Mecp2β* transcript in most tissues, including brain (30,31). Furthermore, using an ES-cell-based differentiation system, Kriaucionis and Bird (31) found that while *Mecp2β* is more abundant in ES cells, the proportion of *Mecp2α* mRNA increases during differentiation.

As a percentage of patients with RTT do not have mutations in exon 3 or 4 of *MECP2*, Mnatzakanian *et al.* (30) screened some of these patients for mutations that might affect the exon 1-encoded open reading frame (ORF). In one patient, a deletion was identified which results in a frameshift in the MECP2B ORF, but does not affect the MECP2A ORF. Although this deletion removes part of the MECP2A 5′untranslated region and could affect its transcription, quantification of mRNA levels showed no alteration of transcript ratios in the patient's whole blood (30). It remains possible that in addition to causing a frameshift in MECP2B, this mutation alters the translation efficiency of MECP2A or affects transcription in brain. If this mutation does only affect MECP2B, it provides evidence that its presence in humans is required to prevent RTT, irrespective of the presence of the MECP2A protein [notably this is not the case in mice; discussed subsequently and (32)].

MOUSE MODELS

A variety of mouse models have been produced which have provided significant advances in our understanding of RTT and of MeCP2 function (Table 1). *Mecp2* heterozygous female mice are viable, fertile, and appear normal well into adulthood (33,34) despite having the same genotype as RTT girls. At ~6 months of age, however, these females begin to show neurological symptoms reminiscent of RTT (34), indicating that the onset of RTT is unlikely to depend upon the developmental stage of affected individuals, as humans and mice develop a similar disease after the same amount of time, despite being at completely different developmental stages. Hemizygous null male or homozygous null female mice appear healthy at birth, but began to display RTT-like phenotypes after ~6 weeks and die at an average age of ~8 weeks of age (33,34). Brain architecture in null mice is grossly normal, although a slight decrease in the size of neurons and an increased packing density in the hippocampus, cerebral cortex, and cerebellum could be identified (25,33). This finding correlates with disease pathology in humans where a decrease in the size of individual neurons and increased packing density are likely contributory factors to the smaller brain size often observed in RTT patients (6–8).

In 2002, Shahbazian *et al.* (35) produced mice expressing a truncated form of MeCP2 often seen in RTT patients. These *Mecp2* mutant males ($MeCP2^{308/Y}$) were aphenotypic until 4–5 months of age, after which time they began to display tremors, kyphosis, and motor dysfunction. Heterozygous females ($MeCP2^{308/X}$) have impaired motor features at 35–39 weeks and show phenotypic variability ascribed to differences in patterns of XCI. Results of learning and memory tests were reported to be normal (35).

Mice in which MeCP2 was deleted in nestin-positive neural precursors (and their progeny, i.e. all neurons and glia) displayed a phenotype indistinguishable from that seen in MeCP2-null mice (33,34), indicating that the primary site of

Table 1. Mecp2 mouse models

Mouse model	Phenotype			Take-home message
	Onset	Survival	Symptoms	
Mecp2 deletion (33,34)	6 weeks (−/y) and (−/−)	10 weeks	Ataxia, reduced motor activity, tremors, hind limb clasping	Lack of MeCP2 causes RTT-like phenotypes in mice; disease onset in females depends upon time, not developmental stage
	6 months (+/−)	Normal lifespan		
Mecp2–Nestin–CRE deletion (33,34)	6 weeks (−/y) and (−/−)	10 weeks	Same as *Mecp2* deletion	RTT is due to absence of MeCP2 in brain
	6 months (+/−)	Normal lifespan		
Mecp2–CamK–CRE deletion (33)	3 months (−/y)	Normal lifespan	Ataxia, reduced nocturnal activity	RTT is due to absence of MeCP2 in post-mitotic neurons
MeCP2 truncation (35)	4 months ($MeCP2^{308}$/y)	90% 10 months	Progressive motor and activity disfunction. Abnormal social interaction. Forepaw movements	Truncated protein found in many RTT cases also produces RTT-like phenotypes in mice
	10 months ($MeCP2^{308}$/MeCP2)	Normal lifespan	Milder, variable phenotypes	
Mecp2β–Tau homozygous transgene expression (32)	9 months	Normal lifespan	Small size, ataxia, tremors	MeCP2 is not essential for early development. Overexpression is detrimental
MECP2 overexpression (43)	Depending upon expression levels From days to weeks	From 3–20 weeks to normal lifespan	Progressive neurological disorder	Overexpression is detrimental Enhanced learning in mildly overexpressing animals
Models we would like to see·				*·and why*
MeCP2 deletion brain region-specific	?	?	?	What symptoms are caused by lack of MeCP2 in specific brain areas?
Postsymptomatic MeCP2 replacement	?	?	?	Can phenotypes be reversed after they appear in mice?
Mecp2α-null *Mecp2β*-null	?	?	?	Do the two different isoforms have different functions?
Bdnf overexpression Dlx5 overexpression (both two-fold)	?	?	?	What aspects of the *Mecp2*-null phenotype are explained by misexpression of these target genes?

action for MeCP2 is in the brain. Furthermore, Chen *et al.* (33) produced mice in which the *Mecp2* gene was only deleted in cells expressing αCamKII, a kinase specifically present in postmitotic neurons. The αCamKII promoter is normally active in postnatal excitatory neurons of the forebrain, hippocampus and brainstem, and to a much lesser degree in the cerebellum (36), although the exact distribution and strength of its activity within the forebrain can vary from one transgenic line to another (33,37). These mice appeared normal until ~3 months of age, at which time they began to display ataxic gait and reduced nocturnal activity. Histological analysis revealed reduced brain weights and smaller neuronal cell bodies in cortex and hippocampus (33). Interestingly, these phenotypes were not seen in the cerebellum where αCamKII is not expressed.

Together with the growing body of protein expression data, these murine studies leave little doubt that the primary (if not the only) site of action for MECP2 function is in neurons. Two recently published studies have taken this conclusion one step further by delimiting the stage of development at which defects in MeCP2-deficient neurons first appear (25,38). By isolating neuronal precursors from murine embryos and then inducing them to differentiate in culture, Kishi and Macklis (25) found no evidence for defects in the proliferation or fate decisions of neuronal precursors in the absence of MeCP2, a finding that contrasts with the situation in the frog *Xenopus laevis* where xMeCP2 is required for embryonic development (39). Rather, these authors found evidence that MeCP2 is important for maturation and maintenance of postmitotic neurons in murine brains (25). Similarly, Matarazzo *et al.* (38) identified defects in neuronal maturation in the olfactory system of *Mecp2*-null mice, which are ascribed to an observed delay in neuronal maturation.

X-CHROMOSOME INACTIVATION

Although it was initially thought that MeCP2 played no role in survival of neurons (20), recent careful studies of XCI patterns in *Mecp2*$^{(+/-)}$ mice have provided evidence to the contrary. Braunschweig *et al.* (27) found that although there was a uniform regional distribution of MeCP2-negative cells in brains of *Mecp2*$^{(+/-)}$ female mice (i.e. those cells in which the X chromosome bearing the wild-type *Mecp2* allele is the inactive X), as a whole the XCI patterns were skewed in favour of inactivating the mutant allele (27). A similar conclusion was reached by Young and Zoghbi (40), who found that the X chromosome bearing the mutant *Mecp2* allele is predominantly the inactive X in the cerebellum and to a lesser extent in the midbrain and cortex of *Mecp2*$^{308/X}$ female mice. In an attempt to provide an explanation for this observation, these authors monitored the survival of hippocampal cultures derived from E18 *Mecp2*$^{308/X}$ embryos. When wild-type and mutant cells were plated out at a 1:1 ratio, it was found that after 7 days the culture consisted of predominantly wild-type neurons. At present it is not clear whether this effect is because of a defect in plating efficiency, survival, or proliferation of MeCP2-deficient neurons and/or neuronal precursors. In any case, however, these studies provide evidence for a previously undetected role for MeCP2 in *ex vivo* hippocampal cultures.

TOO MUCH OF A GOOD THING

As RTT-like symptoms are caused in mice by a deficiency of MeCP2 in neurons, it is important to find out whether re-introducing MeCP2 will rescue these phenotypes. Two different groups have addressed this issue by producing *Mecp2*/*MECP2*-transgenic animals (Table 1). In the first study, *Mecp2β* (Fig. 1) was expressed under the control of the Tau promoter (32). Tau is a major neuronal microtubule-associated protein (41) which is expressed in postmitotic neurons at early stages of differentiation (42). Expression initiates around day 9 of embryonic development, and by 10.75 days Tau is expressed throughout the entire nervous system (42). *Mecp2*-deficient males harbouring one allele of the *tau–Mecp2β* transgene were indistinguishable from wild-type mice (32). Thus, expression of the MeCP2β isoform from day 9 of development in neurons is sufficient to fully rescue the *Mecp2* deletion pheontype in mice. This study resulted in another insight into MeCP2 function when the transgene was crossed onto mice also carrying a wild-type *Mecp2* allele. This genetic combination produces elevated levels of *Mecp2β* in neurons and resulted in severe motor dysfunction, indicating that too much MeCP2 interferes with normal neuronal function.

The other group developed a mouse model in which (MeCP2$^{308/Y}$) mice were made transgenic for the human *MECP2* gene (43). [This transgene presumably expresses both *MECP2A* and *MECP2B* (Fig. 1), although this was not specifically shown in this study.] Transgenic mice developed a progressive neurological phenotype, the onset and lethality of which was correlated with the levels of MECP2 expression. When motor coordination and cerebellar learning were assessed, moderately affected transgenic mice performed better than their wild type littermates. Further, these mice scored higher in hippocampal learning tests than wild-type littermates, and both short-term synaptic plasticity and long-term potentiation (LTP) were found to be enhanced in MeCP2 transgenic mice (43). Deciphering exactly why these transgenic mice display increased learning abilities may provide further clues as to the normal functions of MeCP2 in neurons.

Interestingly, increased *MECP2* expression levels have been identified an autistic patient and one pervasive developmental disorder patient (44). This observation raises the possibility that both deficiency and elevated expression of MECP2 may influence expression of the same set of downstream target genes, the expression of which could also be altered in other neurodevelopmental disorders, such as autism. Indeed, decreased expression of two genes has recently been demonstrated in brains from RTT, autism and Angelman syndrome patients (45)(and see below).

TARGETS OF MeCP2-MEDIATED REPRESSION

MeCP2 possesses strong transcriptional repressor activity, binds to chromosomes and associates with chromatin modifying enzymes (13,14,46,47). It is therefore reasonable to hypothesize that loss of MECP2 in a cell would result in inappropriate expression of a large number of genes (48). Consistent with this assumption are reports in which a number of genes were found to be misexpressed in lymphoblast cell lines derived from RTT patients (49,50). In some cases, the

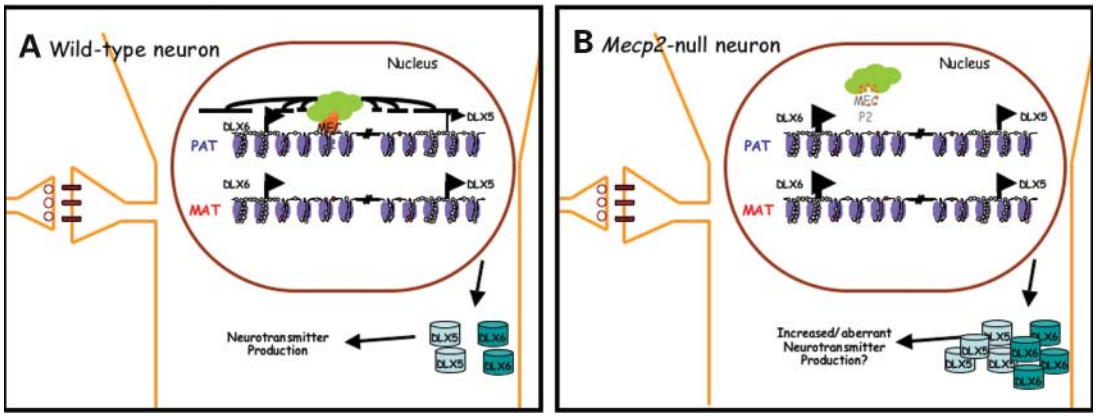

Figure 2. MeCP2 regulation of the *Dlx5* imprinted locus in mice. (**A**) In wild-type neurons MeCP2 binds to sites on the paternal allele of the *Dlx5/6* locus and exerts a repressive influence upon the region, probably through the recruitment of a histone-modifying co-repressor complex (green circles). Transcription of the *Dlx5* gene is reduced from the paternal allele in mouse brain, and is said to show partial imprinting. The *Dlx6* gene is not imprinted. Downstream effects of Dlx5 and Dlx6 proteins in neurons include stimulation of GABA neurotransmitter production. (**B**) In Mecp2-null neurons, expression from the paternal *Dlx5* allele is no longer repressed, resulting in Dlx5 overproduction. Dlx6 transcription is also increased, although this may be due to transcriptional activation by Dlx5, rather than due to a direct result of the absence of MeCP2-mediated repression. Increased levels of Dlx5 and Dlx6 may result in increased or aberrant neurotransmitter production, in turn resulting in neuronal dysfunction.

authors were able to demonstrate binding of MECP2 to the relevant promoters in normal cell lines, indicating that MeCP2 does indeed contribute to silencing of these genes in cells (49). However cell lines are known to have high levels of CpG island methylation that is not seen in primary tissues (51), so the relevance of these target genes to the pathology of RTT remains to be determined.

A number of studies have been published in which global gene expression changes were identified in brain or neuronal tissue (52–54), but these have failed to produce a unified molecular picture of the RTT or *Mecp2*-null brain. What these studies collectively illustrate is that the problem of identifying direct targets or MECP2-mediated repression is not necessarily going to be an easy one to solve using global gene expression (52,54) or proteomics (53) approaches.

One likely class of genes to be direct targets of MeCP2-mediated repression are the imprinted genes (55). Indeed, MeCP2 has been shown to physically associate with the silent alleles of various imprinted genes (56–58). Surprisingly, no loss of imprinting could be found at these loci in *Mecp2*-deficient mice (33,34) casting doubt on a role for MeCP2 in the maintenance of imprinting. Early in 2005, however, convincing evidence finally emerged that MeCP2 is important for imprinting at the *Dlx5* locus (59) (Fig. 2).

Using chromatin immunoprecipitation to identify *Mecp2*-bound sequences in mouse brain, Horike *et al.* (59) identified a sequence located within an imprinted gene cluster on mouse chromosome 6 as a bona fide *Mecp2*-binding site. Subsequent analysis of genes located in this cluster revealed an increase in expression of the imprinted *Dlx5* gene and the neighboring non-imprinted *Dlx6* gene in brains of *Mecp2*-deficient animals which correlated with changes in local and regional chromatin organisation (59) (Fig. 2). Importantly, loss of imprinting at the *DLX5* locus was also found in lymphocyte cell lines derived from three out of four RTT patients. *Dlx5* and *Dlx6* encode homeobox proteins known to regulate the expression of genes involved in neurotransmitter production (60) and are important in various aspects of embryonic development, including brain patterning (61). While it is tempting to speculate that elevated expression of *DLX* genes contributes significantly to the neuronal dysfunction that results in RTT (see 'Models we would like to see' in Table 1), it is important to determine the percentage of RTT patients that show loss of *DLX5* imprinting and how general a phenomenon this is in RTT. It will also be very interesting to determine whether there are any phenotypic differences between those patients who show loss of imprinting at *DLX5* and those who do not.

Phenotypic similarities between RTT and the imprinting disorder Angelman syndrome (OMIM #105830) led another group of researchers to investigate expression levels of another imprinted gene, *Ube3a* (ubiquitin protein ligase E3A, OMIM #601623), in *Mecp2*-deficient animals (45). Angelman syndrome is caused by deficiency or deletion of the maternally expressed *UBE3A* gene (62,63). Using a combination of methods to analyze protein and mRNA levels, Samaco *et al.* (45) found a small but significant decrease in expression of *Ube3a*/*UBE3A* in brains of *Mecp2*-deficient mice and RTT patients. Further, expression of a nearby non-imprinted gene implicated in autism, *Gabrb3*/*GABRB3*, was reduced in the same samples. A similar reduction in *UBE3A* and *GABRB3* expression was also found in brain samples from autism and Angelman syndrome patients, indicating that the similarities in phenotype between these syndromes may have a common molecular aetiology (45). In this case, no evidence for loss of imprinting or for direct MeCP2 binding to either gene was found, although it remains possible that MeCP2-mediated modulation of local chromatin structure (59) may indirectly facilitate expression of *UBE3A* and/and *GABRB3*.

Recently, a candidate gene approach was used to identify the brain-derived neurotrophic factor (BDNF) gene as a bona fide MeCP2 target (64,65) (Fig. 3). BDNF is a neurotrophin required for survival, growth, and maintenance of neurons during development (66). BDNF is known to be

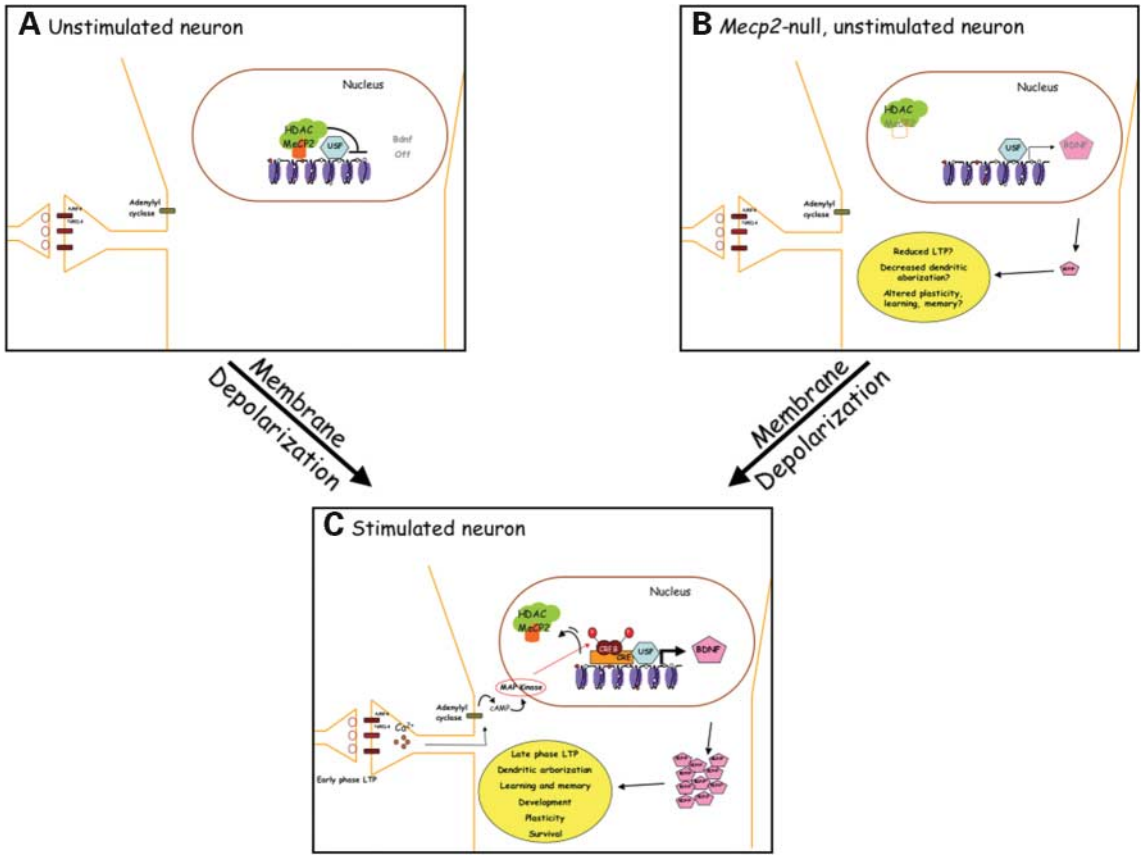

Figure 3. MeCP2 regulation of the neurotrophic factor Bdnf. (**A**) In resting neurons, MECP2 (orange) and its associated co-repressor (here indicated simply as HDAC, in green) represses transcription of the *Bdnf* promoter III. The USF transcription factor (blue) is associated with the promoter irrespective of transcriptional status (64). (**B**) In the absence of MeCP2, repression of *Bdnf* is leaky, resulting in low-level transcription and, presumably, Bdnf protein production that may cause a variety of downstream effects leading to dysfunction at different levels such as reduced LTP, altered learning and memory and decreased dendritic arborization. (**C**) Upon membrane depolarisation (i.e. neuronal stimulation), MeCP2 is displaced from promoter III and *Bdnf* transcription is activated, resulting in Bdnf protein production. Note that *Bdnf* activation is not affected by the presence or absence of MeCP2, and that transcription of *Bdnf* is 100-fold higher in stimulated neurons than in MeCP2-null, unstimulated neurons. *Source*: Adapted from Kandel *et al.* (74).

involved in learning and memory and has the ability to modulate synaptic plasticity by regulating axonal and dendritic branching and remodelling (67–71). MeCP2 was found to associate with and maintain repression of *Bdnf* in resting neuronal cultures (Fig. 3A). Following neuronal depolarization, MeCP2 becomes phosphorylated and disassociates from the *Bdnf* promoter, allowing for full transcription of the gene (Fig. 3C). In the absence of MeCP2, *Bdnf* repression becomes leaky in unstimulated neurons, resulting in a two-fold increase of *Bdnf* mRNA levels (64) (Fig. 3B). This two-fold increase in mRNA levels does not constitute 'activation' of the *Bdnf* gene, as transcript levels in resting *Mecp2*-deficient neurons are still approximately 100-fold lower than those found in activated neurons (64). Rather absence of MeCP2 results in incomplete repression of *Bdnf* (Fig. 3B). No difference in *Bdnf* expression was detectable in depolarised neuronal cultures derived from *Mecp2*-deficient or wild-type mice (Fig. 3C).

Misexpression of *Bdnf* in *Mecp2*-null brains occurs only in certain cell types, and only to a level of about two-fold. This slight increase in *Bdnf* expression, although not enough to be detected using microarray experiments (54), could be sufficient to cause small changes in synaptic plasticity in MeCP2-deficient neurons that may contribute to the overall RTT phenotype (Fig. 3). Interestingly, restricted overexpression of *Bdnf* in the postnatal forebrain of transgenic mice does not result in RTT-like phenotypes (72). Clearly, further studies of *Bdnf* overexpression in mice will be important to determining what part is played by BDNF misexpression in the RTT phenotype (see 'Models we would like to see' in Table 1).

It is now clear that any changes in gene expression resulting from deficiency of MeCP2 vary between cell types (53,64) and between individuals (59) and are at a level that is much lower than was initially expected (48). The lack of a dramatic, global de-repression in the absence of MeCP2 may be because of the fact that it is one of the four known methyl-CpG-binding transcriptional repressors in mammalian cells (10,12,73) and the degree of functional redundancy between them remains to be determined. It now appears that in the absence of MECP2 its target genes are simply not repressed completely, rather than being fully expressed (e.g. Figs 2 and 3). In order to find these genes, we need to be able to distinguish between 'off' and 'not quite off' rather than between 'off'

and 'on.' Although this potentially very subtle difference in gene expression may be difficult to detect using standard techniques, its effects upon neurons within a human brain, in which approximately half of the cells are completely normal, are clearly devastating. Nevertheless it is clear that MECP2 and RTT are, reluctantly, revealing their secrets as long as we in the research community are asking the right questions.

ACKNOWLEDGEMENTS

We are grateful to Nicola Reynolds, Meng Li and Xinsheng Nan for critical reading of the manuscript. I.M.C. is the recipient of a University of Edinburgh School of Biological Sciences PhD Studentship. Our work is funded by the Wellcome Trust and the MRC.

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