Reversibility of functional deficits in experimental models of Rett syndrome

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Abstract

Mutations in the X-linked MECP2 gene are the primary cause of the severe autism spectrum disorder RTT (Rett syndrome). Deletion of MeCP2 in mice recapitulates many of the overt neurological features seen in humans, and the delayed onset of symptoms is accompanied by deficits in neuronal morphology and synaptic physiology. Recent evidence suggests that reactivation of endogenous Mecp2 in young and adult mice can reverse aspects of RTT-like pathology. In the current perspective, we discuss these findings as well as other genetic, pharmacological and environmental interventions that attempt phenotypic rescue in RTT. We believe these studies provide valuable insights into the tractability of RTT and related conditions and are useful pointers for the development of future therapeutic strategies.

Introduction

RTT (Rett syndrome) is a paediatric neurological disorder with a delayed onset of symptoms and is a leading cause of severe mental retardation in girls. The primary cause of RTT is mutations in the X-linked gene MECP2 [1] and mutations at this locus are also found in patients with other neurological conditions including milder forms of learning disability, neonatal encephalopathy, autism and X-linked mental retardation [2]. The onset of overt symptoms typically occurs during late postnatal development (infancy) and for this reason RTT and related conditions are considered to be pervasive neurodevelopmental disorders. The orthodox view is that abnormalities in brain development during critical periods of growth and maturation will produce aberrations in the nervous system, with resultant complex neurological and psychiatric features, and that these defects are essentially irreversible in adults. However, a number of studies in animal disease models ranging from Down’s syndrome [3,4], neurofibromatosis type 1 [5,6], tuberous sclerosis [7–9], Rubinstein–Taybi syndrome [10,11], fragile X syndrome [12,13], Angelman syndrome [14] as well as RTT [15] are beginning to demonstrate an unexpected propensity for phenotypic reversal, even in adult mice (reviewed in [16]). The current perspective considers RTT and various strategies that have been adopted to investigate the concept of phenotypic reversal in the Mecp2 mutant brain (Table 1).

MeCP2 is a nuclear protein that was first discovered through its affinity for DNA sequences containing methylated 5′-CpG-3′ dinucleotides [17]. It is a member of a small group of MBPs (methylated DNA-binding proteins) that can act as transcriptional repressors [18]. While MBPs have been linked to a variety of human diseases, MeCP2 was discovered to be of critical significance in the severe autism spectrum disorder RTT [1]. RTT is suspected to be almost exclusively caused by de novo germ cell mutations in the human MECP2 gene [19]. The MECP2 gene is X-linked and RTT occurs in girls that are heterozygous for the mutated MECP2 allele [20]. Boys acquiring the mutant allele are much more severely affected, presenting infantile encephalopathy and typically not surviving infancy. In contrast, females develop symptoms after a period of 6–18 months apparent normal development. A marked deceleration of head growth is then associated with the onset of autistic features, severe mental retardation and many characteristic symptoms common to the disorder such as abnormal breathing, stereotyped hand movements, motor deficits and scoliosis. Like other autism spectrum disorders and indeed neurodevelopmental disorders generally, RTT patients show a heightened susceptibility to developing seizures which are then difficult to treat [21]. The differences between the observed symptoms in males and in females can be explained in terms of the proportion of cells in the nervous system expressing the mutant allele. While all MeCP2-containing cells will express the mutant allele in males, due to random X-chromosome inactivation, the female brain will develop into a mosaic network of cells with some expressing the mutant allele and others the normal allele. In this way, the pathology associated with the mutant allele is diluted (at the network level) in the female brain, albeit for direct cell autonomous actions of the mutation.

RTT-like symptoms in MeCP2 mutant mice

Owing to its monogenic nature, RTT has been subjected to vigorous experimental investigation with the aim of understanding the underlying pathology and neuronal dysfunction in RTT as well as providing insights into the pathophysiology of autism spectrum disorders more...
Table 1 | Rescue and therapeutic strategies in Mecp2 mutant mice

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Figure 1 | Reactivation of Mecp2 prolongs survival and reverses symptoms in Mecp2-stop mice
(A) Top: diagram showing early symptom onset and lethality in male Mecp2-stop mice. Bottom: survival plot showing that delayed activation of Mecp2 by tamoxifen (TM)-induced Cre-mediated stop cassette excision reduced lethality and prevented the onset of neurological symptoms. (B) Top: female mice that are heterozygous for normal Mecp2 allele do not show early death but develop symptoms (hypoactivity, tremor, irregular breathing, etc.) which increase in severity but then stabilize. Symptom progression is curtailed and reversed in adult female mice upon tamoxifen treatment. Wt, wild-type. Period of tamoxifen treatment in (A) and (B) is shown by hatched boxes. Adapted from [15] with permission from AAAS.

generally. Several genetic mouse models of RTT have been developed through interruption of murine Mecp2, and these models accurately recapitulate the cardinal symptoms that characterize RTT in humans [15,22–25]. For instance, Mecp2-null male mice develop motor impairment, tremor, breathing abnormalities and limb stereotypes [15,22,23]. Like the human condition, mice show an apparent normal early development before the onset of overt symptoms, with males showing an early symptom onset, an aggressive disease progression and early death (~10–20 weeks). Females heterozygous for Mecp2 show a more delayed onset of symptoms. The symptom severity increases over a period of weeks to months but then, as in human females, these stabilize and the mice show an apparently normal lifespan (Figure 1).

MeCP2 is expressed in a range of tissues but is especially abundant in post-mitotic neurons. Mice lacking MeCP2 in neurons show overt RTT-like symptoms whereas mice in which the expression of MeCP2 is driven in neurons alone show a normal phenotype [24]. While MeCP2 is present at low levels in astrocytes, and MeCP2 deficiency in these cells may confer subtle non-cell autonomous actions on neuronal phenotype [26,27], a body of evidence points to the overt RTT-like symptoms being due mainly to MeCP2 deficiency in the nervous system and neurons in particular. More refined experiments investigating the functional consequences of MeCP2 absence or dysfunction in specific cell types and brain structures are ongoing [28–30] (S. Cobb, J. Guy and A. Bird, unpublished results) and point to MeCP2 deficiencies in different neuronal populations conferring specific aspects of RTT pathology. However, the majority of studies have focused on global deletion or truncation of Mecp2 [22,23,25,31,32]. Behavioural studies in these mice have revealed altered gait and motor defects including hypoactivity, hind limb claspning and rotarod and swimming impairments. Such mice also display an anxiety phenotype including reduced exploration, increased thigmotaxis and altered plus and zero maze behaviour. Motor dysfunctions limit the application of certain cognitive tests, but impairments in fear conditioning and novel object recognition have been reported [31,32]. In the Mecp2<sup>308</sup> model, which expresses a truncated allele of Mecp2 and displays significantly milder symptoms, mice show impairments in hippocampal-dependent spatial memory as well as social memory [33].

Synaptic and structural deficits in RTT
In addition to gross neurological symptoms, RTT in patients and MeCP2 deficiency in mice are known to be associated with a number of structural changes in the brain as well as changes in cellular and synaptic physiology. The most conspicuous feature in RTT patients is a reduced brain size and weight as well as more subtle changes in neuronal packing density and aspects of cellular morphology such as reduced dendritic branching complexity and changes in spine density and morphology [34–37]. Similar changes are reported in Mecp2-null mice [38–41] where there is evidence for both cell autonomous and non-cell autonomous alterations in neuronal morphology with respect to the cellular expression of MeCP2 [42]. As well as cellular morphology, MeCP2 level
is reported to regulate the number of excitatory synaptic connections formed [43]. Electrical studies have revealed relatively modest changes in the electrical properties of cortical neurons [44] and more pronounced changes in other regions such as the brain stem and locus ceruleus [45,46]. However, most studies report robust changes in synaptic signalling, notably excitatory and inhibitory amino acid transmission [44,46,47]. In addition to such changes in the frequency and amplitude of synaptic events, several studies have reported deficits in both short- and long-term forms of synaptic plasticity [15,33,47,48]. Interestingly, synaptic plasticity appears normal in young Mecp2-null mice [15,48,49], but shows impairment when tested in older mice upon symptom onset [15,48]. The precise mechanisms underlying the involvement of MeCP2 in regulating morphological and function aspects of synaptic signalling remain to be identified.

**Treatment strategies and reversibility of RTT phenotype**

Despite detectable changes in the neuronal morphology and physiology at the single cell and synapse levels as well as alterations in the expression of genes and various neurochemical markers [50], the structure of the nervous system appears intact at a gross level and there is little evidence for overt large-scale structural malformation or neuronal death, suggesting that RTT is not a neurodegenerative disorder. Instead, RTT is classically considered to be a dysfunction of neuronal maturation or, following development, an inability to maintain a mature phenotype. MeCP2 is expressed widely, but is most abundant in neurons of the mature nervous system [40]. MeCP2 is also present in astrocytes and other non-neuronal cell types in the brain, albeit at much lower levels [51]. While a loss of MeCP2 function in astrocytes may lead to altered release of neurotrophic factors, changes to dendritic outgrowth [26,27] and contribute to non-cell autonomous aspects of RTT pathology, deletion and neuron-specific expression of Mecp2 studies in mice show that the dominant mutant phenotype is principally due to the absence of MeCP2 in neurons [22–24]. That neurons not expressing MeCP2 display long-term survival raises the possibility that the introduction of normal MeCP2 might restore function and, thereby, reverse deficits seen in RTT. Another possibility is that MeCP2 may be essential for neuronal development during a specific time window, after which damage caused by its absence is irreversible. A third scenario is a combination of the above whereby certain RTT-like features can be rectified in the mature nervous system if cells were to begin expressing MeCP2, while other features are critically dependent on the presence of MeCP2 during essential developmental processes and thus intractable by simple restoration of MeCP2 beyond a critical period.

**Delayed activation at the endogenous locus**

To explore these issues and test directly whether the phenotypes observed in Mecp2-null male mice and Mecp2+/− heterozygous female mice can be reversed by restoration of the Mecp2 gene, Guy et al. [15] engineered a mouse line in which the endogenous Mecp2 gene was silenced by the introduction of a lox-stop cassette. Moreover, the silenced Mecp2 gene was enabled to be conditionally activated by stop cassette deletion upon tamoxifen injection. Such a treatment resulted in Cre recombinase-mediated stop cassette excision and delayed activation of Mecp2 at its endogenous locus and, importantly, under the control of its own promoter and regulatory elements. Using this system, delayed activation of the Mecp2 gene in male mice with advanced RTT-like symptoms caused dramatic reversal of most overt features and prevented early death. When Mecp2 was activated prior to the onset of neurological symptoms (~3 weeks), the treatment prevented the onset of symptoms as well early death (Figure 1A). In female mice, a more accurate model of RTT in humans, activation of Mecp2 in fully adult animals largely or fully reversed most of the overt RTT-like phenotype (Figure 1B). In addition to a phenotypic rescue of gross observational phenotypes, reactivation of Mecp2 in symptomatic adult mice also reversed deficits in hippocampal synaptic plasticity. Studies to assess the propensity for reversal of other behavioural, physiological and brain structural deficits are ongoing but the experiments to date suggest that, in mice at least, the profound defects caused by a lack of MeCP2 are reversible if MeCP2 is made available, even in fully adult animals. This has important implications as it supports the concept of phenotypic reversibility in RTT and suggests that it might be possible to treat the disease even after the onset of symptoms. The results also have important implications in terms of RTT and perhaps other disorders described as being ‘neurodevelopmental’ in nature and thus implying that the symptoms result from aberrations in neural development. Specifically, the results show that neurons can develop in the complete absence of MeCP2 but that the dysfunction associated with its absence can be rectified at a later stage simply by restoring the protein. While these results do not provide definitive confirmation, we argue that the findings nevertheless rule out RTT being purely a neurodevelopmental disorder and that the reversibility might be explained in terms of MeCP2 having a role in the maintenance of a mature phenotype [52] (a neuromaintenance disorder) or in permitting normal plasticity within a mature nervous system.

Another strategy to investigate the effectiveness of delayed activation of Mecp2 used a transgene approach whereby mice were engineered in which the endogenous Mecp2 was deleted and a Mecp2 transgene was driven by a range of promoters with differing spatial (whole brain and forebrain specific) and temporal (embryonic and postnatal) profiles [53]. This study revealed embryonic expression to enhance lifespan by approx. 6 months whereas lines generating postnatal (P0–30) MeCP2 expression produced a more modest enhancement of lifespan (4 weeks) as well as delayed motor impairment and prevention of obesity. The likely difference between the more limited extent of phenotypic reversal seen in this study and the reactivation study above [15] is that the latter results
in an activation of the endogenous Mecp2 gene under its own promoter and regulatory machinery and thus the protein will presumably be produced and maintained in the appropriate cell types and at the appropriate levels. Thus, despite MeCP2 protein being produced at a later postnatal time-point in the Guy study [15], it is more likely to be produced in the appropriate places and in appropriate amounts to result in a more robust rescue of phenotype. This has important implications for the likely application of gene therapy strategies should this become a feasible therapeutic approach in the future (see below).

Other genetic approaches
A number of other genetic manipulation studies have revealed insights into the necessity or otherwise of MeCP2 during development. By targeting MeCP2 to the tau locus to achieve selective but early post-mitotic neuronal expression, Mecp2-null mice do not develop detectable symptoms, suggesting that expression of Mecp2 in post-mitotic neurons is sufficient to prevent or alleviate the RTT phenotype [24]. While expression of Mecp2 at levels close to or slightly above endogenous (wild-type) levels do not appear to result in mice developing overt symptoms, higher levels of Mecp2 expression leads to detrimental motor and other effects [24].

Gene therapy
An obvious potential strategy for treating RTT is to adopt a gene therapy-based approach and to deliver MECP2 to replace defective or deficient protein. The genetic rescue data in mice suggest that such a strategy would hold some promise. Several groups are active in this area but to date there are no reports that viral-based delivery of Mecp2 to the brain of Mecp2-null mice can rescue any aspects of the RTT phenotype. While conceptually, gene therapy is appealing, the strategy in humans would require targeting MECP2 to the correct cells and at the correct levels. This is on top of the major challenges of delivering therapeutic genes to the nervous system in the first place. There is evidence that elevated levels of MECP2 may be detrimental since patients with duplication of Xq28 in regions spanning the MECP2 locus show a range of neurological features including decelerated head growth, ataxia, seizures and mental retardation [54–56]. Similarly, mice overexpressing MeCP2 show behavioural changes, some of which are different from those reported in mice lacking MeCP2. Mice with a modest overexpression of MeCP2 show enhanced motor coordination, a reduced anxiety phenotype and increased context-dependent fear conditioning [57]. Mice expressing higher (2–4) fold levels of MeCP2 (targeted to the tau locus) display tremors and motor dysfunction [24]. Clearly, the dosage issue is going to be pertinent for attempts to reintroduce MeCP2 as a strategy. The reactivation study [15] shows that RTT-like neurological defects due to absence of the mouse Mecp2 gene can be effectively rectified by delayed restoration of Mecp2 when driven by its endogenous promoter and regulatory elements and presumably maintained at the correct cellular levels. It is proposed that essential MeCP2 target sites in neuronal genomes are encoded principally by patterns of DNA methylation so that newly synthesized MeCP2 molecules are able to home to their correct chromosomal positions (methyl-CpG patterns) to resume their role as interpreters of the DNA methylation signal [17,58]. The limits within which MeCP2 levels must be maintained in order to confer normal function remains to be systematically explored, but the available evidence is that modest overexpression will not result in serious detrimental consequences [24,59]. Initial attempts to design vectors for delivery of Mecp2 have confirmed the endogenous Mecp2 promoter to be a sensible choice in driving cell type appropriate expression and thus avoiding overt ectopic patterns of expression [60]. While this initial study showed the effectiveness of viral vectors in achieving widespread appropriately targeted expression of the transgene and demonstrated that the expression at a cellular level can promote dendritic branching in vitro, it did not address the dosage of MeCP2 at the level of the single cell. The crucial question as to whether viral-based intervention is successful in reversing systems and behavioural defects in Mecp2-null mice remains unanswered.

Pharmacological approaches
As an alternative to targeting MeCP2, many groups have argued that identifying factors that are downstream to MeCP2 function and targeting those pharmacologically is the most sensible approach to developing rational therapies in RTT. It is certainly the case that low molecular mass entities (classical ‘drug’ molecules) are unlikely to ever restore or replace the function of MeCP2. However, the problem at present is that the precise role of MeCP2 is unknown, and even the concept of MeCP2 having discrete ‘target genes’ whose expression becomes aberrant in the absence of functional MeCP2 may not be correct [61]. If MeCP2 truly functions to sense/interpret the DNA methylation signal then the action of MeCP2, or more specifically, the precise dysfunction caused by its absence, will be uniquely dependent on the methylation status within a given neuronal type or indeed individual neuron. Moreover, high abundance of MeCP2 together with the ubiquity of DNA methylation suggests that candidates for targeting therapeutically will be multiple and diverse. That said, several attempts (http://www.rsrt.org/) are being made to test the effectiveness of existing drug molecules based on our current rather limited understanding of the underlying biology of MeCP2 function.

Changes in the levels of BDNF (brain-derived neurotrophic factor) signalling is consistently reported in the Rett brain [62,63] and genetic potentiation of BDNF signalling is reported to ameliorate key symptoms in the Mecp2-null mouse [64]. Based on this link between a loss of MeCP2 and BDNF dysregulation, Ogier et al. [64a] tested the ampakine CX546, a positive modulator of AMPA receptors and known to enhance BDNF levels, in
**Mecp2-null mice.** This study focused on respiratory function and described the restoration of normal breathing patterns and respiratory minute volume upon CX546 treatment. This is a significant finding as respiratory abnormalities are an especially problematic and indeed a dangerous feature of RTT symptoms [65]. Though different ampakines vary in their effectiveness to potentiate BDNF, the study is nevertheless consistent with the hypothesis that elevations of BDNF are associated with improved symptoms and supports the concept of targeting BDNF signalling in RTT. It will be interesting to find out whether ampakines improve other features of the Mecp2-null phenotype. Analogous studies in a knock-in model of Huntington’s disease show ampakine-induced BDNF up-regulation to reverse deficits in synaptic plasticity and improve impairments in long-term memory but to have no detectable effect on locomotor activity impairments [66].

Another growth factor that is important in neuronal maturation and as a regulator of synaptic plasticity is IGF-1 (insulin-like growth factor 1). Mecp2-null mice and RTT patients have elevated levels of the IGF-binding protein 3, the consequence of which is predicted to be an overall reduction in IGF-1 signalling [67]. With this in mind, Tropea et al. [68] have tested whether systemic administration of IGF-1 can affect dendritic and synaptic maturation and reverse key aspects of the RTT phenotype in Mecp2-null mice. At the cellular level, IGF-1 was found to increase PSD-95 (postsynaptic density protein of 95 kDa) labelling, cortical spine density and produce a very modest but significant increase in the amplitude of excitatory synaptic currents. IGF-1 treatment partially restored brain weight towards wild-type levels and at the organismal level the authors report a significant increased lifespan, improved locomotor activity and more modest improvements in cardiac and respiratory function.

Another pharmacological strategy aimed at ameliorating breathing dysfunction in RTT is to target noradrenergic signalling. Bioamine levels including that of noradrenaline, serotonin, dopamine and several major bioamine metabolites are reported to be reduced in post-mortem RTT biopsies from several brain regions including cortex, basal ganglia and thalamus [69,70]. Deficits in bioamine levels also accompany the appearance of breathing abnormalities in Mecp2-null mice which also show a reduction in tyrosine hydroxylase staining in the medulla [71]. Monoamines are important regulators of brainstem function and therefore the antidepressant desipramine, which boosts noradrenaline signalling through blocking noradrenaline uptake, has been tested in Mecp2-null mice. These studies reveal a delayed genesis of breathing abnormalities in young animals and a substantial amelioration in breathing abnormalities (apnoeas) when administered to mice that were already symptomatic [72,73]. In addition to improved respiratory function, the desipramine treatment also extended the lifespan of Mecp2-null mice (from a modest increase up to double) but failed to alter growth curves or, surprisingly, levels of noradrenaline [73].

Another transmitter system that shows alterations in the Rett brain is the cholinergic system. Several reports suggest impaired cholinergic function such as a reduction in the synthesising enzyme choline acetyltransferase and the binding of the vesicular transporter [74]. This led to the proposal that a diet enriched in choline, considered the rate limiting step in the biosynthesis of acetylcholine, might boost cholinergic function and be beneficial in RTT. This concept has been tested experimentally in Mecp2-null mice that were provided with choline during early postnatal development by delivery through maternal milk [75]. The results of this study were a modest improvement in locomotor activity and motor coordination in males and increased grip strength in females. However, the effects were very subtle and the treatment did not alter contextual or cued fear conditioning.

In addition to targeting downstream systems that are dysregulated in the absence of functional MeCP2, another pharmacological approach would be to target, upstream, the specific mutation resulting in the MeCP2 deficiency. In the special case of nonsense mutations which result in premature stop codons, drugs which promote read-through of nonsense mutations would be a rational and attractive therapeutic approach. Using HeLa cells transfected with a range of commonly occurring MECP2 nonsense mutations, Brendel et al. [76] have shown that the aminoglycoside antibiotic gentamicin can produce a read-through efficiency in the range of 10–22% [76]. While this is clearly an in vitro assay system, the results are nevertheless encouraging given that nonsense mutations are very common in RTT [77]. However, this has to be offset by the fact that the adverse effects of gentamicin can be common and severe, especially at the high concentrations required to impair ribosomal proofreading and promote read-through of nonsense mutations. That said, alternative drugs are being developed to target genetic disorders of nonsense mutations [78] and this strategy has the added advantage that it would be unlikely to result in problems of overexpression.

### Environmental factors

RTT is a genetic disease but the mutations occur in a key epigenetic mediator and therefore the consequences of epigenetic factors in the aetiology of RTT, including the onset and severity of symptoms, are important considerations. While modification of environment may have useful application in the management of children with RTT [79], detailed studies on the precise relationship between environment, for example stressors, and the precipitation of RTT symptom onset or effect on symptom severity remain to be investigated fully. However, the effect of altered environmental conditions on the phenotype of Mecp2-null mice has been the subject of several recent investigations. Enrichment of a rodent rearing environment through provision of sensory, social and motor stimuli is reported to alter a wide variety of proteins and genes in the brain [80,81]. In addition, environmental enrichment has also been shown to produce beneficial effects in a range of models of brain disorders [82]. Two independent studies have shown the provision of an enriched environment to Mecp2 mutant mice to affect brain volume, brain chemistry and several behavioural tasks. Kondo et al.
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Funding

We are grateful to the Medical Research Council, Wellcome Trust, Rett Syndrome Research Trust and Scottish Rett Syndrome Association for support.
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Received 1 December 2009
doi:10.1042/BST0380498