Rett Syndrome: Crossing the Threshold to Clinical Translation

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Abstract

Lying at the intersection between neurobiology and epigenetics, Rett syndrome (RTT) has garnered intense interest in recent years, not only from a broad range of academic scientists, but also from the pharmaceutical and biotechnology industries. In addition to the critical need for treatments for this devastating disorder, optimism for developing RTT treatments derives from a unique convergence of factors, including a known monogenic cause, reversibility of symptoms in preclinical models, a strong clinical research infrastructure highlighted by an NIH-funded natural history study and well-established clinics with significant patient populations. Here, we review recent advances in understanding the biology of RTT, particularly promising preclinical findings, lessons from past clinical trials, and critical elements of trial design for rare disorders.

Progress in Identifying Potential RTT Therapeutics

RTT is a severe neurodevelopmental disorder resulting from mutations in the X-linked gene encoding methyl-CpG-binding protein 2 (MeCP2) [1]. Progress in understanding the pathophysiology of RTT and in identifying potential therapies has outpaced that in many other neurodevelopmental disorders due, in part, to the availability of rodent models with good construct and face validity [2–4]. These include strains of mice carrying either *Mecp2*-null or hypomorphic alleles or human disease-causing mutations [2, 4], as well as an *Mecp2*-

null rat model (SAGE Labs). In addition, some of the core symptoms of RTT, such as abnormal breathing, are more readily quantifiable and translate more directly from mice to humans compared with the complex behavioral abnormalities that define more prevalent disorders, such as nonsyndromic autism. Over the past few years, studies of the biology of MeCP2 (Box 1) and the consequences of MeCP2 loss for neural circuit function and behavior have led to the identification of potential therapeutic strategies [3–8], including: (i) molecular genetic approaches that target *MECP2* itself, ranging from gene and protein replacement therapy to development of novel tools for activating the wild-type allele on the inactive X chromosome; (ii) pharmacologic approaches that target mechanisms downstream of *MECP2* to restore excitatory–inhibitory synaptic balance in specific neural circuits, including some drugs that are now in early-stage clinical trials in patients with RTT (Figure 1; see Table S1 in the supplemental information online for the figure references).

Genetics and Clinical Features of RTT

The *MECP2* gene is X linked and RTT mutations arise predominantly in the paternal germ line. Given that the gene is subject to X chromosome inactivation, most affected individuals are female heterozygotes who are somatic mosaics for normal and mutant *MECP2*. In rare cases, males can be born with an *MECP2* mutation derived from the mother who either has favorable X chromosome inactivation patterns or gonadal mosaicism. However, because males have only one X chromosome, many such individuals are more severely affected than females and die, often early [9]. The prevalence of RTT is estimated at 1 in 10 000 live female births [10], corresponding to approximately 15 000 affected children and women in the USA and 350 000 worldwide. The disorder is diagnosed based on history and clinical presentation, and approximately 95% of individuals with a RTT diagnosis have a confirmed mutation in *MECP2* [10]. While hundreds of mutations in *MECP2* have been identified, eight hotspot mutations account for more than 60% of all cases [11].

Girls affected with RTT exhibit apparently typical early postnatal development followed by stagnation of developmental milestones and regression of skills, usually during the second year of life [12]. The hallmark symptoms of RTT include significant verbal and nonverbal communication deficits and the loss of motor skills, including purposeful hand use, which is replaced by almost constant stereotypical movements. Approximately half of affected individuals cannot walk and those who do have a wide-based and unsteady gait that becomes more pronounced with age. Particularly challenging, especially for families, is loss of speech. Autonomic and respiratory problems are frequent and include dysregulation of breathing with periods of hyperventilation, breath-holding, and abnormal cardiorespiratory coupling, gastrointestinal dysfunction, including severe constipation, and cardiac electrical problems, such as a prolonged QT interval. Seizures, anxiety, and orthopedic problems, such as scoliosis, contractures, and fractures, are common. Most individuals affected with RTT live well into adulthood and require total, round-the-clock care.

During the period of neurological regression, it is not uncommon for girls with RTT to exhibit autistic-like behaviors, such as social withdrawal [13]. However, as they get older, they often become very social and interactive. In fact, as noted by Andreas Rett, when he first described the disorder in 1966 [14], many girls with RTT have a penetrating gaze that

they use effectively for communication purposes. Despite the fact that RTT is no longer classified as an autism spectrum disorder (ASD) in the latest (5th) edition of the *Diagnostic and Statistical Manual of Mental Disorders*, an individual with RTT can also receive a diagnosis of ASD if she meets the behavioral criteria.

Although RTT has historically been described as a cognitive disorder, recent data suggest that the girls have strong receptive language [15]. Without the ability to speak or to adeptly use their hands for pointing, typing, or sign language, expressive language is difficult. Evolving strategies in teaching and augmentative communication technologies have resulted in fresh perspectives and attitudes about what individuals with RTT can achieve [16].

Neural Circuit Defects Resulting from Loss of MeCP2

Despite ongoing questions about the normal function of MeCP2, the effects of MeCP2 deficiency on many aspects of brain structure and function are now clear. Histopathological evidence from patients with RTT and *Mecp2* mutant mice shows that loss of MeCP2 does not result in neuronal cell death, axonal degeneration, or other irreversible deficits [17], consistent with the finding that neurological dysfunction in conditional *Mecp2* mutants is largely reversible upon reactivation of silent *Mecp2* alleles [18]. By contrast, numerous structural and functional abnormalities have been identified at the level of brain microcircuits, all of which are potentially reversible. For example, reduced dendritic complexity and spine density are consistent findings in *Mecp2* mutant mice and in postmortem material from patients with RTT [2, 19–21]. *Mecp2* mutants also exhibit decreased expression of multiple neurotransmitters, neuromodulators, transmitter receptors, and transporters required for normal synaptic function [2, 3, 6, 9]. To the degree that these endpoints have been analyzed in human samples, similar deficits have been found, including decreased levels of brain monoamines and their metabolites, decreased cholinergic markers and abnormal patterns of NMDA receptor expression [22–25] (also see references in [9]). These changes may arise in large measure from the failure of activity-dependent mechanisms that depend on intact MeCP2 function and are required to maintain fully differentiated neuronal and synaptic phenotypes [26]; this view is supported by the fact that loss of MeCP2 at any stage of life is deleterious [27, 28]. In addition, abnormal glial function may also have a role [29]. As a result of these molecular and cellular abnormalities, brain microcircuits in *Mecp2* mutants exhibit shifts in excitatory–inhibitory synaptic balance [19], defects in homeostatic synaptic scaling [30], excitatory or inhibitory connectivity [31], and/or changes in intrinsic neuronal excitability compared with controls [32, 33].

Of particular interest is the topology of changes in neural circuit function in the MeCP2-deficient brain. Studies in *Mecp2*-null and heterozygous mice demonstrated that loss of MeCP2 results in a regional pattern of dysfunction characterized by excitatory hypoconnectivity in many forebrain structures and hyperconnectivity in the caudal brainstem compared with wild-type mice [34] (Figure 2). For example, shifts in excitatory–inhibitory synaptic balance towards reduced excitation and/or increased inhibition have been documented in all cortices examined thus far, including somatosensory, visual, motor-frontal, and medial prefrontal (mPFC) [35–38]. These regions also exhibit marked reductions in the expression of the immediate early gene product Fos, a surrogate marker of

neuronal activity [34]. By contrast, brainstem structures, including the locus coeruleus, nucleus tractus solitarius, and ventrolateral medulla, exhibit shifts towards synaptic or intrinsic hyperexcitability [32, 39], increased Fos expression [34], and enhanced excitatory activity in respiratory motor nerves [40]. An exception to this dichotomy between forebrain and brainstem is the hippocampus, which is hyperexcitable in *Mecp2* mutants due, at least in part, to a loss of excitatory synaptic drive to inhibitory interneurons [41] and increased network synchrony [42].

This regional pattern of functional hypo- and hyperconnectivity accords well with the clinical picture of RTT; that is, cognitive and behavioral deficits consistent with cortical hypofunction coupled with paroxysmal events in brainstem control of respiratory and autonomic outflow. However, the prevalence of seizures in RTT seems at odds with the fact that excitatory synaptic drive onto pyramidal neurons is reduced in cortical circuits in MeCP2-deficient mice. On the other hand, increased network synchrony, even in the face of reduced excitatory connectivity may be a key factor driving epileptiform discharges in RTT [42]. Given the importance of the forebrain in regulating brainstem autonomic, respiratory and somatomotor outputs, the interplay between cortical hypofunction and brainstem hyperactivity likely has a key role in the pathophysiology of RTT. Thus, a major goal, and challenge, in therapy development for RTT is to redress excitatory–inhibitory imbalance not only in particular neuronal cell groups, but also across the neuraxis as a whole.

MECP2 and MeCP2 as Therapeutic Targets

Gene Dosage Concerns

The ultimate goal of strategies that target *MECP2* directly would be to normalize expression without affecting the levels of other genes. However, these treatment approaches must carefully consider the consequences of MeCP2 dosage. An excess of MeCP2 in both humans and mice impairs neuronal development and causes severe neurological dysfunction. For example, mice overexpressing MeCP2 display seizures and hypoactivity [42, 43], and boys with *MECP2* duplication syndrome exhibit some phenotypes that are similar to RTT [44–46]. Recent investigations in mice have shown that the syndrome associated with MeCP2 doubling requires two functional gene copies [47]. Accordingly, both gene therapy and small-molecule strategies to normalize *MECP2* gene expression levels must take care to provide enough MeCP2 per cell to impart a therapeutic benefit, while limiting MeCP2 overexpression.

Activating MECP2 on the Inactive X Chromosome by Small-Molecule Approaches

Most mutations in *MECP2* prevent production of functional MeCP2 protein, rather than producing a partially functional or dominant-negative protein [47], suggesting that reactivating the wild-type copy of *MECP2* on the inactive X (Xi) may be a viable approach for treating most forms of RTT. The therapeutic value of reactivating disease genes has been previously demonstrated in the case of the neurodevelopmental disorder Angelman syndrome, in that a dormant but intact copy of the *Ube3a* gene can be pharmacologically activated to replace the mutated active copy of *Ube3a* in a mouse model [48, 49]. Thus, the technology and procedures for identifying gene unsilencing agents are already established.

Towards this goal, *Mecp2*-GFP fluorescent reporter mice offer a valuable tool for assessing allelic activation of *Mecp2* (Figure 3). One can use high-content imaging of neurons from these mice to assess changes in GFP expression in a high-throughput, small-molecule screen. This approach is unbiased and is only limited by cost and the availability of drug-screening libraries. This screening approach cannot discriminate on first pass between compounds that are specific to de-inactivating *Mecp2* or that produce global X de-inactivation, but these possibilities could be easily distinguished with secondary screens and experiments. It will be essential to validate activities in patient iPSC-derived neurons to verify their applicability to humans.

Rather than specifically targeting *MECP2*, some therapeutic approaches might involve reactivating the entire inactive X (Xi). While this approach may seem intuitively less attractive, recent work has shown that the loss of a protein hormone, Stanniocalcin 1 (Stc1) [50], perturbs silencing at a handful of X-linked genes, including *Mecp2*, and produces X reactivation without grossly affecting chromosome-wide gene expression [51]. Thus, approaches that specifically reactivate *MECP2* or that produce more widespread X chromosome reactivation might help to restore normal MeCP2 protein levels and treat RTT. Successful translation from screening to the clinic will depend on whether active compounds are safe, can be easily administered, are diffusible across the blood–brain barrier, and achieve relatively stable MeCP2 restoration broadly across relevant cell types.

Gene Therapy and/or Genome Editing

Another possible therapeutic approach to restoring MeCP2 function is a gene replacement or gene-editing strategy. The recent discovery of adeno-associated virus (AAV) vector designs, such as AAV9, that can achieve widespread gene transfer across the nervous system has opened up the possibility of a translatable gene therapy approach for RTT [52–54]. Two groups have independently demonstrated the potential of gene replacement therapy in RTT model mice, showing that intravenous delivery of an AAV9/MeCP2 vector prolonged the lifespan of MeCP2 knockout mice as well as partially normalized behavioral phenotypes of male and female RTT mice [55, 56]. The challenge of gene therapy is to deliver and express MeCP2 within a narrow range of expression that is therapeutic without resulting in detrimental overexpression. For example, although the AAV9 vector can deliver the *MECP2* gene across the blood–brain barrier to the brain, approximately 100-fold higher gene transfer occurs to the liver, resulting in some liver toxicity [55]. Thus, the greatest challenge for gene therapy is the ability to homogenously deliver the *MECP2* gene, but to avoid overexpression in the context of a mosaic mixture of wild-type and affected cells in RTT females.

In theory, gene or mRNA editing could circumvent problems associated with MeCP2 dosage, since only the mutant *MECP2* would be targeted and MeCP2 would retain all of its endogenous regulation [57, 58]. However, development of a translatable gene-editing approach is confounded by the following unresolved issues: (i) the ability to deliver the nuclease and editing template broadly to all cells; (ii) the relatively low efficiency of gene editing *in vivo* in postmitotic cells; (iii) potential nonspecific nuclease cleavage elsewhere in the genome, especially upon chronic expression of the editing nuclease; and (iv) potential immune responses against the editing nuclease, which would be a nonhuman protein.

Protein replacement is another approach that could conceptually offer the ability to titrate appropriate MeCP2 levels, but this would need to overcome the following obstacles: (i) ensuring the proper post-translational modifications are present; (ii) homogenous and ongoing delivery of the appropriate levels across the blood–brain barrier; and (iii) adequate cell penetration and localization of the supplied MeCP2 to the nucleus. In addition, pharmaceutical compounds have been developed that allow the read through of premature stop codons [59]. Conceptually, this could provide functional MeCP2 from the endogenous active allele, retaining native regulatory elements and circumventing any risk of overexpression-related toxicity. This treatment would only apply to disease-causing *MECP2* mutations that introduce in-frame premature stop codons, representing approximately 35% of patients. Such a strategy was able to provide some full-length MeCP2 in cultured R168X mouse fibroblasts [60]; however, this has not yet been shown to be effective in *in vivo* models.

Therapeutic Targets Downstream of *MECP2*

By using clinically relevant outcome measures, preclinical studies of potential RTT therapeutics have, in a relatively short period of time, produced compelling evidence that signaling pathways well downstream of *Mecp2* can be effectively targeted to ameliorate specific disease symptoms. In general, the pathways that have been targeted fall into three categories: (i) classical neurotransmitter and neuromodulator systems, including noradrenergic, serotonergic, glutamatergic, GABAergic, and cholinergic signaling; (ii) growth factor signaling, including brain-derived neurotrophic factor (BDNF) and insulin-like growth factor 1 (IGF-1); and (iii) metabolic signaling, including the cholesterol biosynthesis pathway and mitochondrial function [3, 4, 6, 61].

Given that loss of MeCP2 results, to varying degrees, in dysregulation of all of these pathways, it is generally thought that pharmacological strategies focused on targets downstream of *Mecp2* will likely require multiple drugs to effectively treat the full spectrum of RTT symptoms. By contrast, patients' quality of life (QoL) would be significantly improved by treatments that ameliorate or reverse even one of the core symptoms of the disease. In this regard, pharmacological improvement of breathing abnormalities is a particularly good example of preclinical findings with high translational potential. Dysregulation of breathing is a core feature of RTT in up to 93% of patients, significantly impacts QoL, and is thought to contribute to early mortality in some patients [62]. In human RTT and mouse models, respiratory dysfunction is characterized by periods of hyperventilation and prolonged respiratory pauses, including breath holds and apneas [62], which can be rigorously quantified using noninvasive plethysmography. Several laboratories have now shown that respiratory abnormalities in RTT mice can be significantly improved by manipulating diverse transmitter or neuromodulatory systems, including glutamatergic, GABAergic, noradrenergic, serotonergic, and neurotrophin signaling, either with experimental molecules or drugs already approved by the US FDA for other indications [62, 63].

Of particular interest are drugs that improve function across multiple symptom domains. One such example is the nonselective NMDAR antagonist ketamine (2-*O*-chlorophenyl-2-

methyl-amino cyclohexanone), which has been independently validated in two different laboratories in two different strains of *Mecp2* mice and is now in a clinical trial with RTT patients (Table 2). The therapeutic potential of ketamine for treating RTT was first demonstrated by Katz and colleagues, who found that treatment of heterozygous female *Mecp2* mutant mice with a subanesthetic dose of ketamine (8 mg/kg) acutely reversed abnormalities in Fos expression and sensorimotor function [34]. More recently, chronic administration of ketamine was also found to improve symptoms and extend lifespan in null male *Mecp2* mutants [64]. The ability of low-dose ketamine to improve function across a broad range of symptoms may be related to its ability to increase cortical network activity, presumably by selective inhibition of GABAergic interneurons [65], as well as to decrease synaptic excitability in brainstem networks important for respiratory and autonomic control [66]. Thus, ketamine and related molecules may be ideally suited to redress the imbalance between cortical and brainstem activity that characterizes the MeCP2-deficient brain (Figure 2). Moreover, in addition to its acute effects on circuit function, work in other disease models has shown that ketamine also rapidly stimulates dendritic growth, BDNF translation, and expression of key synaptic proteins [67, 68], at least in part through activation of mTOR signaling, which is deficient in *Mecp2* mutants [69]. These findings suggest that, in addition to acute rescue of neurological function, ketamine also has the potential to effect long-term synaptic repair in RTT by enhancing structural and functional connectivity, as previously shown in animal models of depression and stress [70].

Clinical Trials: Resources, Possibilities and Challenges

The United States RTT Natural History Study

Clinical trials in rare diseases are confounded by the limited, often heterogeneous, pool of affected individuals, and difficulty selecting endpoints with a large effect size [71–75]. However, observational natural-history studies have been useful to understand the range of manifestations and progression of other rare diseases, and to establish valid and reliable short-term and long-term outcome measures or biomarkers [76, 77]. Therefore, the United States RTT Natural History Study (USNHS) was conceived in 2003 to acquire longitudinal baseline data in preparation for clinical trials. The objectives of the USNHS are to evaluate the current RTT diagnostic criteria, to examine phenotype–genotype correlation, and to understand the evolution of developmental milestones and associated features, such as seizures, scoliosis, gastrointestinal issues, and breathing dysfunction. At each visit, physicians administer two commonly used RTT instruments (the Clinical Severity Score, and the Motor-Behavioral Assessment [78, 79]) and health-related QoL measures for both caregivers and, by proxy, RTT participants. Several lessons have been learned from the USNHS that can now help inform clinical trial design. For example, the study revealed some phenotype–genotype correlations [11, 80] that could aid in stratifying patients into more homogenous subgroups for clinical trials. The study also showed that most RTT sequelae are not static over time [81, 82], presenting a significant challenge for crossover trials conducted over several months.

Lessons from Past Trials

Over the past three decades, 16 studies of treatment effect have been conducted in RTT (Table 1). Nine others are either underway or in prerecruitment status (Table 2). Many of the completed studies were handicapped by critical design flaws, which can serve as lessons and warnings for future trial design. Remarkably, only three were parallel, randomized, double-blind, placebo-controlled trials (RCT). Five others were crossover studies, a design in which subjects initially receive either the active drug or a placebo, and then switch to the opposite group. Although most studies reported improvement in some outcome measures (Table 1), these have not been independently validated and none have resulted in the use of these treatments in clinical practice.

The crossover design can be problematic in RTT, as highlighted by the naltrexone study [83]. The researchers tested the hypothesis that a period-by-treatment interaction existed before and after the 30-day washout period. Although the half-life of naltrexone is less than 1 day, the researchers found a carryover effect that confounded analysis. Changes, particularly in behavioral outcome measures, may outlast the drug in an unpredictable manner. The same authors conducted the folate-betaine study, a balanced allocation, parallel RCT, which is the largest and longest RCT to date in RTT [84]. The authors recognized methodological issues in their naltrexone study; accordingly, they altered the design to exclude young participants, who are in a period of rapid change, and selected a longer, parallel design, as opposed to a crossover design. Also recognizing the strong placebo effect in parent reporting of outcome measures, they implemented numerous objective measures, including laboratory, polygraphic, neurophysiological, anthropometric, nutritional, and clinical assessments. The only objective finding was improved head growth in the treatment group; however, this effect apparently reflected the overrepresentation of a 'mild' *MECP2* mutation in the active treatment group, highlighting the need for balanced allocation based on genetic characteristics.

In an attempt to shorten the clinical trials process, a recent IGF-1 study in patients with RTT [85] used the highest dose of IGF-1 already approved for other indications, rather than applying to the FDA for permission to use higher doses. However, this study concluded that, due to the complex pharmacokinetics of IGF-1, the FDA-approved maximum dosing regimen was inadequate for a Phase II study [85], highlighting the need for rigorous dose exploration in both preclinical and clinical trials.

Trial Designs

Given that the standard clinical trial process requires thousands of subjects and many years to complete, rare disease researchers have attempted to streamline this process. Open-label designs require fewer participants, all of whom are guaranteed to receive the medication, and have been used for most studies in RTT. However, this model is confounded by multiple sources of bias, most notably the placebo effect, which was 63% in one RTT clinical trial [86]. Consequently, the results of these studies can be uninterpretable. In rare diseases, historical controls have been sufficient for FDA approval of an investigational drug. Since the placebo effect can be large, an objective historical control with good reliability must be chosen if it is to be used in lieu of a placebo group. Another strategy, the adaptive design,

incorporates participant covariate values and prior responses to treatment. Response-adaptive trials and sequential designs offer the ability to recruit fewer participants overall, and minimize the number who receive placebo [87]. Crossover designs necessitate longer trials and pose the risk of carryover effect; as an alternative, Bayesian statistics can help incorporate previous information (e.g., from natural history studies) into the clinical trial design, improving statistical power and limiting the number of subjects needed for a trial [88]. Alternative trial designs can increase the possibility of type 1 error and, thereby, the approval of medications that are not in fact safe and efficacious. However, systematic postmarketing studies (i.e., additional clinical trials of safety and effectiveness after a drug has been approved for use) can attenuate this risk in a rare disease.

Concluding Remarks and Future Directions

This is a promising time for the RTT field as researchers move closer to understanding the basic biology of MeCP2 and there are more and more examples of interventions that improve or reverse symptoms in mouse models. By definition, therefore, this is also a time for caution, because the expectations of families affected by RTT must be managed appropriately (see Outstanding Questions). Based on a wealth of experience with other disorders, the chance that any particular treatment will translate from preclinical RTT models to humans is predicted to be low. However, with increased attention to rigorous preclinical trial design in RTT [2], it is hoped that success in translation will improve. Although streamlining the clinical trials process could result in more drugs being brought to market, the great risk is that many will lack true efficacy unless adequate safeguards are in place. The RTT field will also face the challenge of which trials to run, given a relatively small patient pool and funding limitations. Fortunately, with recent incentives from the FDA for companies to invest in drug development for orphan indications, there is new hope that the RTT field will be able to attract the kind of large-scale funding that is required to run clinical trials. Nonetheless, as preclinical studies generate more and more promising results, the need to prioritize clinical trials will become increasingly important and will require a high degree of coordination within the RTT community and with industry partners. If a concerted global effort can be made in optimizing preclinical research, clinical trial design, and prioritization goals, pooled resources and shared methodology could result in efficacious treatments for RTT in the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Trends

Studies of RTT mouse models have convincingly demonstrated that neurological disability caused by loss of methyl-CpG-binding protein 2 (MeCP2) function is reversible to a significant degree.

Recent insights into the biology of MeCP2 and its role in regulating interactions between DNA and repressor protein complexes seemed poised to resolve longstanding controversies about the role of MeCP2 in transcriptional control.

The knowledge that reintroduction of *Mecp2* can restore circuit functionality in mouse models of RTT has spurred the investigation of gene replacement and gene reactivation strategies as comprehensive and potentially transformative treatment approaches for RTT.

Pharmacologic strategies targeting neurotransmitter and neuronal growth factor signaling pathways have proven highly effective at improving neurological function in mouse models of RTT.

The natural history of RTT is becoming increasingly well defined, facilitating the identification of clinically measurable endpoints for therapeutic trials.

Box 1. Function of MeCP2

MeCP2 is a basic nuclear protein that is highly expressed in the brain [89]. Its amino acid sequence is conserved in vertebrate evolution, being 95% identical between humans and mice. Functional studies have identified a DNA-binding domain (MBD) as the major determinant of chromosome binding through its affinity for short sequences in the genome that contain 5-methylcytosine (mC) [90]. Methylation of the cytosine pyrimidine ring follows DNA synthesis and primarily affects the two base-pair sequence CG, which becomes a major target of MeCP2 binding [91, 92]. However, other methylated sites are now known and some of these also bind MeCP2. In particular, the sequence mCA, which is abundant in neurons but rare in other cell types, is established as a target for MeCP2 [93, 94]. In addition, the oxidized derivative of mC, hydroxymethylcytosine (hmC), is also abundant at CG sites in the brain and is elevated at transcriptionally active genes and their regulatory regions [95]. MeCP2 does not bind to hmCG, suggesting that this chemical change switches the mCG site to a form that cannot interact with the protein [94, 96]. In the genome, both mCG and mCA are broadly distributed, but are absent at CpG islands, which surround the promoters of most genes [97]. Accordingly, MeCP2 binding to the brain genome is relatively uniform, but dips sharply at CpG islands [91, 98].

Binding to DNA is evidently an essential part of MeCP2 function, because mutations that compromise MBD function cause RTT [99]. MeCP2 interacts with other partner macromolecules, but so far only one such protein–protein interaction has been experimentally linked to RTT. A discrete domain within the C-terminal half of the protein binds to the two closely related co-repressor complexes NCoR and SMRT (hence 'NCoR/ SMRT Interaction Domain' or NID) [100] and mutations that disrupt binding cause RTT. The importance of DNA and co-repressor interactions is highlighted by the mutational spectrum underlying RTT. Of the many documented disease-causing mutations, missense mutations are particularly informative because they accurately pinpoint important functional domains. The distribution of RTT missense mutations is strikingly nonrandom, being largely confined to regions of the gene that encode the MBD and the NID [101]. A simplistic explanation for this observation is that MeCP2 forms a bridge between methylated DNA and the co-repressor complexes, and disruption of the bridge at either end results in RTT [100].

While there is a depth of biochemical and genetic evidence favoring the idea that MeCP2 represses transcription [100, 102, 103], analysis of gene expression in MeCP2-deficient brains does not reveal simple derepression of genes [104, 105]. Instead, large numbers of modest transcriptional changes are observed, both positive and negative. Analysis of multiple published and novel gene expression data sets uncovered a subtle but consistent upregulation of long genes in the MeCP2-deficient brain [94]. Given that many brain-specific genes are long, it is possible that modestly deregulated expression of thousands of such genes compromises brain function. By contrast, a separate study suggests that genes with more bound MeCP2 are either up- or downregulated in its absence [98]. However, both studies agree that non-CG methylation (e.g., mCA) makes a disproportionately large contribution to this effect.

Several other hypotheses have been advanced to explain MeCP2 function. For example, it has been proposed, based on functional studies, that MeCP2 is an activator of transcription [105–107], a regulator of miRNA processing or splicing [108, 109], a facilitator of chromosome looping or compaction [110, 111], or a regulator of several other aspects of cellular metabolism. A way of unifying these disparate potential functions is to propose that MeCP2, similar to some other relatively unstructured protein molecules, serves as a coordinating platform for multiple different interactions. In other words, MeCP2 might be an important 'multifunctional hub' for many pathways that support brain function [112].
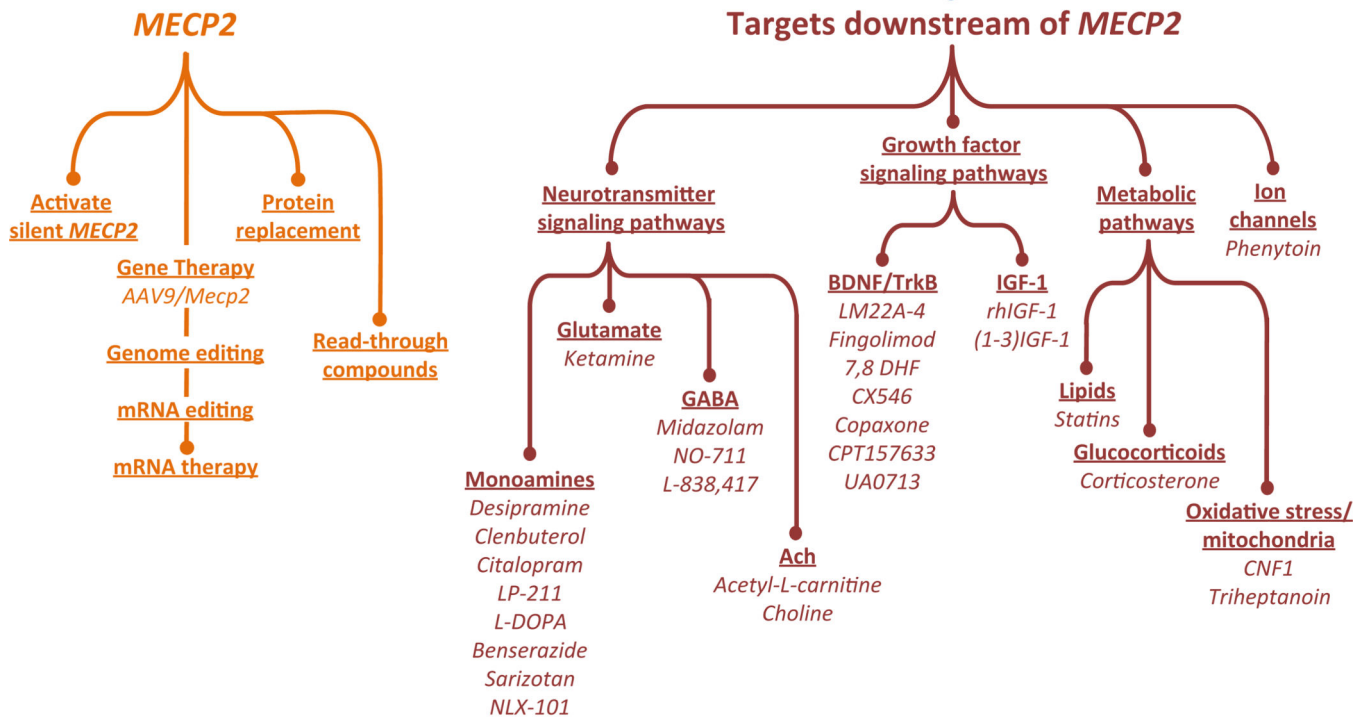
Outstanding Questions

Can the apparent imbalance between cortical hypoconnectivity and brainstem hyperexcitability in the MeCP2 deficient brain be addressed by pharmacological strategies aimed at restoring the excitatory–inhibitory synaptic balance? Will this require combination therapies targeting multiple neurotransmitter and/or neuromodulator signaling pathways?

Can gene replacement or *MECP2* reactivation strategies be developed that are effective and safe for human translation? In particular, will it be possible to titrate MeCP2 levels within the relatively narrow range required for healthy brain function?

What criteria will be used to prioritize the selection of candidate therapeutics that advance to clinical trials in patients with RTT? Presently, candidate molecules are being proposed at a rate that exceeds the patient population and resources needed to study them using the traditional clinical trial model.

What is the optimal clinical trial design to study a rare neurological disease? An optimal design would incorporate expected delays between improved neuronal function and measurable changes in clinical symptoms or behavior, the likelihood of effect outlasting treatment cessation, and patient-specific data, such as genotype.

Targets and treatment strategies

MECP2

Targets downstream of MECP2

Activate silent MECP2

Protein replacement

Gene Therapy
AAV9/Mecp2

Genome editing

mRNA editing

mRNA therapy

Read-through compounds

Neurotransmitter signaling pathways

Growth factor signaling pathways

Metabolic pathways

Ion channels
Phenytoin

Glutamate
Ketamine

GABA
Midazolam
NO-711
L-838,417

BDNF/TrkB
LM22A-4
Fingolimod
7,8 DHF
CX546
Copaxone
CPT157633
UA0713

IGF-1
rhIGF-1
(1-3)IGF-1

Lipids
Statins

Glucocorticoids
Corticosterone

Monoamines
Desipramine
Clenbuterol
Citalopram
LP-211
L-DOPA
Benserazide
Sarizotan
NLX-101

Ach
Acetyl-L-carnitine
Choline

Oxidative stress/ mitochondria
CNF1
Triheptanoin

Trends in Neurosciences

Figure 1. Therapeutic Targets and Potential Pharmacological Strategies Currently Being Explored in Animal Models for the Treatment of Rett Syndrome

Underlined headings indicate therapeutic targets; compounds that have been reported in the literature to be effective in improving behavioral outcome measures or physiological function *in vivo* are shown in italics (see Table S1 in the supplemental information online for the figure references).
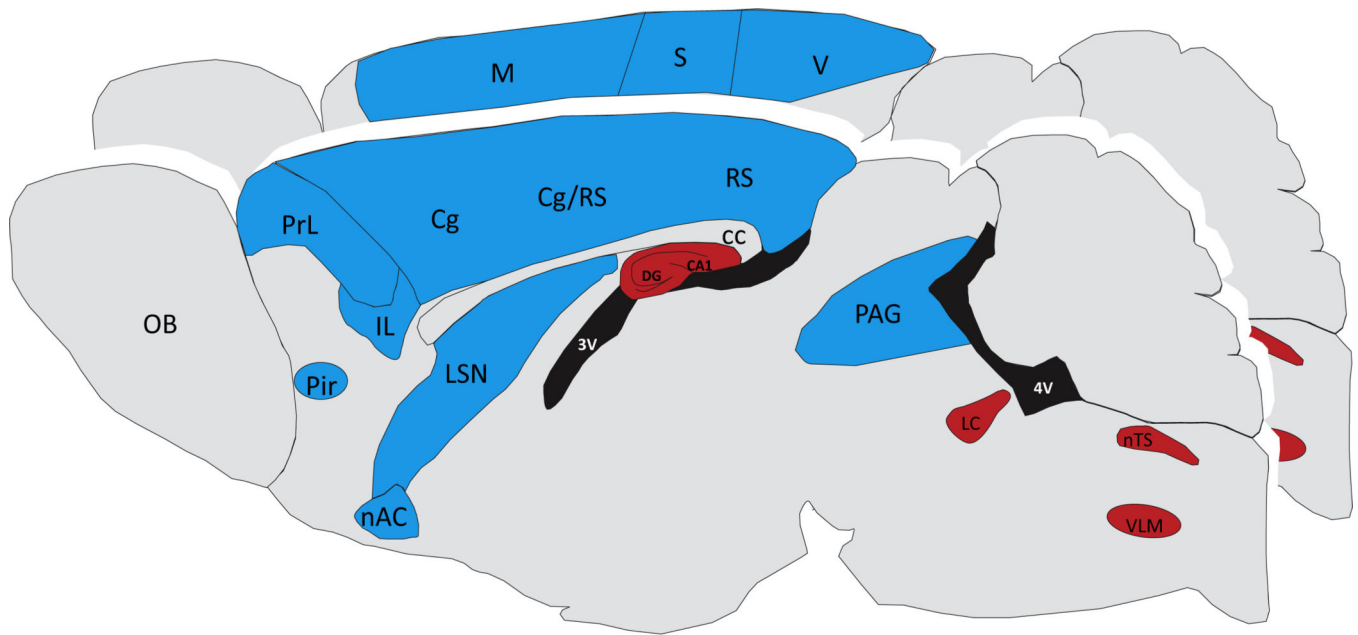
Trends in Neurosciences

Figure 2. Neural Circuit Dysfunction in the Methyl-CpG-Binding Protein 2 (*Mecp2*) Mutant Brain

Colors indicate brain regions in which *Mecp2* mutant mice exhibit a shift in either neuronal or synaptic activity towards decreased (blue) or increased (red) excitation compared with wild-type controls. This schematic summarizes findings from numerous laboratories and is based on electrophysiological recordings of intrinsic neuronal activity, synaptic activity, and/or population activity, as well as Fos mapping of neuronal activity. Abbreviations: 3 V, third ventricle; 4 V, fourth ventricle; CA1, cornu ammonis; cc, corpus callosum; Cg, cingulate; DG, dentate gyrus; IL, infralimbic cortex; LC, locus coeruleus; LSN, lateral septal nuclei; M, motor cortex; nAC, nucleus accumbens; nTS, nucleus of the solitary tract; OB, olfactory bulb; PAG, periaqueductal gray; Pir, piriform nucleus; PrL, prelimbic cortex; RS, retrosplenial cortex; S, somatosensory cortex; V, visual cortex; VLM, ventrolateral medulla.
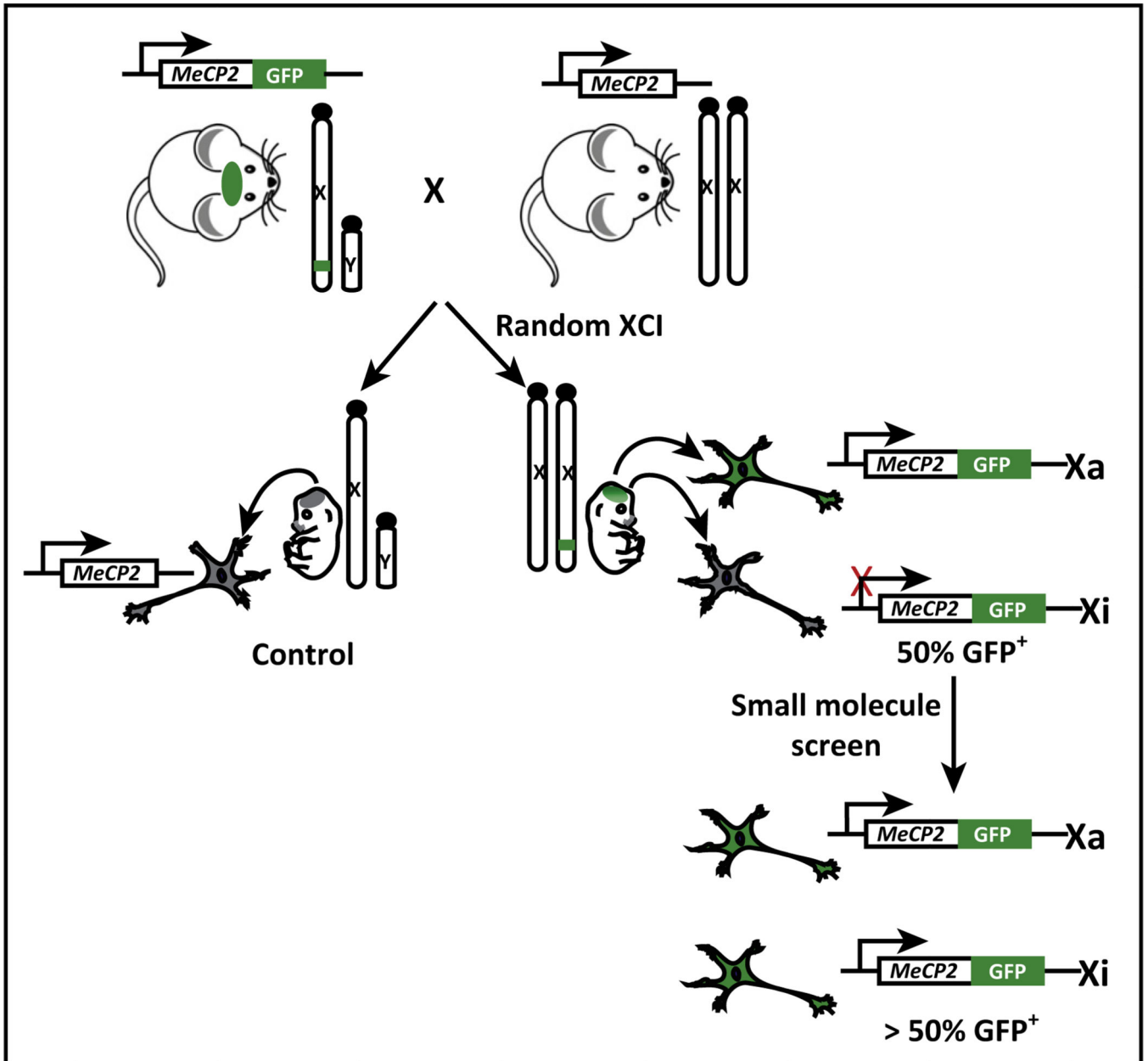
Trends in Neurosciences

Figure 3. High-Content Small-Molecule Screening Strategy to Detect Methyl-CpG-Binding Protein 2 (*Mecp2*) Reactivation

Neurons harvested from Embryonic day (E)15.5 embryos produced in matings between hemizygous *Mecp2*-GFP males and wild-type females are used to screen for *Mecp2* de-inactivating compounds. Of the neurons derived from female embryos, approximately 50% will be GFP+ due to random X chromosome inactivation (XCI). Positive hits result in an increase in the proportion of GFP-labeled neurons. Neurons derived from nontransgenic

male embryos serve as negative controls. GFP reporter mice are available from Jackson Laboratories (*Mecp2*$^{tm3.Bird}$/*J*; Reference #014610).

Table 1

Completed Clinical Trials[a,b]

Intervention	Proposed Mechanism	N	Year	Design	Reported Outcomes (Improved, Worsened, No Change)	Refs
Ketogenic diet	Induce ketosis	7	1986	Open label, uncontrolled	% seizure control, EEG features, stereotypies, ambulation, bruxism, breathing dysregulation, social interaction, clinical lab values	[113]
Bromocriptine	Stimulate dopamine receptors	10	1990	Double-blind, randomized, placebo-controlled, partial cross-over	Gross/fine motor development, cognition, socialization (Portage Guide for Early Education)	[114]
Naltrexone	Opiate receptor antagonist	25	1994	Double-blind, randomized, placebo-controlled, cross-over	Breathing regulation, clinical stage, Bayley, Peabody, Gesell, Vineland, Motor-Behavioral Assessment, EEG, CSF	[84]
L-Carnitine	Increase metabolic substrate	35	1999	Double-blind, randomized, placebo-controlled, cross-over	Motor-Behavioral Assessment (behavioral/social, orofacial/respiratory subscales) Hand Apraxia Scale, Patient Well-Being Index	[115]
L-Carnitine	Increase metabolic substrate	21	2001	Open label	RS: SSI (communication, energy level, bruxism), sleep efficiency, alertness, Hand Apraxia Scale, SF-36	[116]
Folate/Betaine	Enhance MeCP2 binding	73	2009	Double-blind, placebo-controlled, parallel	Head circumference, overall improvement by parental report in <5 years, Motor-Behavioral Assessment, height, weight	[84]

Intervention	Proposed Mechanism	N	Year	Design	Reported Outcomes (Improved, Worsened, No Change)	Refs
Folinic acid	Increase CSF folate	25	2009	Open label, allocated based on low CSF folate	Seizure frequency, clinical exam	[117]
Folinic acid	Increase CSF folate	12	2011	Double-blind, placebo-controlled, cross-over	EEG, Hagberg Stage, Motor-Behavioral Assessment, Hand Apraxia Scale, Modified Symptom Severity Score, Overall Well-Being Index, Dependency Scale, CSF 5-MTHF, seizure frequency, clinical exam	[86,118]
Creatine	Increase metabolic substrate	18	2011	Double-blind, placebo-controlled, cross-over	Motor-Behavioral Assessment, clinical lab values	[119]
ω-3 PUFAs	Antioxidant	42	2011	Open label	CSS, oxidative stress markers	[120,121]
IGF-1 (Boston)	Enhance growth factor signaling pathways	12	2012	Phase I, open label	Apnea Index, RSBQ (fear/anxiety), ADAMS (social avoidance subscale), EEG alpha asymmetry, CSS, Motor-Behavioral Assessment	[85]
IGF-1 (Italy)	Enhance growth factor signaling pathways	6	2012	Open label	Seizure frequency, CGI, EEG	[122]
ω-3 PUFAs	Antioxidant	20	2012	Single-blind, placebo-controlled	CSS, oxidative stress markers	[123]
NNZ-2566[c]	Unclear; possibly enhance growth factor signaling pathways	60	2014	Unbalanced (high-dose, low-dose, placebo), double-blind, placebo-controlled, parallel	Motor-Behavioral Assessment, CGI, VAS, EEG spikes, Modified Apnea Index, Behavior, Autonomic function, CSS, Vineland	

Intervention	Proposed Mechanism	N	Year	Design	Reported Outcomes (Improved, Worsened, No Change)	Refs
EPI-743[c]	Augment glutathione biosynthesis	24	2014	Double-blind, placebo-controlled, parallel	Head circumference, CSS, RSBQ, PedsQL, Respiratory Disturbance Index, oxidative stress	
ω-3 PUFAs	Antioxidant	66	2014	Single-blind, placebo-controlled	CSS, myocardial function, oxidative stress markers	[124]

[a] Outcome measures in blue were reported as improving, those in red were reported as worsening and those in black were reported as not changing. Single case reports and retrospective case series are not included in this table.

[b] Abbreviations: ADAMS, Anxiety, Depression, and Mood Scale; CGI, Clinical Global Impression; CSF, cerebrospinal fluid; CSS, Clinical Severity Score; EEG, electroencephalogram; PedsQL, Pediatric Quality of Life Inventory; RSBQ, Rett Syndrome Behavioral Questionnaire; RS, SSI, Rett Syndrome, Symptom Severity Index; SF-36, Short-form 36 items; VAS, Visual Analog Scale; ω-3 PUFAs, ω-3 polyunsaturated fatty acids.

[c] Studies completed; no published data available.

Table 2

Active, Recently Completed, and Pending Clinical Trials[a,b]

Intervention	Proposed Mechanism	N	Status	Design	Outcome Measures
IGF-1	Enhance growth factor signaling pathways	30	Recruiting	Double-blind, placebo-controlled, cross-over	Kerr, EEG, RSBQ, ADAMS, ABC, CGI, VAS, Vineland
Glatiramer acetate (Israel)	Increase BDNF	10	Stopped	Open label	EEG, seizure frequency, sleep diary, height, weight, respiratory regulation, Kerr and Naidu severity scores
Glatiramer acetate (New York)	Increase BDNF	20	Active, not recruiting (4/20/2015)	Open label	EEG, gait, autonomic, visual attention, behavior, QOL
Dextromethorphan[b]	NMDA receptor antagonist	60	Recruiting	Double-blind	Mullen, Vineland, Screen for Social Interaction
Desipramine	Inhibit norepinephrine reuptake	36	Completed (8/6/2015)	Unbalanced (high-dose, low-dose, placebo), double-blind, placebo controlled, parallel	Respiratory regulation
Fingolimod	Increase BDNF	6	Recruiting	Phase I	Serum and CSF BDNF levels
Triheptanoin	Increase metabolic substrate	10	Pending	Open label, challenge-dechallenge	Seizure frequency, dystonia
Ketamine	NMDA receptor antagonist	30	Recruiting	Double-blind, placebo-controlled	Respiratory regulation, cardio-respiratory coupling, EEG, auditory evoked potentials, RSBQ, RBSR
Lovastatin	Modulate cholesterol synthesis	20	Recruiting	Open label	Gait velocity, visual attention and memory, visual pursuit, respiratory regulation, EEG, QOL

[a] Abbreviations: ABC, Aberrant Behavior Checklist; ADAMS, Anxiety, Depression, and Mood Scale; BDNF, brain-derived neurotrophic factor; CGI, Clinical Global Impression; CSF, cerebrospinal fluid; EEG, electroencephalogram; RBSR, Repetitive Behavior Scale-Revised; RSBQ, Rett Syndrome Behavior Questionnaire; VAS, Visual Analog Scale; ω-3 PUFAs, ω-3 polyunsaturated fatty acids.

[b] One stage of this study is completed; no published data available.