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Publication Date

2020-10-01

DOI

10.1016/j.neuroscience.2020.04.037

Peer reviewed

Published in final edited form as:

Neuroscience. 2020 October 01; 445: 190–206. doi:10.1016/j.neuroscience.2020.04.037.

Intellectual and Developmental Disabilities Research Centers: A Multidisciplinary Approach to Understand the Pathogenesis of Methyl-CpG Binding Protein 2-related Disorders

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Abstract

Disruptions in the gene encoding methyl-CpG binding protein 2 (MECP2) underlie complex neurodevelopmental disorders including Rett Syndrome (RTT), *MECP2* duplication disorder, intellectual disabilities, and autism. Significant progress has been made on the molecular and cellular basis of *MECP2*-related disorders providing a new framework for understanding how altered epigenetic landscape can derail the formation and refinement of neuronal circuits in early postnatal life and proper neurological function. This review will summarize selected major findings from the past years and particularly highlight the integrated and multidisciplinary work done at eight NIH-funded Intellectual and Developmental Disabilities Research Centers (IDDRC) across the US. Finally, we will outline a path forward with identification of reliable biomarkers and outcome measures, longitudinal preclinical and clinical studies, reproducibility of results across centers as a synergistic effort to decode and treat the pathogenesis of the complex MeCP2 disorders.

Keywords

neurodevelopmental disorders; translational; animal models; biomarkers; signaling pathways

INTRODUCTION

Recent discoveries on the molecular basis of *MECP2*-related disorders have provided a new framework for understanding how an altered epigenetic landscape can lead to a variety of neurodevelopmental disorders including Rett Syndrome (RTT), *MECP2* duplication disorder, intellectual disabilities and autism. These insights highlight the importance of chromatin regulation in the formation and refinement of neuronal circuits in early postnatal life and in their maintenance for proper adult neurological function.

MECP2 is an X-linked gene that encodes methyl-CpG binding protein 2 (MeCP2), which binds to methylated DNA and regulates gene expression at the local and global level. MeCP2 interacts with multiple proteins including the histone deacetylase co-repressor complexes SIN3A, NCOR and SMRT amongst other chromatin remodeling proteins. In addition, MeCP2 affects chromatin remodeling, alternative splicing and micro-RNA processing. Although present in somatic cells, MeCP2 is most abundant in neuronal cells where its levels are 5- to 10 fold higher than in other cell types, and it has cell-autonomous as well as non-cell-autonomous effects. MeCP2 itself is dynamically regulated in response to neural activity and experience during postnatal life inserting additional layers of complexity to its role in brain maturation and function. MeCP2 affects successive phases of brain development including prenatal neurogenesis, development and refinement of neuronal circuits, and maintenance of adult neural function including sensory integration (Lyst and Bird, 2015).

In 1999, Zoghbi and colleagues reported the exciting discovery that *MECP2* mutations cause Rett Syndrome (RTT, Amir et al., 1999). Due to X chromosome inactivation, the majority of

affected subjects are females who are mosaic for mutated and wild-type *MECP2* expression. Males carrying a *MECP2* mutation display a more severe phenotype and often do not survive infancy. RTT is characterized by apparently normal early development followed by developmental regression or stagnation that includes progressive loss of acquired motor and language skills, acquisition of stereotyped repetitive hand movement, muscle hypotonia, respiratory dysfunction and severe cognitive impairment. Seizures, anxiety, and orthopedic problems often appear at the end of the regression phase. Duplications spanning *MECP2* cause a serious condition known as *MECP2* duplication syndrome (MDS) characterized by infantile hypotonia, autistic features, cognitive deficits, gait abnormalities, seizures, and recurrent infections. Mutations of some other genes can cause disorders that share some features of RTT but they are clinically distinct disorders; namely Forkhead Box protein G1 (FOXG1) and Cyclin-Dependent Kinase-Like 5 (CDKL5). These discoveries have enabled the generation of animal models with good construct and face validity and allowed understanding of the neurobiological basis of these three distinct disorders (Eagleson et al., 2007; Siegenthaler et al., 2008; Wang et al., 2012a,b). Research into the neurobiological mechanisms behind RTT and RTT-like disorders has progressed quickly in recent years thanks to the convergence of NIH-funded natural history study, well-established clinics, and basic research in animal models and patient-derived iPSCs.

This review summarizes where MECP2 research currently stands, reviewing selected major findings from the past few years and particularly highlighting work done in NIH-funded Intellectual and Developmental Disabilities Research Centers (IDDRC) across the US. The review also highlights the importance of IDDRC-supported integrated and multidisciplinary approach to understand the pathogenesis of the complex MeCP2 disorders.

MECP2 PROTEIN FUNCTION

There are two aspects to dissecting the MeCP2 function, first understanding what it does at the molecular level, and then identifying its targets. Baker and colleagues from the Baylor college of Medicine (BCM) IDDRC used data from male patients with different mutations and varying severity to develop mouse models and characterize critical domains of the protein. Studying two human mutations that either disrupt the protein at amino acid 270 or 273 allowed them to pinpoint the importance of a new AT-hook domain that is disrupted in the more severe earlier truncation. The discovery of this second AT-hook domain and the homology of MeCP2 to HMGA DNA-bending proteins support a role for MeCP2 in altering chromatin structure (Baker et al., 2013).

In their publication in Biochemistry (2016), lead investigator Michael Brenowitz at Einstein College of Medicine together with IDDRC colleagues Aristea Galanopoulou and John M. Greally have dissected the mechanisms underlying its molecular interaction with DNA and its ability to specifically bind to sites of epigenetic modification. In particular they showed that MeCP2 specificity and cooperativity to DNA binding is sensitive to both the nature of the bound DNA and the concentration and types of surrounding ions whose concentrations change with neuronal development raising the possibility of a novel and direct mechanism by which the function of MeCP2 might be developmentally regulated (Khrapunov et al., 2014).

Over the last two decades, several laboratories have tried to identify transcriptional targets of MeCP2 to gain insight into the etiology of RTT. However, target identification is confounded by the cellular heterogeneity of the brain. The identification of transcriptional targets is also convoluted by the widespread binding patterns of MeCP2 to methylated cytosines, hydroxymethylated cytosines, or unmethylated GC-rich regions throughout the genome (Connolly and Zhou, 2019). Further, although RTT predominantly affects heterozygous females, identifying gene expression changes in neurons lacking functional *Mecp2* in a mosaic female brain is another challenge. Only recently, two RNA sequencing studies focused on investigating gene expression changes in murine and human female RTT brains (Renthal et al., 2018; Johnson et al., 2017), strengthening the idea that MeCP2 dysfunction alters gene expression changes in a cell-type specific and MeCP2 mutation-specific manner (Johnson et al., 2017).

Greenberg's laboratory uncovered evidence that at genome-wide level, MeCP2 functions to temper the expression of genes in a gene-length-associated manner, possibly by binding to methylated CA sites within long genes. Interestingly, long genes as a population are enriched in neuronal functions and selectively expressed in the brain (Gabel et al., 2015). They provided further evidence that MeCP2 represses transcription by binding within transcribed regions of genes and this repressive effect is proportional to the total number of methylated DNA binding sites for MeCP2 within each gene. These findings suggest a model in which MeCP2 represses transcription of long neuronal genes that contain many methylated binding sites by impeding transcriptional elongation (Kinde et al., 2016). Data from Lin and colleagues also showed enriched binding of MeCP2 on non-CG methylation and a correlation between that binding and gene expression changes. Their data pointed to gene expression changes in both long and short genes and in both directions (Chen et al., 2015). However, a re-analysis of large datasets from different transcriptome profiling technologies has not identified a preferential misregulation of long genes in MeCP2 datasets, suggesting that amplification-based transcriptomic technologies can lead to overestimations of long gene expression changes (Raman et al., 2018). Recently, Greenberg's laboratory reported that in the absence of MeCP2 the process of transcriptional initiation is likely increased especially for genes that are highly methylated and long (Boxer et al., 2020).

Another relevant study from the Zhou laboratory at the IDDRC at the Children's Hospital of Philadelphia and University of Pennsylvania (CHOP/Penn) further highlighted that the effects of MeCP2 on gene expression is rather complex. They engineered genetically modified mice whereby nuclear MeCP2 can be labeled with biotin in a Cre-dependent manner. To understand the molecular impact of RTT-associated mutations on neuronal cell type-specific gene expression *in vivo*, they also developed knock-in mice bearing one of two frequent RTT missense mutations with different clinical severities, T158M and R106W. When combined with Fluorescence-Activated Cell Sorting (FACS), this strategy allows for the isolation of neuronal nuclei from targeted cell types. By examining MeCP2-mediated gene expression changes in different types of neurons, they identified underlying transcriptional features that are cell type specific and correlate with the severity of the MeCP2 mutation. They also found that MeCP2-dependent repression of long genes is not observed in nascent RNA transcripts, suggesting the presence of post-transcriptional compensation of RNAs in MeCP2 mutant-expressing cells (Johnson et al., 2017). Due to the

fact that hundreds of genes are subtly altered in MeCP2 mutants, it has been difficult to identify uncontroversial MeCP2 targets and to fully make sense of the large variability of differentially expressed genes. Nevertheless, the MeCP2-biotin tag approach circumvents X-linked cellular mosaicism and allows profiling the transcriptome of neighboring wild-type and mutant neurons in females, thereby discerning cell and non-cell autonomous transcriptional effects (Johnson et al., 2017).

While studying knock-in mice carrying RTT-associated common mutations, Zhou and colleagues also found that mutations in the methyl-CpG binding domain (MBD) of MeCP2, such as T158M, T158A and R106W, impair MeCP2 binding to DNA, and concomitantly destabilize MeCP2 protein in an age-dependent manner (Goffin et al., 2011; Lamonica et al., 2017; Johnson et al., 2017). Interestingly, genetic elevation of MeCP2 T158M expression ameliorates multiple RTT-like features, including motor dysfunction and breathing irregularities, in both male and female mice. These improvements are accompanied by increased binding of MeCP2 T158M to chromatin. Notably, overexpression of MeCP2 T158M in transgenic mice does not lead to the development of RTT-like phenotypes, ruling out the possibility that MeCP2 T158M shows dominant negative effects (Lamonica et al., 2017). Together, these findings demonstrate that reduced levels of MeCP2 T158M at least partially underlie RTT pathology, and provide a proof-of-principle that pharmacologic elevation or stabilization of MeCP2 protein represents a novel approach to treat patients with MBD missense mutations.

Among the multiple known functions of MeCP2, its role in modulating RNA splicing is less well understood. Chang's laboratory at the Waisman IDDRC took several unbiased approaches to investigate how MeCP2 may regulate splicing, what splicing changes are caused by the loss of MeCP2, and what functional consequences are caused by altered splicing. Using ChIP-seq and co-immunoprecipitation followed by mass spectrometry, they discovered that MeCP2 physically interacts with several modulators of RNA splicing, including LEDGF and DHX9. These interactions are disrupted by RTT causing mutations, suggesting that they may play a role in RTT pathogenesis. Consistent with the idea, deep RNA sequencing revealed misregulation of hundreds of splicing events in the cortex of *Mecp2* knockout (KO) mice. To reveal the functional consequence of altered RNA splicing due to the loss of MeCP2, they then focused on the regulation of the splicing of the flip/flop exon (two alternatively spliced exons, termed flop and flip, situated between the L3- and the M4-coding exons, Sommer et al., 1990) of *Gria2* and other AMPAR genes and found a significant splicing shift in the flip/flop exon toward the flop inclusion, leading to a faster decay in the AMPAR gated current and altered synaptic transmission in cortical neurons in the *Mecp2* KO mice. Importantly, they designed an engineered splicing factor that specifically targets the flip/flop exons, and showed that it is sufficient to rescue the defects in flip/flop splicing, AMPAR current and altered synaptic transmission in *Mecp2* KO neurons (Li et al., 2016). Interestingly, widespread aberrations in alternative splicing was recently demonstrated in response to neuronal activity, elicited either in vitro by potassium chloride (KCl) or in vivo by kai-nic acid (KA) in the hippocampi of Mecp2 KO mice (Osenberg et al., 2018). These results further advance our understanding of the molecular function of MeCP2 and reveal potential drug targets for future therapies.

EFFECTS OF MECP2 DYSFUNCTION ON NEUROGENESIS AND NEURONAL MATURATION

Growing evidence indicates that disruption of MeCP2 function negatively impacts early developmental stages including neurogenesis, migration, and patterning (Feldman et al., 2016). In both the embryonic and adult brain, a critical step in neurogenesis is neuronal maturation. Zhao's laboratory at the Waisman IDDRC has discovered that MeCP2 plays critical roles in the maturation step of new neurons during neurogenesis through the regulation of expression of a microRNA, miR-15a (Gao et al., 2014). MeCP2 is known to regulate the expression of brain-derived neurotrophic factor (BDNF), a potent neurotrophic factor for neuronal maturation. Nevertheless, how MeCP2 regulates BDNF expression and how MeCP2 deficiency leads to reduced BDNF expression remain unclear. Overexpression of miR-15a inhibits dendritic morphogenesis in immature neurons. On the other hand, a reduction in miR-15a has the opposite effect. They further showed that miR-15a regulates expression levels of BDNF, and exogenous BDNF could partially rescue the neuronal maturation deficits resulting from miR-15a overexpression. Finally, inhibition of miR-15a could rescue neuronal maturation deficits in MeCP2-deficient adult-born new neurons. These results demonstrate a novel role for miR-15a in neuronal development and provide a missing link in the regulation of BDNF by MeCP2 (Gao et al., 2014).

MeCP2 is a reader and interpreter of DNA methylation across the genome. A better understanding of how extracellular signals access MeCP2 to generate adaptive functional outputs will provide valuable insights into how such a critical epigenetic interface influences normal and abnormal development and function of the mammalian nervous system. Chan's laboratory used the well-established experimental system of adult neurogenesis to investigate the central role of stimulus-induced MeCP2 phosphorylation. They discovered that S421 is phosphorylated in proliferating adult neural progenitor cells (aNPCs) in response to growth factors (i.e. FGF2/EGF) and linked to cell cycle. MeCP2 physically interacts with aurora kinase B (AurkB) directly regulating the cell cycle-linked S421 phosphorylation in aNPCs through the Notch signaling pathway, downstream effector of S421 phosphorylation. These results not only provide the first genetic evidence that the precise control of MeCP2 phosphorylation plays an important role in regulating adult neurogenesis, but also lend mechanistic insights into how MeCP2 phosphorylation may regulate adult neurogenesis. Prior to this study, S421 phosphorylation has been detected exclusively in post-mitotic neurons. By discovering S421 phosphorylation in a dividing cell type and defining its upstream signaling pathway, its direct kinase, and its downstream effector pathway that are completely different from those known in post-mitotic neurons, these results further support the idea that S421 phosphorylation is a general molecular switch accessible to diverse stimuli through different signaling pathways with important functional outcomes in different cell types (Li et al., 2014).

Functions of MeCP2 during early neurogenesis affect neuronal migration and cortical patterning. Data, however, have demonstrated that NPCs derived from *Mecp2* KO mice exhibit delayed corticogenesis with respect to migration from the subventricular and ventricular zones into the cortical plate (Bedogni et al., 2016). These findings raise the

possibility that these early defects may contribute to the derailing of neuronal circuits during postnatal life.

MECP2 AND SYNAPTIC PLASTICITY

MeCP2 deficiency causing RTT has a major negative impact on formation and stabilization of synapses and synaptic plasticity (Johnston et al., 2015). Mutant mouse models have been generated with a global deletion of MeCP2 from all neurons and selectively from specific neuronal subtypes and glia or at different stages of development. Deletion or re-expression lines have served as a powerful tool to study the common principles underlying synaptic defects in RTT.

Brain growth slows in the neonatal period due to failure of synapse proliferation, and pathological examination of nasal biopsy samples from girls with RTT compared to controls showed that olfactory receptor neurons failed to form synapses with neurons in the olfactory bulb (Ronnett et al., 2003). Analysis of postmortem human brain from girls with RTT of different ages show increased concentrations of *N*-methyl-D-aspartate receptors (NMDA) and α-amino-3-hydroxy-5-m ethyl-4-isoxazolepropionic acid (AMPA) type glutamate receptors in frontal cortex and caudate-putamen in younger girls with fewer receptors in those older than 8 years of age (Blue et al., 1999a,b). A similar age-dependent pattern of increased NMDA and AMPA receptors followed by a decline in number was present in the frontal cortex of Mecp2 deficient mice compared to wild-type controls (Blue et al., 2011).

NMDARs are critical for many forms of learning and memory, in part due to their activity dependence and contribution to synaptic integration and plasticity (Paoletti et al., 2013). The GluN2 subunit composition determines the decay kinetics of the NMDARs, which undergo an experience-dependent switch from GluN2B to GluN2A at cortical synapses during early postnatal development (Carmignoto and Vicini, 1992). The Fagiolini laboratory demonstrated that in the absence of MeCP2 the time course of NMDAR maturation in visual cortex is differentially affected depending on cell type, being slower in pyramidal neurons and faster in PV positive inhibitory cells in complete contrast to WT littermate mice (Mierau et al., 2016). Reducing GluN2A expression in mutant mice prevented the premature NMDAR maturation in PV cells and rescued RTT cortical phenotype. These results suggest that MeCP2 dysfunction alters excitatory transmission in a cell-specific manner.

Glutamate, the major excitatory neurotransmitter in the brain, is elevated in both cerebrospinal fluid (CSF) and brain of girls with RTT (Hamberger et al., 1992; Lappalainen and Riikonen, 1996; Horska et al., 2009) and in *Mecp2* KO mice (Pozo and Goda, 2010). Analysis of sleep stages and brain levels of glutamate using continuous monitoring of both EEG and intracerebral glutamate levels using an intra-cerebral electrode in mecp2-deficient mice showed markedly disrupted sleep with long periods of wakefulness compared to controls (Johnston et al., 2014). Brain glutamate normally rises during wakefulness in rodents, and then falls during sleep but in the MeCP2-deficient mice glutamate rose to much higher than normal levels during wakefulness before falling (Johnston et al., 2014). These findings support the hypothesis that Mecp2 deficiency causes a failure of synaptic scaling, a form of homeostatic synaptic plasticity, in the developing brain (Pozo and Goda, 2010).

Homeostatic synaptic plasticity is a mechanism that allows neuronal circuits to offset excessive excitation or inhibition. Conditional knockout of *Mecp2* in mice has been shown to impair synaptic scaling mediated by changes in levels of GluR2 subunits of the AMPA receptor (Qui et al., 2012). These findings are consistent with the observations that high levels of glutamate co-exist with high levels of glutamate receptors in developing human and mouse brains with a RTT mutation.

A defect in homeostatic plasticity may also be caused by MeCP2 dysfunction in GABAergic neurons (Chao et al., 2010; He et al., 2014; Ure et al., 2016). As a chromatin-associated protein, MeCP2 affects the expression of a large number of GABAergic related genes (Chao et al., 2010; Durand et al., 2012). As such, deletion or re-expression of *Mecp2* from GABAergic neurons (using *Viaat-Cre*; *Dlx5/6-Cre*, PV-Cre or Sst-Cre mouse lines) disrupt or recover cortical function respectively (Chao et al., 2010; Durand et al., 2012; Goffin et al., 2014; He et al., 2014; Ure et al., 2016). In *Mecp2* KO mice, calcium binding protein parvalbumin (PV) is significantly upregulated across multiple cortical areas, such as V1, A1, S1 and M1 (Durand et al., 2012; Mierau et al., 2016; Krishnan et al., 2015; Patrizi et al., 2019, Morello et al., 2018). This is accompanied by premature switch in NMDA receptor composition (Mierau et al., 2016) and excessive excitatory inputs converging onto PV-expressing interneurons (Morello et al., 2018; Sigal et al 2019). Hyper-complex PV-positive large basket cells are also hyper-connected onto the soma of pyramidal neurons (Durand et al., 2012; Mierau et al., 2016; Patrizi et al., 2019). An increase in the density of calretinin (CR)- and PV-positive cells across S1, M1 and V1 cortex has also been reported by one study (Tomassy et al., 2014). Together these contribute to decreasing output firing of pyramidal neurons across cortical regions very early on in the progression of the disorder as measured both *in vitro* and *in vivo* electrophysiological and anatomical analysis. As regression is completed, there is a significant downregulation of GAD65 and GAD67 GABA synthesized enzymes as well as CR and somatostatin (SST) markers, suggesting a possible homeostatic attempt to dampening the strength of GABAergic circuits in *Mecp2* KO mice (Chao et al., 2010; Durand et al., 2012; Krishnan et al., 2015; 2017; Patrizi et al., 2019). It is interesting to note that loss of MeCP2 function in excitatory neurons expressing vesicular glutamate2 transporter (Vglut2), causes some features (e.g. anxiety, tremors) not seen in mice lacking the protein in inhibitory neurons, whereas there were some overlapping phenotypes (Meng et al., 2016). Neuron-specific rescue experiments revealed the surprising finding that rescue in Vglut2-expressing cells corrected the phenotypes in female mice, whereas a rescue in Viaat-expressing cells was most robust in male mice. These data highlight the network differences in male (null) versus female (mosaic) mice, and underscore the importance of studying female mice to find ways to modulate the course of RTT (Ure et al., 2016; Meng et al., 2016).

MECP2 AND GLIA

Over the past few years, non-neuronal cells such as glia have also been shown to play important roles in the pathology of RTT.

Dysfunction of astrocytes in RTT

As astrocytes are multi-functional regulators of brain metabolism, and directly supply neurons with substrates for oxidative phosphorylation (Stobard and Anderson, 2013), their dysfunction may cause aberrant metabolic support to the brain. Alterations of mitochondria redox status and increased oxidative stress has been reported both in patients and animal models of RTT (Kriaucionis et al., 2006; De Felice et al., 2012; Grosser et al., 2012; Shulyakova et al., 2017; Muller 2019; Neul et al., 2020). It spans from increased mitochondrial activity and oxygen consumption, over exaggerated mitochondrial reactive oxygen species (ROS) release and cytosolic/mitochondrial redox imbalance to disturbed neuronal network function and a facilitation of disease progression (De Filippis et al., 2015; Valenti et al., 2017). Yet, the link between MECP2 mutations and the redox imbalance found in RTT is not completely clear. Interestingly, cultured astrocytes from *Mecp2* KO mice exhibit higher number of mitochondria and oxidative stress than wild-type astrocytes (Bebensee et al., 2017), while *Mecp2* knock-down astrocytes exhibit elevated expression of mitochondrial respiration chain proteins and a lower activity of complexes I and II (Dave et al., 2019). The use of RhoGTPases activators such as cytotoxic necrotizing factor 1 (CNTF1) is sufficient to reduce atrophy of astrocytes, improve brain metabolism and brain bioenergetic markers, and ameliorate significantly the overall neurobehavioral phenotype in mouse models of RTT (De Filippis et al., 2012, 2015). The systemic redox imbalance and oxidative stress are not limited to RTT but have been reported also in blood samples of MECP2-duplication syndrome (Signorini et al., 2016) or CDKL5 deficiency disorder (Leoncini et al., 2015). Hence, targeting cellular redox balance might represent a potential therapeutic approach to improve neuronal network function.

Interestingly, it was demonstrated that selective re-expression of *Mecp2* in astrocytes is sufficient to rescue breathing phenotypes in mouse models (Lioy et al., 2011) but the role of astrocytes in RTT has not yet been validated in human cells. Chang's laboratory at the University of Wisconsin-Madison, IDDRC differentiated human RTT induced pluripotent stem cells (iPSCs) into astrocytes (Williams et al., 2014). They demonstrated that wild-type human astrocytes express detectable levels of MECP2, and that mutant human astrocytes carrying 3 different RTT mutations have an adverse influence on the morphology and function of wild-type neurons. The hallmark pathologies observed in RTT human autopsy samples and in RTT mouse models of small neurons, shorter total neurite length, and fewer terminal ends were also observed in this study. Moreover, they developed a neuron/astrocyte co-culture system that validated previous findings from RTT mouse models and it revealed that both cell types contribute independently and additively to the same morphological deficit in neurons. Finally, the study tested the efficacy of two candidate drugs (full length IGF-1 and the GPE tripeptide) that are currently in clinical trials in RTT patients at the cellular level in the neuron/astrocyte co-culture. Surprisingly, the effect of these drugs on neuronal morphology was dependent on the genotype of the astrocytes in these co-cultures. These results suggest that disease-specific iPSCs and their derivatives are useful *in vitro* platforms for studying disease mechanisms and testing candidate drugs.

To identify novel cell autonomous phenotypes in RTT astrocytes, the Chang laboratory used astrocytes differentiated from congenic pairs of human RTT patient specific iPSCs to

demonstrate that both the spontaneous and the pharmacologically evoked cytosolic calcium activities are abnormal in mutant RTT astrocytes. A similar phenotype was also identified in astrocytes derived by *Mecp2* KO mice. Interestingly, they demonstrated that the abnormal calcium activities in astrocytes lead to excessive activation of extrasynaptic NMDA receptors (eNMDARs) on neighboring neurons and increased network excitability as a direct consequence of the loss of *Mecp2* (Dong et al., 2018). In the same year, Mandel's group show that *Mecp2*-negative astrocytes derived either from *Mecp2* KO mice or Mecp2 Het mice fail to increase synaptic modulation as in WT mice and that the calcium signals in astrocytes are severely weakened (Rakela et al., 2018).

Microglia in RTT

MeCP2 is expressed in microglia and related mononuclear phagocytes and regulates their functions, but the pathological role of microglia in RTT remains controversial (Maezawa and Jin, 2010; Derecki et al., 2012; Wang et al., 2015; Horiuchi et al., 2017; Schafer et al., 2016; Jin et al., 2015; Cronk et al., 2015). Derecki et al. (2012) and Cronk et al. (2015) provided *in vivo* data supporting that microglial abnormalities drive RTT progression. They showed that *Mecp2*-KO mice, which usually die at 8–10 weeks, became almost normal and lived to nearly one year after their brains were populated with wild-type myeloid cells/microglia by a bone-marrow transplant approach. However, some key results were not reproduced in a recent study (Wang et al., 2015), making the role of microglia unsettled. On the other hand, a recent gene array study in microglia derived from heterozygous (Het) female mice, carrying one *Mecp2*-null allele, shows that genes involved in innate immunity and macrophage activation are differentially expressed both at pre-phenotypic (5 weeks) to phenotypic phases (24 weeks), suggesting the dysfunction of MeCP2 can lead to dysregulation of inflammatory responses contributing to some aspects of the progression of disease pathogenesis (Zhao et al., 2017).

IDDRC investigators at two sites, Boston Children's Hospital and University of California Davis, have made significant progress in clarifying the role of microglia in RTT. Schafer et al. (2016) examined the retinogeniculate system in *Mecp2* null mice throughout disease progression. They found that microglia excessively engulfed and removed presynaptic inputs during the last stage of the disorder. Surprisingly, the selective manipulation of *Mecp2* expression in microglia did not impact this synaptic pruning phenotype or any other manifestation of the disease in the mice. Combining these results with the previous finding that lack of *Mecp2* weakens single fiber synapse strength (Noutel et al., 2011), microglia appear to target circuits made vulnerable during the early stages of the disorder and dismantle them in the final stages. Importantly, microglia do this independent of their own expression of *Mecp2*.

Horiuchi et al. (2017) from the University of California Davis conducted a study demonstrating a critical role for microglia in RTT. They found that ablation of CX_3CR1, a key microglial receptor mediating neuron-microglia interaction, substantially improved respiratory and motor functions and prolonged the lifespan of *Mecp2*-KO mice. Interestingly, CX_3CR1 ablation also restored the microglial morphology and quantity to wild-type levels in multiple brain regions. It remains to be determined if CX_3CR1 ablation

improves microglial function and morphology via a cell-autonomous mechanism or by blocking the detrimental non-cell autonomous influences from abnormal MeCP2-deficient neurons. The other interesting and somewhat surprising finding was that complete (Cx_3-cr $^{-/-}$) and partial ($Cx_3cr1^{-/+}$) ablation of CX_3CR1 nearly equally attenuated disease severity. The Cx3cr1-EGFP targeted mutation line, which is equivalent to $Cx_3cr1^{-/+}$ used in this study, has been frequently used for studying microglial function *in vivo* because microglia are labeled with EGFP. This result should raise awareness that Cx3cr1-EGFP microglia may behave differently from and by no means represent wild-type microglia.

The epigenomic-bioenergetic hypothesis, proposed by Wallace and Fan (2010), states that perturbation of the epigenome causes various pathologies via disrupting the coordinated expression of bioenergetic genes to reduce mitochondrial function. Consistent with this hypothesis, another study from University of California Davis IDDRC investigators found that MeCP2 deficiency impairs microglial mitochondrial function, and causes over-production of mitochondrial reactive oxygen species (Jin et al., 2015). Interestingly, severe mitochondrial structural damage was only found in microglia and not in astrocytes or neurons in *Mecp2*-KO mice. While mitochondrial abnormalities may be global as a consequence of MeCP2 deficiency, the observation that mitochondrial structural damage is only seen in microglia suggests highlighted vulnerability of microglia to prolonged or high oxidative stress leading to structural changes in proteins, lipids, and DNA (Dai et al., 2014).

This selective susceptibility perhaps is not surprising considering that microglia are at the forefront of coping with the brain microenvironment (Hammond et al., 2018) and should be strongly dependent on epigenetic regulation (Cheray and Joseph, 2018). Moreover, microglia are constantly motile and robustly reactive (Nimmerjahn et al., 2005; Davalos et al., 2005), for which efficient bioenergetic regulation is of utmost importance. Failure of the MeCP2-directed epigenetic-bioenergetic pathways would be devastating to microglial function and survival, a fundamental mechanism that may underlie microglial abnormalities and premature death in RTT models (Maezawa and Jin, 2010; Horiuchi et al., 2017; Jin et al., 2015; Cronk et al., 2015; Derecki et al., 2012).

FROM MECP2 TO EXCITATORY/INHIBITORY IMBALANCE AND NEURONAL CIRCUIT DEFECTS

Neurons communicate and get organized into circuits through such synapses, thereby gaining the capability to process information from the outside world. Abnormalities in synaptic excitation (E), inhibition (I), and excitation/inhibition ratio (E/I) in cortical circuits greatly affect both dynamics and information processing of neuronal circuits, and are widely considered to be the root cause of the plethora of symptoms characterizing RTT during development and adulthood.

Anatomical and functional abnormalities have been well described in multiple neuronal circuits in both cortical and subcortical brain regions and have highlighted a distinct phenotype based on the area analyzed: excitatory hypoconnectivity in forebrain regions and hyperconnectivity in brainstem compartments (Kron et al., 2012). A shift in excitatory-inhibitory balance in favor of inhibition has been extensively reported in somatosensory,

visual, auditory, motor, insular and prefrontal cortex by *in vitro* and *in vivo* analysis (Dani et al., 2005; Durand et al., 2012; Krishnan et al., 2017; Wood and Shepherd, 2010; Gogolla et al., 2014; Sceniak et al., 2016). It has been suggested that this early shift is driven by an early structural and functional hyper-maturation of fast-spiking parvalbumin-positive cells (PV) inhibitory interneurons and perineuronal nets enwrapping them (Durand et al., 2012; Krishnan et al., 2015; Patrizi et al., 2019; Sigal et al., 2019) and a concurrent immaturity of excitatory synapses (Mierau et al., 2016). Using super resolution fluorescence imaging, Sigal et al (2019) also recently showed that in Mecp2 KO mice, PV cells receive weaker recurrent inhibitory inputs and stronger thalamocortical excitatory inputs in the primary visual cortex. Overall, there is growing evidence that PV circuits are functionally and structurally upregulated throughout the progression of the disorder. Future work should be devoted to a comprehensive functional characterization of other inhibitory cell types (CR, SST, VIP) across selective cortical areas in relation to the progression of the disorder.

On the other hand, brainstem regions display synaptic hyperexcitability and increased expression of immediately early gene c-Fos (Kron et al., 2012). The hippocampal circuits set themselves apart by exhibiting hyperexcitability due to a loss of excitatory drive onto inhibitory circuits (Calfa et al., 2015). Some of these changes are present very early on in the postnatal development and precede the full onset of RTT phenotype and behavioral regression.

How the imbalance between excitatory and inhibitory transmission in multiple brain circuits drives the onset of epileptic seizures is still puzzling. The late onset of epilepsy likely reflects the miswiring and rewiring of neuronal circuits and the failure of homeostatic synaptic plasticity mechanisms that accompany the progression of the disorder. Rather than acting like a simple break preventing over-excitation of neuronal circuits, GABAergic transmission function critically determines the fine-tuning of a cortical network and its information flow across cortex by enhancing or suppressing dynamically along feed-forward and lateral connections, depending on the stimulus processing demand and the spatial and temporal constraints (Womelsdorf et al, 2007). A study by Lu and colleagues from the BCM-IDDRC revealed that both null males and heterozygous (Het) female RTT mice have abnormally elevated synchrony in the firing activity of hippocampal CA1 pyramidal neurons, an impaired homeostatic response to perturbations of excitatory-inhibitory balance, and decreased excitatory synaptic response in inhibitory neurons (Lu et al., 2016).

Many of these studies have been conducted in *Mecp2* KO mice rather than in *Mecp2* Het female mice, a mosaicism model for MeCP2 as found in RTT patients. As MeCP2 is differentially regulated during development in males and females (Kurian et al., 2007), and DNA methylation states are sex-specific (Lister et al., 2013; Keown et al., 2017), circuits maturation and refinement may also be differentially regulated in Mecp2 Het females in comparison with null males. Indeed, the rescue in either inhibitory or excitatory neurons gives different results in null males versus the mosaic females underlying the complexity of excitatory/inhibitory balance dysfunction and the network differences in RTT model (females) versus *Mecp2* null mice (Ure et al., 2016; Meng et al., 2016). Despite the onset of neurological symptoms in *Mecp2* Het females in young adulthood (8–12 weeks) and therefore much later compared to patients (first 6–18 months), their behaviors clearly show

multiple and robust RTT phenotypes stable across backgrounds such as anxiety-like behavior, fear memory, breathing abnormalities with some phenotypes specific to one of the backgrounds such as acoustic startle and prepulse inhibition, and weight gain (Samaco et al., 2013). In an ethologically relevant behavior, Shea and colleagues have recently demonstrated that *Mecp2* Het females fail to learn a simple maternal care behavior performed in response to their pups' distress cries and this impairment appeared to critically involve PV inhibitory neurons in the auditory cortex. Similar to what has been found in null mice in other cortical regions, PV networks and relative perineuronal nets (PNN) undergo precocious and over maturation. While both excitatory and inhibitory PV cells adapt their response to pup calls during maternal learning through disinhibition in WT females, mutants lack such maternal experience-dependent plasticity specifically to vocal signals from pups. In *Mecp2* Het, PV spiking activity fails to decrease, preventing the increase of excitatory drive (Krishan et al., 2017; Lau et al., 2020). Similarly, accelerating rotarod task for 2 consecutive days in *Mecp2*$^{tm1.1Jae}$ mouse line, was accompanied by a shift of PV expression to a higher state in M1 (Morello et al., 2018). Together these studies confirm significant cortical defects of experience-dependent plasticity in mice models of RTT that persist past early development and affect adult behavior.

MECP2 AND CORTICAL PROCESSING

As the previous section detailed, excitatory/inhibitory balance is disrupted in RTT animal models, across all cortical areas tested so far (Katz et al., 2016). Alterations of synaptic physiology ultimately lead to miswiring of circuits and drive abnormal sensory, motor, and cognitive function. In the past few years, increasing attention has been given to the evaluation of sensory processing in visual and auditory cortices in RTT patients as a non-invasive and quantitative probing of cortical function. Sensory evoked potentials can be elicited using passive presentation of a sensory (auditory, visual, or somatosensory) stimulus, without requiring any sedation, overt effort or a behavioral response on part of the subject, making them suitable for severely impaired populations, such as RTT patients.

Visual cortical processing in murine and human RTT

Researchers at Boston Children's Hospital IDDRC have found analogous deficits in visual processing in mouse models and humans. In 2012, Durand et al. reported a regression in visual acuity in *Mecp2* KO mice, measured both behaviorally and electrophysiologically, coupled with decreased spontaneous and evoked single unit activity in the visual cortex. Following up on this result, LeBlanc et al. (2015) found that *Mecp2* heterozygous female mice also exhibited reduced visual acuity and altered shape of the visual evoked potential (VEP) waveform. Strikingly, VEPs measured in girls with RTT displayed the same morphological changes, diminished and protracted waveform, and these aspects were impacted by disease stage and mutation type. Analysis of VEPs in response to a range of spatial frequencies revealed that the RTT group also exhibited reduced spatial acuity. These results paralleled the previous findings of Saunders and colleagues (1995) showing reduced amplitude of VEP in response to low spatial frequencies in a small group of RTT subjects.

Visual circuit abnormalities in RTT have also been revealed by another independent study investigating how the absence of *Mecp2* in mice impacts experience-dependent plasticity in the visual cortex (Krishnan et al., 2015). The critical period for ocular dominance plasticity was moved forward in *Mecp2* null mice, opening and closing precociously, as measured using optical imaging in the visual cortex following monocular deprivation. This critical period is a time during which experience helps shape circuits in the visual cortex, enabling the matching of orientation tuning between the two eyes. In *Mecp2* null mice, binocular matching of orientation preference was disrupted, providing an example of how the altered critical period may impact vision in RTT. Interestingly, these changes in visual plasticity and function were accompanied by an acceleration of parvalbumin interneuron maturation, fitting with other reports (Durand et al., 2012, Patrizi et al., 2019; Lau et al., 2020).

Auditory cortical processing in murine and human RTT

Auditory processing is another sensory domain being actively investigated in RTT. Language and communication are severely impaired in RTT, but it is unclear if basic auditory processing is compromised as well. Auditory Event Related Potentials (ERP) have been measured in mouse models of RTT with the aim of assaying cortical function and determining the cellular basis for deficits. Mice carrying the T158A mutation were found to display delayed and diminished ERPs in response to white noise stimulation, but only while symptomatic (after P30) (Goffin et al., 2011). A study by the same group removed and re-expressed *Mecp2* specifically from different cell types in order to determine the cellular origins of these ERP deficits (Goffin et al., 2014). They found that loss of *Mecp2* from forebrain GABAergic neurons was sufficient to produce the ERP deficits and restoration of *Mecp2* expression to either PV or SST-expression interneurons was able to partially rescue these deficits (Goffin et al., 2014). Notably, the same research group used a transgenic approach to increase MeCP2 T158M expression and found that elevation of MeCP2 T158M protein level significantly ameliorates numerous RTT-related phenotypes, including the restoration of auditory ERP response, highlighting the feasibility and validity if using auditory ERP as a biomarker for RTT (Lamonica et al., 2017).

Researchers at the Einstein College of Medicine IDDRC, Children’s Hospital at Montefiore, and the University of Rochester are using ERPs to assess auditory function in females with RTT, using simple tones that are the building blocks of speech perception to semantic processing. Foxe and colleagues (Foxe et al., 2016) report findings on auditory function in 14 girls with genetically confirmed RTT and 22 age-matched neurotypical controls (ages: 3.9–21.1 years). Here they tested the ability to distinguish the frequency of two tones by presenting a stream of standard 503 Hz tones occasionally interspersed with a higher-pitched deviant tone of 996 Hz, and measuring the mismatch negativity response (the MMN, derived by subtracting the auditory evoked brain response (AEP) to the deviants from the AEP to the standards). The MMN indexes the brains ability to discriminate between sounds and, despite being recorded in passive listening conditions, is highly associated with behavioral discrimination.

The data revealed a clear MMN response in the Rett group, although this was both delayed and protracted in duration compared to an age matched control group. Furthermore, the base

AEP was highly anomalous in the Rett girls in both this and a subsequent study (Brina et al., 2019), and further pointed to slowing of auditory responsiveness in this group. These data suggest preserved but atypical ability to process pitch changes in RTT. A similar approach was used to test the cortical representation of auditory stimulus duration for fast versus slow presentation rates (ranging from ~2 stimuli per second to õne stimulus every two seconds) (Brima et al., 2019). Small differences in stimulus duration (of 80 ms) evoked MMN responses in the RTT group at the fastest presentation rate. However, when stimuli were presented at slightly slower rates, this response appeared largely abolished in the RTT group. So, while RTT patients can decode deviations in auditory duration, the span of auditory sensory memory is severely curtailed, with likely implications for the processing of speech and language. These findings are similar to what was found in the visual system, where clear VEP responses were present and modulated by spatial frequency in individuals with RTT, but waveform amplitude was diminished and protracted and the threshold of visual acuity was decreased (LeBlanc et al., 2015). What is more, auditory atypicalities of delayed and reduced sensory evoked responses have been observed in rodent models of Rett (Schoups et al., 2001; Liao et al., 2012).

AEPs also allow the probing of impairments in speech perception. Peters et al. (2015, 2017) were the first to quantify changes in gamma band power in response to familiar and novel voices or own names versus other names in a small cohort of children with Rett and MDS. Although AEPs from all patients clearly indicate the ability to discriminate between the two stimuli, yet the relative changes in gamma power are in opposite directions, suggesting that under- versus over-expression of the MECP2 protein has a differential impact on cortical processes. Interestingly, the bigger amplitude of the AEPs found in MDS patients was associated with higher social functioning. Recently, Key and colleagues (2019) reported that girls with RTT use not-canonical circuits (right vs left parietal hemisphere) to distinguish familiar versus not familiar words.

It is noteworthy that event related evoked potentials are also abnormal in *CDKL5* and *FOXG1* deficient mouse and patients (Wang et al., 2012a,b; Boggio et al 2016; Mazziotti et al., 2017; Demarest et al 2019) further supporting the use of evoked potentials as quantitative and not invasive probing tools for better understanding the pathophysiology of RTT, MECP2 duplication and related disorders. However, a consortium approach, in which systematic longitudinal studies of significantly larger samples could be coordinated, would significantly improve the ability to capture the progression of the disorders in such heterogeneous patient populations, and link neural function with clinical phenotype, disease stage, and so forth to progress the possibility of ERP/EEG data serving as critical biomarkers of disease state and treatment efficacy, an idea that is addressed in more detail below.

BIOMARKERS FOR MECP2 RELATED DISORDERS

There is an urgent need for objective, quantitative, non-invasive and translational biomarkers for early diagnosis of disorder status, its progression over time and potential response to therapeutic interventions. Ideally, each domain impacted by the disorder should be identified by one or more selective biomarkers for cross-reference, accuracy and evaluation of possible

interaction across domains. In addition to ERPs described above, recently Fagiolini's lab and colleagues have shown that pupillometry and heart rate variation (HRV), two proxies of spontaneous arousal fluctuations, combined with deep machine learning, allow early and highly accurate diagnosis of RTT across species (Artoni et al., 2019). RTT is characterized by deficits in cholinergic neuromodulation, attention and altered regulation of autonomic arousal from very early on during the disorder. By taking advantage of deep machine learning, they trained neural networks to recognize patterns of spontaneous arousal fluctuations typical from mice with heightened cholinergic sensitivity (ConvNetACh) and used them to then successfully detect impaired fluctuations in both *Mecp2* KO and *Mecp2* Het mice, even before mice became symptomatic. Similar defects were also detected in CDKL5 KO adult mice (Artoni et al., 2019). More importantly, by retraining only the last layers of ConvNetACh with arousal data directly from RTT patients, they generated a neural network (ConvNetPatients) capable of predicting RTT (Artoni et al., 2019). The cross-species reproducibility of this approach, the non-invasive nature of arousal proxies, such as pupillometry and HRV, and the intrinsic flexibility of deep neural networks can allow trained networks to be adapted to rare patient data, significantly increasing the translational value to these rapidly collected biomarkers of arousal.

PRECLINICAL EFFICACY OF NEW THERAPIES

Existing knowledge of the molecular, cellular and circuit mechanisms underlying postnatal development, plasticity and mature function of auditory and visual sensory areas and the preservation across species of the fundamental processes represent a powerful tool in the quest of dissecting the role of MeCP2 in RTT and the identification of possible intervention strategies. Progress in understanding the neurobiological basis of RTT has led to the development of numerous promising therapeutic strategies. Therapies can generally be categorized as either (1) targeting the downstream cellular pathways and circuits disrupted by MeCP2 deficiency or (2) gene therapy approaches that target the MECP2 gene directly itself. The robust efficacy of several of such interventions at the preclinical level has created great hope in the Rett community for treatments for the condition (Clarke and Addala Sheikh, 2018; van Karnebeek et al., 2016).

Here we will discuss several treatments relating to the first (deep brain stimulation, low dose ketamine, and valproic acid) and second (gene therapy and X chromosome reactivation) categories.

Benefits of deep brain stimulation

Deep brain stimulation (DBS) is a therapy that involves implanting electrodes in the brain that emits electrical stimulation to reset abnormal signaling; much like a pacemaker acts to normalize a disrupted heartbeat. DBS can alleviate motor dysfunction in disorders like Parkinson's disease (Huang et al., 2018) and dystonia (Hu and Stead, 2014) and may improve cognition too, as in the case of Alzheimer's disease (Aldehri et al., 2018). DBS is appealing because it can be directed to different brain targets depending on the symptoms and it is relatively safe and reversible.

Individuals with RTT suffer from motor and cognitive dysfunction, making DBS a therapy worth pursuing. Studies by BCM-IDDRC investigators using DBS in mouse models of RTT show promise. Forniceal DBS rescued contextual fear memory and spatial learning and memory in adult *Mecp2* heterozygous female mice (Hao et al., 2015). DBS also rescued *in vivo* hippocampal long-term potentiation and neurogenesis, providing a putative mechanism for the behavioral benefits. A follow-up study explored the effect of forniceal DBS on hippocampal circuits in the RTT mouse model and found a positive effect on CA1 pyramidal synchrony, homeostatic plasticity, and excitatory synaptic responses in inhibitory neurons (Lu et al., 2016).

A study by Pohodich and colleagues from BCM-IDDRC shed some light into the mechanisms behind the well-established DBS benefits. In particular, they assessed gene expression and proteome changes following forniceal DBS in wild-type mice, *Mecp2* KO and *Mecp2* Het Mice showing that DBS upregulates genes involved in synaptic function, cell survival, and neurogenesis and normalized expression of ~25% of the genes altered in *Mecp2*KO mice. The restored genes in *Mecp2*KO mice were mainly enriched in neural functions, including components of synapses such as *Gad2* (Glutamate Decarboxylase 2) and *Grin2d* (Glutamate Ionotropic Receptor NMDA Type Subunit 2D). Variation of GABA synthesis and NMDA receptor signaling have been implicated in the circuit dysfunction observed in these mice (Chao et al., 2010, Durand et al., 2012, Ure et al., 2016), suggesting that DBS acts through physiological pathways improving plasticity (Pohodich et al., (2018).

These findings support the argument that RTT is a neural circuit disorder and show that DBS could represent a strategy to renormalize the activity of circuits and improve cognition. Much work still remains, including determining the ideal frequency and duration of treatment and confirming long-term safety in a pediatric population.

MANIPULATION OF NMDAR SIGNALING: LOW DOSE KETAMINE

Substantial evidence demonstrates that NMDARs are abnormal in RTT, in both human tissue (Blue et al., 1999a,b) and mice (Asaka et al., 2006; Blue et al., 2011; Durand et al., 2012; Mierau et al., 2016). In an attempt to normalize glutamatergic signaling, the NMDAR antagonist ketamine is being explored as a potential treatment for RTT. In 2012, Kron et al. rescued hypoexcitability of the forebrain and sensorimotor gating deficits in heterozygous Mecp2 female mice with a single treatment of low-dose ketamine. This subanesthetic dose has been shown to specifically reduce NMDAR activity onto parvalbumin fast-spiking inhibitory neurons (Kinney et al., 2006; Behrens et al., 2007; Picard et al., 2019), a cell population whose maturation is accelerated in the absence of *Mecp2* in mice, leading to increased innervation of pyramidal cells and an overall silencing of the visual cortex (Durand et al., 2012; Mierau et al., 2016; Patrizi et al, 2019).

Based on this evidence, Patrizi et al. from the IDDRC at Boston Children's Hospital set out to perform a preclinical trial for chronic ketamine treatment in *Mecp2* KO mice, testing the ability to prevent the onset of symptoms by early administration (from P15 on) and the ability to rescue symptoms with later administration (from P30 on) (Patrizi et al., 2016). Both treatment paradigms were well-tolerated and caused no adverse side effects. Lifespan

was extended and many RTT-related symptoms were improved, including hindlimb clasping, motor coordination, and breathing. Visual regression was slowed and visual cortical activity and connectivity were normalized. These effects of ketamine are likely a result of preferential binding to highly active NMDARs on parvalbumin interneurons, dampening their inhibitory effect on pyramidal cells and restoring normal levels of excitation in the cortex.

The next step for mouse preclinical work is to test in heterozygous female mice, the closer model for human RTT. As Picard et al have shown, attention should be paid when administering low dosage ketamine to females as the response may be affected by estrous cycle (Picard et al., 2019). Short treatment of low-dose ketamine is currently being scheduled in patients in an ongoing Phase 2 clinical trial at the Cleveland Clinic and Case Western Reserve University (www.clinicaltrials.gov, NCT02562820). The results of this trial combined with the preclinical work in mice will soon yield a definitive answer of whether low-dose ketamine is an effective treatment for RTT syndrome and will help to further elucidate the neurobiological mechanisms behind its actions.

Valproic acid (VPA) treatment in RTT

VPA, also call ed valproate, is a drug commonly used to treat bipolar disorder, epilepsy, and to prevent migraines (Chiu et al., 2013). While its mechanism of action is not completely understood, it is known to be a histone deacetylase inhibitor, reactivates transcription of a wide range of genes and it has been implicated in reopening synaptic plasticity in adult visual cortex (Göttlicher, 2004; Lennartsoon et al., 2015).

IDDRC investigators at the University of Wisconsin, Madison and Kyushu University in Fukuoka, Japan tested whether VPA could positively impact the condition of symptomatic *Mecp2* KO mice (Guo et al., 2014). When six-week-old *Mecp2* KO mice were injected daily with VPA for 2 weeks, the mice exhibited improvement in some RTT-related symptoms and a subset of downregulated genes were restored to near-normal levels. On the other hand, VPA has been used to treat RTT patients in limited clinical studies, mainly in order to manage seizures, and the results are mixed (Faulkner and Singh, 2013). One study reported no improvements from VPA (Huppke et al., 2007) while another found that seizures were significantly reduced (Krajnc et al., 2011). Another reported an increased risk of bone fracture after VPA treatment (Leonard et al., 2010). Thus it is still unclear whether VPA positively impacts RTT-related neurological symptoms and more work may be needed.

Gene therapy/viral vector-mediated gene transfer in RTT

Gene therapy or introduction of a healthy copy of *MECP2* into the brain, represents a potential one-time treatment that could address the root causes of RTT. Viral vector-mediated *MECP2* gene transfer has been used in recent years for basic science research as well as proof-of-concept studies for the therapeutic reversal of RTT in mice. However, conceptually, *MECP2* gene therapy is challenging for two primary reasons: (1) To create a broadly efficacious, safe gene therapy approach, researchers must create vectors that restore MeCP2 activity in unhealthy cells while avoiding a MeCP2 duplication-like phenotype (Lombardi et al., 2015); (2) Depending on the treatment age and injection route (in mice),

researchers may have difficulty achieving moderate or high levels of transduction efficiency with widespread delivery throughout the brain (Lombardi et al., 2015; Gadalla et al., 2013).

Despite these conceptual challenges, three labs have independently shown that adeno-associated viral (AAV) vector-mediated MECP2 gene transfer can extend lifespan and reverse or delay behavioral abnormalities in mouse models of RTT (Gadalla et al., 2013; Garg et al., 2013; Matagne et al., 2017). In short, Gadalla et al. demonstrated that intracerebral administration of single-stranded (ss) AAV9/CBA-*MECP2* in *Mecp2*-null neonates can extend lifespan while improving multiple phenotypic readouts, including rearing frequency, poor treadmill performance, and poor locomotor scores during open field tests (Gadalla et al., 2013). Intravenous (IV) administration of self-complementary (sc) AAV9/MeP229-*MECP2* also extended lifespan in juvenile *Mecp2* KO mice but induced acute liver toxicity in WT mice (Gadalla et al., 2013). More recently, Matagne et al. published experiments showing that codon-optimized *MECP2* gene transfer (also under a truncated *MECP2* promoter) extends survival of *Mecp2*-null mice and delays the onset of behavioral deficits in rotarod and open field tests (Matagne et al., 2017). Concurrently with Gadalla et al., Garg and colleagues showed that scAAV9-MeP730-*MECP2* extended the median survival of Mecp2-null mice and improved scores for nest building, rotarod, and inverted grid tests for female RTT mice (Garg et al., 2013).

Yet another independently developed viral vector has provided further evidence that *MECP2* gene transfer can be therapeutic *in vivo* (Tai et al., 2016). This lentiviral vector was developed as a tool to help understand the role of post-translational sumoylation of MeCP2 (addition of small ubiquitin-like modifier proteins called SUMOs). Although the authors' gene transfer studies were part of a larger goal to elucidate a specific MeCP2-dependent signaling pathway, the *in vivo* experiments in *Mecp2* conditional (inducible and brain region-specific) knockout mice provided an opportunity to observe downstream behavioral responses. More specifically, gene transfer of SUMO-modified MeCP2 (but not a sumoylation-resistant MeCP2 variant) improved long-term potentiation (LTP), social interaction, and memory retention relative to those of negative controls (Tai et al., 2016).

A legitimate concern of *MECP2* gene transfer is that it will induce neurological abnormalities in animals expressing supraphysiological levels of MeCP2 in transduced cells (Lombardi et al., 2015; Gadalla et al., 2013). Indeed, AAV9/MeP229-*MECP2* has recently been shown to increase severity scores for limb clasping and abnormal gait in treated WT and *Mecp2* KO mice after intraCSF administration, while an AAV9/MeP229-GFP vector at the same dose and route was well-tolerated (Sinnett et al., 2017). Importantly, a recently developed second-generation vector (AAV9/MeP426-*MECP2*) provides tighter control of transgene expression and – at low intraCSF doses – can extend Mecp2-null survival without inducing the above-mentioned behavioral abnormalities (Sinnett et al., 2017; Gadalla et al., 2017). Ongoing vector design efforts will seek to further improve the therapeutic index of MECP2 gene transfer (Sinnett et al., 2017). Together these studies have catapulted gene therapy for RTT to the forefront of interventions. Under the sponsorship of rettSyndromeResearchTrust and AveXis, a clinical trial testing AVXS-201 is under preparation pending the approval of the U.S. Food and Drug administration.

Reactivating the silent healthy copy of MECP2

Another approach for treating RTT at the root of its cause is to reactivate the normal copy of MECP2 that resides on the inactive X (Xi) chromosome. While this method avoids the risk of MECP2 overexpression, there is the possibility of complications due to activating many other genes on the Xi chromosome. The ideal method would be to selectively awaken the silent copy of MECP2 rather than the entire X chromosome. Although it's still early, ongoing research is making progress in identifying pharmacological targets for reactivation. New female mouse model with a mutation in the regulator of X chromosome inactivation (Tsix) has been recently generated allowing to skew the MeCP2 levels in the brain and bringing a useful tool to do dose–response types of analysis with candidate compounds (Carrette et al., 2018).

Many laboratories are actively screening to identify new and selective molecules to induce Xi chromosome reactivation (Lessing et al., 2016; Sripathy et al., 2017). A screen in mouse fibroblasts looked for small molecules that reactivate the entire Xi chromosome (Lessing et al., 2016). They found that drugs targeting the Aurora kinase and DNA methylation pathways work in synergy to reactivate genes on the X chromosome, including *Mecp2*. Another recent shRNA screen in mouse fibroblasts identified 30 genes that normally repress the other copy of *Mecp2* (Sripathy et al., 2017). These genes clustered into 7 functional groups and 6 were members of the BMP/TGF-B pathway. Later, the same team showed that small molecule inhibitors acting against previous identified targets, can reactivate Xi-linked *Mecp2* in non-dividing cultured RTT neurons and in cerebral cortical neurons of adult mice, demonstrating that reactivation of Xi-linked *Mecp2* can occur in post-mitotic brain neurons (Przanowski et al., 2018). As far as we know, to date, the number of cells in which *Mecp2* has been fully restored has been quite limited. Recently, it was demonstrated that a mixed modality approach including an antisense oligonucleotide (ASO) against Xist, the noncoding RNA responsible for X inactivation, and an inhibitor of DNA methylation, can achieve a 30,000-fold reactivation of *Mecp2* (Carrette et al., 2017).

TACKLING MDS

In pursuit of therapeutic intervention for *MECP2* duplication disorder Zoghbi and colleagues at the BCM-IDDRC targeted the MeCP2 protein itself. First, using genetics they showed that restoring MeCP2 levels to normal rescues duplication-like phenotypes in the MDS mouse model. To explore a strategy that has the potential for translation, they used antisense oligonucleotides that target the human MeCP2 protein encoded by the human transgene. They discovered that normalizing MeCP2 levels in adult mice reversed all the duplication phenotypes including anxiety, autism-like features, seizures, synaptic plasticity, EEG abnormalities, and gene expression changes (Sztainberg et al., 2015). These findings inspired new studies to identify druggable targets that modulate MeCP2 levels. From a genetic screen of kinases and phosphatases, they identified four targets that modulate MeCP2 levels and discovered a key residue on MeCP2, Serine 216, that is phosphorylated by two of the targets Hipk1 and Hipk2 which in turn affects MeCP2 stability (Lombardi, et al., 2017). These data suggest that genetic screens are likely to reveal modulators of MeCP2 that are potentially druggable. Moreover, the strategy will also allow identification of targets

that can increase MeCP2 levels, which might help people with T158M mutation given the recent data from the Zhou lab in the IDDRC at CHOP/Penn (Lamonica et al., 2017).

FROM BENCH TO BEDSIDE

The promising interventions that improve or reverse symptoms in mouse models give great hope to the RTT community that deciphering the basic biology of MeCP2 is in our reach. Yet no treatment targeting the downstream consequences of *MECP2* mutations is considered to be widely effective (Glaze et al., 2017; Glaze et al., 2009; Khwaja et al., 2014; O'Leary et al., 2018). A multi-site clinical trial for gene therapy directly targeting MECP2 expression is under preparation and may be launched as early as the end of 2020 pending U.S Food and Drug administration approval. However, the number of completed, ongoing or planned clinical trials is continually rising (currently as we are writing we are at 59, https://clinicaltrials.gov/ct2/results?term=rett+syndrome), and with each one comes a better understanding of what may or may not be worth pursuing further. A review by Katz et al. (2016) summarizes completed clinical trials and their outcomes and discusses how the field can learn from past trials and plan for the future. Some of the most recently completed clinical trials include IGF-1, NNZ-2566, EPI-743, ω−3 PUFAs, Glatiramer acetate (Copaxone), statins, and Desipramine. Ongoing and recruiting trials include Sarizotan, Ketamine, Fingolimod, Dextromethorphan, and Cannabidiol. The existing knowledge of the molecular, cellular and circuit mechanisms underlying postnatal development, plasticity and mature function of sensory areas, and the preservation across species of the fundamental processes, represent a powerful tool in the identification of novel intervention strategies. Unsuccessful clinical trials must be used to design better ones closely paralleling the intervention done at the preclinical level (when, how long and how much) and with more sensitive outcome measures (Sahin et al., 2018).

PATH FORWARD

The past few years have been filled with discoveries leading to greater insight into how *MECP2* mutations lead to RTT. From basic biological research, to translational and preclinical studies, and all the way to clinical research and clinical trials, each step is crucial in the quest to treat RTT effectively. Going forward, it will be necessary to prioritize several approaches in order to maximize success in achieving these goals.

First, reliable biomarkers and outcome measures must be identified that can be directly translated from animal models to patients. This is critical in order to accurately assess the efficacy of new treatments being tested and currently a major limitation in clinical trials. Second, because RTT is a dynamic disorder involving stagnation and regression of development in early postnatal life, studies should be designed whenever possible as longitudinal to analyze trajectories rather than taking snapshots in time both in animal models and in patients. Third, reproducibility of results must be improved by conducting studies at multiple locations and with a significantly higher number of subjects examined. This applies to pre-clinical and clinical studies.

The progress made in the last few years is the result of a global effort to decode this complex disorder by the synergistic collaboration among scientists, advocacy groups and funding agencies. NIH funded IDDRC centers represent a great opportunity to lead and train the next generation of clinicians and researchers.

It takes a village to raise a child, it takes the world to cure MECP2 disorders.

ACKNOWLEDGEMENTS

This work was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development U54 Grant (HD090260) to the Human Clinical Phenotyping Core of the Rose F. Kennedy Intellectual and Developmental Disabilities Research Center (SM); Rett Syndrome Research Trust grant to SG, and an NIH grant 4T32HD040127-15 to SS. Indirect administrative support for SJG was provided by Research to Prevent Blindness to the UNC-CH Department of Ophthalmology IDDRC center grant (U54HD090256) (QC); NIH NINDSROS1NS095959 and Children's Hospital Boston Intellectual And Developmental Disabilities Research Center (BCH IDDRC 1U54HD090255) (MF); U54HD086984 (ZZ).

Abbreviations:

CDKL5	Cyclin-Dependent Kinase-Like 5
CR	calretinin
FOXG1	Forkhead Box protein G1
IDDRC	Intellectual and Developmental Disabilities Research Centers
KO	knockout
MBD	methyl-CpG binding domain
MDS	*MECP2* duplication syndrome
MECP2	methyl-CpG binding protein 2
RTT	Rett Syndrome
BDNF	brain-derived neurotrophic factor
NPCs	neural progenitor cells
NMDA	N-methyl-d-aspartate receptors
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
PV	parvalbumin
CSF	cerebrospinal fluid
SST	somatostatin
Het	heterozygous
PNN	perineuronal nets
VEP	visual evoked potential

ERP	auditory event related potentials
WT	wild-type
AAV	adeno-associated viral

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