

## Research Awardees: 2003

### Research Awards

Bruria Ben Zeev, M.D. and Eva Gak Ph.D.

Reiko Maki Fitzsimonds, Ph.D.

Michael Greenberg, Ph.D.

Ege T. Kavalali, Ph.D.

Solomon L. Moshé, M.D.

Gert Jan C. Veenstra, Ph.D.

Christopher L. Woodcock, Ph.D.

Jerry Yu, Ph.D.

Xinyu Zhao, Ph.D. and Fred H. Gage, Ph.D.

J. Julius Zhu, Ph.D.

### Post-Doctoral Fellowship Awards

Shaun Cowley, Ph.D.

Amit Mehta, Ph.D.

### Miscellaneous Awards

Hannah Kinney, M.D.

Michael Friez, Ph.D.

Tony Charman, Ph.D.

Noriyuki Kishi, M.D., Ph.D.

### Research Awards

Bruria Ben Zeev, M.D.

Eva Gak, Ph.D.

Sheba Medical Center, Israel

"Molecular diagnosis of Rett Syndrome: an alternative scheme"

1-Year Award: \$50,000

Research Sponsor: Israel Rett Syndrome Center

Final Report (November 2005)

During the past five years, more than 100 patients have been admitted to the Israeli RTT cohort including patients with classical and atypical RTT, PSV, EEF and FF variants, patients with Angelman-like features and with other clinical indications reminiscent of RTT. Most of the cohort members have been diagnosed using standardized clinical criteria by the same pediatric neurologist at the Neuropediatric Clinic at the Sheba Medical Center, where they continue to attend for clinical follow-up. Our molecular backing of RTT includes analyses of MECP2 coding region, MLPA, XCI. Within this framework, we identified 55 patients with MECP2 sequence variations, including mutations at the known CpG hot-spots, 3'-end microdeletions and large rearrangements encompassing MECP2 gene region, as well as novel sequence variations. The latter include two maternally inherited variations that were present in conjunction with imbalanced XCI, wherein the variant X was preferentially activated in the patients and inactivated in the asymptomatic mothers, thus suggesting that these variations could be related to RTT phenotype. In addition, we detected a rare TG deletion of exon 1 splice-donor in two other classical patients. These efforts however did not provide diagnosis for the remainder of our cohort, including at least 10 patients with classical RTT and several atypical cases with strong indication of RTT.

In attempt to resolve the remainder of the cases, we developed a quantitative assay providing estimates of MECP2\_e1 and \_e2 expression levels in peripheral blood, which has been implemented in patients with known MECP2 mutations as well as patients with no mutation findings, the latter including few classical patients who have been referred to us by RSRF and Berge Minassian from the Hospital for Sick Children, Toronto. We observed that various mutations had distinct effects on MECP2 expression levels in the peripheral blood. Patients with the splice-donor mutation had significantly lower levels of both MECP2 isoforms, patients with deletions and nonsense mutations had slightly lower levels of MECP2\_e1 or \_e2, while patients with missense mutations and in-frame deletions had normal MECP2 expression levels. Preferential XCI additionally contributed to the inter-individual differences in MECP2 expression levels. Several classical and atypical patients with no previous mutation findings were found with lower MECP2 expression levels, which prompt to persist and search the regulatory regions of this gene for yet unknown mutations. For the meantime, these findings suggest that blood MECP2 expression levels reflect the patients' genetic and epigenetic status and may be used as an additional index of RTT diagnosis.

## Lay Summary

This study essentially aims at a subset of RTT patients in whom no coding sequence mutation has been identified by previous sequence analysis. It is built on the premise that genetic and epigenetic aberrations encompassing the MECP2 gene region could explain the entirety of clinical subtypes included or related to RTT. Thus, it suggests an alternative functionally based strategy that could potentially uncover novel MECP2 mutations in RTT. To date, the majority of classical RTT and to lesser extent atypical RTT have been associated with mutations in the MECP2 coding region. It is anticipated that the mutations in the MECP2 regulatory sequences, particularly the 5' and 3' UTRs, are responsible for an additional part yet undiagnosed RTT cases. Monitoring MECP2 expression level in patients' blood samples could reveal these types of aberrations, reflected in quantitative changes in MECP2-mRNA. A finding of a reduced MECP2-mRNA will thus indicate the presence of a regulatory mutation that could be traced in-retrospect to the genomic level by means of DHPLC analysis of MECP2 genomic fragments generated from 5' and 3' UTRs. Other putative regulatory mutations that involve intron-exon splice junctions will be delineated on the RNA level using highly specific TaqMan probes enabling the detection of low RNA populations. Alongside, we intend to foster fluorescent in-situ hybridization (FISH) assay to enable the detection of large deletions in the MECP2 chromosomal segment that may have been omitted from PCR-based methods. We will also include X-inactivation studies in order to evaluate potential consequences of epigenetic events on expressivity of MECP2 aberrations as revealed in diversity of RTT phenotypes. The ultimate resolution of this study may lead to a very exclusive subset of RTT patients, in whom the involvement of MECP2 has been consistently excluded and a search for another disease locus might be initiated.

## Abstract

We propose to develop a novel diagnostic strategy incorporating RNA-based quantitative PCR technology together with genomic-based approaches DHPLC and FISH, that may enable to extend the spectrum of MECP2 mutations that are

functionally relevant to the Rett Syndrome (RTT) and associated clinical presentations. To this end, we propose to:

1. Determine the relative MECP2 transcript quotient in peripheral blood that is most consistent with the presence of a regulatory mutation. This can be accomplished by monitoring MECP2 expression levels versus several housekeeping genes in patients with C-terminal deletions or other known regulatory mutations in comparison to normal female and male subjects.
2. Implement the above approach in a broader panel of classical and atypical RTT patients with no apparent mutation findings, in order to demarcate samples with potentially novel regulatory mutations.
3. Identify novel regulatory mutations in patients with altered MECP2 expression by means of comparative DHPLC analysis of MECP2 5' and 3' UTR genomic fragments generated from patients and ethnically matched controls.
4. Characterize putative splice-site mutations by means of Real-Time PCR using differential intron specific probes.

Develop differential region-specific FISH probes for the deletion of large deletions in the MECP2 chromosomal region.

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Reiko Maki Fitzsimonds, Ph.D.

Yale University School of Medicine

"Relationship between synaptic structure and plasticity in dendritic neuropathologies associate with Rett Syndrome"

2-Year Award: \$100,000

Research Sponsor: The Farmer Family Foundation

Final Report (November 2005)

Brain cells communicate with each other and store information by modulating the efficiency or strength of electrical signals sent from one neuron to the next. In the laboratory it is possible to measure how "strong" the connections are using a variety of methods including recording the electrical signals (electrophysiology) as well as measuring the levels of proteins (biochemistry) that are necessary for allowing these electrical signals to pass from one neuron to the next. Using the brains from genetically engineered Mecp2-null mouse of Rett Syndrome, we performed electrophysiological and biochemical analyses to determine what, if any, changes occurred in neuronal communication in mice that were pre-symptomatic (young mice) versus those who exhibited motor and cognitive symptoms (older mice) that are very reminiscent to those observed in girls with Rett Syndrome. Interestingly, under baseline conditions, both younger and older Mecp2-null mice were indistinguishable from normal littermates. However, when neurons were challenged to respond to repetitive activity (thought to be analogous to learning) while the neurons of younger pre-symptomatic Mecp2-null mice were able to respond like their wild-type littermate control neurons, neurons from older, symptomatic Mecp2-null mice were impaired. Biochemically, we found that there are age-dependent abnormalities in the protein levels of a class of neurotransmitter receptors, the NMDA receptors, which are known to be critical for enabling neurons to change the strength of communication as a function of activity. NMDA receptors have been previously shown to be critical for some forms of learning and memory. The most interesting outcome of these studies however, is that many aspects of

neuronal function are in fact preserved in the Mecp2-null mice, and that subtle and specific impairments in information encoding and storage may underlie myriad of neurological dysfunctions of Rett Syndrome.

## Lay Summary

Neurons communicate with each other at specialized sites of contact called synapses. Activity-dependent changes, or plasticity, of the strength of neuronal signaling play a critical role in shaping developing neural circuits as well as modifying information transmitted and encoded in existing neuronal connections. Neurons throughout the mammalian central nervous system receive the majority of their excitatory synaptic inputs onto small protrusions known as dendritic spines. Rett Syndrome (RTT) is characterized by striking alterations in the organization of dendritic processes and synaptic connectivity that undoubtedly contribute to the devastating cognitive and behavioral deficits. The neuropathology of RTT points to significant deficits in neuronal development, including reduced dendritic complexity and spine densities. Establishing a functional relationship between such morphological changes in neuronal architecture and synaptic transmission or plasticity in animal models of RTT holds the immediate promise of identifying targets for the development of new therapeutic approaches or effective strategies for the amelioration of RTT symptoms. Whether the structural pathologies contribute significantly to the cognitive deficits through changes in synaptic function and plasticity will begin to be directly addressed by experiments in the present proposal. By using cortical neurons in culture prepared from Mecp2-knockout mice, we hope to understand the details of cellular and physiological abnormalities observed in RTT, and importantly, directly evaluate in vitro the potential for restoring neuronal structure and function in neurons lacking Mecp2.

## Abstract

The development of elaborate dendritic arbors is necessary for the formation of necessary synaptic contacts that are the sites of information processing and encoding. Rett Syndrome (RTT) is characterized by marked reductions in the development of axo-dendritic connections thought to result from mutations in the Mecp2 transcription factor. Experiments aimed at whether pathological reorganization of dendritic arborization or aberrations in synaptic structure, composition or stability has significant functional consequences on neurotransmission and plasticity are central to understanding the neurobehavioral phenotype of RTT. We propose to examine the role of Mecp2 in dendritic function, with a focus on the physiological consequences of altered dendritic morphology on synaptic transmission and plasticity. Specifically, cortical neurons cultured from the Mecp2-null mouse model of RTT will be used to directly address the role of Mecp2 on (1) morphological organization of dendritic arbors, (2) the localization of functionally relevant pre- and postsynaptic signaling machinery, and (3) the modulation of synaptic function and activity-dependent plasticity. Simultaneous paired- and triple- whole-cell patch clamp recordings from monosynaptically connected glutamatergic cortical neurons allow us to directly address the cellular mechanisms underlying changes in synaptic efficacy. Fluorescence time-lapse imaging of functionally recycling pools of synaptic vesicle (labeled with the styryl dye FMI-43) and retrospective immunocytochemistry of neurons will be performed. In combining all of these approaches, we are able to thoroughly examine the relationship between morphological changes in neuronal structure, such as alterations in synapse density or dendritic arborization, and synaptic function. A second series of experiments aimed at using in vitro gene transfer methods to re-introduce wild-type Mecp2 to cultured cortical neurons lacking Mecp2 will directly address whether rescue of aberrant morphology or function is possible.

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Michael Greenberg, Ph.D.

Children's Hospital Boston

"Neuronal Activity-Dependent Regulation of MeCP2"

2-Year Award: \$100,000

Research Sponsor: The Massachusetts Rett Syndrome Association

Final Report (November 2005)

Mutation of the MECP2 gene has been found to result in 80-85% of Rett Syndrome (RTT) cases, and the protein encoded by this gene is thought to function to suppress gene activation in neurons. We have found that MeCP2 acts to inhibit expression of the Bdnf gene, which encodes a secreted protein capable of regulating neuronal survival and function. In response to neuronal firing, MeCP2 is modified by phosphorylation and is released from the Bdnf gene. These results suggest that MeCP2 acts to regulate genes induced by neuronal activity, and dysregulation of this function may contribute to RTT disease. However, the sites of phosphorylation on MeCP2 were unknown. To begin to address the role of MeCP2 phosphorylation in both normal development and disease, we have identified a number of novel sites of phosphorylation on the MeCP2 protein. One phosphorylation site was found to be induced in response to neuronal activity. Furthermore, mutation of this site interfered with the normal regulation of the Bdnf gene. These studies have provided an increased understanding of MeCP2 function in neurons, and further study of this regulatory mechanism may ultimately provide new opportunities for the development of RTT therapeutics.

### Lay Summary

Mutation of the MeCP2 gene has been found to result in 80-85% of Rett Syndrome (RTT) cases. The protein encoded by this gene is thought to function to suppress gene activation in neurons, however, as yet, no significant changes in gene expression have been reported in animal models of RTT. We have found that MeCP2 acts to inhibit expression of the BDNF gene, which encodes a secreted protein capable of regulating neuronal survival and function. In response to neuronal firing, MeCP2 is modified by phosphorylation and is released from the BDNF gene. These results suggest that MeCP2 acts to regulate genes induced by neuronal activity, and dysregulation of this function may contribute to RTT disease. To begin to address the role of MeCP2 phosphorylation in both normal development and disease, we propose to identify the sites of phosphorylation on the MeCP2 protein and generate reagents that could be used to monitor this modification both in the developing nervous system and in RTT samples. In addition, we propose experiments to begin to evaluate the effect of these modifications on MeCP2 function. These studies should provide increased understanding of MeCP2 function, give insight into gene expression induced by neuronal activity, and may provide new opportunities for the development of RTT therapeutics.

### Abstract

Mutations in MeCP2, a methyl-CpG-binding protein that functions as a global transcriptional repressor, are a major cause of Rett Syndrome (RTT), an X-linked progressive neurological disorder. While the selective inactivation of MeCP2 in neurons is sufficient to confer a Rett-like phenotype in mice, the specific functions of MeCP2 in postmitotic neurons are not known. We have found that MeCP2 binds to a site in BDNF promoter III just 3' to the site of transcriptional initiation and functions to repress expression of the BDNF promoter III-dependent transcription. These findings indicate that MeCP2 plays a key role in the control of activity-dependent gene expression and suggest that the deregulation of this process may underlie the pathology of RTT. To begin to test this hypothesis, we propose 1) To characterize the sites of calcium-dependent MeCP2 phosphorylation, 2) To develop phosphorylation site-specific antibodies that will recognize the phosphorylated form of MeCP2, and 3) To assess the effect of phosphorylation on MeCP2 activity. These experiments should provide insights into MeCP2 function and may suggest novel therapeutic strategies to alleviate RTT disease.

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Ege T. Kavalali, Ph.D.

University of Texas Southwestern Medical Center at Dallas

"A role for MeCP2 in synaptic function"

2-Year Award: \$99,880

Research Sponsor: Ford Motor Company

Final Report (November 2005)

In humans, mutations in the gene MeCP2 have been found to result in a neurodevelopmental disorder called Rett Syndrome (RTT). While it is believed that MeCP2 functions as a transcriptional repressor, there is currently no direct link between the loss of function in MeCP2 and the pathogenesis of RTT.

Based on the neurological phenotypes seen in RTT, we hypothesized that MeCP2 plays a role in the maintenance and/or regulation of synaptic transmission between central neurons. To examine this, we carried out a number of electrophysiological and imaging experiments on neurons cultured from hippocampal brain regions of MeCP2 knockout mice. Dissociated hippocampal cultures provide an experimentally amenable system to study the basic properties of synaptic communication between neurons. Using electrophysiological recordings, we found a specific decrease in the frequency of spontaneous excitatory synaptic transmission in neurons lacking MeCP2 compared to wild type controls. We also found that the number of functional synaptic vesicles (membranous organelles that contain neurotransmitters) in MeCP2 knockout neurons were similar to wild type controls. We also examined the dynamic properties of synaptic transmission in the MeCP2 knockout cultures by measuring responses to train stimulation and observed a faster synaptic depression and slower response recovery after synaptic depression in the knockout neurons.

We then investigated whether these functional defects can be ascribed to loss of MeCP2's function as a transcriptional repressor. 24 hour treatment with drugs that impair DNA methylation and histone deacetylation produced similar functional changes in wild type neurons but were blocked in neurons lacking MeCP2 suggesting a role for MeCP2 in the control of neurotransmitter release through the regulation of presynaptic genes.

Thus these results show that basic synaptic abnormalities observed with the loss of MeCP2 are due to MeCP2's role as a transcriptional repressor and not an outcome of aberrant neuronal development. We think this is an important finding that argues against the 'neurodevelopmental' characterization of the disorder and instead suggests that MeCP2 acts as a bona fide regulator of synaptic transmission through its transcriptional repressor activity.

## Lay Summary

Rett Syndrome (RTT) is a childhood neurodevelopmental disorder that accounts for one of the leading causes of mental retardation and autistic behavior in females. In general, individuals affected with RTT experience normal development up to the age of 6 ; 18 months at which time affected children then fail to acquire new skills and enter a period of motor skill regression. With time, the RTT defects become more pronounced and include a wide range of neurological defects (mental retardation, autism-like behavior, seizures, disturbances of sleep, problems with gait, decelerated head growth, and stereotypical hand movements). Recent work has demonstrated that RTT is an X-linked dominant disorder that in most instances results from mutations in the Methyl-CpG-binding protein 2 (MeCP2) gene. The MeCP2 gene normally encodes a protein that binds to DNA and represses gene transcription. These mutations are predicted to impair MeCP2 function that then result in genes being turned on in an inappropriate manner. While MeCP2 mutations have been identified in the majority of RTT cases, there is currently no direct link between loss of function of MeCP2 and the pathogenesis of RTT. Studies looking at the brains of RTT patients as well as recent animal models of the disease have not found major neuropathological abnormalities or neuronal loss. This suggests that the MeCP2 mutations are producing subtle alterations in the brain that will have to be studied with functional assays. Our primary goal in this

project is to identify functional deficiencies at the level of individual neurons or their synaptic connections that may lead to symptoms associated with this disorder. Analysis of MeCP2 function is important in identifying neurological deficits. Furthermore, identification of a deficiency in synaptic function may facilitate development of therapeutic approaches for the treatment of RTT.

## Abstract

The major objective of this proposal is to analyze the role of the MeCP2 mutations on maintenance and regulation of synaptic transmission between central neurons. We hypothesize that alterations of MeCP2 function may lead to abnormalities in synaptic transmission due to impairment in formation and/or stability of synaptic connections between neurons. The relationship between MeCP2 alterations and synaptic function may be an indirect effect, due the regulation of expression of synaptic proteins, or a direct effect of MeCP2 in synaptic architecture since it has recently been demonstrated to be localized in the postsynaptic density. We have obtained preliminary evidence suggesting that MeCP2 mutations alter synaptic transmission compared to wild type MeCP2 in primary hippocampal neurons. We have identified alterations in both presynaptic and postsynaptic function. The goal of this project is to extend these observations to elucidate the functional consequences of MeCP2 dysfunction. To achieve this goal we propose three specific aims: 1. Evaluate synaptic transmission deficiencies in neuronal cultures obtained from MeCP2 knockout mice. 2. Study the functional impact of expressed MeCP2 mutations in neuronal cultures obtained from MeCP2 knockout mice. 3. Further characterize synaptic transmission deficiencies in primary 'wild type' hippocampal neurons transfected with either wild type or mutant MeCP2 constructs. There is currently no information on the functional deficiencies at the level of individual neurons or their synaptic connections that may lead to symptoms associated with this disorder. Identification of a deficiency in synaptic function may lead to recognition of drug targets and facilitate development of therapeutic approaches for the treatment of RTT.

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Solomon L. Moshé, M.D.

Albert Einstein College of Medicine

"Clinical investigation of autonomic dysfunction in Rett children under evaluation for epilepsy"

1-Year Award: \$49,173

Research Sponsor: Julian Robertson

Final Report (November 2005)

Rett Syndrome (RTT) results in abnormal regulation of vital functions such as breathing, heart rate and blood pressure. These functions are regulated automatically by a specialized circuits and brain regions known collectively as the autonomic nervous system. RTT is also often associated with seizures and epilepsy. Even in the absence of RTT, seizures can impact on the control of the autonomic nervous system. The autonomic manifestations of seizures in RTT have not been clearly delineated. This question is important because clinical observations suggest that autonomic dysfunction in RTT is associated with disturbances of the cardiac cycle and respiration that increase the risk of sudden death. We suspect that the underlying abnormality of autonomic function associated with RTTS results an increased susceptibility to seizure-induced disruptions of vital functions, and increased the risk of death or impairment in children with RTT.

We studied the impact of seizures on the autonomic nervous system in children with RTT and in relatively homogeneous group of patients without RTT, those temporal lobe epilepsy, with a particular emphasis on the regulation of the heart rate. Patients were recruited from those admitted to an epilepsy monitoring unit for evaluation and management of their seizures.

Our preliminary results suggest that in patients without RTT, seizures can result in transiently increased activity of the autonomic nervous system followed by a more prolonged decrease in autonomic activity that characteristically outlasts the seizure. We have as of yet monitored too few patients with RTT to compare their seizures to patients with temporal lobe epilepsy. We plan to continue monitoring epilepsy patients with and without RTT to better characterize seizure-related autonomic changes, their potential to precipitate hemodynamically significant arrhythmias, and their impact upon respiratory patterns.

## Lay Summary

Rett Syndrome (RS) results in abnormal regulation of vital functions such as breathing, heart rate and blood pressure. RS is also often associated with seizures and epilepsy. Even in the absence of RS, seizures can impact on the control of vital functions. Vital functions are regulated automatically by specialized circuits and brain regions in the nervous system known collectively as the autonomic nervous system. Seizures can disrupt normal function in the autonomic nervous system. In fact, sudden unexpected death in epileptic patients (SUDEP) is strongly suspected to result from seizure induced disruption of vital functions by disrupting activity of the autonomic nervous system.

We suspect that the underlying abnormality of autonomic function associated with RS results in an increased susceptibility to seizure-induced disruptions of vital functions, and increased the risk of death or impairment in children with RS. We propose to study children with RS to determine the effects of seizures on the regulation of vital function as a first step in developing treatments intended to reduce the disability associated with RS.

## Abstract

Rett Syndrome (RS) is associated with epileptiform EEG and seizures and with autonomic nervous system (ANS) disturbances in cardiac cycle and respiration. Seizures produce changes in ANS activity ranging from mild alterations like flushing, sweating and piloerection to severe increases in blood pressure and cardiac arrhythmias. In cases of 'sudden unexpected death in epileptic patients' (SUDEP), it is strongly suspected that seizures cause dangerous autonomic over-activity sufficient to trigger lethal cardiac arrhythmias or respiratory arrest. The effect of seizures on autonomic function may be most significant in individuals or organisms predisposed to autonomic dysfunction, as is the case with RS. For RS children, clinical observations suggest seizure-triggered destabilization of autonomic function may contribute significantly to increased morbidity and mortality. We propose to noninvasively monitor autonomic function (blood pressure, EKG, respiratory rate, skin conductance) and EEG in RS children in order to determine (1) baseline autonomic abnormalities, (2) the evolution of autonomic defects during the diurnal cycle, and (3) the impact of seizures on autonomic function. Improved understanding of the autonomic dysfunction in RS and the impact of seizures on autonomic instability has the potential to identify therapeutic targets to improve morbidity and mortality in RS.

## "Autonomic Consequences of Seizures in a Rett Murine Model"

Pilot Study: \$25,000

We propose a limited set of pilot experiments to determine the feasibility of detailed physiological recordings in adult mice. We propose to conduct a series of pilot studies that progressively increase implanted instrumentation to determine the number and types of implantable devices, and the evolution of the autonomic response following surgery. We will initially implant wild type mice with EEG electrodes consisting of screw electrodes in the skull, and EKG electrodes consisting of wires implanted in the chest wall. Next, we will implant mice with EEG, EKG, and diaphragmatic EMG electrodes. In parallel with the development of the electrode implantation surgery, we will also implant an electronic arterial BP catheter, that will be externalized to a connector sutured to the skin. Finally we adapt the



technique for implantation of the renal nerve electrodes from the procedure in use with the rat to a method suitable for the mouse. We expect that chronically monitoring EEG and EKG signals from MECP2 mice will be readily achievable, since we have achieved chronic EEG recordings from rat pups weighing ~25g. Diaphragmatic EMG recordings, renal nerve recordings, and BP catheter insertion and maintenance have been achieved in adult rats and will be evaluated through a limited pilot study in mice.

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Michael G. Rosenfeld, M.D.

University of California, San Diego

"MeCP2 and CoREST Target Genes in Developing CNS"

2-Year Award: \$99,946

Research Sponsor: The Coca-Cola Company

Final Report (November 2005)

Defining the molecular epigenetic strategies that underlie neurodevelopment is a central problem in contemporary molecular neurobiology, and critical to understanding new degenerative diseases. The ability to effectively perform the analyses required to decipher this code during development in a mouse model system depends, in part, on an approach that can accurately locate all DNA binding factors, factor-modifying enzymes, and all coactivators and corepressors using the limited quantity of tissue that is available. We first successfully generated a human 20,000K array of 40 mer oligonucleotides representing human promoters, for use in a "location" analysis using a new strategy referred to as ChIP-DASL-chip array, and demonstrated the efficacy of the technology works at the 20K primer pair level, providing clear results. We have initially applied the technology to the study of an aspect of transcriptional activation/repression events including the study of the CoREST/StBP complex with the 20K array. Tiling a number of genomic loci have revealed that promoters can be thought of in "cohorts" that use similar but distinct, cofactors/modifications, documenting a diversity of epigenetic behavior in control of specific target genes. Based on the hypothesis that histone modifications are a "code, and consistent with the prediction that the balance between activation and repression is reflected in the methylation status of histone H3, we found that activated promoters can exhibit the presence of tri-Me H3K4 in 90% of the promoters, but it does not determine whether a gene is active. Silenced genes exhibit tri-Me H3K9 on their promoters. Thus, a full 10% of promoters harboring the tri-Me H3K4 mark remain Pol II-negative. This platform has now permitted us to look at CoREST as an associated initial evaluation of demethylase and several methyltransferases. CoREST, which as a corepressor of REST target genes, can also employ MeCP2 in repression events. Our data have provided insights into critical aspects of gene regulation, indicating that components of REST-mediated gene silencing events exert a separate, broader role in modulating activation of gene expression programs revealing the multiple, functionally distinct roles of components of the CoREST/CtBP complex. This link between gene activation/repression events involving a specific demethylase/CoREST complex provides a new way to explore an aspect of MeCP2-dependent events in gene regulation.

## Lay Summary

Rett syndrome, a disorder characterized by severe mental retardation and autistic features has been extensively investigated, with 80% of Retts patients harboring mutations in the MeCP2 gene. We have recently found that MeCP2 can associate with a specific corepressor referred to as CoREST. CoREST is a SANT domain protein that can be recruited to a zinc finger transcriptional repressor referred to as REST/NRSF. We have recently discovered that CoREST is recruited to a subset of REST/regulating genes, and with recruitment of MeCP2 and specific histone methyltransferases, causes silencing of a subset of REST repressed genes. CoREST is highly expressed in the

developing and mature nervous system, associating with other transcriptional factors. A critical issue is to understand the potential gene program modified by MeCP2, and we wish to specifically explore the relationship of this program to that regulated by the CoREST corepressor. To this end we are developing a murine promoter array that will permit a "genome-wide" analysis of gene targets by a technique referred to as chromatin immunoprecipitation on chip analysis (ChIP-on-chip). By conducting this analysis during development of the murine central nervous system, we hope to begin to identify intriguing candidates for more detailed physiological studies, and perhaps provide insights into the abnormalities in Rett Syndrome.

## Abstract

To further understand the molecular basis of Rett Syndrome it is important to formulate the models that account for the role of epigenetics in the developing brain. Our goal is to identify the cohort of MeCP2-regulated genes and linking them to regulation by CoREST, a transcriptional corepressor that interacts with SuVAR and MeCP2, by utilizing an unbiased method to isolate genomic sites directly bound by physiological levels of MeCP2 in living cells. On the premise that MeCP2 has an ability to modify chromatin structure via interaction with components of gene-silencing machinery and the selective brain functions, we propose to identify direct brain specific target genes of MeCP2 and MeCP2-interacting partners, particularly CoREST, using an open-ended screening approach. We will experimentally attack the connection between transcriptional status of MeCP2 and CoREST direct targets and timing of these events in normal brain development. On the basis of obtained information we will further assess the changes in the transcriptional status of these genes in existing models that might underlie the RTT phenotype. This investigation would likely provide novel targets of potential significance for diagnostic, prognostic and therapeutic approaches.

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Gert Jan C. Veenstra, Ph.D.

Nijmegen Center for Molecular Life Sciences, The Netherlands

"MeCP2-dependent epigenetic stability"

2-Year Award: \$100,000

Research Sponsor: Sheila Johnson

Final Report (November 2005)

To design strategies to treat Rett Syndrome (RTT) or to alleviate its symptoms, it is essential to understand why and how mutations in MeCP2 lead to disease. Whereas it is clear that MeCP2 is able to switch off genes, it is not known how this leads to disease, as no major differences in gene regulation have been found in for example the mouse model for RTT. We are testing the possibility that, although most cells regulate genes normally in the absence of MeCP2, the cells may be less stable in the way they commit themselves to switching genes on and off. We are investigating the influence on epigenetic stability (stability in gene regulation) using embryonic cells from *Xenopus laevis*, which is an ideal model system for this type of research question. We have elucidated the genomic structure of MeCP2 in *Xenopus* and determined the precise sequence of two MeCP2 isoforms. Furthermore, we have established a robust antisense knockdown of MeCP2. We will continue to work on MeCP2 and epigenetic stability of cellular differentiation and gene regulation for at least another three years. Our ability to allocate funds to this project is a direct result of the work funded by the RSRF. We will employ the antisense knockdown of MeCP2 in a number of ways. First, we will further examine the contribution of MeCP2 to epigenetic stability of cell fates. Second, we will also use the knockdown-rescue assay to test xMeCP2 mutants in a functional assay. Third, we will examine the developmental regulation of xMeCP2 binding to methylated DNA and the potential involvement of post-translational modifications in this phenomenon.

## Lay Summary

Rett Syndrome is caused, in at least 80% of the cases, by mutations in the MeCP2 gene. To design strategies to treat this debilitating disease and alleviate its symptoms, it is essential to understand why and how mutations in MeCP2 lead to Rett Syndrome. Whereas it is clear that MeCP2 is able to switch off genes, it is not known how this leads to disease, as no major differences in gene regulation have been found in for example the mouse model for Rett Syndrome. We want to test the possibility that although most cells regulate genes normally in the absence of MeCP2 the cells may be less stable in the way they commit themselves to switching genes on and off. Such a reduced epigenetic stability (stability in gene regulation) may cause some individual cells to change too easily, resulting in random, aberrant gene regulation events. This may disrupt neural networks without causing major changes in gene expression in the cell population at large. We propose to investigate this mechanism using embryonic cells from the South African toad *Xenopus laevis*, which is the model system of choice to examine how MeCP2 influences the way cells are induced to adopt different gene expression patterns. In particular we want to determine (1) the extent to which neural cells respond more readily to outside signals in the absence of MeCP2, (2) the extent to which rare, aberrant changes in gene regulation occur in individual cells in the presence or absence of MeCP2, and (3) the extent to which MeCP2-dependent defects in gene regulation stability can be suppressed by other molecules, which is relevant to other drug screening efforts. We expect that the proposed research will shed more light on how mutations in MeCP2 cause Rett Syndrome, and will open a new avenue to screen drugs for their ability to suppress defects in epigenetic stability in Rett Syndrome patients.

## Abstract

Rett Syndrome is a debilitating X chromosome-linked disease that involves defects in brain maturation, which is caused, in at least 80% of the cases, by mutations in the MeCP2 gene. To design therapeutic strategies, it is essential to understand why and how mutations in MeCP2 lead to Rett Syndrome. While it is clear that MeCP2 is a powerful transcriptional repressor, it is not known how a lack of gene repression by MeCP2 leads to a neural phenotype, as microarray gene expression profiling experiments have revealed no major transcriptional defects in the brains of the mouse model for Rett Syndrome. We want to determine the extent to which MeCP2 contributes to the epigenetic stability of gene expression patterns. A reduced epigenetic stability may cause individual cells to respond to extracellular signals inappropriately, resulting in stochastic, aberrant induction events. This may disrupt neural networks without causing major changes in gene expression in the cell population at large. We propose to test this hypothesis using *Xenopus* animal cap cells, which is a very powerful embryo-derived model system to look at induction events. Using antisense technology, we want to test (1) the extent to which neural cells respond more readily to extracellular signals in the absence of MeCP2, (2) the extent to which ectopic and aberrant induction events occur in individual cells in the presence or absence of functional MeCP2, and (3) the extent to which MeCP2-dependent defects in epigenetic stability can be suppressed or enhanced by chemical compounds or through overexpression of other proteins. We expect that the proposed research will shed more light on Rett Syndrome pathogenesis and will open a new avenue to screen drugs for their ability to suppress defects in MeCP2-dependent epigenetic stability in Rett Syndrome patients.

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Christopher L. Woodcock, Ph.D.

University of Massachusetts, Amherst

"3D Conformation of Chromatin-MeCP2Complexes"

2-Year Award: \$90,000

Research Sponsor: Provident Bank

Final Report (November 2005)

We know that most cases of Rett Syndrome are caused by a defect in the MeCP2 protein that can be as small as a single amino acid change, resulting in interference with proper binding of the protein to methylated DNA. In living cells, MeCP2 encounters not naked DNA but the DNA-protein complex known as chromatin, and we have found that MeCP2 induces dramatic increases in chromatin compaction which may well be connected with its in vivo function. Our work has focused on the details of the interaction between MeCP2 and chromatin, especially the architecture of the compact state and the molecular interactions that lead to this compaction.

Our analyses have included both biochemical and structural measurements of the interactions between purified recombinant human MeCP2 (and its key mutations) and defined chromatin arrays using direct imaging by electron microscopy in combination with molecular tools, has produced the following major results:

1. Like most proteins that bind specifically to DNA, MeCP2 also binds non-specifically (in this case to unmethylated DNA and chromatin containing unmethylated DNA). This property is thought to be advantageous in vivo since it speeds the protein to its target methylation sites. In our experiments, an excess of unmethylated competitor DNA enables us to observe specific binding to the target methylated DNA and chromatin.
2. Under these conditions, there is, as predicted, no interaction between DNA and MeCP2 carrying mutations that disable the domain that binds to methylated DNA (MBD) and cause the most severe RTT symptoms. However, with chromatin we observe three levels of interaction. The first two levels occur whether or not the DNA is methylated. The weakest is observed with MeCP2 mutations that disrupt the MBD and cause the most severe RETT symptoms. The next level is also non methylation specific and occurs with normal MeCP2. As expected, the third and most pronounced interaction occurs between normal MeCP2 and methylated chromatin. All three interaction levels result in chromatin compaction, and imaging experiments to define the changes in chromatin architecture that accompany each level are in progress. These findings indicate a new and unexpected role of the MBD in chromatin binding and compaction which may have important implications for MeCP2 function in normal and RTT patients.

## Lay Summary

The majority of Rett Syndrome (RTT) cases can be traced to defects in a single protein, MeCP2. This protein is known to be involved in gene silencing, and there is strong evidence for a multiple step process that results in regions of the genome becoming compact and inaccessible to the machinery that allows genes to be expressed. Our preliminary work has led to the hypothesis that MeCP2 binding directly induces an immediate and dramatic increase in compaction of genes, which in itself is likely to prevent gene expression. If this hypothesis is correct, then MeCP2 has an additional mechanism of silencing genes, by directly altering the 'architecture' of the genetic material to which it binds.

Evaluating this hypothesis will include determining the precise mechanism by which MeCP2 binds to and compacts defined chromatin arrays (chromatin is the DNA-protein complex that is the native in vivo substrate for MeCP2). We will also examine any changes in the ability of the most common RTT MeCP2 mutants to compact chromatin. A unique feature of this proposal, and the preliminary work that led to it, is the use of direct electron microscopy (EM) imaging to determine the 3D conformation of individual chromatin arrays. For examining chromatin conformation, direct imaging often provides more insight into architectural changes than biophysical and biochemical techniques.

A detailed understanding of the effects of MeCP2 and MeCP2 mutants on chromatin in fully defined conditions will be essential for generating and evaluating effective therapies for RTT.

## Abstract

Preliminary work has produced the surprising and potentially important finding that the binding of MeCP2 to defined chromatin arrays induces a dramatic change in 3D conformation, resulting in a marked increase in compaction. Unlike the chromatin compaction induced by histone H1, which is highly dependent on salt concentration and produces a zig-zag conformation, MeCP2 compaction is independent of salt concentration, and proceeds through edge-to-edge contact between nucleosomes. Further, MeCP2 compaction appears not to require DNA methylation.

Previous work by others has demonstrated that the gene silencing function of MeCP2 can occur by the recruitment of histone deacetylase (HDAC) complexes, followed by hypoacetylation of core histones leading to the generation of local areas of 'heterochromatin'. The new findings suggest a dual mechanism of heterochromatin formation by MeCP2, one is a direct consequence of MeCP2 binding to chromatin, the other indirect via HDAC recruitment.

This proposal is aimed at understanding the molecular mechanism(s) by which MeCP2 compacts chromatin, and the impact of MeCP2 mutants known to be important to Rett Syndrome (RTT). A primary technique to be used in the proposed research is direct imaging by electron microscopy (EM), complemented by appropriate biochemical and biophysical tools. EM has the advantage over 'bulk' techniques that the 3D conformation of chromatin is accessible, including locations and orientations of nucleosomes in individual chromatin arrays.

Specific aims include: determining which domain(s) of MeCP2 contribute to its ability to compact chromatin; determining the relative contributions of nucleosomal and linker DNA in MeCP2 binding; and, in view of the report that MeCP2 can displace MeCP2 from chromatin, determining whether H1 and MeCP2 share the same or overlapping binding sites on chromatin.

The results will provide fundamental new information of the interactions of MeCP2 with chromatin, information that can be drawn on as potential therapies for RTT are formulated and evaluated.

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Jerry Yu, Ph.D.

University of Louisville

"Pulmonary Reflexes in Rett Syndrome"

2-Year Award: \$100,000

Research Sponsor: Robert C. & Adele R. Schiff Foundation

Final Report (November 2005)

Supported by the Foundation, we have made significant progress in testing the hypothesis that one or more types of airway sensory receptors may be defective in Rett Syndrome patients and this defect may account for the abnormal breathing pattern. To achieve the goal, we first established techniques for assessing single unit activities in four different types of airway receptors [slowly adapting receptors (SARs), rapidly adapting receptors, C-fiber receptors, and

high threshold A delta fiber receptors]. We also established a way to assess airway reflex sensitivity, including the Hering-Breuer reflex and the pulmonary chemoreflex. Except for SARs, no other airway sensory receptor has been recorded in mice. Thus, establishment of this technique was essential for the investigation of whether airway receptors are defective in RTT mice. Our studies demonstrate that mice possess all four types of airway sensors and reflex effects similar to those described in larger species. Our preliminary data also indicate that strength of the Hering-Breuer reflex is reduced in spontaneous breathing RTT mice. In anesthetized, open chest, mechanically ventilated mice, we recorded single unit activity from the SARs at different levels of constant pressure lung inflation, and showed that the receptor stimulus response curve (SARs activity plotted against airway pressure) shifted to the right in RTT mice. In addition, many SARs in RTT mice were inactive at the airway inflation pressure of 10 cm H<sub>2</sub>O, contrasting with SARs in normal mice, which were all active at a pressure of 10 cm H<sub>2</sub>O. Clearly, there is an increase in activation threshold of SARs in the RTT mouse model, suggesting there is a functional defect in this receptor. Such receptor behavior explains the less effective Hering-Breuer reflex observed in spontaneously breathing mice. It also can account for the slow deep breathing pattern observed in anesthetized RTT mice.

## Lay Summary

Rett Syndrome is caused by mutations in the MeCP2 gene, producing neurodevelopment problems in young females. Those with the disease develop mental retardation, loss of motor skills, and profound abnormalities in breathing. Optimal breathing control relies on a variety of sensory information. Feedback arising from the lung, transmitted by afferents in the vagus nerves, is probably one of the most important determinants of breathing performance. There are four parallel vagal afferent pathways originated in the lung that influence the breathing control system. Activating or inhibiting each one of the pathways will alter breathing with various patterns. It is clear that respiratory control is under significant influence of vagal afferents, and the abnormal respiratory pattern observed in Rett patients might be explained by alteration of the vagal afferent control system. Using newly developed, state-of-art electrophysiological, immunohistochemical and morphological techniques, we will examine this possibility. We will determine which of the four respiratory control pathways are out of order functionally in the Mecp2 knock out mouse (a model that resembles the Rett patient). If a functional abnormality is identified, we will examine whether the sensory receptor is abnormal structurally. The studies will provide important information to understand the pathophysiology of the abnormal breathing in Rett Syndrome.

## Abstract

Rett Syndrome (RTT) is a progressive neurodevelopmental and X-linked dominant mutation disorder that causes abnormal breathing pattern, such as intermittent hyperventilation, apnea, and air swallowing. Breathing critically depends on sensory feedbacks for optimal control. The sensory information from the lung, transmitted in the vagal afferents, provides one of the most important feedbacks. There are four types of sensory receptors in the lung: slowly adapting receptors (SARs), rapidly adapting receptors (RARs), C-fiber receptors (CFRs), and high threshold A $\delta$  fiber receptors (HT-AFRs). Stimulation of SARs produces breath-holding and apnea. Activating RARs augments breathing. CFR activation produces apnea followed by rapid shallow breathing. Stimulating HT-AFRs evokes hyperpnea and tachypnea. Thus, it is hypothesized that the abnormal respiratory pattern observed in RTT is partly due to alteration of the vagal afferent control system. Using electrophysiological techniques we will determine whether the reflex system initiated from each of the four sensory receptors is defective in the Mecp2 knock out mouse (a model that resembles the Rett patient). If a functional abnormality is identified, we will further examine whether the sensory receptor is abnormal structurally by use of neural tracing, immunochemistry and confocal microscopy. The results will help elucidate underlying neural mechanisms for abnormal breathing in RTT.

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Xinyu Zhao, Ph.D.

University of New Mexico

Fred H. Gage, Ph.D.

Salk Institute

"Function of MeCP2 in postnatal neurogenesis and neuroprogenitor cell biology"

2-Year Award - \$99,987

Research Sponsor: Anonymous

Final Report (November 2005)

Rett syndrome is primarily caused by mutation of MeCP2, a methyl-CpG binding protein (MBD) that binds to methylated DNA in the genome and facilitates transcriptional repression. Using a mouse knockout model, we analyzed the role of MeCP2 in neurogenesis during postnatal development, a period comparable to the onset of symptoms in human Rett patients. Though we found that MeCP2 was not critical for the generation of new neurons, both in culture and in mammalian brains, MeCP2 mutant mice displayed abnormal presynaptic protein expression and distribution, and had a smaller hippocampus. To determine whether MeCP2 was critical for postnatal neuronal maturation, we grafted GFP-expressing retrovirus into the hippocampus of 4-week-old mice to label dividing neural stem and progenitor cells, and analyzed the new neurons at 4-weeks post-infection. We found that new neurons generated in postnatal MeCP2 mutant mice had reduced dendritic spine densities and increased variations in spine distribution compared to wild type mice, suggesting deficits in neuronal maturation. Our work demonstrates that MeCP2 is not critical for generating new neurons in postnatal brains but may be essential for their maturation. Exploring the role of MeCP2 in the maturation and maintenance of new neurons in postnatal brain is a crucial step towards understanding the etiology of Rett Syndrome.

### Lay Summary

MeCP2 belongs to a family of methyl-CpG binding proteins (MBDs) that, by binding to the methylated-CpG in the genome, regulate gene expression. Currently, no clear mechanism for the neurological deficits in MeCP2-deficient (MeCP2 KO) mice and humans (Rett Syndrome) have been found. We have recently demonstrated that one of the MBDs, MBD1, plays an important role in neurogenesis and neural stem cell function. Since MeCP2 deletion results in postnatal neurological deficits, we would like to determine whether the MeCP2 KO mice have deficits in postnatal neurogenesis and neuroprogenitor cell function similar to those we have discovered in MBD1 mice. Such studies will

help to elucidate the mechanism underlying the neurological deficits in Rett Syndrome, and help to unravel the biological functions of DNA methylation and MBDs in neural system. We propose to analyze postnatal neurogenesis in MeCP2 KO mice. We will also isolate and determine if MeCP2 KO neuroprogenitor cells have reduced neurogenesis and increased genomic instability as seen in MBD1 cells. Furthermore, to find out the molecular basis for the neurological deficits in MeCP2 KO mice, we will determine which genes are expressed differentially between normal and MeCP2 KO neuroprogenitor cells and neurons. Finally, we are generating MeCP2 and MBD1 double knockout mice to determine if MBD1 can functionally compensate MeCP2 in mice brain.

## Abstract

MeCP2 belongs to a family of methyl-CpG binding proteins (MBDs) that, by binding to the methylated-CpG in the genome, regulates gene expression. The functions of MBDs are not well understood but are currently being intensively investigated. We have recently demonstrated that one of the MBDs, MBD1, plays an important role in neurogenesis and neuroprogenitor cell function. Currently, no clear mechanism for the neurological deficits in MeCP2-deficient (MeCP2 KO) mice and humans (Rett Syndrome) have been found. We would like to determine whether the MeCP2 KO mice have deficits in postnatal neurogenesis and neuroprogenitor cell function similar to those we have discovered in MBD1 mice. Such studies will help to elucidate the mechanism underlying the neurological deficits in Rett Syndrome, and help to unravel the biological functions of DNA methylation and MBDs in neural system. We propose to first assess postnatal neurogenesis of MeCP2 KO mice housed in both conventional cages and in enriched environment. We will also isolate neuroprogenitor cells from MeCP2 KO mice and determine their proliferation and neural differentiation capacity, and their genomic stability. If we have found differences, we will analyze the gene expression profile of MeCP2 KO neuroprogenitor cells. In addition, because MeCP2 is highly expressed in postmitotic neurons, we will microdissect out the neurons from MeCP2 KO mice brain and analyze their gene expression profiles to search for a molecular basis for the neurological deficits. Finally we are generating MeCP2 and MBD1 double knockout mice to determine if there is functional compensation between these two MBDs in the nervous system.

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J. Julius Zhu, Ph.D.

University of Virginia

"Aberrant synaptic AMPA-R trafficking in MECP2 knockout mice"

2-Year Award: \$100,000

Research Sponsor: Drs. A. Bruce and Daphne Thomas

Final Report (November 2005)

Patients with RTT often have difficulty in remembering, talking and behaving appropriately. It is generally believed that the brain learns, remembers, and executes many other cognitive behaviors by controlling the strength of synapses - the connections between neurons. The synaptic strength can be changed due to the movement in and out of synapses of a small number of proteins known as AMPA receptor proteins. Mutations in the MeCP2 protein were recently identified as the primary cause of RTT. But do mutations of MeCP2 cause the aberrant AMPA receptor trafficking and defects in synaptic function? It is now clear that the genetic defects of signaling molecules in small GTPases Ras and Rap signaling pathways can lead to severe mental retardation. Our preliminary results indicate that Ras signaling, which controls synaptic delivery of AMPA receptors, is impaired in Mecp2 knockout mice. Our recent results suggest that impaired synaptic trafficking of AMPA receptors is due primarily to the reduced expression of Ras in Mecp2 knockout mice. We are currently confirming this notion with a variety of experimental approaches combining a number of



electrophysiology, imaging, genetics, biochemistry, cell and molecular biology techniques. Identifying the signaling pathway that causes aberrant synaptic function in Mecp2 knockout mice should suggest concrete molecular targets that novel pharmacological and genetic therapies may be developed to treat patients with RTT more efficaciously.

## Lay Summary

Patients with Rett Syndrome often have difficulty in remembering, talking and behaving appropriately. It is generally believed that the brain learns, remembers and executes many other cognitive behaviors by controlling the strength of synapses, the connections between neurons. The synaptic strength can be changed due to the movement in and out of synapses of a small number of proteins known as AMPA receptor proteins. Mutations in the MECP2 gene were recently identified as the primary causes of Rett Syndrome. But do mutations of MECP2 cause the aberrant AMPA receptor trafficking and defects in synaptic function? It is now clear that the genetic defects of signaling molecules in small GTPases Ras and Rap signaling pathways can lead to severe mental retardation. Our preliminary results indicate that Ras and Rap signaling, which controls AMPA receptor trafficking at synapses, is impaired in MECP2 knockout mice. Here we propose to test the hypothesis that mutations of MECP2 cause misregulation of the expression of molecules involved in Ras and Rap signaling pathways. In this proposal, we plan to characterize the aberrant Ras and Rap signaling in MECP2 knockout mice. In addition, we will examine whether expressing recombinant Ras and Rap mutants can restore normal AMPA-R trafficking in these animals. Our hypothesis will be tested using several state-of-the-art techniques, including recombinant DNA delivery, multiple whole-cell recordings and two photon laser scanning microscopy. It is expected that understanding the aberrant signaling in MECP2 knockout mice should suggest novel efficacious pharmacological and genetic therapies for patients with Rett Syndrome.

## Abstract

Synaptic trafficking of glutamate receptors plays a key role in regulating synaptic plasticity and the process may underlie cognitive behaviors, such as learning and memory. It is believed that disrupting glutamate receptor trafficking at synapses may lead to mental disorders, which are prominent in patients with Rett Syndrome. However, whether glutamate receptor trafficking is altered in these patients is still unclear. Our recent studies indicate that small GTPases Ras and Rap control synaptic delivery and removal of AMPA-sensitive glutamate receptors respectively. Our preliminary results have shown that Ras and/or Rap signaling is impaired in MECP2 knockout mice. Thus, we decided to investigate whether Ras- and Rap-regulated AMPA receptor trafficking is aberrant in MECP2 knockout animals. Our central hypothesis is that mutations of MECP2 cause misregulation of the expression of molecules involved in Ras and Rap signaling pathways. In this proposal, we plan to characterize the aberrant Ras and Rap signaling in MECP2 knockout mice. In addition, we will test whether expressing recombinant Ras and Rap mutants can restore normal AMPA-R trafficking and synaptic plasticity in these animals. The findings from this project should suggest the appropriate molecular targets to which novel pharmacological and genetic therapies can be designed to efficaciously treat patients with Rett Syndrome.

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## Post-Doctoral Fellowship Awards

Shaun Cowley, Ph.D.

Fred Hutchinson Cancer Research Center

Mentor: Robert N. Eisenman, Ph.D.

## "Determining the role of the Co-repressor mSin3A in Mecp2 function"

2-Year Award: \$100,000

Recipient of the Alan P. Wolffe Memorial Fellowship

Research Sponsor: Adam (Buddha) Lavey / Reading Rock, Inc.

Final Report (November 2005)

Mecp2, the gene most commonly mutated in Rett syndrome patients, is thought to function by inactivating other genes. It is aided in this role by the recruitment of a second gene product called mSin3A. Over the past two years with support from the RSRF I have been investigating the many roles of mSin3A.

The main job of mSin3A is to serve as a bridge between 'repressor' proteins which bind to DNA and help switch genes off, and enzymes called HDACs, which actually provide the silencing activity. In order to do this mSin3A has a number of docking sites known as 'PAH domains' that function as molecular 'Velcro'. Repressors, such as mec2, are able to bind directly to mSin3A via these PAH domains and recruit it to DNA thus allowing the associated HDACs to switch genes off. As part of a collaborative team I was able to determine the first molecular structure, an actual picture, of a 'PAH domain' bound to a repressor. Using this detailed picture I was also able to distinguish which parts of the PAH domain are required to bind other proteins and how the specificity of such interactions are maintained. We are hopeful that this in-depth study will provide a useful example in trying to understand the many thousands of protein-protein interactions that occur within the cell nucleus.

In addition to this test-tube analysis of mSin3A function, we have also generated a mutant mouse lacking the mSin3A gene to better understand its biological role. Analysis of our mSin3A knock-out mouse model has demonstrated a requirement for mSin3A in the developing embryo and for the development of many other tissues including the cells of the immune system. A similar mutant mouse lacking the Mecp2 gene has been demonstrated to mimic many of the symptoms of patients with Rett syndrome, such as tremors and decreased physical ability. As part of an on-going study we are breeding heterozygous mSin3A mice (mice with only one functional copy of the gene) with Mecp2 knock-out animals and analyzing their off-spring. If mSin3A activity is important for Mecp2 function we might expect mice lacking both Mecp2 and one copy of mSin3A to have an increased severity of their neurological symptoms.

### Lay Summary

Rett Syndrome (RTT) is a devastating neurological disorder that effects predominantly young girls, depriving them of motor functions and basic communication skills. Most RTT cases are associated with mutations in a gene called MeCP2, which functions by restricting the activity of many other genes. Thus, the mutation of this single gene within young girls can have an adverse effect on many other genes, resulting in RTT. It has been proposed that for MeCP2 to reduce gene activity it must physically interact with another gene product called mSin3A. I will assess the importance of mSin3A for Mecp2 function by cross breeding mice with reduced levels of mSin3A to mice deficient for Mecp2. Mice, which lack Mecp2, exhibit many of the symptoms associated with human RTT patients, such as tremors and decreased physical ability. I will examine mutant mice that lack both Mecp2 and Sin3A for increased severity of RTT like symptoms. As a complementary strategy to assess the role of mSin3A in Mecp2 function I will use cells taken from mSin3A deficient mice and grow them in culture. If the ability of Mecp2 to inhibit gene activity is dependent upon mSin3A then I predict this will be lost in cells lacking mSin3A. Utilizing our knowledge of normal Mecp2 and the mutated variants found in RTT patients, I can test for a correlation between the ability of Mecp2 to cooperate with mSin3A physically, in the test tube, with its function in both normal and mSin3A deficient cells.

### Abstract

Rett Syndrome (RTT) is caused by mutations in a protein called Mecp2, a methylated-DNA binding transcriptional repressor. Consistent with this role, Mecp2 has been shown to recruit the co-repressor mSin3A and associated histone

deacetylases. However, it remains to be demonstrated how important the integrity of the mSin3A co-repressor complex is for gene silencing by Mecp2 in vivo. To address this question I will use two related approaches that take advantage of the mSin3A conditional knock-out (cKO) mice and cells I have generated. Initially, I will cross heterozygous null mSin3A and Mecp2 mice to test for a genetic interaction. To test for a functional overlap I propose to cross mSin3A heterozygotes with hypomorphic and heterozygous Mecp2 mice. By reducing the titer of mSin3A in mice with attenuated Mecp2, which develop RTT-like symptoms, I anticipate an increase in severity or acceleration of their phenotype. I plan to examine the mice for differences in their physical attributes such as brain size, ataxia, nocturnal activity and weight. Because the effects of mutated Mecp2 manifest themselves in the nervous system I will also attenuate mSin3A specifically, to study its role in postmitotic and developing neurons, using CaMKIIa and Nestin-Cre transgenic mice respectively. In a second approach, I intend to analyze the ability of wild-type Mecp2 and a number of clinically relevant mutants to repress transcription in wild-type and mSin3A null cells. If Mecp2 transcriptional repression is dependent, at least in part, upon mSin3A I would expect to observe a reduction in activity. By better understanding Mecp2 function and the nature of RTT associated mutations we can begin to design rational therapies for the prevention and cure of RTT.

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Amit Mehta, Ph.D.

Stanford University

Mentor: Mark Schnitzer, Ph.D.

"Minimally invasive in vivo imaging of hippocampal neurons"

3-year Award: \$153,000

The Rett Syndrome Research Foundation Fellow of the Life Science Research Foundation

Lay Progress Report (August, 2004)

At the beginning of this year, my colleagues and I moved from Lucent Bell Laboratories to the Clark Center at Stanford University. We have since reassembled our instruments, one-photon and two-photon fluorescence microscopes that can be fitted with micro-optic probes.

As of the 2003 RSRF meeting, I had obtained images deep within live mammals. Brain tissue movement due to respiration and circulation limited the exposure times, preventing longer exposures due to blurring. To address this, I designed and constructed a feed-forward device which uses low-coherence interferometry to detect fine movements of the brain surface. This information will be used to move a macroscopic objective lens such as to keep a constant brain section in focus although the tissue continues to move. This project is now in its late stages of testing.

In parallel with instrumentation development, I have been working towards imaging studies of neuronal activity. Such studies require activity-sensitive fluorescent probes and electrophysiological measurements of individual cells. In order to integrate these approaches with our imaging technology, I have worked in parallel on two tracks. First, I have become proficient with the use of sharp microelectrodes to record single-unit activity in the cerebellum. To further hone this expertise and to become proficient in patch electrode recordings, I will be spending several weeks in the lab of my collaborator, Nigel Emptage, at Oxford University. Second, I have worked towards use of pressure injections of Calcium-sensitive dyes into Cerebellar neurons. To date, we have imaged small populations of putative granule cells using two-

photon microscopy.

## Lay Summary

Rett Syndrome (RTT) is a devastating neurological disorder that effects predominantly young girls, depriving them of motor functions and basic communication skills. Most RTT cases are associated with mutations in a gene called MeCP2, which functions by restricting the activity of many other genes. Thus, the mutation of this single gene within young girls can have an adverse effect on many other genes, resulting in RTT. It has been proposed that for MeCP2 to reduce gene activity it must physically interact with another gene product called mSin3A. I will assess the importance of mSin3A for Mecp2 function by cross breeding mice with reduced levels of mSin3A to mice deficient for Mecp2. Mice, which lack Mecp2, exhibit many of the symptoms associated with human RTT patients, such as tremors and decreased physical ability. I will examine mutant mice that lack both Mecp2 and Sin3A for increased severity of RTT like symptoms. As a complementary strategy to assess the role of mSin3A in Mecp2 function I will use cells taken from mSin3A deficient mice and grow them in culture. If the ability of Mecp2 to inhibit gene activity is dependent upon mSin3A then I predict this will be lost in cells lacking mSin3A. Utilizing our knowledge of normal Mecp2 and the mutated variants found in RTT patients, I can test for a correlation between the ability of Mecp2 to cooperate with mSin3A physically, in the test tube, with its function in both normal and mSin3A deficient cells.

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## Miscellaneous Awards

Hannah Kinney, M.D.

Harvard Medical School

"Serotonin in the Rett Medulla"

1-Year Award: \$50,000

Research Sponsor: Family and Friends of Lauren Stevens

### Lay Summary

The Rett child and adult frequently exhibit irregular patterns of breathing which appear to cause discomfort and which may, in some cases, be dangerous. The control of breathing requires a complex interaction of neurons in the brainstem. This interaction is mediated by chemicals that are produced in specific neurons in the brain. Serotonin is one of these chemicals. Its action by appropriate neurons requires enzymes, transporters and receptors in appropriate groups of neurons within the respiratory control circuit. The chemical mediators in the brain, such as serotonin, have potent and specific effects on brain function. Their effectiveness depends upon being produced in appropriate neurons at the correct times. Regulatory proteins, such as MeCP2, ultimately control the expression of the chemical mediators in specific cells. It is known that MeCP2 is deficient in some neurons in the Rett brain. In this study we hypothesize that there is an abnormality of serotonin synthesis, transport and/or receptors in neurons involved in respiratory control, and that these neurons are also deficient in MeCP2. If a specific deficiency in the serotonin is identified, specific drugs to normalize breathing could be designed, thereby improving the quality of life of patients and potentially reducing their risk for sudden death.

### Abstract

We hypothesize that MeCP2 deficiency influences serotonin's role in the control of respiration causing breathing irregularities in Rett Syndrome (RTT). Using autoradiography applied to frozen brainstems from RTT and control autopsies, we will determine the level of expression of serotonin receptors and transporters in the caudal raphe nucleus and its major projection sites to brainstem nuclei involved in respiratory control. Using immunocytochemistry the same respiratory-related nuclei will be examined for tryptophan hydroxylase (the enzyme required for serotonin synthesis) and for MeCP2, the regulator protein that is deficient in RTT. The identification of specific abnormalities in serotonin synthesis, uptake and/or receptor binding may help determine strategies for correction of the breathing irregularities in RTT.

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Michael Friez, Ph.D.

Greenwood Genetic Center

Investigating MLPA technology to screen for exonic deletions in MECP2 negative cases

\$10,000

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Tony Charman, Ph.D.

Institute of Child Health, London, UK

"A Detailed Genotype-Phenotype Correlation Study in Rett Syndrome"

2-Year Award: \$99,437

Research Sponsor: Family Friends of Jessie Lebson

#### Lay Progress Report (August, 2004)

At the beginning of this year, my colleagues and I moved from Lucent Bell Laboratories to the Clark Center at Stanford University. We have since reassembled our instruments, one-photon and two-photon fluorescence microscopes that can be fitted with micro-optic probes.

As of the 2003 RSRF meeting, I had obtained images deep within live mammals. Brain tissue movement due to respiration and circulation limited the exposure times, preventing longer exposures due to blurring. To address this, I designed and constructed a feed-forward device which uses low-coherence interferometry to detect fine movements of the brain surface. This information will be used to move a macroscopic objective lens such as to keep a constant brain section in focus although the tissue continues to move. This project is now in its late stages of testing.

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#### Lay Summary

Research into Rett Syndrome, a disorder that profoundly affects brain development in females, moved into a new era with the recent discovery that up to 80% of cases are caused by mutations in a specific gene (MECP2). The extent to which the presence or absence of a mutation in this gene, or the precise nature of the mutation, affect the natural history of the clinical disorder is not yet clear. We aim to address this question by carrying out mutation detection screens in 3 large groups of Rett Syndrome females in whom the clinical presentation has been detailed with greater precision than has been the case in previous studies. We hope that the findings will contribute to our understanding of the pathology that underlies Rett Syndrome (holding out the possibility for gene therapeutic strategies in the future). However they will also inform clinical decision making by improving diagnostic accuracy and advice on prognosis and likely outcome as well as choosing the most efficacious strategies for each individual patient.

#### Abstract

At least 80% of classical cases of Rett Syndrome are caused by mutations in the gene encoding MECP2. This protein binds to methylated CpG dinucleotides in DNA and probably functions in the control of transcription competence of numerous chromosomal loci. The effect of mutations in MECP2 on the function of the protein are beginning to emerge, but the extent to which the presence or absence of an MECP2 mutation, or the precise nature of the mutations, affect the natural history of the clinical disorder is not yet clear. Previous studies that have attempted to identify genotype-phenotype correlations have been limited by low sample sizes and poor characterization of the physical, developmental

and behavioral phenotype. Few consistent associations have thus far emerged. We aim to address this question by carrying out mutation detection screen in MECP2 and measuring X-inactivation ratios, in 3 large cohorts of Rett Syndrome patients. Using a combination of detailed systematic clinical assessment information and parental questionnaire information (including information from a newly-developed questionnaire with proven ability to discriminate between typical Rett Syndrome behaviors and those found in other individuals with severe and profound developmental disability) we will be able to characterize the phenotype more accurately than has been previously achieved. In addition the size of the cohorts available (N=100 with clinical assessment information, N=300 with questionnaire information) and the number of classic (N=220) and atypical/variant (N=80) cases will allow for a stronger test of genotype-phenotype associations than in previous studies. The identification of phenotypic differences between patients with different mutations will aid in the development of models of the neuropathological processes that occur in the disorder (holding out the possibility for gene therapeutic strategies in the future). It will also aid clinicians now to improve diagnostic and prognostic accuracy, and decide on the best therapeutic strategy for each individual patient.

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"Molecular Controls Over Differentiation of Cortical Projection Neurons from Neural Precursors: Normal and MECP2 -/-"

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Rett syndrome is a disease of brain development that causes mental retardation and autistic behavior in girls (one in every 10,000 to 15,000 girls), as well as in a small group of boys. Recent research has revealed that a defect in a gene called the methyl-CpG-binding protein 2 (MECP2), which encodes a protein that suppresses expression of other genes (transcriptional repressor), causes Rett syndrome. MECP2 mutant mice display several symptoms that are similar to those of Rett patients. Although it is evident that the symptoms of these mutant mice are attributable to the lack of the MECP2 gene in the brain, we know little about how the MECP2 gene works in the brain, and why mutation of the MECP2 gene causes predominantly neurological symptoms, despite the fact that the MECP2 gene is expressed throughout the entire body.

My work has added invaluable insights in the role of MECP2 in nerve cell maturation in the brain. I investigated how mutation in MECP2 gene may cause the symptoms of Rett syndrome using MECP2 genetically modified mice which lack MECP2 gene. First, I investigated which cells express MECP2 in the brain, using mouse brains and cells grown in dishes in the laboratory. I found that MECP2 is expressed in nerve cells (neurons), not in glia. Furthermore, my studies show that MECP2 is expressed in more mature neurons rather than in immature moving neuronal precursors, indicating that MECP2 is involved in the maturation and maintenance of neurons.

Although my data strongly indicate that MECP2 is involved in the maturation and maintenance of neurons, MECP2 has also been detected at low levels during embryonic development, and experiments using frog eggs showed that MECP2 controls production of neurons. However, the idea that MECP2 controls cell fate decisions does not mesh with the symptoms of Rett syndrome, because no report shows that there is decreased number of neurons in the patients. To

investigate whether MECP2 is involved in events of initial neural development in mammals, I developed a mouse neural progenitor/stem cell culture system that can investigate these events. My results, using cells from MECP2 mutant mice, indicate that MECP2 mutation does not affect initial neural development events in mice, suggesting that MECP2 plays a different role in mammals (including humans) than it does in frogs.

I next investigated the thickness of the high level brain area called the cerebral cortex (or neocortex) in MECP2 mutant mice. I measured the thickness of the neocortex, which is composed of six layers and is responsible for high level cognitive functions. There is significant reduction of thickness in the neocortex of MECP2 mutant brains. Why do MECP2 mutant brains appear to have a thinner cortex? Two possibilities are 1) loss of neurons; and 2) reduced size and/or complexity of neurons. When I measured the cell density in each layer of each genotype, the cell densities of layers II/III, IV, V, and VI in MECP2 mutant mice are significantly higher than those in wild-type mice, suggesting that the reduced thickness of cortex in MECP2 mutant mice is due to reduced size of neurons, rather than to loss of neurons. In agreement with this hypothesis, I performed direct cellular analysis showing that pyramidal neurons in layer II/III in MECP2 mutant mice are smaller and their dendrites are less complex than those in wild-type mice.

In addition, I am finalizing neuronal transplantation experiments to further investigate potential abnormalities of both cell size and amount/quality of branches they send (dendritic complexity) by neurons of MECP2-null mice. By transplanting MECP2 mutant neuroblasts genetically labeled with a special genetic green color (GFP fluorescence) into wild-type brains, I can address whether the cell size and dendritic complexity of MECP2 mutant neurons is comparable or abnormal compared with wild-type neurons, and, if abnormal, determine whether this is due to MECP2 in neurons themselves, or dependent on the environment. Although some of the results are not yet final, transplanted MECP2 mutant layer II/III pyramidal neurons are smaller and less complex even in the wild-type environment than those of transplanted wild-type neurons, indicating that wild-type environment does not rescue the phenotype of transplanted MECP2 mutant neurons. Thus, MECP2 in neurons themselves is the central reason for their abnormalities.

Taken together, my data indicate that MECP2 is involved in the maintenance and maturation of brain neurons, including their connections, and the stabilization of neurons with long axons, rather than the early development or movement of neurons as the brain is initially formed. I am now beginning experiments to investigate these issues directly at the molecular level.

## Lay Summary

Rett syndrome is a disease of brain development that causes mental retardation and autistic behavior in girls. Recent experiments have revealed that a defect in a gene called the methyl-CpG-binding protein 2 (MECP2) causes Rett syndrome. There is evidence from human autopsies that neurons in the cerebral cortex that make long-distance connections between the two hemispheres of the brain express MECP2 at high levels during brain development. In addition, recent work using MECP2 mutant mice has shown that mutation of the MeCP2 protein in neurons is sufficient to cause neuronal dysfunction in various parts of the brain including the cerebral cortex; these animals manifest symptoms that are similar to those of Rett syndrome. However, the specific neuronal abnormalities have not been elucidated. The cerebral cortex is the most complex structure of the brain and is responsible for high functions unique to humans; even a subtle abnormality of connections of this complex structure can cause diseases affecting behavior and cognition, such as Rett syndrome. The development of cortical connection neurons is critical to cortical function; the role of MECP2 mutation in this process may be central to understanding the neurobiological basis of Rett syndrome. The molecular mechanisms underlying development of cortical connection neurons, and the specific role of MECP2 in these processes, remain largely unclear. Therefore, I propose to investigate directly the molecular mechanisms of cortical connection neuron development and effects of MECP2 mutation on this process. I propose to: 1) investigate the effects of MECP2 on development of connections between the two hemispheres of the cerebral cortex using MECP2 mutant mice and analysis of MeCP2 production in select populations of cortical connection neurons during normal development; and 2) investigate developmental events that follow MeCP2 production and function, using precursor cells and purified cortical connection neurons in cell culture experiments. This knowledge will not only add to our understanding of the neurobiological basis of Rett syndrome and the mechanisms underlying connection neuron development in the cerebral cortex, but also may contribute to the potential for molecular and therapies for neurological disorders involving neuronal compromise and loss, including Rett syndrome.

## Abstract



Rett syndrome is a neurodevelopmental disorder and one of the causes of mental retardation and autistic behavior in girls. Recent work has revealed that a defect in the methyl-CpG-binding protein 2 (MECP2) gene causes Rett syndrome.æ There is evidence from human autopsy material that cortical projection neurons express MECP2 at high levels during development.æ In addition to the gene discovery, recent mouse genetic work has demonstrated that, in MECP2 mutant mice, a MeCP2 deficiency in neurons is sufficient to cause neuronal dysfunction in neocortex, hippocampus, and cerebellum, with manifesting symptoms that mimic those of Rett syndrome.æ However, the specific neuronal abnormalities have not been elucidated.æ The neocortex is the most complex structure of the brain and gives us unique talent, but even a subtle disruption of cortical connectivity can cause behaviorally and cognitively significant diseases, such as Rett syndrome.æ The development of cortical projection neurons is critical to cortical connectivity; the role of MECP2 mutation in this process may be central to understanding the neurobiological basis of Rett syndrome.æ The molecular mechanisms underlying development of cortical projection neurons, and the specific role of MECP2 in these processes, remain largely unclear.æ Therefore, I propose to investigate directly the molecular mechanisms of callosal projection neuron development and effects of MECP2 targeted gene deletion ("knockouts") on this process.æ I propose to: 1) investigate the effects of MECP2 on development of interhemispheric connections using MECP2 knockouts and analysis of MeCP2 expression in select populations of projection neurons during normal development; and 2) investigate developmental events downstream of MeCP2 expression, using cultured neural precursors and purified cortical projection neurons.æ This knowledge will not only add to our understanding of the neurobiological basis of Rett syndrome and the mechanisms underlying projection neuron development in neocortex, but also may contribute to the potential for molecular and cellular therapies for neurological disorders involving neuronal compromise and loss, including Rett syndrome.

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