

## Research Awardees: 2005

### Research Awards

#### Gene Therapy Collaboration

John Aletta, Ph.D.

Ian Marc Bonapace, Ph.D.

Uta Francke, M.D.

Aristea Galanopoulou, M.D., Ph.D.

Peng Jin, Ph.D.

David Katz, Ph.D.

Janine LaSalle, Ph.D.

Jeffrey Macklis, M.D., D.H.S.T

David Paterson, Ph.D.

Thomas Seyfried, Ph.D.

Yi Eve Sun, Ph.D.

### Post-Doctoral Fellowship Awards

Feixia Chu, Ph.D.

Gregory Pelka, Ph.D.

Georg Stettner, M.D.

### Research Awards

#### Gene Therapy Collaboration:

Development of Gene Therapy Strategies for Rett Syndrome

\$357,060

David B. Levin, Ph.D.

Department of Biology, University of Victoria

Lay Progress Report (August 2006)

Rett syndrome, which is the second leading cause responsible for mental retardation in girls, has been determined to be caused by the change of a single but very important gene--Methyl-CpG binding protein 2 (MeCP2) gene. There is no current treatment approach available. Mutations in the *mecp2* gene result in an MeCP2 protein that fails to regulate gene expression, resulting in "arrested development" of neuronal cells. A reasonable approach may be to deliver the normal gene back with the hope of rescuing the phenotype. We are part of an international collaborative research effort to develop a gene therapy strategy for Rett syndrome. Three research groups (Dr. David Levin and Dr. Kerry Delaney, at the University of Victoria, in Victoria, British Columbia; Dr. Jame Ellis and Dr. Peter Dirk, Hospital for Sick Children, University of Toronto, Toronto, Ontario; and Dr. Jude Samulski, Gene Therapy Centre, University of North Carolina, Chapel Hill, North Carolina) are working together to investigate parameters essential to the development of a viable gene therapy method. Our focus is on the use of virus-based vectors to deliver normal MeCP2 to neurons in the brains of mice with mutated *mecp2*, with the goal of restoring normal development of neuronal cells. Expected outcomes of our research collaboration include: i) delineation of the timing of neuro-development and architecture of neocortical cells in the brains of MeCP2-null mice; ii) characterization of the mechanisms that regulate the timing and tissue-specificity of *mecp2* gene transcription; iii) identification of which isoform of MeCP2 (MeCP2E1 or MeCP2E2) works best as a therapeutic agent. Our collaboration with the other members of the team will also help determination of which vector system (Retrovirus, Lentivirus, Adeno-Associated Virus, and/or neural stem cells) offers the best potential for gene therapy delivery. Successful demonstration of the gene therapy in the MeCP2-null mouse model will provide data of fundamental importance for developing gene therapy for human RTT children.

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James Ellis, Ph.D.

Hospital for Sick Children, University of Toronto

Lay Progress Report (August 2006)

Rett syndrome is caused by a mutation in the MeCP2 gene. This type of genetic disease may be treated by "gene therapy" that introduces the normal gene into affected cell types. Here, we propose to deliver the normal MeCP2 gene into brain cells using a specially designed retrovirus. This retrovirus vector will be tested in a mouse model of Rett syndrome, an objective that raises challenges that we have carefully identified and will resolve as follows. First, there are two different proteins encoded by the gene, called MeCP2E1 and MeCP2E2, and their relative importance is not yet well defined. To address this, we have generated retrovirus vectors encoding each protein separately. Second, MeCP2 must be carefully regulated because over-expression causes neurological disease. Therefore, we will test our existing ubiquitously expressed vectors in Neural Stem Cells (NSC) from MeCP2 null mice, and create novel MeCP2 minigenes regulated by their normal MeCP2 control elements. Third, gene therapy itself entails some risk that the virus may integrate into a chromosomal region that activates an oncogene and causes cancer. We have developed safety-enhanced lentivirus vectors that use "insulator" elements to prevent this insertional activation. Fourth, MeCP2 null mice are difficult to obtain in large numbers. To limit the number of mice required, we will culture "neurospheres" to produce expanded numbers of Neural Stem Cells (NSC), each of which generate many neurons when induced to differentiate. The NSC will be infected with MeCP2 retrovirus vectors, differentiated into neurons in culture, and the level of MeCP2 proteins tested. Fifth, delivery of the virus back into the brain can be performed in two different ways. NSC grown in culture can be manipulated outside the body ("ex vivo") and then the cells transplanted back into the brain where they will ultimately generate neurons with normal MeCP2. Alternatively, the virus can be delivered directly into a region of the brain where NSC normally reside ("in vivo"). We will compare both delivery methods to determine which is optimal for Rett syndrome gene therapy.

Our anticipated outcome is a proof of principle that gene therapy of NSC can produce neurons expressing correct levels of MeCP2. To this end, we have extensive experience in designing gene expression cassettes and gene therapy vectors for use in stem cells. Moreover, we already culture human and mouse NSC and deliver these cells into fetal mouse brains in utero using ultrasound-guided injection. We have convincing preliminary results demonstrating MeCP2 gene transfer, and proven expertise with all the procedures required for this therapeutically important study of gene therapy for Rett syndrome.

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Jude Samulski, Ph.D.

Center for Gene Therapy, University of North Carolina

Lay Progress Report (August 2006)

One strategy for treating the Rett Syndrome may be to deliver a therapeutic gene to the brain of an affected individual. As a carrier for the therapeutic gene, adeno-associated virus (AAV) seems to be an excellent choice. This virus is unique in that it is not harmful to the human beings and has been tested in phase I clinical trials for several genetic disorders such as cystic fibrosis and hemophilia. At the moment, 8 different serotypes of AAV have been developed into gene carriers. In this study we aim to find a suitable serotype that can efficiently deliver genes to mouse brain. Genetic evidence supports that a MeCP2 gene mutation is a major cause of RTT. MeCP2 appears to maintain normal brain development by controlling its downstream targets. Based on this hypothesis, we have focused on reversing the abnormal control of the downstream targets by introducing the correct MeCP2 gene in an animal model. To achieve a safe and effective level of MeCP2 with AAV, we have focused on developing and optimizing a novel regulation system to help control MeCP2 gene expression. Over the past funding period, we have made significant progress as summarized below:

Identify an ideal AAV vector that specifically and efficiently transduces neurons globally. Using various serotype AAV vectors carrying a green fluorescent protein gene as a marker, we have generated preliminary data collaborating results obtained by others that serotypes 1 & 5 appear to transduce similar number of cells (avg. 3000), compared to AAV 2 (avg. 40) in the brain. The observation indicates that the current serotype vectors, while capable of mediating gene delivery, may not be optimal for efficient and global transduction of neurons and therefore for gene therapy of brain diseases. To develop more efficient carriers for gene delivery, we have taken a directed evolution approach which involves shuffling the available AAV serotypes to generate a library of AAV mutants. Our results support the notion that AAV mutants with remarkably enhanced delivery efficiencies for a particular target can be generated. We anticipate that such a powerful technology will allow us to create AAV mutants with highly improved efficiency of gene delivery to the brain.

Characterize a novel regulatable AAV expression vectors based on alternative splicing. We continue to use a novel regulation system based on alternative splicing to develop and optimize a marker AAV that specifically and efficiently transduces neurons globally.

Once steps 1 and 2 above have been completed, we will proceed with this system to deliver MeCP2 gene into mouse brain. We hope that the results obtained from these studies will start to develop a therapeutic approach for treating Rett

syndrome.

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## Lay Summary

Rett syndrome, which is the second leading cause responsible for mental retardation in girls, has been determined to be caused by the change of a single but very important gene--Methyl-CpG binding protein 2 (MeCP2) gene. There is no current treatment approach available. Mutations in the MECP2 gene result in an MeCP2 protein that fails to regulate gene expression, resulting in "arrested development" of neuronal cells. A reasonable approach may be to deliver the normal gene back with the hope of rescuing the phenotype. We propose an international collaborative research effort to develop a gene therapy strategy for Rett syndrome. Three research groups (Dr. David Levin and Dr. Kerry Delaney, at the University of Victoria, in Victoria, British Columbia; Dr. James Ellis and Dr. Peter Dirk, Hospital for Sick Children, University of Toronto, Toronto, Ontario; and Dr. Jude Samulski, Gene Therapy Center, University of North Carolina, Chapel Hill, North Carolina) will work together to investigate parameters essential to the development of a viable gene therapy method. The three groups will investigate the use of virus-based vectors (Retrovirus, Lentivirus, and Adeno-Associated Virus) as well as transduced neural stem cells, to deliver normal MeCP2 to neurons in the brains of mice with mutated *mecp2*, with the goal of restoring normal development of neuronal cells. Expected outcomes of the research collaboration include: i) delineation of the timing of neuro-development and architecture of neocortical cells in the brains of MeCP2-null mice; ii) characterization of the mechanisms that regulate the timing and tissue-specificity of *mecp2* gene transcription; iii) identification of which isoform of MeCP2 (MeCP2E1 or MeCP2E2) works best as a therapeutic agent; iv) determination of which vector system (Retrovirus, Lentivirus, Adeno-Associated Virus, and/or neural stem cells) offers the best potential for gene therapy delivery. Successful demonstration of the gene therapy in the MeCP2-null mouse model will provide data of fundamental importance for developing gene therapy for human RTT children.

## Abstract

We propose an international collaborative research effort to develop a gene therapy strategy for Rett syndrome. Three research groups work together to investigate parameters essential to the development of gene therapy for Rett syndrome. Dr. David Levin and Dr. Kerry Delaney (University of Victoria, in Victoria, British Columbia) will develop transgenic mice that both constitutively express a Yellow Fluorescent Protein and carry an MeCP2-null mutation. These mice will be used to characterize neuronal development and architecture in MeCP2-null mice using two-photon laser scanning microscopy (Delaney lab). The Levin lab will develop Lentivirus vectors that express recombinant MeCP2 proteins fused with fluorescent proteins and cell penetrating peptides, which will be used to evaluate if Lentiviruses can deliver functional MeCP2 to the brain and if recombinant MeCP2 can restore neuronal development in MeCP2-null mice. Dr. Jame Ellis and Dr. Peter Dirk (Hospital for Sick Children, University of Toronto, Toronto, Ontario) will investigate in vivo transfer of recombinant MeCP2, or ex vivo transduction of cultured neural stem cells, using Retroviruses or Lentiviruses to deliver MeCP2 isoforms to the brains of MeCP2-null mice. They will identify and characterize cis-acting elements that regulate the timing and/or tissue-specificity of MeCP2 expression in neuronal cells and develop safety-enhanced Lentivirus vectors that express MeCP2 isoforms under their endogenous transcriptional control elements. Dr. Jude Samulski (Gene Therapy Centre, University of North Carolina, Chapel Hill, North Carolina) will investigate the use of Adeno-Associated Virus (AAV) for gene therapy delivery. Dr. Samulski and his team will develop a novel mechanism for regulating the expression of recombinant MeCP2 in mouse neocortical cells using alternative splicing. The three labs will collaborate in the effort to develop a gene therapy strategy for Rett syndrome by sharing information, expertise, materials, and methods. Where appropriate, students or research personnel from each lab will travel to other labs to learn specific techniques and/or use specific equipment that will benefit their research. In the first year of funding, each lab will focus on developing their respective projects, but multiple interactions between the labs will be initiated as the projects mature. Knowledge generated by the Levin & Delany labs of the neuro-development timing and architecture of neocortical cells in MeCP2-null mice will greatly facilitate experiments in the Ellis and Samulski labs. Comparisons of Retrovirus, Adeno-Associated Virus, Lentivirus, and transduced neural stem cells, expressing recombinant MeCP2 isoforms, will determine which system has the best potential as a viable gene therapy delivery agent. Mini-gene cassettes consisting of MeCP2-regulatory elements delineated by the Ellis lab, or the alternative splicing strategy that will be developed in the Samulski lab, will be important in the development of gene therapy delivery systems with optimal developmental timing and tissue-specific expression.

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John Aletta, Ph.D.

University of Buffalo

Regulatory Control of Cellular MeCP2 Function

\$100,000

Research Sponsor: Ford Motor Company

Lay Progress Report (August 2006)

Experiments in the Aletta lab are aimed at understanding the cellular and molecular regulation of neuronal differentiation and development. Cell signals initiated by neurotrophins (NGF and BDNF) are involved in many aspects of neuronal development. We believe that one target of neurotrophin-mediated signals may be MeCP2. Neurotrophin activation of two kinds of regulatory enzymes known as kinases and methyltransferases is capable of producing chemical modifications, phosphorylation and methylation respectively, of MeCP2. We would like to know if, and how, these chemical changes of MeCP2 interact in living cells. By using easily manipulated cellular models of cell signaling and gene regulation to elucidate the biological consequences of neurotrophin-mediated changes in MeCP2 phosphorylation and methylation, our goal is to determine the normal cellular mechanisms that regulate MeCP2 function. It is hoped that this new information will be useful in the development of future pharmacological interventions for Rett Syndrome.

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Lay Summary

Although Rett Syndrome is now clearly known to be a neurodevelopmental disease involving a variety of mutations in the MECP2 gene that encodes methyl-CpG-binding protein 2 (MeCP2), the regulation of the normal function of the protein product of the gene is not well understood. The research proposed in this application seeks to learn more about the cellular and molecular interactions of MeCP2. We will utilize well defined neuronal models called cell lines that allow us to pose biological questions using living cells in a similar developmental state as those just beginning to take on neuronal properties in embryonic animals. We aim to examine the chemical modifications that can regulate MeCP2 function during this process of neuronal development. The main hypothesis of this proposal is that there are two opposing chemical changes that direct the function of MeCP2 as a regulator of neuronal gene expression. By using functional cellular assays of both of the chemical modifications and their effects on MeCP2 function as a gene regulator, we expect to learn more relevant information about the basic cellular signals that direct MeCP2 to carry out its normal cellular jobs. Increasing our understanding of how MeCP2 works normally is necessary to provide the insight for why the gene mutations cause the symptoms of the disease. Ultimately, the description of the contributions of the chemical changes that regulate MeCP2 and the means by which the changes are brought about may provide potential cellular targets for drug intervention and new prospects for the development of clinical therapeutic interventions.

## Abstract

The role of MeCP2 in gene silencing is well documented. Many unanswered questions remain, however, regarding the details of the cellular regulation of MeCP2 function. Chemical modifications of MeCP2 mediated by regulatory enzymes in response to electrical activity and/or other developmental cues may play very important roles in MeCP2 function. Recent experimental evidence implicates neuronal activity in the de-repression of BDNF transcription by phosphorylation of MeCP2. My laboratory has found that MeCP2 is also post-translationally modified by arginine methylation as well. The purpose of this project is to examine how these post-translational modifications interact to regulate MeCP2 binding to gene promoters and subsequent effect on transcriptional control. We will determine the effects of phosphorylation and arginine methylation on the ability of MeCP2 to bind to the BDNF exon III promoter by ChIP analysis as well as the silencing of exon III dependent transcription measured by RTPCR. We will also determine whether these post-translational modifications affect MeCP2 binding to and silencing of a second neurotrophin-sensitive gene, peripherin. The accomplishment of the aims of this project will provide functional information about the role of MeCP2 post-translational modifications in the context of cell signaling pathways during early stages of neuronal differentiation.

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Ian Marc Bonapace, Ph.D.

University of Insubria (Italy)

The isolation of protein complexes involving MeCP2 and Np95: Understanding their roles in the structural organization of heterochromatin

\$100,000

Research Sponsor: Pro Rett Ricerca

## Lay Summary

Mutations in the MeCP2 gene account for approximately 70-80% of the genetic alterations found in the Rett Syndrome (RS), although a small proportion of clinically well-defined RS patients (ca 5-10%) do not appear to have MECP2 mutations. MeCP2 binds preferentially to methylated DNA (DNA that has undergone a modification called methylation) and is mainly concentrated in the condensed regions of the genome. The accepted paradigm for its transcriptional repression activity involves the formation of protein complexes that bind to the regulatory regions of target genes, inactivating them. No definitive answer, however, has yet been achieved. MeCP2 is a protein that acts at the level of the structural organisation of the dense chromatin areas that are assembled at the time of DNA replication. By this means, MeCP2 can provide physical barriers to the assembly of gene activating complexes. MeCP2 interacts with Np95, a chromatin modulator, that is essential for DNA replication and chromatin structure. We hypothesise that Np95 and MeCP2 could be part of protein complexes required for the condensation of large areas of chromatin and for transcriptional silencing, which are established and propagated during replication of the cells. The goal of this project is to study the role of MeCP2-Np95 interaction for the organisation of chromatin and for the regulation of gene expression. We will study if MeCP2 by its own or in cooperation with other biochemical partners has a role in the structural and functional organisation of the chromatin areas in which it is located and if MeCP2 mutants, known to be involved in Rett Syndrome, have lost these properties. These studies will be important to assess if the action of MeCP2 on the structural organisation of chromatin is needed for the regulatory mechanism of the protein in differentiated neuron cells. It will also allow finding new proteins that interact with MeCP2 and that might cooperate with this protein for the maintenance of the correct regulation of MeCP2 dependent genes in neurons.

## Abstract

The accepted paradigm for the transcriptional repression activity of MeCP2 involves formation of a corepressor complex (involving MeCP2, Sin3a and HDAC1), that targets promoters and inactivates gene expression. MeCP2, however, is a potent chromatin-condensing protein that mediates assembly of novel chromatin structures. MeCP2 is, indeed, highly concentrated in heterochromatin in the mouse in vivo. These highly methylated, condensed and repressed heterochromatic domains, rich in MeCP2, are present either in constitutive heterochromatin or are interspersed with relatively decondensed euchromatic and transcriptionally active regions. MeCP2 could exert an important role in the organization of epigenetic modifications and chromatin structures that are built at the time of heterochromatin replication. MeCP2 could be part of complexes that participate in the organization and stability of this chromatin domain. Np95 is an essential S phase protein and novel chromatin binding protein that directly interacts with histones (H3>>H1>H2B) both in vivo and in vitro. Double immunostaining for Np95 and chromatin-bound PCNA, a marker of DNA replication sites, revealed that Np95 was almost exclusively co-localized with chromatin-bound PCNA throughout the nucleus in early S phase (euchromatin replication timing) and partly in mid-S phase (pericentric heterochromatin replication timing). Np95 does not seem to be directly involved in DNA replication as part of the DNA synthesizing machinery, like PCNA, but is presumably involved in other DNA replication-linked nuclear events. Functional ablation of Np95 by RNAi experiments shows that this protein is essential for heterochromatin replication. There is a great deal of evidence to indicate that the structural organization of heterochromatin is determined at the time of replication, in mid-late S phase, and that associated multi-protein complexes are required to enable the stable epigenetic inheritance of a heterochromatic state that includes large-scale chromatin condensation, transcriptional silencing, sister-chromatid cohesion and kinetochore function. Our preliminary experiments indicate that Np95 and MeCP2 interact with each other both in vitro and in vivo. Np95 is by itself unable to bind methylated DNA. However, MeCP2 can recruit Np95 to modified DNA, and its presence stabilizes the nucleoprotein complexes formed by the methyl-binding protein. We hypothesize that Np95 and MeCP2 could be part of multi-protein complexes required for large-scale chromatin condensation, transcriptional silencing of both constitutive and facultative heterochromatin and the stable inheritance of heterochromatin domains, which are established and propagated during replication. In this view, negative control of gene expression mediated by MeCP2 would arise also from its contribution to the proper organization of chromatin domains. Correct localization to methylated CpG islands and recruitment of histone modifying enzymes would, however, also be necessary for its action. The aim of this project is to study protein complexes involving MeCP2 and Np95 that are implicated in the structural organization of heterochromatin. In summary, the objectives are to:

1. Identify components of the SRA domain complex(es);
2. Study the effect of MeCP2 and the components of the Np95-MeCP2 complex on the structure of heterochromatin during replication and differentiation;
3. Solve the 3D structure of the SRA domain and study its interaction with MeCP2 as well as other proteins in the identified complexes.

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Uta Francke, M.D.

Stanford University

Is DLX5 a target of MeCP2 and, therefore, involved in Rett Syndrome?

\$27,500

Research Sponsor: Eastern Development, LLC

[Lay Summary](#)

When changes in a gene on the X chromosome were identified in Rett patients in 1999, a lot was already known about the function of this gene called MeCP2. Its product, methyl CpG binding protein 2, binds to DNA especially at sites carrying a particular mark called "methylation". A result of the binding event is to shut down genes in the neighborhood. What are the targets of MeCP2? "Imprinted genes" are only expressed from the maternal or the paternal copy. A methylation mark is used to distinguish the two copies. Therefore, MeCP2 is a logical candidate to translate the methylation mark into parent-of-origin specific gene expression. Given the paucity of information about the true targets of MeCP2 binding in the brain, the recent report of DLX5 (distal-less homolog 5), a purportedly imprinted gene, is potentially exciting. The claim that DLX5 is a direct target of MeCP2 is based on a series of observations that are not tightly linked in a coherent way. We have available a collection of lymphoblastoid cell lines from 20 individuals with Rett Syndrome and different mutations in MECP2 gene, as well as from 33 unaffected first and second degree relatives. We propose detailed studies of DLX5 and DLX6 expression in these cell lines and hope to determine whether MECP2 plays a role in the regulation of these genes. To extend these studies to the brain, we will study DLX5/6 expression in dissected brain regions of two different strains of mice that are lacking MeCP2 expression and compare the results with their normal siblings.

## Abstract

Rett Syndrome (RTT) is caused by new mutations in the X-linked gene MECP2 that encodes methyl-CpG binding protein 2. The mutations cause loss of function of the MeCP2 protein. Because the MECP2 gene is subject to X-inactivation, affected females are mosaics for cells with normal MECP2 expression and cells lacking MECP2 function completely. MeCP2's function is to repress transcription of methylated genes in vitro. Therefore, it has been hypothesized that MeCP2 dysfunction causes abnormal expression of genes that affect post-natal neuronal development. While large-scale dysregulation of gene expression has not been observed, potential targets include imprinted genes, because their uniparental expression is controlled by parent-of-origin-specific methylation patterns. A recent claim in the literature that the developmental gene DLX5 is imprinted and that its imprinting status is modulated by MeCP2 could have implications in understanding the molecular basis of RTT if the findings reported in this paper can be confirmed. We therefore, propose experiments on a larger series of human and mouse tissues, including clonal lymphoblastoid cell lines from RTT females with either one or the other X chromosome active. Specifically, we will address the question whether DLX5 and the neighboring gene DLX6 are imprinted in humans and mice, and whether allele-specific expression patterns are altered in tissues from MeCP2 deficient humans and mice. To evaluate the hypothesis that not allele-specific imprinting, but differential chromatin loop formation - affecting both alleles - could control DLX5 expression levels in different brain regions, we will compare the absolute expression levels of both genes in defined regions of the brain in MeCP2-deficient male mice and normal littermates. We expect that the results of these studies will provide evidence for or against the hypothesis that DLX5 plays a role in the pathogenesis of Rett Syndrome.

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Aristea Galanopoulou, M.D., Ph.D.

Albert Einstein College of Medicine

The role of the GABAergic system of the substantia nigra in the motor dysfunction of Rett Syndrome

\$100,000

Lay Progress Report (August 2006)

Among the cardinal symptoms of Rett syndrome are the abnormal movements and motor function. The substantia nigra



(SN) is one of the important nuclei involved in motor control. Neuropathologic studies on brains from patients with Rett syndrome demonstrate abnormalities in the SN. The differentiation of the SN is controlled by GABAA receptors and estrogens, the signaling pathways of which in certain cases interact. In this study we are testing, in Rett mice with mutated MECP2 gene, the hypothesis that there is an abnormal functional maturation of the GABAA receptors in the SN, which results in broader dysfunction of GABA and estradiol signaling during the early postnatal development of the SN, affecting calcium regulated gene expression. The information obtained from these studies will allow us to select drugs and interventions that may restore the normal pattern of maturation of the SN and hopefully improve the symptomatology in patients with Rett syndrome.

## Lay Summary

Among the cardinal symptoms of Rett Syndrome are the abnormal movements and motor function. The substantia nigra (SN) is one of the important nuclei involved in motor control. Neuropathologic studies on brains from patients with Rett Syndrome demonstrate abnormalities in the SN. The differentiation of the SN is controlled by GABAA receptors and estrogens, the signaling pathways of which in certain cases interact. In this study we will test, in Rett mice with mutated MECP2 gene, the hypothesis that there is an abnormal functional maturation of the GABAA receptors in the SN. Furthermore, we will test whether this results in broader dysfunction of GABA and estradiol signaling during the early postnatal development of the SN, affecting calcium regulated gene expression. The information obtained from these studies will allow us to select drugs and interventions that may restore the normal pattern of maturation of the SN and hopefully improve the symptomatology in patients with Rett Syndrome.

## Abstract

Rett Syndrome is associated with significant neuropathological changes in the substantia nigra, which have been implicated in the pathogenesis of the abnormal movements and motor function. The developmental and sex-specific differentiation of the substantia nigra neurons is controlled by factors determining the developmental window under which GABAA receptors remain capable of eliciting depolarization-dependent activation of calcium sensitive signaling processing. One such factor is the neuronal specific potassium chloride cotransporter KCC2, which appears to be controlled by neuronal specific transcriptional silencers, such as MECP2 and NRSF. In the proposed experiments, we plan to use the mice developed by Dr. Bird and test the following hypothesis: (1) the switch of GABAA receptors from depolarizing to hyperpolarizing occurs earlier in MECP2-/+ and MECP2 -/y mice compared to same sex and age MECP2 +/+ and MECP2 +/- mice due to higher levels of KCC2 expression in the substantia nigra. (2) As a result, the GABAA receptor mediated control of calcium sensitive processes and differentiation of the substantia nigra differs in MECP2 -/y and MECP2 +/- mice compared to respective controls. The results of this study will be useful in guiding future experiments aiming at restoring the normal maturation and differentiation of the substantia nigra in Rett Syndrome and hopefully ameliorate the symptomatology.

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Peng Jin, Ph.D.

Emory University

Role of MeCP2 in small RNA-mediated gene regulation and identification of MeCP2-associated genomic regions using whole-genome BAC array

\$100,000

Research Sponsor: Boston Wharf Company

## Lay Progress Report (August 2006)

Rett Syndrome is a neurodevelopmental disorder mainly caused by mutations in the X-linked gene MECP2 and primarily affects females. MeCP2 is thought to selectively bind methyl-CpG-binding dinucleotides in mammalian genome and to block gene expression. Recent studies have shown that small RNAs (~20nt) play important role in transcriptional silencing, particularly in plant and yeast. In mammalian cells it has been shown that small RNAs could lead to DNA methylation and transcriptional repression. Mutations in MeCP2 affect its ability to block gene expression and may lead to aberrant patterns of gene expression in RTT. The predominant manifestation of central nervous system dysfunction in RTT suggests that MeCP2 plays critical roles in the development and stability of neurons. However, the genomic regions associated with MeCP2 remain to be defined and how the mutations in MeCP2 alter their association is still unclear. Small RNAs have been shown to play important roles in gene regulation. We are examining whether MeCP2 utilize small RNAs to repress transcription. In addition, we have tested NimbleGen CGH array and will use this array to identify the genomic target regions of

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## Lay Summary

Rett Syndrome (RTT) is a neurodevelopmental disorder mainly caused by mutations in the X-linked gene methyl-CpG-binding protein (MeCP2) and primarily affects females. MeCP2 is thought to selectively bind methyl-CpG dinucleotides in mammalian genome and to block gene expression. Recent studies have shown that small RNAs (~20 nt) play important role in transcriptional silencing, particularly in plant and yeast. In mammalian cells, it has been shown that small RNAs could lead to DNA methylation and transcriptional suppression. Mutations in MeCP2 affect its ability to block gene expression and may lead to aberrant patterns of gene expression in RTT. The predominant manifestation of central nervous system dysfunction in RTT suggests that MeCP2 plays critical roles in development and stability of neurons. However, the genomic regions associated with MeCP2 remain to be defined and how the mutations in MeCP2 alter their association is still unclear. In the proposal, we will use a newly developed array that covers the whole human genome to identify the genomic target regions of MeCP2. These studies should provide insight into the molecular pathogenesis of Rett Syndrome.

## Abstract

Rett Syndrome is an X-linked dominant neurodevelopmental disorder caused by mutations in MeCP2, encoding methyl-CpG protein 2 (MeCP2). MeCP2 is thought to selectively bind methyl-CpG dinucleotides in mammalian genome and to function as transcriptional repressor in vivo by interacting with histone deacetylase (HDAC). Recent studies have shown that small RNAs (~20 nt) play important role in transcriptional silencing, particularly in plant and yeast. In mammalian cells, it has been shown that small RNAs targeted to certain promoter regions could lead to DNA methylation and transcriptional suppression. Interestingly, MeCP2 was shown to bind to small interference RNAs in vitro, which raises the possibility that MeCP2 might be involved in small RNA-mediated gene regulation. Several targets of MeCP2, including BDNF and DLX5, have been identified. MeCP2 have been implicated in neuronal activity-dependent transcriptional regulation as well as chromatin remodeling and genomic imprinting. Despite these advances, the genomic regions associated with MeCP2 remain to be systematically defined and how the mutations in MeCP2 alter their association is still unclear. The goals of this proposal are to determine whether MeCP2 is involved in small RNA-mediated gene regulation and identify the genomic target regions of MeCP2 using both CpG island array and whole-genome BAC tiling array.

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David Katz, Ph.D.

Case Western

Maturation of BDNF-dependent respiratory neurons in MeCP2 mutants

\$88,000

Research Sponsor: CIBC World Markets Corporation

### Lay Summary

In addition to cognitive and motor impairments, Rett patients can suffer from severe problems with control of breathing, heart rate and blood pressure. However, the causes of these dysfunctions are unknown. Previous studies in my laboratory demonstrated that development of several key groups of neurons that control cardiorespiratory function is regulated by a specific protein called Brain Derived Neurotrophic Factor (BDNF). Moreover, work in other laboratories has shown that BDNF is controlled by another protein, MeCP2, which is encoded by the gene responsible for Rett. Therefore, we hypothesize that abnormalities in the expression and/or function of BDNF may contribute to the difficulties in cardiorespiratory control that afflict many Rett patients. The proposed research is therefore designed to define the role of MeCP2 in maturation of cardiorespiratory neurons whose development and function is regulated by BDNF.

### Abstract

In addition to cognitive and motor impairments, Rett patients can suffer from a spectrum of cardiorespiratory disturbances that include severe respiratory dysrhythmias, prolonged QT interval and diminished cardiac vagal tone and cardiac baroreflex sensitivity. However, the cellular and molecular pathogenesis of these disturbances is unknown. MeCP2, the gene responsible for Rett, is a transcriptional regulator of Brain Derived Neurotrophic Factor (BDNF), which is required for development and function of critical subsets of cardiorespiratory control neurons. We hypothesize, therefore, that abnormal development or signaling in these populations contributes to cardiorespiratory dysfunctions in Rett. The proposed research is designed to test this hypothesis by defining 1) survival and maturation of BDNF dependent chemoafferent, baroreceptor and brainstem catecholaminergic neurons and 2) expression and release of BDNF by cardiorespiratory neurons in MeCP2-/- mutant mice. It is hoped that improved understanding of the molecular pathogenesis of cardiorespiratory dysfunction in Rett will lead to development of new therapies as well as shed light on basic mechanisms of the disease.

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Janine LaSalle, Ph.D.

UC Davis

## Investigation of novel MeCP2 target genes regulating neuronal maturation

\$100,000

Research Sponsor: American Commercial Claims Administrators, Inc.

## Lay Progress Report (August 2006)

Rett syndrome is caused by mutations in the gene MECP2 that encodes a protein, methyl-CpG protein 2 (MeCP2). Elevated MeCP2 expression is acquired in individual neurons within the brain beginning in infancy and progressing throughout childhood. The function of MeCP2 in the developing brain is unclear at this stage, but the mutations in Rett syndrome and the Mecp2 deficient mouse model provide evidence that MeCP2 is essential for mature neuronal function.

MeCP2 is predicted to be a regulator of other genes in maturing neurons, but finding these genes is complicated by the complexity of cells and genes in the brain. In a previous study funded by the RSRF, a group of genes, called "ID" for "Inhibitors of Differentiation" were found to show altered expression when MeCP2 activity was blocked in neuronal cultures. ID genes are well-characterized regulators of cell differentiation (maturation) and the increased expression of these genes could help explain the why neurons appear to be immature in Rett syndrome brains.

Our recent publication (Peddada et al., Human Molecular Genetics, 2006) has demonstrated significantly increased levels of all four IDs (ID1, ID2, ID3, ID4) in Rett syndrome brain and Mecp2 deficient mouse brain. To further test the hypothesis that IDs are important in the pathogenesis of Rett syndrome, we are breeding Mecp2 deficient mice to Id1 and Id3 deficient mice to determine if reducing the dosage of ID genes may lessen the severity of disease.

## Lay Summary

Rett Syndrome is caused by mutations in the gene MECP2 that encodes a protein, methyl-CpG protein (MeCP2). Elevated MeCP2 expression is acquired in individual neurons within the brain beginning in infancy and progressing throughout childhood. The function of MeCP2 in the developing brain is unclear at this stage, but the mutations in Rett Syndrome and the Mecp2 "knockout" mouse model provide evidence that MeCP2 is essential for mature neuronal function. MeCP2 is predicted to be a regulator of other genes in maturing neurons, but finding these genes is complicated by the complexity of cells and genes in the brain. In a previous study funded by the RSRF, a group of genes, called "ID" for "Inhibitors of Differentiation" were found to show altered expression in the presence of a MeCP2 "decoy." ID genes could help explain the why neurons appear to be immature in Rett Syndrome brains. In the current proposal, the ID genes (ID1, ID2, ID3, and ID4) will be further characterized and tested as potential MeCP2 target genes. Changes in ID expression levels will be examined in Rett Syndrome brain samples and brains of the Mecp2 "knockout" mouse model at different ages to determine if increased ID proteins are a characteristic of Rett Syndrome and autism cases without MECP2 mutations. If these studies are successful in showing increased ID expression in Rett Syndrome, these proteins could be promising targets for rationale drug design. Importantly, ID inhibitors are currently being developed for cancer therapy.

## Abstract

Rett Syndrome is an X-linked dominant neurodevelopmental disorder caused by mutations in MECP2, encoding methyl-CpG protein 2 (MeCP2). MeCP2 selectively binds to methylated CpG residues and is hypothesized to be an essential repressor of genes that regulate neuronal maturation in the postnatal central nervous system. In order to determine potential novel MeCP2 target genes during neuronal maturation, genome-wide expression analysis was performed. MeCP2 decoy transfection during neuronal maturation resulted in the dysregulated expression of an interesting class of genes called ID (Inhibitors of DNA binding or Differentiation), which are involved in control of cell proliferation and differentiation. Aim 1 of this proposal seeks to confirm and further characterize the effect of MeCP2 blocking on the ID gene family of transcriptional regulators. In the second aim, the expression of ID proteins will be tested directly on brain

tissue samples by quantitative immunofluorescence and LSC on tissue microarrays. The mouse tissue microarray will include cerebral samples from ~20 Mecp2-null and wild-type littermate controls at different developmental time points. The normal developmental expression of each ID protein. The human tissue microarray will be constructed from post-mortem brain samples from RTT patients with and without MeCP2 mutations, autism samples, and age-matched controls. The expression of all four ID proteins will be tested for significant expression differences in all patient samples compared to controls. These studies are expected to determine if altered expression of the ID transcription factors play an important role in the pathogenesis of RTT.

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Jeffrey Macklis, M.D., D.H.S.T

Harvard/Massachusetts General Hospital

Identification and analysis of target genes for the Rett Syndrome transcriptional repressor MECP2 in the developing cerebral cortex projection neurons

\$100,000 Research Sponsor: Rett Syndrome Association of Massachusetts

Lay Progress Report (August 2006)

Recent research has revealed that a defect in a gene called MECP2, which encodes a protein that suppresses expression of other genes ("transcriptional repressor"), causes Rett syndrome. The discovery of MECP2 mutations as the overwhelming cause of Rett syndrome enabled a new era of cellular and molecular analysis, and understanding of the mechanisms of Rett syndrome. An important next research goal will be to find the specific target genes that MeCP2 regulates in individual affected nerve cells, because MeCP2 normally regulates its target genes during development and function of the nervous system, and abnormal expression of target genes directly or indirectly causes Rett syndrome.

Our previous work demonstrates 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. These previous results show that "pyramidal neurons" in cerebral cortex layer 2/3 in Mecp2 mutant mice are smaller and their dendrites are less complex than those in normal mice. In addition, our recent work using genetic and physical chimeric mice (mixing one type of neuron with another type of brain), shows that a normal environment does not eliminate the abnormalities of transplanted Mecp2 mutant neurons, indicating that the lack of MeCP2 in neurons themselves (rather than in surrounding cells lacking MeCP2) is the central reason for their abnormalities.

Based on our previous work, we have pursued two complementary approaches for the identification and molecular analysis of target genes of MeCP2 in these important cortical neurons (callosal projection neurons; CPN), using both microarray and chromatin immunoprecipitation (ChIP) approaches. Using 'microarrays', we can investigate expression levels of many genes in cells at once. By comparing gene expression patterns in normal neurons with those in neurons from Mecp2 mutant mice, we found that 18 genes were significantly increased and 21 genes were decreased in Mecp2-mutant ("null", lacking Mecp2 function) CPN. One of them is increased 6.2-fold in Mecp2-null CPN compared to wild-type CPN. We tentatively named this gene MeCP2 Target Gene 1 (Mtg1). To confirm this result, we performed RT-

PCR, another method to investigate gene expression. Importantly, we could detect the gene expression difference only when we used pure CPN, but not when we used the whole cortex, demonstrating that the use of pure cell populations can detect new direct or indirect MeCP2 target genes hidden by cellular heterogeneity.

We have also pursued the identification of MeCP2 target genes using a pure cell population of CPN by a ChIP-based approach. ChIP is an approach to determine the location on chromosomes where molecules like MeCP2 bind, and thus, which genes they regulate. So far, we have found approximately 200 MeCP2 binding DNA fragments. Using the mouse genome database, we mapped those DNA fragments onto the genome. The MeCP2 binding sequences we obtained are distributed in special areas on each of the mouse chromosomes. Approximately 14% of these DNA fragments were found to be within 20 kb "upstream" from a transcriptional start, suggesting that MeCP2 may bind these regions and control the expression of these genes in normal CPN.

We are now confirming these results from the microarray experiments using other methods, such as RT-PCR, and ChIP analysis.

## Lay Summary

Recent research has revealed that a defect in a gene called MECP2, which encodes a protein that suppresses expression of other genes ("transcriptional repressor"), causes Rett Syndrome. The discovery of MECP2 mutation as the cause of Rett Syndrome enabled a new era of cellular and molecular analysis and understanding of mechanisms of Rett Syndrome. An important next research goal will be to find the specific target genes that MECP2 regulates in individual affected nerve cells, because MECP2 normally regulates its target genes during development and function of the nervous system, and abnormal expression of target genes directly or indirectly causes Rett Syndrome. Our previous work demonstrates 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. These previous results show "pyramidal neurons" in cerebral cortex layer II/III in MECP2 mutant mice are smaller and their dendrites are less complex than those in normal mice. In addition, our recent work using genetic and physical chimeric mice (mixing one type of neuron with another type of brain), shows that a normal environment does not eliminate the abnormalities of transplanted MECP2 mutant neurons, indicating that MECP2 in neurons themselves (rather than MECP2 in surrounding cells) is the central reason for their abnormalities. Based on our previous work, we propose two complementary approaches for the identification and molecular analysis of target genes of MECP2 in these important cortical neurons, using both microarray approaches and chromatin immunoprecipitation (ChIP) approaches. Using 'microarrays', we can investigate expression levels of many genes in cells at once. By comparing gene expression patterns in normal neurons with those neurons from MECP2 mutant mice, we can find which genes are abnormally expressed in MECP2 mutant mice. ChIP is an approach to determine the location on chromosomes where molecules like MECP2 bind, and thus, which genes they regulate. We will use established approaches in our lab to purify the major population of layer II/III projection neurons which are affected by MECP2 mutation, so we study exactly the nerve cells that are affected, instead of mixed cells that can confuse analysis. Our proposed experiments will not only add to our understanding of molecular and pathological mechanisms of Rett Syndrome, but will also potentially contribute to future therapeutic and/or preventative strategies.

## Abstract

The identification in 1999 of mutation of the MECP2 gene (nominally a transcriptional repressor) on the X chromosome as the cause of Rett Syndrome in 1999 enabled a new era of cellular and molecular analysis and understanding of Rett Syndrome pathophysiology. An important next research goal will be to identify the specific target genes of MECP2 in specifically affected neuronal populations. This direction is critical, because MECP2 is a transcriptional regulator, and abnormal expression of its target genes in specific neuronal circuitry quite likely directly or indirectly causes the phenotype. Our previous work has elucidated that MECP2 is involved in neuronal maturation and maintenance, rather than cell fate decisions, and that these effects are cell autonomous. These previous results show that layer II/III cortical projection neurons are specific and centrally involved cellular population in which to investigate MECP2 function. They increasingly express MECP2 as they mature, and MECP2 mutation affects their soma size and dendrite complexity (Kishi and Macklis, 2004). In recent unpublished work using genetic and physical chimeric mice, we find that these defects are cell autonomous (Kishi and Macklis, in preparation, 2005). Based on our previous work, I propose new complementary approaches for the identification and molecular analysis of target genes of MECP2 in this important

interhemisphere cortical projection neuron population - callosal projection neurons (CPN), using both microarray approaches and chromatin immunoprecipitation (ChIP) approaches. Now that we have identified a particular neuron type with a cell autonomous phenotype, we can use established methods in our lab for fluorescence-activated cell sorting (FACS) and purification of CPN, the major population of layer II/III projection neurons. Using extensive work on molecular controls over CPN differentiation, we will investigate MECP2 transcriptional targets in a directed manner in these specific neurons that are centrally involved in the CNS abnormalities and MECP2-null phenotype. Our proposed experiments will not only add to our understanding of molecular and pathological mechanisms of Rett Syndrome, but will also potentially contribute to future therapeutic strategies.

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David Paterson, Ph.D.

Children's Hospital, Boston

The medullary serotonergic system and respiratory dysfunction in Rett Syndrome

\$25,000 (Pilot Project)

## Lay Summary

Rett Syndrome (RTT) is a disorder of brain development and is one of the most common causes of mental retardation in females. RTT patients also have heart irregularities and problems with breathing. Most RTT cases are caused by a genetic abnormality that results in a non-functioning form of methyl-CpG Binding Protein 2 (MeCP2). The MeCP2 protein plays an important role in brain development, and controls the level of another protein that stimulates neuron growth, brain derived neurotrophic factor (BDNF). BDNF is particularly potent stimulator of serotonin (5-HT) neuron growth and survival. 5-HT neurons located in the medulla oblongata of the brainstem are involved in the unconscious control of breathing and heart rate, and damage to these 5-HT neurons causes breathing and heart rate irregularities. These observations suggest the possibility that in RTT, dysfunctional MeCP2 causes abnormal levels of BDNF in the brain during development, which subsequently cause abnormal development of medullary 5-HT neurons, ultimately resulting in heart and breathing irregularities. In support of this idea, we previously observed that the amount of serotonin transporter (SERT, a marker of 5-HT neuron function) in the dorsal motor nucleus of the vagus (DMX, a region of the medulla that plays an important role in the regulation of breathing and heart rate) was abnormal in RTT patients. Based upon these observations, we hypothesize that 5-HT abnormalities in the DMX contribute to the breathing and heart dysfunction observed in RTT. In this study we propose to characterize further this 5-HT abnormality by examining the level of 5-HT<sub>1A</sub> receptors, another marker of 5-HT function, in the DMX of RTT and control cases. In addition, we propose to examine the level of BDNF, and markers of BDNF function, in the DMX of the same RTT and control cases, to test the hypothesis that abnormal levels of BDNF are present in the brains of RTT patients during development, and are responsible for the 5-HT abnormalities observed. Finally, we propose to examine the distribution and levels of these same markers in the medulla oblongata of a mouse model of RTT (MeCP2<sup>2308/y</sup> mice), which have an abnormal MeCP2 gene. The proposed studies will provide insight into the developmental abnormalities in RTT medulla that may be responsible for heart and respiratory dysfunctions observed, and potentially provide a basis for new treatments.

## Abstract

Rett Syndrome (RTT) is a progressive neurodevelopmental disorder and is one of the most common causes of mental retardation in females. In addition, RTT patients demonstrate a spectrum of cardiorespiratory disorders. The majority of RTT cases are caused by mutations in the methyl-CpG Binding Protein (MeCP2) gene, which regulates expression of brain derived neurotrophic factor (BDNF), a potent stimulator of serotonin (5-HT) neuron growth and survival. BDNF also

promotes the synthesis of the astrocyte-produced neurotrophin S100B, which is released by 5-HT1A receptor activation. 5-HT neurons in the medulla oblongata form the "medulla 5-HT system", which regulates cardiorespiratory function. Abnormalities in this system are associated with respiratory dysfunction and sudden infant death syndrome (SIDS). These observations suggest the possibility that MeCP2 mutations resulting in altered BDNF expression, and thus abnormal development of 5-HT neurons, may be at least partly responsible for the cardiorespiratory dysfunction associated with RTT. In support of this idea, we previously observed age-related abnormalities in serotonin transporter (SERT) binding density in the dorsal motor nucleus of the vagus (DMX), which plays an important role in the regulation of autonomic and respiratory function, in RTT patients. Based upon these observations, we hypothesize that developmental abnormalities in the medullary 5-HT system contribute to cardiorespiratory dysfunction observed in RTT. In this study, we propose to characterize further the medullary 5-HT abnormality in RTT by examining the developmental distribution and density of 5-HT1A receptors in the DMX and raphe obscurus (Rob) of RTT and control cases. In addition, we propose to examine the expression of BDNF, trkb, and S100B in the same RTT and control cases to test the hypothesis that these are abnormally expressed in RTT, and putatively responsible for the medullary 5-HT abnormalities. Finally, we propose to examine the distribution and density of these same markers in the medullae of MeCP2308/y mice, an animal model of RTT, for comparison to the human data. The proposed studies will provide insight into the developmental abnormalities in RTT medulla that may be responsible for autonomic and respiratory dysfunction observed, and potentially provide a basis for therapeutic strategies.

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Thomas Seyfried, Ph.D.

Boston College

Gene-environmental interactions in the metabolic control of Rett Syndrome in Mecp2 mice using a ketogenic diet

\$77,000

Research Sponsor: Connors Family Charitable Gift Fund

Lay Progress Report (August 2006)

We conducted the first evaluation of the ketogenic diet on behavioral, metabolic, and neurochemical parameters in adult Mecp2 308/Y Rett mice and in their normal male littermates. The ketogenic diet we administered was KetoCal®, a nutritionally balanced soybean oil-based diet for managing seizures in children with epilepsy. The ketogenic diet is effective in managing seizures and improving behavior in some children with Rett syndrome. KetoCal® was administered to the mice in restricted amounts as recommended for use in children with epilepsy. The normal and the Rett mice were separated into two groups: 1) a high carbohydrate, low fat standard chow diet group fed unrestricted (SD-UR), and 2) the KetoCal® diet group restricted to achieve a 25% body weight reduction (KC-R). The number of normal and Rett mice in each dietary group was 7 and 4, respectively. We measured; 1) food intake and body weight as indicators of metabolic state, 2) rotorod performance, anxiety (exploration of novel environment), proprioception, and behavioral seizures as indicators of motor coordination and behavior, and 3) cerebellar lipid composition as an indicator of neural structure and cellular integrity. We found that body weight in the SD-UR groups was significantly higher ( $P < 0.01$ ) in Rett mice ( $40.4 \pm 2.2$  g) than in normal mice ( $34.4 \pm 0.8$  g), despite similarities in food intake. These findings suggest that the basal metabolic rate is abnormal in the Rett mice. The Rett mice showed no behavioral seizures, proprioceptive defects, or neurodevelopmental abnormalities when compared to the normal mice. However, rotorod performance (time on bar) in the SD-UR groups was significantly less in the Rett mice ( $6.2 \pm 1.8$  sec) than in the normal mice ( $32.9 \pm 9.0$  sec) ( $P < 0.01$ ). Although the restricted KetoCal® diet significantly improved rotorod performance in the normal mice ( $58.1 \pm 1.9$  sec), KetoCal® had no significant effect on rotorod performance in the Rett mice ( $5.9 \pm 2.4$  sec). KetoCal® reduced anxiety in the normal mice, but did not reduce anxiety in the Rett mice. No differences were found between the normal and Rett mice for the content or distribution of cerebellar gangliosides, neutral lipids (cholesterol, phosphatidylethanolamine, phosphatidylcholine, cerebrosides, and ceramides), and acidic lipids (cardiolipin,



phosphatidylserine, phosphatidylinositol, and sulfatides). We conclude that KetoCal® did not improve motor coordination or reduce anxiety in the Rett mice relative to normal mice, and that the motor and behavioral abnormalities in the Rett mice were not associated with noticeable changes in the content or distribution of cerebellar lipids. Studies are in progress to determine if the abnormality in basal metabolic rate in Rett mice is related to abnormalities in mitochondria.

## Lay Summary

Rett Syndrome (RS) is a debilitating progressive neurodevelopmental disorder afflicting mostly females and involves microcephaly, reduced muscle tone, repetitive hand movements, anxiety, autistic-like behavior, and seizures. The disorder has been linked to mutations in the MECP2 gene. We propose that RS may ultimately involve disruptions of brain energy homeostasis and that the ketogenic diet (KD) might manage aspects of the disease phenotype. The KD is a high fat, low carbohydrate diet used to control refractory seizures in children with epilepsy and also produces clinical benefits in patients with RS. The KD controls seizures through multiple integrated changes of inhibitory and excitatory neuronal systems. Shifts in the availability of brain energy metabolites (glucose and ketone bodies) produce changes in gene-linked metabolic networks that manage the seizure disorder. Our goal is to study the effect of KetoCal®, a medically approved KD for children with epilepsy, in Mecp2 knockout mice. The Mecp2 mice exhibit classical phenotypic characteristics of RS. We anticipate that the KetoCal® ketogenic diet will reduce seizure susceptibility, improve motor coordination, and reduce anxiety in Mecp2 mice. Our recently published findings show that the ketogenic diet with caloric restriction manages seizures and improves behavior in mice with idiopathic generalized epilepsy. In addition, the diet up-regulates neuropeptide Y and GABA synthesis related to seizure suppression. No prior studies, however, have evaluated the influence of the KD on behavior or brain gene expression in mice with RS. Our proposed research will identify novel therapeutic strategies and improve the quality of life for children with RS.

## Abstract

We propose that Rett Syndrome (RS) may ultimately involve disruptions of brain energy homeostasis and that the ketogenic diet (KD) might manage aspects of the disease phenotype. We will test our hypothesis in Mecp2 knockout mice fed KetoCal®, a nutritionally balanced and medically approved ketogenic diet for children with epilepsy. The Mecp2 mice exhibit several phenotypic characteristics of the RS and can be used to evaluate therapeutic effects of the KetoCal® ketogenic diet. We recently showed that seizure management in epileptic mice with restricted ketogenic diets involves multiple integrated changes of inhibitory and excitatory neuronal systems. Our proposed studies will involve Mecp2 knockout mice under three dietary conditions, a) a control standard mouse chow diet fed ad libitum or unrestricted, b) the KetoCal® diet fed unrestricted, c) the KetoCal® diet fed in restricted amounts. Aim 1 will analyze myoclonic seizure susceptibility, motor function, and anxiety in Mecp2 mice under the three dietary conditions. Aim 2 will analyze gene expression of NPY and NPY receptors (y2 and y5) and SIRT1 histone deacetylase in cortex and hippocampus of Mecp2 mice. Aim 3 will analyze brain protein levels of the B3 subunit of the GABA receptor, GABRB3, and the GABA synthetic enzyme glutamic acid decarboxylase. These studies can identify metabolic regulatory networks that might underlie the antiepileptic and anxiolytic action of the KetoCal® ketogenic diet in Mecp2 mice and have translational benefit to the clinic.

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Yi Eve Sun, Ph.D.

UCLA

MeCP2 glycosylation and phosphorylation, the Yin and Yang aspects of MeCP2 post-translational modifications

\$100,000

Research Sponsor: Robert and Adele Schiff Foundation

## Lay Summary

In most cases Rett Syndrome (RTT) is caused by mutations in the gene encoding for the methyl-CpG binding protein, MeCP2. MeCP2 binds to methylated DNA and often leads to inhibition of gene expression. Many RTT associated mutations of MeCP2 occur in either its DNA binding or the transcription repression domain, which underscores the importance of the gene regulation function of MeCP2. Recent studies suggest the cell environmental factors as well as intracellular signaling pathways may also dynamically influence the ability of MeCP2 to inhibit gene expression, which might provide additional therapeutic targets for RTT. Our own studies indicate that MeCP2 can be both phosphorylated and glycosylated on similar serine/threonine residues in response to increase neuronal electric activity. The competitive nature of these two modifications on similar serine/threonine residues of many other factors that regulate gene expression has been shown to lead to opposing effects on turning on and shutting off genes. Therefore we propose to examine the potential Yin & Yang relationship of MeCP2 glycosylation and phosphorylation in response to extracellular stimuli. Our study will likely provide new insight into RTT etiology as well as new therapeutic approaches for RTT.

## Abstract

In most cases Rett Syndrome (RTT) is caused by mutations in the gene encoding for the methyl-CpG binding protein, MeCP2. MeCP2 binds to methylated DNA and often leads to inhibition of gene expression. Many RTT associated mutations of MeCP2 occur in either its DNA binding or the transcription repression domain, which underscores the importance of the gene regulation function of MeCP2. Although the identities of the misregulated genes that cause neuronal dysfunction and the disease symptoms in RTT have not been revealed, recent studies suggested that the DNA binding property as well as the gene regulation function of MeCP2 could be dynamically regulated by extracellular factors and intracellular signaling pathways through posttranslational modifications of the MeCP2 protein. Recently, we have found that MeCP2 is a phosphoprotein with serine/threonine (S/T) phosphorylation at multiple sites under normal conditions. Increased neuronal electric activity causes both increases and decreases of MeCP2 at different S/T sites, resulting in a decrease in the gene inhibition function of MeCP2. In addition, we found that MeCP2 may also be glycosylated by O-linked N-acetylglucosamine (O-GlcNAc) on S/T residues and that depolarization could increase O-GlcNAc glycosylation of MeCP2. Given that phosphorylation and O-GlcNAc glycosylation may target same S/T residue, it was previously reported that these two types of modification of transcription factors usually exert opposing effects on the gene regulation function of those transcription factors. We propose to examine the potential Yin & Yang relationship of MeCP2 glycosylation and phosphorylation by mapping the modulated S/T residues before and after membrane depolarization and studying regulation and the functional consequences of site-specific MeCP2 glycosylation and phosphorylation. Such study may provide new insight into RTT etiology as well as new potential therapeutic approaches.

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Post-Doctoral Fellowship Awards

Feixia Chu, Ph.D.

UCSF

Mentor: Barbara Panning and A.L. Burlingame

## Unraveling aberrant epigenetic gene regulation in Rett Syndrome using mass spectrometry

\$100,000

## Lay Progress Report (August 2006)

RTT has recently been attributed to mutations in a gene encoding the methyl CpG binding protein 2 (MeCP2). MeCP2 interacts specifically with methylated DNA CpG islands via its methyl-binding domain (MBD), and its transcriptional repression domain (TRD) recruits a co-repressor complex, including Sin3A and histone deacetylases. Deacetylation of core histones results in chromatin compaction and transcriptional repression of the target genes. Furthermore, transcriptional repression in vivo could be relieved by deacetylase inhibitor trichostatin A (TCA), underlining the MeCP2-orchestrated chromatin structure alteration as an essential component in this gene expression regulation process. I have set off to use mass spectrometry based proteomic approach to identify changes in posttranslational histone modifications and in global protein expression patterns in MeCP2 knock-out mouse brain. A direct comparison of mass spectrometric analysis on histones at the peptide level has revealed a substantial discrepancy in histone posttranslational modifications between wt and ko mouse, including an up-regulation on H4 Lys20 trimethylation, and a down-regulation on acetylation of histone H4 Lys31 and phosphorylation of H4 Ser47. More comprehensive data processing and analysis is currently underway.

## Lay Summary

Rett Syndrome (RTT) is a dominant neurodevelopmental disorder that affects about 1/10,000 to 1/15,000 females. RTT has recently been attributed to mutations in a gene encoding the methyl CpG binding protein 2 (MeCP2). MeCP2 interacts specifically with methylated DNA CpG islands via methyl-binding domain (MBD), and its transcriptional repression domain (TRD) recruits a co-repressor complex, including Sin3A and histone deacetylases. Deacetylation of core histones results in chromatin compaction and transcriptional repression of the target genes. Furthermore, transcriptional repression in vivo could be relieved by deacetylase inhibitor trichostatin A (TCA), underlining the MeCP2-orchestrated chromatin structure alteration as an essential component in the gene expression regulation process. However, it remains to be revealed if there are other aberrant transcriptional gene regulation in RTT cells, what other histone posttranslational marks MeCP2 employs to ensure the stable gene transcriptional repression and which set of proteins are upregulated due to the aberrant transcriptional gene regulation in RTT cells. We propose to use the cutting-edge techniques in mass spectrometry and cell biology to identify changes in posttranslational histone modifications and in global protein expression patterns in Rett Syndrome (RTT) cells. These studies will substantially contribute to our mechanistic understanding of this disease, the development of more accurate diagnosis, and efficient therapy.

## Abstract

Rett Syndrome (RTT) is mainly (~80%) caused by mutations in a gene encoding the methyl CpG binding protein 2 (MeCP2). MeCP2 binds methylated DNA and represses gene transcription by recruiting histone deacetylases and modifying chromatin structure. It is therefore likely that MECP2 loss-of-function mutants fail to recruit effectors for appropriate spatial and temporal modifications on nucleosomes, which results in abnormal regulation for a set of genes. We will use mass spectrometry-based proteomic approach to compare histone posttranslational modification patterns and expressional proteomes of normal and RTT cells. The results from this systematic, unbiased approach will yield valuable information about defects in nucleosome posttranslational modifications in RTT cells, and provide insight into the epigenetic mechanisms employed by MeCP2 to regulate gene expression. The discovery of differentially expressed proteins in RTT cells will shed light on key effectors that directly cause the phenotype of Rett Syndrome, and develop our understanding of how mutations in this ubiquitously expressed, general transcriptional factor cause the brain-specific phenotype of RTT.

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Gregory Pelka, Ph.D.

Children's Medical Research Institute, Australia

Mentor: John Christodoulou

Investigation of the impact of regionalized Mecp2 deficiency and the manifestation of the RTT phenotype using chimera analysis

\$100,000

## Lay Summary

Rett Syndrome (RTT) is a neurodevelopmental disorder of early childhood, with a prevalence estimated to be 1:10,000 to 22,000 and believed to be one of the most common genetic causes of mental retardation in females. In classic RTT, apparently normal development is seen in females prior to the onset of learning and mobility impairments at 6 to 18 months of life. Approximately 80% of affected females have mutations in the Methyl-CpG-Binding Protein 2 (Mecp2) gene, which is predominantly expressed in the central nervous system.

The generation of mouse models by deletion or truncation of Mecp2 which emulate many social and behavioral aspects seen in RTT patients have provided a basis for further investigations. We have generated Mecp2 deficient embryonic stem (ES) cells which also express a marker gene, enabling mutant cells to be easily identified. We will introduce these ES cells into 3.5 day old mouse embryos which will result in the production of chimeric (containing two genotypes) mice. The ability of the mutant ES cells descendants to contribute to different brain regions and their effect on surrounding cells will be studied. We also hope to pinpoint which brain regions are critical in the manifestation of the RTT phenotype. This will be addressed by correlating the development of RTT features in these chimeric mice with the pattern of Mecp2-deficient cell colonization of their brains.

This research will reveal the previously unrecognized potential role of Mecp2 in early development of the nervous system. The correlation of chimera phenotypes and corresponding mutant cell distribution will identify possible regions in the brain where loss of Mecp2 activity is most critical. This knowledge will provide the foundation for devising therapeutic paradigms whereby tissue-specific treatment can be targeted to the affected brain parts.

## Abstract

To analyze the developmental potency of mutant cells comprehensively, and to determine if Mecp2-null mutation acts in a cell-autonomous manner we have generated Mecp2-deficient embryonic stem (ES) cells placing a promoterless lacZ reporter gene under the control of the Mecp2 promoter. The expression of the lacZ transgene detected by a positive reaction to X-gal histological staining revealed precisely which cells or tissues had an active Mecp2 promoter but were deficient in Mecp2 protein.

Through the introduction of these ES cells into host blastocysts we will generate chimeras which will enable us to (Aim 1) study the impact of Mecp2 deficiency on the development of the central nervous system (CNS) by chimera analysis. This will reveal any limitations on mutant cells in their ability to contribute to regions of the CNS revealing the unrealized contribution of Mecp2 to development, given the genes widespread expression in early development. We will also investigate the phenotypes presented by chimeric mice to (Aim 2) correlate the manifestation of disease phenotype with the chimerism of the brain in mice. This will enable us to pinpoint Mecp2 critical regions of the brain directly involved in eliciting the specific features associated with RTT.

Preliminary results from E16.5 chimeric embryos and adult chimeras have indicated the effectiveness of our Mecp2lacZ-TRD-3ter ES to contribute to different brain regions and manifest quantifiable traits correlated to their regionalized brain distribution. A greater understanding of the role of Mecp2 in development coupled with the identification of brain

regions directly associated with RTT features will provide the foundation for devising therapeutic paradigms whereby tissue-specific treatment can be targeted to the affected brain parts.

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Georg Stettner, M.D.

Georg August University, Germany

Mentor: Peter Huppke

Analysis of breathing abnormalities in Rett patients and MeCP2 knockout mice and development of therapeutic strategies

\$100,000

Lay Progress Report (August 2006)

Most girls suffering from Rett syndrome (RTT) develop severe breathing abnormalities, which have a significant influence on the life quality of the affected girls and also their families. Furthermore, breathing arrhythmias are seen as a main cause of sudden and unexpected death in RTT. Since only little is known about the mechanisms leading to these breathing abnormalities, the aim of our research project is to understand the causes of this breathing disorder to have a starting point for the development of effective future therapies.

During the first year of RSRF funded research we investigated the breathing abnormalities in a RTT mouse model, the so-called *Mecp2*<sup>-/-</sup> knockout mouse. We used a working heart-brainstem preparation (WHBP) that represents the entire neuronal breathing network located in the mice brainstem. We found that brainstem preparations of RTT mice display the breathing abnormalities seen in living RTT mice and moreover, that these abnormalities are strikingly similar to those seen in RTT patients. We could further demonstrate that in RTT mice the early expiratory phase, also called postinspiratory phase, was longer and in its duration highly variable when compared to control mice. The cause for these findings might be a defect in control mechanisms located in neurons (nerve cells) of the brainstem. Further experiments are necessary to proof this hypothesis. These results are of high clinical interest since most of the respiratory abnormalities in RTT patients like repetitive apneas, breath holding spells, Valsalva's maneuvers, and loss of speech are potentially linked to a defect in the brainstem respiratory network.

We also investigate the breathing pattern of RTT girls from different age groups and with different MECP2 mutations by using a mobile monitor. This allows us to perform the measurements at home in the patients' usual environment avoiding hospitalization. So far, 12 girls with RTT have been studied. We will go on and monitor at least 25 patients. These data will illustrate the typical breathing pattern of RTT patients in their daily environment and together with results from the RTT mice breathing data will be the basis for designing a clinical trial to treat breathing abnormalities in RTT girls.

Lay Summary

Breathing abnormalities are seen in almost all Rett patients. Periods of fast or very deep breathing are followed by long

periods without breathing activity. Most parents are sure that their girls suffer under irregular breathing. Furthermore, in a number of patients they lead to epileptic fits and in others to faints. They also have been associated with the sudden unexplained death that is seen in some Rett patients. These are therefore several reasons to try to treat these breathing abnormalities. In order to do so we need to understand the underlying causes. Göttingen offers an expert environment for these investigations. The Department of Pediatric Neurology takes care of more than 100 Rett patients and has concentrated on clinical and molecular research on Rett Syndrome. The Department of Physiology has international expertise in breathing physiology. In our project we plan to investigate the breathing patterns of several Rett girls from different age groups and with different MECP2 mutations. We will do this with a mobile monitor that will be taken home by the patients for 24h. We will be able to study the breathing in the normal environment and we avoid hospitalization. Data analysis will include the description of the type of breathing abnormalities; see if they are specific for a certain age or mutation and if they are related to epileptic fits or epileptic activity in the EEG. However, the study of the brainstem function in detail is very limited in patients. Therefore we will extend our experiments to MECP2 knockout mice. In the Department of Physiology the intra-arterially perfused working heart-brainstem preparation (WHBP) is a well established method that enables us to directly investigate the breathing on the level of the brainstem. It also allows studying the effect of drugs on brainstem function. The ultimate aim is to use these experimental results to establish therapeutic approaches to control the breathing dysfunction in Rett patients.

## Abstract

Towards the end of the regression period most patients with Rett Syndrome develop breathing abnormalities that have in many of them a major influence on life quality. Little is known about the underlying physiology of these breathing abnormalities. What we know comes from investigations in sleep laboratories. However, Rett patients usually do not cope well with an unfamiliar environment and therefore we do not know whether these results really reflect the conditions in daily life. In our project we want to analyze the breathing abnormalities of Rett patients in their normal environment using a mobile sleep laboratory recorder. This recorder allows us to analyze for 24h the breathing activity, blood oxygenation, ECG and EEG during normal activities. The aim is to establish typical breathing patterns for different disease stages, see if there is a genotype/phenotype correlation and analyze EEG patterns during the breathing abnormalities.

However the experiments in humans will only allow us to describe the breathing abnormalities. We will not be able to investigate directly the brainstem dysfunction. In a second set of experiments we will therefore extend our experiments to the MECP2 knockout mouse model. Using the intra-arterially perfused working heart-brainstem preparation (WHBP) we will analyze if these mice do have a disturbed breathing phenotype at all and can serve as a valid model. If this is the case, this model will enable us to study the pathophysiology of breathing abnormalities in Rett Syndrome on the level of the brainstem. The model also allows studying the influence of factors like oxygen level of CO<sub>2</sub> as well as drugs on the breathing activity. In the end we will transfer the results from the animal model to Rett patients for therapeutic approaches.

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