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Epigenetics and Neural Developmental Disorders: Washington DC, September 18 and 19, 2006

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Abstract

Neural developmental disorders, such as autism, Rett Syndrome, Fragile X syndrome, and Angelman syndrome manifest during early postnatal neural development. Although the genes responsible for some of these disorders have been identified, how the mutations of these genes affect neural development is currently unclear. Emerging evidence suggest that these disorders share common underlying defects in neuronal morphology, synaptic connectivity and brain plasticity. In particular, alterations in dendritic branching and spine morphology play a central role in the pathophysiology of most mental retardation disorders, suggesting that common pathways regulating neuronal function may be affected. Epigenetic modulations, mediated by DNA methylation, RNA-associated silencing, and histone modification, can serve as an intermediate process that imprints dynamic environmental experiences on the “fixed” genome, resulting in stable alterations in phenotypes. Disturbance in epigenetic regulations can lead to inappropriate expression or silencing of genes, causing an array of multi-system disorders and neoplasias. Rett syndrome, the most common form of mental retardation in young girls, is due to l mutation of *MECP2*, encoding a methylated DNA binding protein that translates DNA methylation into gene repression. Angelman syndrome is due to faulty genomic imprinting or maternal mutations in *UBE3A*. Fragile X Syndrome, in most cases, results from the hypermethylation of *FMR1* promoter, hence the loss of expression of functional FMRP protein. Autism, with its complex etiology, may have strong epigenetic link. Together, these observations strongly suggest that epigenetic mechanisms may play a critical role in brain development and etiology of related disorders. This report summarizes the scientific discussions and major conclusions from a recent conference that aimed to gain insight into the common molecular pathways affected among these disorders and discover potential therapeutic targets that have been missed by looking at one disorder at a time.

Keywords

Epigenetic; DNA methylation; chromatin; development; Rett Syndrome; Fragile X syndrome; Angelman syndrome; autism; neuronal maturation; synaptogenesis

INTRODUCTION

Neural developmental disorders are highly heterogeneous constellation of disorders both in terms of etiology and clinical manifestations, and therefore post a great challenge in

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understanding their etiology and therapeutic development. Past effort has focused on identifying the genetic basis of these disorders with minimal success. Autism spectrum disorders (ASDs, including autism, Asperger disorder, Rett Syndrome, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified) and related Fragile X syndrome and Angelman syndrome are complex neurological disorders that manifest during early postnatal neural development. Although the genes responsible for some of these disorders have been identified, how the mutations of these genes affect neural development is currently unclear. It is now becoming clear that many of these disorders share common underlying defects in neuronal morphology, synaptic connectivity and brain plasticity. In particular, alterations in dendritic branching and spine morphology play a central role in the pathophysiology of most mental retardation disorders,[1] suggesting common pathways regulating neuronal function may be affected.

Genomic DNA in eukaryotic cells exists in the form of chromatin, and is associated with histones and other chromatin proteins. The term “epigenetics” defines all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself, which only alter phenotype without changing genotype.[2] Three systems, including DNA methylation, RNA-associated silencing, and histone modification, are used to initiate and sustain epigenetic modulation. Epigenetic modulations, such as DNA methylation, within promoter regions (CpG island) could result in heritable silencing of genes without changing their coding sequence. It has been proposed that epigenetic modulations could serve as an intermediate process that imprints dynamic environmental experiences on the “fixed” genome, resulting in stable alterations in phenotype and has long been recognized as an epigenetic silencing mechanism of fundamental importance.[3,4] Disturbance in epigenetic regulations can lead to inappropriate expression or silencing of genes, causing an array of multi-system disorders and neoplasias. Rett syndrome, the most common form of mental retardation in young girls, is due to l mutation of MeCP2, a methylated DNA binding protein that translate DNA methylation into gene repression.[5–8] Also several inherited syndromes are due to faulty genomic imprinting (parent-specific, monoallelic expression of a gene), including Angelman’s syndrome, Prader-Willi syndrome, and Beckwith-Wiedemann syndrome.[9,10] Fragile X Syndrome, in most cases, results from the hypermethylation of Fmr1 gene promoter hence the loss of expression of functional FMRP protein. Recently, mutation in MBD1, another methylated CpG binding protein, was found in a group of autistic patients.[11] Together, these observations strongly suggest that epigenetic modulation plays a critical role in brain development and its normal functions.

OUTLINE OF THE MAIN QUESTIONS COVERED BY THE MEETING

The time of onset and clinical pathology of these neural developmental disorders suggest that common pathways regulating neuronal development may be affected. The genes involved in these disorders overlap in many cases. Existing conferences emphasize just the basic biology of epigenetic regulation, or focus on one disorder at a time, therefore a meeting that links these disorders together may provide insight into the common molecular mechanisms among them and point to potential therapeutic interventions that have been missed by looking at one disorder at a time. Therefore a conference titled “Epigenetics and Neural developmental disorders” was held at Washington DC in September 2006. This conference, sponsored by GTCBio, compared four related neural developmental disorders, Rett Syndrome, Fragile X syndrome, autism, and Angelman syndrome, with focus on the epigenetic links of these disorders. The meeting was therefore divided into four sessions centering around four model disorders: (1) RTT resulted from mutation of MeCP2, a central player of epigenetic regulation. (2) Fragile X syndrome due to a lack of functional FMRP that is regulating and is regulated by epigenetic mechanism. (3) Autism that have strong epigenetic link but with no identified responsible genes; and (4) Angelman syndrome that is due to imprinting loss of functional UBE3A. In addition, the

meeting also discussed the functions of epigenetic regulation in prenatal and postnatal neural development and other developmental disorders with unknown etiology, such as schizophrenia.

DETAILED DISCUSSIONS OF THE SPECIFIC TALKS

Session I. Rett Syndrome

Moderator: Janine LaSalle, PhD, University of California Davis

Rett Syndrome: A Complex Neurodevelopmental Disorder Linked to MeCP2 Mutation: Walter E. Kaufmann, M.D, Kennedy Krieger Research Institute, Johns Hopkins University.

Dr Kaufmann first gave an overview of RTT, a postnatal neurodevelopmental disorder that occurs in 1:8000 to 1:15000 female births and the second most common form of severe mental retardation in females. RTT patients are apparently normal at birth, with postnatal arrest of development including deceleration of head growth, loss of achieved purposeful hand skills but replaced with stereotypical hand wringing, ataxia, cognitive impairment, and social withdrawal. After this initial regression, the symptoms stabilize with improved social contact and eye gaze, but gradual slowing of motor functions.[12] However, the clinical features of individual RTT patients vary significantly from mild to severe. At the most severe end of RTT spectrum are the male patients (Rettoids) that are characterized by many features similar to female patients but with more severity, including moderate to severe early postnatal progressive encephalopathy, central ventilation/respiratory insufficiency, abnormal tone and movements, and intractable seizure. MeCP2 mutation is responsible to 90% classic RTT cases and 50–60% atypical RTT cases.[12] There are about 200 mutations reported with most common mutations at about 10 sites clustered in two regions of MeCP2 protein, methyl-CpG binding domain (MBD) and trans-repression domain (TRD). In addition to MeCP2, mutation in DLK5/STK9, a kinase that may phosphorylate MeCP2, is responsible for certain RTT cases with early onset of seizure. Dr. Kaufmann then discussed the neurobiological correlates of RTT, including reduced brain growth[13] and smaller and more compact neuronal morphology.[14] Several functional characteristics of MeCP2 suggest a role in synaptic development and function, including its neuronal expression pattern, neuronal activity dependent regulation of BDNF, induction by hippocampal kindling, and developmental expression pattern coinciding with neuronal maturation.[15] Using direct lymphocyte samples, Dr. Kaufmann has shown RTT patients have reduced histone H3 acetylation at K-14, and this change correlates with head growth impairment.[16] Using P19 cells as a model, he found that during RA-triggered differentiation, there is an increased 75KDa form but decreased 50KDa form of MeCP2, along with increased acetylation of H3. He proposed during early postnatal development, direct targets of MeCP2, such as BDNF, in conjunction with other synaptic signals have a particularly strong effect on the process of dendritic pruning. Marked reduction in MeCP2 function and deficient afferent input, in neurons carrying a mutated *MECP2* allele, impairs appropriate dendritic expansion. The abnormality extends and worsens during dendritic pruning because of the abnormally high levels of MeCP2 targets (i.e, BDNF) and additional neurotransmitter disturbances (glutamate receptor activity). Finally, in the context of this conference, he discussed that MeCP2 deficiency at postnatal synaptic developmental stages may be involved in other developmental disorders such as Angelman, autism, and other mental retardation. Neurological phenotype associated with MeCP2 deficiency would depend on timing and nature of the deficiency.

Neurobiology of Rett Syndrome: Mary Blue, Kennedy Krieger Research Institute (KKRI), Johns Hopkins University

Dr. Blue suggested that a lack of MeCP2 disrupts neuronal circuits in the brain. Human brain development decelerates during the first postnatal year, partially due to a neuronal maturation process called dendritic pruning. Using radioactive ligand binding assay, Dr. Blue has found that expression levels of NMDA glutamate receptors are significantly higher in two year old RTT patients, but lower in 10 year old RTT patients, compared to age-matched controls. At both ages, synaptic densities are lower and the number of NMDA receptors per synapse are higher, with younger patients showing a more dramatic deficit suggesting younger patients are more affected by MeCP2 deficiency during the peak of synaptic maturation.[17] This observation is consistent with previous findings that RTT brains have elevated Glutamate.[18–20] On the other hand, increased expression of GABA receptors have been found in postmortem RTT brains and RTT brains also have increased expression of DLX5 that leads to increased activity of GABA synthetic enzymes. Therefore a lack of MeCP2 not only affects excitatory but also inhibitory transmitters in the brain. Since most RTT patients are heterozygote females, Dr. Blue's group has compared *Mecp2* heterozygote mice with null and WT mice and found that there is no difference in either the total number of neurons or the cortical volume among wildtype, HET and null mice. However, in older HET mice (24–95 week of age), 68% of neurons express wildtype MeCP2 compared to 50% at 7–9 week of age. Her current work aims to determine whether this increase is due to increased neurogenesis, reactivation of MeCP2 expression from the inactivate X-chromosome, or developmental delay. Nevertheless, such a change may explain the more neurologically stable period seen in many Rett patients.

Long-term Neurodevelopmental Consequence of Mice Expressing a Truncating Mutation: Amy Palmer, Johns Hopkins University

Dr. Palmer's presentation showed that MeCP2 truncation mutant (TM, or *Mecp2*$^{y/308}$) mice have a similar defect as the Mecp2 null mouse model; however, the cellular dynamics are different between these two mouse models. The olfactory system provides a good model for studying neurogenesis and neuronal maturation because its laminar organization allows the detection of abnormalities. Also, cellular markers for mature (OMP) and immature neurons (GAP-43) are readily available. Using BrdU to label a cohort of proliferating neurons, Dr. Palmer showed that at two weeks post labeling, there is an early block (presymptomatic) in maturation during the time of initial synaptogenesis. Additionally, following the initial neuronal defect there is subsequent compensation prior to the onset of symptoms. However, this compensation did not involve cell death as shown by TUNEL studies. Following the onset of the described neuronal defect, axonal morphology was compromised in the olfactory bulb due to a reduction in mature olfactory neurons at four weeks. During adult ages when animals are at a late-symptomatic stage, the olfactory system continues to show signs of compensation by increasing cell proliferation. This is also reflected in the olfactory bulb where glomeruli, which were significantly smaller than wildtype controls later regained a size comparable to wildtype animals. Additionally, the disruptions in axonal morphology did not persist into adulthood. An assessment of activity at twelve weeks suggests that adult TM animals have a normal level of synaptic activity. These studies suggest that the olfactory system is plastic and conductive to neurodevelopmental compensation/rebounding. Furthermore, *mecp2* truncation or deficiency disrupts synaptogenesis and neuronal maturation during early postnatal ages.

Role of Phosphorylation in Regulation of Mecp2 Function: Keping Hu, Nemours, A.I. duPont Hospital for Children and NIH/NIA

Dr. Hu focused on two central questions underlying MeCP2 mediated gene regulation: whether MeCP2 forms a stable complex with other proteins and what the functions of MeCP2 phosphorylation are. Although some studies have suggested that MeCP2 forms a stable complex with histone deacetylase, Sin3, and Brahma-associated SWI/SNF complex,[21–23]

other studies proposed that MeCP2 does not form stable complex with other proteins.[24] To solve this controversy, Dr. Hu, using immunoaffinity purification coupled to mass spectrometry protein sequence analysis, found that MeCP2, purified from mouse brain, does not stably associate with any other proteins.[25] It has been shown that KCl-induced neuronal depolarization leads to phosphorylation of MeCP2 and its subsequent release from *Bdnf* promoter.[7,8,26] To determine which amino acid reissues in MeCP2 are phosphorylated, Dr. Hu purified MeCP2 from mouse brains and found that native MeCP2 ran as two bands in an SDS gel. He identified that MeCP2 is phosphorylated at 7 Serines in the upper band and 5 Serines in the lower band. In contrast to S-421, whose phosphorylation correlates with neuronal activation and is responsible for the up shift of MeCP2 in SDS gel,[26] Ser80 phosphorylation of MeCP2 is decreased in response to increased neuronal activity and phosphor-S-80 is found in both upper and lower bands. Transfection of neurons with Ser80 mutant (S80A) MeCP2 plasmid leads to attenuated induction of *Bdnf* expression upon neuronal activation. Therefore, increased neuronal activity correlates with the overall level of phosphorylated MeCP2, but inversely correlates with phosphorylation at Ser80. Phosphorylation at different sites of MeCP2 may have distinct functions, which may explain why MeCP2 mutations lead to variable phenotypes.

Rett Syndrome: A Rosetta Stone for Understanding Autism?: Janine LaSalle, University of California, Davis School of Medicine

Dr. LaSalle proposed that MeCP2 may be the important epigenetic link between Rett Syndrome, autism and Angelman syndrome that share common phenotypes. Prior evidence has shown that genomic 15q11-13 maternal duplications were found in 1–3% of autism and 15q11-13 loci have been linked to autism. However, direct molecular evidence for epigenetic alterations in autism was lacking. Using human neurodevelopmental disorder brain tissue microarray followed by laser scanning cytometry, Dr. LaSalle's group found that 80% of autism brain tissue had reduced MeCP2 expression.[27] In addition, reduced MeCP2 expression was also found in majority of tissue samples from Prader-Willi, Angelman, and Down's syndromes patients. In conclusion, reduced MeCP2 expression is a frequent but not unique characteristic of the Autism frontal cortex. Since high MeCP2 expression is a marker for neuronal maturation, she hypothesized that disorders affecting neuronal maturation would likely also indirectly affect MeCP2 expression. In fact, her lab found that reduced MeCP2 expression in autism frontal cortex correlates with aberrant MeCP2 promoter hypermethylation, which is more prominent in the younger juvenile samples than adult samples. Interestingly, this correlation seems specific to autism, because it is absent for Down's patients.[27]

Because 15q11-13 also contains a cluster of genes encoding GABA receptors that have important roles in neurodevelopment, *GABRB3*, *GABRA5*, and *GABRG3*, Dr. LaSalle's lab analyzed the expression of these receptors on a large number of frozen postmortem human tissues.[28] They found that unlike normal individuals, AS and PWS patients had a paternal bias in expression of these three genes. In addition, one Rett and three autism brain samples showed a loss of normal biallelic expression for one or more of these three genes, which also correlates with a lower expression level of *GABRB3* expression. The observation of positive correlation between MeCP2 and *GABRB3* protein levels prompted her lab to test whether MeCP2 may positively regulate *GABRB3* expression. Indeed, they found that MeCP2 binds to at least two regions of *GABRB3* and *GABAG3*, but not *GABRA5*. Bisulfate sequencing data indicate moderate CpG methylation in the regions with high MeCP2 binding in *GABRB3* intron 3, and high level of CpG methylation in corresponding intronic regions of *GABRG3*. Several of the methylation CpG sites are next to AT-runs that are high affinity sites for MeCP2.[33] In conclusion, epigenetic defects in autism samples may lead to the overlapping phenotypes with RTT and AS.

Epigenetic Gene Regulation in Neuronal Survival and Maturation: Guoping Fan, Department of Human Genetics, University of California, Los Angeles

Dr. Fan investigates how DNA methylation is involved in neuronal differentiation and survival by using mice with Dnmt1 deleted in specific cell types at different developmental stages. These mice are generated by using mice with conditional *Dnmt* gene deletion crossed with mice that express Cre recombinase under specific promoters. For example, Nestin-Cre mice express Cre in neural stem cells (NSC) and therefore the entire CNS; Emx1-Cre mice express Cre in only the cortex and hippocampus; CamK-Cre mice express Cre in post mitotic neurons in the forebrain; Wnt1-Cre mice express Cre in neural crest derivatives including PNS. Nestin-cre;Dnmt1 CKO mice are neonatal lethal because that DNA hypomethylation in the CNS disrupts vital functions such as neural control of respiration. During embryonic development, neurogenesis precedes gliogenesis, and the timing of gliogenesis (at about E14) is correlated with the activation of STAT signals. Dr. Fan found that there was increased expression of STAT1 in $Dnmt1^{-/-}$ CNS, which correlated with the demethylation of STAT1 promoter and increased association with active histone modification on Stat1 promoter. In addition, there was precocious astroglial differentiation in E11.5 $Dnmt1^{-/-}$CNS neural precursor cells (NPCs) both in vitro and in vivo, and this precocious differentiation was mediated by elevated STAT1 and STAT3 activation. Therefore, DNA methylation controls the timing of astrogliogenesis. Conditional knockout mice of *Dnmt1* with the Emx1-Cre live to adulthood; however these mice have severe cortical degeneration and significantly high levels of apoptotic cell death in both embryonic and adult brains. Dr Fan bred these mice with transgenic mice that express EGFP in specific cortical layers (e.g., M4-*egfp*; Emx1-Cre; $Dnmt1^{2lox/2lox}$) and used these triple transgenic approaches to mark the demethylated cell population in developing and adult cortex. He found that $Dnmt1^{-/-}$ neurons exhibit defects in both cell morphology and neuronal excitability. Dr. Fan's group is currently determining direct vs. indirect effect of DNA demethylation on neuronal gene expression using gene expression profiling in specific neurons (e.g., FACS purified subpopulation of M4+ cortical neurons); genome-wide methylation analysis in control and mutant neurons; and bioinformatics analysis to connect demethylation in gene promoters with gene activation. The combination of these powerful mouse genetic models and state of art high throughput approaches will provide critical information on how DNA methylation mediated epigenetic mechanisms regulate brain development and functions.

Session II. Fragile X Syndrome

Moderator: Peng Jin, Emory University School of Medicine

Molecular Pathway Determination, and Therapeutic Development for Fragile X Syndrome: Steve Warren, Department of Human Genetics, Professor of Pediatrics, Professor of Biochemistry, Emory University. Keynote Speaker.

Dr. Warren's keynote presentation began with comprehensive summary of molecular basis and clinical phenotypes of Fragile X syndrome, the most frequently inherited form of mental retardation. The 5′ untranslated region of *FMR1* presents highly polymorphic CGG-repeat in general population. Three classes of *FMR1* alleles are associated with repeat length; normal alleles ranging from typically 7–55 triplets, carrier premutation alleles ranging from 55–200 triplets and full mutation alleles showing greater than 200 triplets which give rise to the disorder. In the case of full expansion of repeats, epigenetic silencing of *FMR1* due to methylation of CGG repeats and upstream CpG islands occurs, resulting in the loss of the encoded protein, FMRP. Loss of FMRP is detrimental owing to its critical role in neuronal function from mediating control of local protein synthesis in neuronal processes. Abnormal dendritic spines observed from both fragile X syndrome patients and *Fmr1*-knockout mice further strengthen the notion that FMRP is involved in synaptic maturation and spine-pruning through regulating type I mGluR-stimulated local protein synthesis at the dendrites. In this

regard, consequence of FMRP loss is the over-translation of mRNAs which normally associates with FMRP and thus leads to exaggeration of mGluR-stimulated processes as seen in fragile X syndrome patients. This model is further supported by the fact that treatment with mGluR antagonists can rescue behavioral and neurological defects in both mouse and *Drosophila* models of fragile X syndrome.[29,30] Dr. Warren discussed a more recent progress in fragile X syndrome where in an attempt to further depict the molecular etiology and normal function of FMRP, Dr. Warren's group undertook a 2,000 compound drug screen which implicated FMRP in the GABAergic inhibitory pathway and identified additional nine compounds that were able to rescue the *dFmr1*-deficient phenotype in flies. This work potentially could provide further insight into therapeutic trials and discovery of new treatments.

Characterization and Pharmacological Rescue of a Drosophila Model for Fragile X Syndrome: Thomas A. Jongens, Department of Genetics, University of Pennsylvania School of Medicine

Dr. Jongens discussed his research using a *Drosophila* model for fragile X syndrome, which was developed based on loss-of-function mutants of *dfmr1* and studies have shown neuronal and behavioral phenotypes that are similarly observed in actual fragile X patients. Phenotypes include alterations in circadian rhythms, synaptic branching, and courtship behaviors but cognitive defects were not monitored previously. Based on fragile X mouse model data which show enhanced mGluR activity, Dr. Jongens' group explored the possibility that similar mGluR misregulation might occur in the fly model. Taking a pharmacological approach, Dr. Jongens' group used mGluR antagonists which work in vivo in mammals and whose binding pockets have been well characterized.[29] One antagonist, 2-methyl-6-(phenylethynyl) pyridine (MPEP) was extensively used in this study which is an antagonist of mammalian group I subtype 6 mGluR. When examining the ability of MPEP treatment to restore normal courtship behavior in *dfmr1* mutant males, treatment of MPEP resulted in a significant increase in naïve courtship levels. However, they did not observe learning defects in flies lacking *dfmr1* activity and treatment of MPEP did not have any effect in observed learning phenotype. Dr. Jongens' group also examined the effect of MPEP treatment on mushroom bodies (MBs) associated with learning and memory, but mGluR antagonists could not rescue morphological defect of MBs in fragile X mutant flies. These findings suggest potential therapeutic approach to improve cognitive defects and behavioral symptoms seen in fragile X patients by similar modulation of mGluR activity.

Role of MicroRNA Pathway in Fragile X Syndrome: Peng Jin, Department of Human Genetics, Emory University

Although specific structural interactions between mRNAs and FMRP have been identified, Dr. Jin has sought out to unravel the unclear mechanism by which FMRP regulates the translation of mRNAs. MicroRNAs (miRNAs) are a class of non-coding RNAs that are thought to control translation of specific mRNA targets by base-paring with imperfect complementary sequences in the mRNA 3′ untranslated region. Mature miRNAs are single-strands of 20–25 nucleotides and are generated from processing of ~70 nucleotide stem-loop precursors by Dicer, a dsRNA-specific RNAse III enzyme. Members of PIWI-PAZ-domain protein (Argonaute) family facilitate the downstream processing and functions of miRNAs that are incorporated into RNA-induced silencing complex (RISC) in which some of its components are also used by small interfering RNAs (siRNAs). Previous works suggest the biochemical link between FMRP and components of the RNAi pathway. *Drosophila* gene *dFmr1* has been shown to interact with *Argonaute 2* (*AGO2*) and RISC. Also, FMRP does associate with miRNAs in both *Drosophila* and mammals. In particular, FMRP has been shown to interact with mammalian Argonaute protein, eIF2C2, a component of miRNA-containing mRNP complexes. Furthermore, association of FMRP with Dicer activity suggests its possible involvement in the

processing of miRNA precursors. Dr. Jin showed the association of human FMRP with endogenous miRNAs both in its mature forms and precursor transcripts, mammalian Argonaute protein eIF2C2, as well as dicer activity in vivo.[31] The idea that FMRP interacts with components of miRNA pathway was further validated genetically using *Drosophila* as a model system. He proposed a model where the use of miRNAs as a mechanism to generate synaptic tags that transiently mark a synapse after activation, allowing for local protein synthesis-dependent synaptic strengthening as previously has been implicated for FMRP.

Fragile X and DNA Methylation: Karen Usdin, NIH/NIDDK

Dr. Usdin's interests lie in the mechanism responsible for CGG·CCG-repeat expansion in the *FMR1* gene and the subsequent abberrant gene expression that results. Her group and others have shown that the FMR1 promoter region binds transcription factors such as NRF-1, Sp1/3, CREB and USF1/2 in cells from unaffected individuals and carriers of premutation alleles (55–200 repeats). This binding is not seen in carriers of full mutation alleles (>200 repeats) where the gene is heterochromatinized and transcriptionally silent. Premutation alleles generate abnormally high *FMR1* mRNA levels. Such alleles are associated with Fragile X tremor and ataxia syndrome and premature ovarian failure. It has been suggested that the elevated FMR1 levels result from changes in the TSS usage seen in premutation carriers. This in turn may be the result of a more open chromatin architecture related to the ability of CGG-repeats to exclude nucleosomes in vitro. It has also been suggested that the new 5′ UTRs contribute to the poor translatability of the *FMR1* mRNA from premutation alleles. However, Dr Usdin's group has shown that in the *Fmr1* premutation mice her group has generated, elevated levels of *Fmr1* mRNA occur in the absence of TSS changes. Moreover, no nucleosome exclusion is evident in human premutation carriers. The murine premutation transcript is also poorly translated even though the TSS remains unchanged.[32] It may be that the translation difficulties in both humans and mice result from the stable RNA hairpins that the Usdin group have shown to be formed by the CGG-repeat 38. Dr Usdin's group has also shown that the translation difficulties lead to regional deficiencies of FMRP in the premutation mouse brain.[32] This may explain why human premutation carriers show some but not all of the symptoms of Fragile X mental retardation syndrome seen in full mutation carriers. Dr. Usdin's group has also shown that CGG-RNA hairpins are substrates for the human Dicer enzyme 38 raising the possibility that the gene silencing seen in full mutation carriers is triggered by the expanded CGG-repeat via some sort of RNA interference-type mechanism.

Regional Rates of Cerebral Protein Synthesis in Fragile X Mice: Carolyn Beebe Smith, Laboratory of Cerebral Metabolism, National Institute of Mental Health

Dr. Smith's research focuses on studying the underlying causes of brain dysfunction in genetic mouse models of inherited forms of mental retardation. Phenotypes associated with fragile X mice are: hyperactivity, enlarged testicles, abnormal dendritic spine morphology, increased energy metabolism in brain, susceptibility to audiogenic seizures and mild deficits on spatial learning tasks. In her study, she utilizes an in vivo quantitative autoradiographic method that she developed for measuring regional rates of cerebral protein synthesis (rCPS) in experimental animals. Applying this technique, her group strives to give insight in the precise role of FMRP in neuronal function by determining rCPS in vivo in adult wild type and *Fmr1* null mice. Her results from quantitative autoradiographic rCPS method show a substantial decrease in rCPS in both WT and *Fmr1* null mice between the ages of four and six months which might reflect the process of synaptic pruning during young adulthood. However, she demonstrated a regionally selective elevation in rCPS in *Fmr1* null mice particularly in hippocampus, hypothalamus and thalamus. From these observations, one can infer FMRP as a negative regulator of protein synthesis and the observed regional selectivity of elevated rCPS may reflect FMRP distribution in vivo.

Session III. Autism

Moderator: Elizabeth Powell, University of Maryland

Roles of Plasminogen Related Molecules in Forebrain Maturation and Circuit Formation: Elizabeth M. Powell, Departments of Anatomy & Neurobiology and Psychiatry, University of Maryland School of Medicine

During brain development, orderly generation of neurons defines the phenotypic properties of the neurons. In mammalian brains, glutamatergic and GABAergic neurons (ratio 6:1) comprise the cortical circuits. Disruption of GABAergic interneuron migration and differentiation could alter the balance of excitatory to inhibitory neurotransmission, leading to behavioral deficits. Despite significant progress made in this field, many critical questions remained to be answered. One family of factors that play an important role in brain development is hepatocyte growth factor/scatter factor (HGF/SF). HGF belongs to a large family of Kringle-Serine Protease super family that also includes plasminogen, u-PA, t-PA, and prothrombin. HGF is expressed as inactive pro-HGF and its activation requires urokinase plasminogen activator receptor (uPAR). Inactivation of the *uPAR* gene leads to reduced levels of HGF and a 50–65% reduction in cortical GABAergic interneurons beginning at embryonic day 16.5 (E16.5).[33] Dr. Powell has shown previously that HGF/SF mediates interneuron migration and that the of uPAR−/− mouse has an overall decrease in $GABA^+$ interneurons in the parietal cortex, which is attributable both to a drastic reduction in the number of parvalbumin $(PV)^+$ cells and to a decrease in detectable GABA immunoreactivity in the PV^+, calretinin $(CR)^+$, and somatostatin $(SST)^+$ subtypes. To confirm that uPAR affects interneuron development through the HGF pathway, Dr. Powell confirmed that uPAR−/− mice have decreased levels of HGF and its receptor MET in the forebrain. In addition, by crossing uPAR−/− mice with GFAP-HGF mice, Dr. Powell created rescued uPAR−/− mice with increased HGF expression.[34] In these rescued mice, both GABA+ and PV+ GABAergic interneuron defects of uPAR−/− mice were recovered, indicating that the interneuron deficits in uPAR−/− mice are due to defect in HGF pathway and that HGF, MET and uPAR systems are critical for interneuron development. Reduced interneuron number lead to behavioral deficits in mice, including increased seizure susceptibility (8% uPAR−/− mice), increased anxiety, and reduced cognitive (executive) functioning. These behavioral phenotypes of the uPAR−/− mouse are similar to human autism spectrum disorders. Addition of HGF can reverse seizure susceptibility and social interaction abnormalities, demonstrating that HGF, MET and uPAR systems may have a critical role in neurodevelopmental disorders.

Designing Mouse Behavioral Tasks Relevant to the Symptoms of Autism: Jacqueline N. Crawley, Laboratory of Behavioral Neuroscience Intramural Research Program, NIH/NIMH

Autism is a neurodevelopmental disorder that is behaviorally defined by three core symptoms: 1. Aberrant reciprocal social interactions; 2. Qualitative impairments in social communication; and 3. Stereotypes, repetitive, ritualistic behaviors, narrow or restricted interests. To develop effective mouse models for autistic research, it is important to know which of the above symptoms are the most critical to model and also have analogies in mice. However, researchers should keep in mind that there may never be “autistic mice.” Rather, we are generating mouse behavioral assays that face validity to the symptoms of autism, mouse models that are used to test hypotheses and to provide translational tools to evaluate treatment efficacy. To develop effect models, we need to first understand the Social Behaviors of *Mus musculus.* Dr. Crawley described the approach she and collaborators have developed to model the number one core symptom of autism, sociability. One of them is “Automated Social Approach Apparatus” for detecting social interaction in mice.[35] Using this 3 chamber-device, she showed that three different commonly used inbred strains of mice, despite age differences, all spend significantly more time in the chambers that housed stranger mouse, rather than in empty chambers. In

addition, the time spent in the mouse chamber, but not number of entries, positively correlate with true social interaction, rather than exploration. Dr. Crawley cautioned that other parameters that may mask the social behavior tests should be considered by researchers, such as general health, neurological reflexes, sensory abilities, and motor abilities. She gave one example to show that the social interaction test can be used in forward genetics to identify social interaction genes among different strains of mice. The number two core symptom of autism is social communication. Mice communicate primarily with olfactory pheromones and ultrasonic vocalizations. Therefore, the tests are designed to look for reduced responses to olfactory social cues or fewer ultrasonic vocalizations and inappropriate responses to ultrasonic vocalization. Olfactory habituation/dishabituation to test social smells has been developed and used to test Galanin overexpressing transgenic mice.[36] The number three core symptom of autism is stereotyped repetitive behaviors and narrow restricted interests. Mice can be videotaped and scored for spontaneous stereotyped jumping, grooming, reduced exploration, repetitive behaviors, and restricted interests. Using Morris water maze and T-maze spatial tasks, mice can be tested for failure to make change in a search strategy. Holeboard exploration task in an Accuscan Pokemon holeboard apparatus can be used to determine whether mice have restricted interests. Dr. Crawley's group continues to ask how we can best model the defining features of autism in mice.

Methyl-CpG Binding Proteins and Autism Spectrum Disorders: Xinyu Zhao, Department of Neuroscience, University of New Mexico School of Medicine

Methyl-CpG binding proteins (MBDs) translates DNA methylation into gene repression.[37] Dr. Zhao's presentation focus on the function of two of the MBDs in postnatal neurodevelopment: MeCP2 and MBD1. Using postnatal hippocampus as model system, they found that, while MeCP2 was not critical for the production of immature neurons in the dentate gyrus (DG), the newly generated neurons exhibited pronounced deficits in neuronal maturation, includingdelayed transition fromimmature (Doublecortin positive) into a more mature stage (NeuN positive) stages. Using retroviral-mediated gene delivery to mark single new neurons, they found that newly matured neurons in *Mecp2* KO mice have reduced dendritic spine density, suggesting that MeCP2 plays a central role in neuronal maturation, a role that is likely to be mediated through epigenetic control of expression pathways that are instrumental in both dendritic development and synaptogenesis. Although extensive in vitro analyses have suggested a role for MBD1[38] in transcriptional repression,[39] chromatin assembly,[40,41] and heterochromatin structure maintenance, the biological function of MBD1 is not well understood. By generating and analyzing *Mbd1*$^{-/-}$ mice, Dr. Zhao's group demonstrated a novel and clear role for MBD1 and DNA methylation in adult brains. At cellular level, *Mbd1*$^{-/-}$ NSCs have reduced neuronal differentiation and proliferation and increased genomic stability. At animal level, *Mbd1*−/− mice have reduced neurogenesis and learning. In addition, she found that *Mbd1*$^{-/-}$ mice have characteristics that are similar to autistic phenotypes, such as reduced social interaction, increased depression and anxiety, abnormal serotonin activity and HPA axis regulation. Together with the recent discovery of *MBD1* mutation in small number of autism patients, MBD1 may regulate multiple pathways linked to autism and ASDs.

Identification of Autism Candidate Genes Through Proteomic Profiling in Drosophila Melanogaster: Lawrence T. Reiter, Department of Neurology, University of Tennessee Health Science Center

Identification and understanding the functions of disease genes in humans is complex and difficult. *Drosophila melanogaster* has proven to be a valuable model organism for studying the molecular pathway of disease genes. To use *Drosophila* for human disease gene study, it helps if there is a fly counterpart highly related at the protein level to the human gene (Homophila database: <http://superfly.ucsd.edu/homophila/>). If mutant alleles already exist

with P-elements in or near the gene of interest, and/or there is obvious phenotype for mis-expression using GAL4/UAS system, it will facilitate the genetic screening process. Dr. Reiter presented how his group uses *Drosophila* to search for proteins regulated by UBE3A, an E3 ubiquitin ligase protein that catalyzes ubiqutination of proteins targeted for degradation by the ubiquitin proteasome system (UPS). *UBE3A* maternal gene deletion or mutation results in Angelman syndrome (AS) (see Session IV). It has been proposed that failure of UBE3A to target proteins for degradation is a key aspects of the pathogenesis of AS. Dr. Reiter made transgenic flies that express human UBE3A using *GAL4-UAS* expression system and crossed these flies to *Heatshock*-GAL4 transgenic flies to create flies that express human UBE3A in the eyes upon 37°C heatshock. The protein extracts were then prepared from the heads of flies and separated by 2-dimensional gel electrophoresis. They found that 19 spots have lower expression levels and 5 spots have increased expression levels in UBE3A expressing flies, compared to control flies. One of the spots down regulated by UBE3A is Rho-Guanine nucleotide Exchange Factor (Pbl). Pbl and its mammalian orthologue Ect2 physically interact with Dube3a/UBE3A proteins, respectively. In *Drosophila* eyes, UBE3A suppresses Pbl activity and fat facets, and de-ubiquitinase enhances the Pbl phenotype. Thus, Pbl activity appears to be regulated by the UPS. Finally, he confirmed that there is indeed mis-regulation of *Pbl/Ect2* in the brains of *Ube3a*−/− mice. Therefore, Ect2 may be a functional target of UBE3A contributing to the human AS phenotype. Dr. Reiter plans to use the *Drosophila* mis-expression system to identify all putative protein targets of UBE3A, Dube3a and Faf and explore the hypothesis that UBE3A regulated substrates may substantially contribute to the pathology of both AS and autism.

Epigenetic Programming by Maternal Care: Moshe Szyf, Department of Pharmacology and Therapeutics, McGill University.

Dr. Szyf and collaborators have found that in the rat good maternal care, which is typified by high licking and grooming (LG), and arched-back nursing (ABN) within the first 6 days of postnatal life leads to increased glucocorticoid receptor (GR) expression in the hippocampus and a reduced stress response in adult offspring, indicating the importance of early social environment on programmed gene expression later in life. The critical questions are how are the long-term effects of maternal care on gene expression maintained into adulthood, and how these differences are transmitted across generations. A splice variants of the GR mRNA containing the exon 1_7 sequence is found predominantly in the hippocampus. The expression of this variant is increased in the offspring of high-LG-ABN mothers or following manipulations that increase maternal licking and grooming, suggesting that the use of this promoter is enhanced as a function of maternal care.[42] Exon 1_7 sequence promoter has a binding site for nerve growth factor-inducible protein A (NGFI-A), a transcription factor also known as egr-1, krox-24, zenk, and zif-268. In the hippocampus, the 5′CpG of NGFI-A binding site is methylated upon birth but demethylated only in high LG pups, not in LG pups.[42] Dr. Szyf proposes that the steady state methylation pattern of a given gene is a dynamic equilibrium between methylase and demethylase activities. Therefore, Dr. Szyf treated adult rats (day 90) with TSA which should cause replication-independent active demethylation[43–45] and found that TSA treatment caused active demethylation of GR promoter and consequently, the GR gene expression in Low LG-ABN offspring was modified to reach the expression of High LG-ABN offspring in the hippocampus which resulted in reduced stress response in Low LG-ABN offspring. On the other hand, treating rats with Methionine that provides substrate of DNA methyltransferease caused active remethylation of the NGFI-A binding site on the GR promoter, reversed the effect of high maternal LG-ABN.[46] Whether a DNA demethylase exists in the adult brain is currently controversial. Dr. Szyf proposed that MBD2 is a DNA demethylase that is responsible for this dynamic equilibrium.[47] First, he shows purified His-dMTase from HEK293 cells exhibits in vitro demethylase activity. Second, ectopic expression of MBD2 causes both global demethylation and demethylation of methylated genes in Panc1

pancreatic cancer cells and nontransformed NIH 3T3 cells. Third, MBD2 localizes with sites of histone acetylation and with HAT CBP in the nucleus of NIH 3T3 cells. Fourth, LG-ABN increases the binding of MBD2 on day 6 to the hippocampal GR promoter. Finally, NGFI-A interacts with MBD2; ectopic expression of NGFI-A increases MBD2 binding to promoter. At this point Dr. Szyf switched his topic to human studies. The state of methylation of rRNA genes determines the level of activity of rRNA gene expression and by extension cellular protein synthesis activity. Dr Szyf found that there is a correlation between the state of methylation of rRNA genes in DNA and aggressive behavior in humans. In another study, he found that suicidal individuals had high methylation of rRNA promoter, as well as higher GR promoter than controls. These data suggests that environmental effect on the epigenome might have profound and long lasting effects on social and psychiatric behaviors and related disorders.

Redox-Dependent Regulation of Methylation by Methionine Synthase: Richard Deth, Pharmacology, Northeastern University

Methionine synthase that catalyzes methylation of homocysteine (HCY) to methionine (MET) is a part of the MET cycle of methylation. Methionine synthase activity can affect DNA methylation through several mechanisms. Oxidative stress inhibits methionine synthase activity and leads to increased HCY and S-adenosylhomocysteine (SAH) levels and SAH is an inhibitor of DNA methyltransferase by blocking the binding to its substrate SAM. On the other hand, inhibition of methionine synthase activity using inhibitors of PI3 kinase leads to decreased DNA methylation of the cyclin D2 gene and increase its transcription. In human SH-SY5Y cells, a neuroblastoma cell line, IGF-1 induced global DNA methylation is blocked by PI3 kinase inhibitors that mediating methionine synthase activity. Inhibition of MS activity can also lead to compensatory reduction of oxidative stress. Brain imaging analyses indicate that the autistic brain shows very little cooperation between brain areas when volunteers try to perform copycat finger movements, suggestion under-connectivity of high cortical functions. Dr. Deth has found that Plasma levels of methionine cycle-related metabolites in association with oxidative stress are abnormal in autism. Autistic children have higher levels of SAH and oxidized glutathione, but significantly lower levels of MET, SAM, HCY, and glutathione. Therefore, he proposes that autism might be a neurological disorder of metabolic origins. Methionine synthase is also used for phospholipids methylation of dopamine D4 receptor. Reduced methionine synthase leads to reduced dopamine D4 receptor stimulated phospholipids methylation therefore may be responsible for neuronal synchronization that leads to reduced attention in autism. He presented a preliminary human study using nasal methylB12/folinic acid that increased DNA methylation in an ADHD subject. Immediately after the drug delivery, the attention has improved and such improvement lasts for more than 1 hour. In summary, genetic polymorphisms, environmental triggers, inflammation and oxidative stress can all affect DNA methylation, which in turn may contribute to autistic phenotypes.

A Comprehensive Model of Gene Regulation in Schizophrenia/Bipolar Disease: SNPs, Promotor Methylation, and Gene Expression Levels for COMT, RELN and DRD2 in the Prefrontal Cortex: Cassandra Smith, Molecular Biotechnology Research Laboratory, Boston University

Schizophrenia (SCZ) is a devastating disorder affecting 1% of the worldwide population (2.2 million in US) and has been linked to over 100 genes throughout the genome, yet it has been difficult to link a single gene to the majority of patients. Reelin (RELN) mRNA has been shown to have abnormal expression in schizophrenia (SCZ) and bipolar disorder (BP). To find a potential mechanism for the down regulation of RELN expression, Dr. Smith's group analyzed gene expression and genomic methylation in post-mortem tissue from the Harvard Brain Tissue Resources Center (HBTRC) of SCZ patients. They found promoter hyper-methylation of the *RELN* gene in the frontal lobe correlated with the pathogenesis of SCZ.[48] These findings have

been confirmed in a larger sample set (n=115)[49] and in a different study using a different sample set from the same brain banks.[50] These results led Dr. Smith's group to investigate the epigenetic modifications in the Catechol-O-methyltransferase (*COMT*) gene. Membrane-bound COMT (MB-COMT) is involved in dopamine catabolism in the human brain, which is linked to a number of psychiatric illnesses. MB-COMT has a functional polymorphism with the Val/Val genotype gives rise to an enzyme with a 3-fold higher enzymatic activity than the Met/Met genotype, and has been linked to SCZ and BD in a large study with 720 patients and 2970 controls,[51] as well as early onset of major depressive disorder and suicide[52] Dr. Smith's group found significant hypo-methylation of the *MB-COMT* promoter and 2.7 times higher expression of *MB-COMT* mRNA levels in the frontal cortex of SCZ and BP patients. They found that in SCZ and BP, but not in controls, hypo-methylated *COMT* promoter is linked to hyper-methylation of *RELN*/*DRD2* promoters and the Val allele. Overall, DNA methylation helps adaptation to variable environments in each generation and compensates for mal-functional polymorphisms, a job the genome can not accomplish in the short term. Environmental factors also impact DNA methylation, and can be more problematic for individuals with susceptibility to genetic disease.

Session IV. Angelman Syndrome

Moderator: Laura Herzing, Northwestern University

Deregulated Expression of Non-coding RNAs in Angelman Syndrome: Marc Lalande, University of Connecticut

Genetic phenomenon of genomic imprinting is clinically manifested in Angelman syndrome (AS) and Prader Willi syndrome (PWS) both of which, in majority (~70%) of cases, result from deletion of human chromosome 15q11-q13. Depending upon the parental origin of inheritance of this deleted region, two different disorders occur. In the case of AS, the deletion is inherited from the mother, which renders the offspring with no functional copy of ubiquitin protein ligase E3A (UBE3A) that is paternally imprinted. On the contrary, PWS results from deletion which is of paternal origin where multiple genes are maternally imprinted. Clinical features of AS include microcephaly, severe mental retardation, 'puppet-like' ataxic gait with jerky arm movements, seizures, EEG abnormalities, hyperactivity and bouts of inappropriate laughter. PWS characteristics involve hypotonia, failure to thrive in infancy and marked obesity resulting from hyperphagia during adulthood. In previous years, mouse models of AS by targeted *Ube3a* inactivation have been created to recapitulate the clinical manifestations of AS patients[53,54] which include: impaired motor function, defects in long-term potentiation, perturbed CaMKII activity, deficits in context-dependent and spatial learning, inducible seizures, abnormal EEG, fast oscillation in cerebellar cortex and sleep disturbance. In order to gain insight into molecular mechanism of imprinting of *UBE3A* gene and its misregulation in AS patients, Dr. Lalande discussed his recent working model using murine P19 embryonic carcinoma (EC) cell line as a model system. *UBE3A* is transcribed predominantly from maternal allele in the brain but is bialleically expressed in most other tissues. This silencing of paternal allele in the brain is mostly likely to be mediated in cis by a large non-coding antisense transcript (*Ube3a-ATS*) that is exclusively expressed from the paternal allele. Dr. Lalande's group showed increased expression of paternal *Ube3a-ATS* as a consequence of maternal transmission of *Ube3a* mutation.[55] Here, they provide an alternative model where antisense is modulated by sense rather than the reciprocal mode of regulation. In addition, using P19 EC cell line, they observed that shRNA-mediated knockdown of *Ube3a* results in increased expression of *Ube3a-ATS* suggesting that *Ube3a* regulates expression of *Ube3a-ATS* in trans is in contrast to the other cases of sense-antisense epigenetic cis-interactions and argues against a major role for *Ube3a-ATS* in the imprinting of *Ube3a*. He proposes a model where *Ube3a* represses *Ube3a-ATS* via a transcriptional or chromatin silencing mechanism and furthermore,

indicates the increased levels of *Ube3a-ATS* observed in AS mouse model may contribute to phenotypic manifestations of this disorder.

Coordinated Misregulation by the Angelman Syndrome Imprinting Control Center (AS-IC): Laura B. Herzing, Department of Pediatrics, Northwestern University Feinberg School of Medicine, Program in Human Molecular Genetics, Children's Memorial Research Center

In Angelman syndrome, frequent deletion spanning 15q11-13 seen in AS patients covers the AS-imprinting center (AS-IC), a component of bipartite control element shown to interact with MeCP2. This regulatory element controls expression of both *UBE3A* gene and the adjacent maternally expressed *ATP10C* gene in the brain. *MECP2*, methyl CpG-binding protein 2, is the mutated gene in Rett syndrome (RS) which has significant phenotypic overlap with AS such as autism, stereotypic movements, ataxic gait and seizures. Mutations in *MECP2* have also been identified in patients with AS and autism, again emphasizing phenotypic overlap seen in these disorders. Work from Dr. Herzing's group concludes that *UBE3A* and *ATP10C* sense and antisense transcription is similarly disrupted in patients with AS-IC or *MECP2* mutations, in *Ube3a* knockout AS-model and *Mecp2*-knockout RS-model animals. From these observations, Dr. Herzing suggests a model where MeCP2 mediates control of gene expression via AS-IC and decrease or alterations in expression of these genes may play a role in phenotypic overlap that is present in RS, AS and autism.

Alterations in CaMKII Phosphorylation can Rescue the Cognitive and Synaptic Plasticity Defects in a Mouse Model for Angelman Syndrome Human: Edwin J. Weeber, Department of Molecular Physiology and Biophysics, Department of Pharmacology, Kennedy Center for Research on Human Development, Vanderbilt University Medical Center

Through biochemical analyses, Dr. Weeber's group showed that calcium/calmodulin-dependent kinase type II (CaMKII) activity is reduced and phosphorylation of inhibitory Thr305 and Thr306 site is increased.[56] In an attempt to address whether increased inhibitory phosphorylation is directly causing major neuronal deficits seen in AS, they introduced an additional mutation at the inhibitory phosphorylation site of CaMKII. By eliminating inhibitory phosphorylation in AS mice, kinase activity was restored to near wild-type levels. Some clinical phenotypes of AS patients are recapitulated in these AS mice. Epilepsy, which is a common feature of AS, can result from reduced CaMKII activity. In this regard, increased seizure propensity of AS mice was reduced in the double mutant mice, suggesting that increased inhibitory phosphorylation of CaMKII contributes to increased seizure propensity of AS mutants. Motor coordination deficits and hippocampus-dependent learning deficits of AS mice and double mutant mice showed a difference in that double mutant mice displayed better performance in all tests suggesting regulation of inhibitory phosphorylation of CaMKII activity is critical for both phenotypes. Lastly, hippocampal long term potentiation (LTP) induction is dependent upon inhibitory phosphorylation of CaMKII and AS mice show severe LTP deficit where double mutants do not, again strengthening the argument that plasticity deficits of AS mice result from inhibitory phosphorylation of CaMKII. These findings are the first to identify altered regulation of CaMKII as a molecular cause of neurobehavioral defects in a human learning disorder.

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