SHARED NEUROBIOLOGY OF AUTISM AND RELATED DISORDERS

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Young Investigator Program
POSTER SESSION A
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Immune Proteins and Glutamatergic Dysfunction in Autism

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Autism is a complex neurological syndrome that is thought to result from a combination of environmental and genetic factors that impact brain development and function. One consistent finding in autism that may affect both brain development and function is an imbalance in glutamatergic neurotransmission (Lam et al., 2006). Genetic studies have found a significant link between autism and genes encoding certain isoforms of ionotropic glutamate receptors (GluRs) (e.g., NMDA, AMPA and kainate receptors; Jamain et al., 2002; Ramanathan et al., 2004; Barnby et al., 2005), and modifications in the levels of specific AMPA receptor subunits have been observed in the cerebellum of patients with autism (Purcell et al., 2001). Alterations in GluRs have also been reported in related disorders, including Rett syndrome (Blue et al., 1999) and tuberous sclerosis (White et al., 2001). However, the molecular and cellular causes of these changes remain elusive.

In parallel, many studies have identified a link between autism and abnormalities in immune genes and/or immune function (Cohly and Panja, 2005). One emerging hypothesis is that infection or other immune challenge, in a genetically predisposed individual, may contribute to the pathogenesis of autism. In support of this hypothesis, maternal infection is a risk factor for autism in humans, and mouse models of maternal immune challenge support a role for the immune response – specifically upregulation of cytokines--in the origins of autism (Patterson, 2002; Shi et al., 2003; Molloy et al., 2006).

Cytokines act in large part by modulating expression of members of the major histocompatibility complex (MHC) class I family of proteins, crucial components of the adaptive immune response. The brain has long been considered 'immune privileged' due to an apparent lack of MHC class I on the surface of neurons. However, recent studies by myself and others have shown that normal, healthy neurons express MHC class I mRNA and protein both at rest and in response to cytokines, and further, that MHC class I unexpectedly performs critical, non-immune functions in the developing and adult brain (Corriveau et al., 1998; Huh et al., 2000; Loconto et al., 2003; Boulanger and Shatz, 2004). In mice genetically deficient for cell surface MHC class I, activity-dependent remodeling of developing visual connections fails, and bidirectional synaptic plasticity in the adult hippocampus is systematically shifted in favor of potentiation: long-term potentiation (LTP) is enhanced, while long-term depression (LTD) is absent. However, more severely immunocompromised mice do not share these defects (Huh et al., 2000), revealing that these changes are not simply an indirect result of immune compromise, but rather reflect a novel, brain-specific role for MHC class I molecules in activity-dependent structural and functional plasticity of developing and adult circuitry.

It remains unclear how MHC class I signals in neurons to affect synaptic plasticity. Much evidence indicates that activity-dependent modulation of the trafficking of GluRs drives changes in synaptic strength in the adult hippocampus (Malenka, 2003; Kennedy and Ehlers, 2006). In particular, insertion of AMPA type glutamate receptors (AMPARs) leads to LTP, while endocytosis of AMPARs leads to LTD. MHC class I is known to regulate trafficking of other transmembrane receptors, notably the transferrin receptor (Pietrangelo et al., 1998), outside the brain. Therefore we hypothesized that neuronal MHC class I may affect synaptic plasticity by altering AMPAR trafficking. To directly test this possibility, we assessed AMPAR levels, distribution, and trafficking in hippocampal slices and acutely dissociated hippocampal neurons from normal (WT) and MHC class I-deficient ($\beta 2m^{-/-}TAP^{-/-}$) animals, using a combination of biochemical, immunocytochemical, and electrophysiological approaches. In the absence of cell surface MHC class I, total, surface, and synaptic levels of AMPARs are indistinguishable from WT in the basal state, consistent with previous reports indicating normal basal synaptic transmission in these animals (Huh et al., 2000). Since synaptic plasticity is strikingly shifted in MHC class I deficient animals, we next monitored AMPAR distribution specifically after induction of plasticity. To maximize the number of synapses undergoing plasticity and permit biochemical and immunocytochemical assessment of AMPAR trafficking, we used a chemical LTD protocol consisting of a brief bath application of NMDA. NMDA induced robust LTD of CA3-CA1 synapses in adult WT mice, as previously described. However, this same protocol instead induced significant LT*P* in MHC class I deficient slices, similar to the aberrant LTP seen in response to low frequency stimulation in these animals (Huh et al., 2000). When applied to acutely dissociated hippocampal neurons in culture, NMDA caused little change in AMPAR distribution in WT neurons, but induced a significant *increase* in cell surface AMPARs in MHC class I deficient neurons. These results suggest that changes in neuronal MHC class I may mechanistically link maternal immune challenge, a risk factor for autism, with glutamatergic dysfunction, a common symptomatic correlate of the disorder. Elucidating this connection may lead to novel, immune-based strategies for the diagnosis, treatment, and prevention of autism.

Potential Pharmacotherapeutic Actions of Taurine in a Mouse Model of Fragile X Syndrome

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Fragile X syndrome is an X-linked dominant disorder and the most common cause of inherited mental retardation. It is caused by trinucleotide repeat expansion in the fragile X mental retardation 1 gene (*FMR1*) at the Xq27.3. The expansion blocks expression of the gene product, Fragile X Mental Retardation Protein (FMRP). The syndrome includes mild to moderate mental retardation and behavioral manifestations such as tactile defensiveness, gaze avoidance, repetitive motor mannerisms, perseverative (repetitive) speech, hyperarousal and it frequently includes seizures. This behavioral phenotype overlaps significantly with autism spectrum disorder.

Our previous investigation of the basis for the increased seizure susceptibility in the mouse model for this disorder (the fragile X mouse) found a reduction in $GABA_A$ receptor expression (El Idrissi et al., 2005). This receptor is a major component of the inhibitory (GABAergic) system and its reduced expression probably contributes to the increased seizure susceptibility observed in the fragile X mouse. We also found a potentially related alteration of the GABAergic system--increased expression of glutamic acid decarboxylase (GAD), the enzyme responsible for GABA synthesis. Furthermore, we have now found that the expression of somatostatin is significantly decreased in the fragile X mouse brain. Somatostatin is a neuropeptide with functions in the central and peripheral nervous system, gastrointestinal tract, pancreas, immune and cardiovascular systems. Somatostatin inhibits the secretion of several hormones and it also has an inhibitory effect on synaptic transmission through inhibition of ionic currents, in that it decreases the probability of a neuron firing an action potential. The reduced expression of this neuropeptide, observed in the fragile X mouse, could explain a number of features of the fragile X syndrome. Here we report that supplementation of drinking water with taurine, a neuroactive amino acid, results in a significant increase in somatostatin expression both in controls and fragile X mice. This treatment reversed some of the features of the fragile X mouse model.

The taurine-induced increase in somatostatin expression was accompanied by significant neurobehavioral improvements in the fragile X mouse. Using two behavioral tests (passive avoidance learning task and open field behavior), we found that the fragile X mice fed with 0.05% taurine for four weeks learned the passive avoidance task significantly faster than controls or untreated fragile X mice. Furthermore, they performed significantly better on tasks that required retention of the learned behavior. In the open field test, the treated fragile X mice showed the highest level of activity as measured by line crossings and time spent in the center field. We found that taurine supplementation decreased the total distance traveled, the overall movement speed, the time the animals spent moving, the number of line crossings, and the number of times the animals entered the center zone, indicating that taurine supplementation reduces the levels of anxiety in these mice.

Additionally, we found that fragile X mice had a significant decrease in the number pancreatic islets, which may be linked to the increased glucose metabolism observed in these mice (Qin et al., 2002). Taurine supplementation to mice resulted in a significant increase in the number of Islets of Langerhans in fragile X mice. Increased testicular volume is a consistent feature of the fragile X syndrome. We found that taurine supplementation significantly reversed the increased testicular volume in the fragile X mice.

A possible mechanism by which taurine increases somatostatin expression could be attributed to the metabolic pathway of taurine biosynthesis which involves the production of cysteamine. Cysteamine is derived from cysteine during co-enzyme A degradation and is a significant intermediate in taurine biosynthesis. Feeding mice taurine leads to a product-mediated inhibition of this pathway and results in accumulation of cysteamine. Cysteamine interacts with the disulfide bonds of somatostatin, rendering the molecule both immunologically and biologically inactive. Our hypothesis is that a moderate increases of cysteamine in taurine-fed mice results in a compensatory increase in somatostatin expression. The evidence for this is that low cysteamine doses mimic, whereas high doses abolish the taurine-induced somatostatin-mediated neurobehavioral and anatomical improvements observed in the KO mice after taurine supplementation. The strategy and progress in testing this hypothesis will be discussed.

Role of Matrix Metalloproteinases in Dendritic Spine Development and Cognitive Performance in Fragile X Mice

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Defects in dendritic spine formation have been found in the brains of patients with Fragile X, suggesting these structures may represent an anatomical and physiological basis for the cognitive deficits associated with this disorder. Dendritic spines are the postsynaptic sites of most excitatory synapses in the brain and changes in their morphology and number influence properties of neuronal circuits. Recently, we found that MMPs can influence dendritic spine development and hence synaptic stability. Active forms of MMP-7 and MMP-9 induce immature dendritic spine profiles in cultured hippocampal neurons as evidenced by dramatic F-actin re-distribution and transformation of mature mushroom-like dendritic spines into less stable filopodia-like thin protrusions. Similarities between immature dendritic spine profiles in hippocampal neurons of Fragile X mental retardation (FMR1) knock out mice, a mouse model for Fragile X, and hippocampal neurons treated with MMP-9 suggest that higher than normal levels of MMP activity in the developing hippocampus of FMR1 KO mice may contribute to the dendritic spine abnormalities. Indeed, MMP-9 activity is up-regulated in the hippocampus of FMR1 KO mice and may be responsible for the immature dendritic spine profile of hippocampal neurons. We show that an MMP-9 inhibitor, minocycline, rescues this abnormal phenotype by inducing the formation of mature dendritic spines in FMR1 KO hippocampal neurons *in vitro.* Moreover, minocycline treatment inhibits excessive MMP-9 activation in the hippocampus of P7 FMR1 KO mice and prevents abnormal development of dendritic spines *in vivo*. We hypothesize that higher than normal levels of MMP activity in the brains of FMR1 KO mice contribute to dendritic spine abnormalities and cognitive dysfunction. Although triggers for enhanced MMP-9 activity are not yet clear, activity-dependent regulation of MMP activity at synapses and synaptic hyperactivity in FMR1 KO mice might result in up-regulation of MMP activity above normal levels. Clinical trials were recently approved for the use of minocycline to treat the human neurodegenerative diseases. We are currently investigating whether minocycline has synapse-protecting effects in developing brain of FMR1 KO mice and if it can reverse related behavioral defects, thus bypassing genetic causes of these abnormalities.

Dynamic Misregulation of Chromosome 15 Autism Candidate Genes in the *Mecp2*-Knockout Mouse

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Mutations in the Rett syndrome (RS) gene *MeCP2* have been identified in Angelman syndrome (AS) and autism, highlighting the phenotypic overlap between these disorders. *UBE3A*, the AS gene, and *ATP10A* are maternally expressed imprinted genes within the autism candidate region 15q12-q13. We have demonstrated that their expression is similarly affected by Angelman Syndrome-imprinting center (AS-IC) deletions and by *MeCP2* mutations in cortex from Rett Syndrome (RS) patients. *UBE3A* and *ATP10A* expression may be regulated by antisense transcription arising from the IC, which may in turn be affected by loss of MeCP2. In the mouse, imprinted expression of these genes is less strict, and effects of loss of Mecp2 are modulated by strain, gender and age.

Objective: To determine how total, allele- and strand-specific expression of imprinted chromosome 15 autism candidate genes is affected by loss of Mecp2 in mouse throughout phenotypic regression, and whether altered expression patterns correlate with individual phenotypic severity.

Methods: *Atp10a* anti-sense transcripts were identified by EST database searches and by direct RT-PCR; polymorphisms were identified by sequencing. Sense and antisense transcripts were generated by strand-specific RT-PCR. Relative gene expression between *Mecp2*-deletion/ wild-type littermates was quantitated using Real-Time RT-PCR. Allele-specific expression was quantitated using sub-cloning and sequencing of RT-PCR products from animals carrying polymorphisms of known parental origin.

Results: In both human and B6x129 mouse postnatal and/or symptomatic male cortex, loss of *MeCP2* is coordinate with a decrease in maternal *UBE3A*, *ATP10A* and in *GABRB3* expression and with an increase in total and relative proportion of maternal antisense *ATP10A* expression. In contrast, total *Atp10a* and *Ube3a* expression increases transiently to normal or above normal levels in most animals in the juvenile/early adult period, prior to overt phenotype development. These animals retain/regain normal allele-specific expression ratios, as do a minority of young animals that do not exhibit early expression decreases, and a minority of older animals that have delayed, milder symptom onset with longer lifespan.
Further variability is found in older female ($Mecp2^{+/-}$) animals and on an outbred background, wherein both total and *paternal* antisense expression is increased, resulting in a net gain of imprint for *anti-Atp10a* expression in these animals.

Conclusions: We provide further evidence that loss of *MeCP2* disrupts regulation of transcripts of autism-candidate genes controlled by the AS-IC. In mouse, the great majority of animals carrying *Mecp2*-knockout alleles exhibit abnormal total, allele- and/or strand-specific expression of *Atp10a* and *Ube3a*. Variability in expression patterns between individuals and throughout symptom development in mouse suggests that normal or elevated *Ube3a* and/or *Atp10a* expression may be protective against neurologic deterioration mediated by loss of Mecp2. On the other hand, elevated expression of these genes is correlated with autism in humans, and a similar elevation in girls with RS during regression may account for the autistic features seen at this time. Aberrant expression of these genes in humans may contribute to the phenotypic overlap between RS, AS and autism, and variability between individuals with RS in the degree to which these genes are affected may account for differences in the progression and severity of AS- and autism-related phenotypes.

Genetics of Tame Behavior in Silver Foxes

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Autism, like several other disorders impairing human social, communicative, and cognitive behavior clearly has a significant genetic component, but efforts to identify human loci are seriously constrained by complexity and heterogeneity of inheritance. For autism, as for many other genetic traits, animal models offer a complimentary approach to human studies. While the full complexity characteristic of human behavior may not be observed in animal models, components of these behaviors can be. Domestication, in particular, dramatically changes social behavior in animals, and these changes are undeniably genetic. The complex suite of modified social behaviors and cognitive abilities that results from domestication specifically includes tameness, the ability to interact with humans in a positive way. The paradigmatic example is provided by the domesticated dog (*Canis familiaris*) and its progenitor species the grey wolf (*Canis lupus*). In contrast to wolves, dogs are eager to establish human contact from a very early age, readily develop a positive emotional attachment to their human companions, and possess enhanced capabilities to read, sense and interpret intent in humans. An understanding of the genetics of these human-like social skills of dogs may provide insight into the mechanisms underlying development of human social behaviors and pinpoint mechanisms impaired in individuals with autistic and other behavioral disorders.

Although these differences between dogs and wolves in their social interactions with humans strongly suggest a genetic basis for these behaviors, studies are seriously constrained by the fact that essentially all dogs possess this phenotype (i.e. it is genetically fixed within the species) and no significant set of populations segregating behavioral differences between the domestic dog and the grey wolf are currently available. More recently, however, another member of the *Canidae* has been domesticated. In an unparalleled experiment at the Institute of Cytology and Genetics of the Russian Academy of Sciences a strain of silver foxes (*Vulpes vulpes*) demonstrating friendly behavior to humans, has been developed as a result of 40 years of selectively breeding foxes for tame behavior. In contrast to wild type foxes, foxes bred for tameness behave much like domesticated dogs, actively seeking out both other members of their own species and humans in a playful, friendly and communicative manner (see http://cbsu.tc.cornell.edu/ccgr/behaviour/index.html). Unlike the modern dog, the speed with which the tame fox population was developed suggests that selection in these foxes may be acting on a few genetic loci that control development of the tame phenotype. Because these genetically determined behavioral differences segregate in very large pedigrees of a single species, they offer an opportunity to map and identify the loci and genes responsible.

Mapping tools developed for the canine genome, have enabled a systematic approach to identify the loci and molecular mechanisms controlling these segregating fox behavioral phenotypes. During the first phase of this project, tools and resources for genetic mapping in foxes were developed, and three quantitative trait loci (QTLs) affecting behavioral variation in these fox strains have been identified.

1) Construction of the first meiotic linkage map of the silver fox. The map has been developed using 320 microsatellites adapted from the canine genome. 286 individuals from 37 fox three-generation pedigrees have been used for map construction. The resulting sex-averaged map covers 16 fox autosomes and the X chromosome with an average inter-marker distance of 7.5 cM.

2) Development of three-generation experimental mapping pedigrees. Tame and wild-type founders were crossbred, and the F1 progeny backcrossed to the tame strain, Each animal from the backcross-to-tame pedigrees has been genotyped with 320 polymorphic markers equally spaced along each fox chromosome.

3) Implementation of a rigorous system for measuring behavior as a truly continuous variable. Behavior of foxes in experimental pedigrees was videotaped under a standardized observation protocol. A comprehensive primary set of objective observations was developed for scoring the physical manifestations of fox behavior during the test. Statistical analysis, including principal-components analysis, was used to dissect out the independent, resegregating traits underlying the phenotypic variation expressed in these multiple correlated observations. PC1 accounts for approximately 30% of the total variation in behavior among foxes and is highly significantly related to the expected additive genetic distance between the populations.

4) Undertaking QTL analysis of the first set of the backcross-to-tame pedigrees. Significant peaks associated with PC1 have been identified on the three fox chromosomes.

The silver fox presents a well established large animal model, intermediate between rodents and primates in biological complexity. Identification of the genes and loci influencing social behavior in these foxes is anticipated to provide new insights into, and candidate genes for human disorders of social behavior.

Maternal Immune Activation Impairs Extinction of the Conditioned Eyeblink Response in the Adult Offspring.

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Maternal infection is a known risk factor for mental disorders such as schizophrenia and autism. In an animal model, injection of poly(I:C), a synthetic double-stranded RNA that evokes an anti-viral inflammatory response, into pregnant mice produces several behavioral, histological and transcriptional changes in the adult offspring. We are investigating classical eyeblink conditioning, a behavior that is abnormal in autistic subjects, in the adult offspring of poly(I:C)-injected mice. Pregnant mice are injected polyI:C or saline on embryonic day 12.5, and adult offspring from each group are tested in a delay eyeblink conditioning paradigm. Compared to saline group, the poly(I:C) group shows impaired extinction of the conditioned response. Auditory brainstem responses and tail-flick latency are similar in both groups, indicating normal auditory and somatosensory functions. The impaired extinction in poly(I:C) group suggests that perservative motor behavior, a characteristic of autism, is induced in the offspring by maternal immune activation.

Immune Molecules and Cortical Synaptogenesis: Possible Implications for the Pathogenesis of Autism

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Autism spectrum disorder is a severe neurobehavioral syndrome with a heterogeneous phenotype. The prevalence of autism may be as high as 1/150 and its incidence is progressively increasing. Although there is clearly a strong genetic component to autism, its incidence also appears to be influenced by a wide range of environmental factors. Many of these factors have in common the ability to alter immune function. Since MHCI molecules mediate the immune response and are present on neurons in the developing brain, it is possible that changes in MHCI expression lead to cellular changes that contribute to autism.

Recently, my laboratory has started experiments to test the hypothesis that MHCI molecules play a role in the initial establishment of cortical connectivity in addition to their documented role in later postnatal plasticity in the visual system. MHCI protein is present at the right place and right time to be involved in both the initial stages of cortical synaptogenesis and later stages of synapse elimination. MHCI proteins are present in small clusters in axons and dendrites of cortical neurons at all stages of development. The proportion of MHCI clusters in the plasma membrane (sMHCI) increases with age. In addition, MHCI proteins are present in axonal and dendritic growth cones, suggesting that MHCI may influence the initial stages of synapse formation. Interestingly, the distribution of sMHCI is higher proximally than distally at 3 and 8div, but becomes uniform across dendrites at 14div. This local sMHCI density inversely correlates with glutamatergic synapse density, which is lower proximally and higher distally at 8div.

Manipulation of sMHCI molecules indicates that MHCI negatively regulates the initial establishment of cortical connectivity. sMHCI was knocked-down by transfecting neurons with siRNAs against β2m, the light chain that is required for export of MHCI out of the endoplasmic reticulum. Dissociated cortical neurons were transfected with β2m siRNA at 5div, before most synapses have formed, and glutamatergic synapse density was quantified at 8div, during the peak of synaptogenesis. β2m and sMHCI protein decreased to approximately 50% of their original levels by three days after transfection. To our surprise, the density of glutamatergic synapses increased by about 70% as a consequence of this knockdown. This increase in excitatory synapse number suggests that young cortical neurons have the capacity to form many more synapses than they normally do and that sMHCI molecules actively suppress the number of connections established during the initial stages of cortical development. This increase in synapse number appears to be a direct effect of sMHCI knockdown, and not a direct effect of decreased β2m signaling, since the addition of exogenous soluble β2m does not rescue the increase in synapse number. Moreover, this increase in synapse density caused by sMHCI knockdown is specific for excitatory synapses since there is a slight decrease in inhibitory synapse number in siRNA-transfected neurons. Overall, these results suggest that MHCI molecules normally act to limit the formation of excitatory, but not inhibitory, synapses.

In order to determine if MHCI bidirectionally regulates glutamatergic synapse number, sMHCI expression was increased by transfecting neurons with MHC-H2Kb-CFP for 48 hours from 6-8 div. MHCI-CFP overexpression caused a 33% <u>decrease</u> in excitatory synapse density. Since this density of synapses is less than that at 5div, these results indicate that sMHCI not only limits the ability of neurons to establish connections but it also actively causes existing synapses to be eliminated. We are especially excited about these results since they demonstrate a novel function for any transmembrane protein during the initial period of synaptogenesis.

Our results imply that environmental factors that initiate an immune response could dramatically affect connectivity in the developing brain and thereby alter cognition. Since cytokines potently regulate MHCI expression in the immune system, cross the blood-brain barrier in the early postnatal period, and are upregulated in the brains of autistic children, it is possible that cytokines alter synaptic connectivity in the developing brain by altering sMHCI levels. Consistent with this hypothesis, preliminary data from my laboratory shows that IL-6, but not IFNγ, alters both sMHCI levels and the density of glutamatergic synapses. We are currently testing the effects of several additional cytokines known to be upregulated in the cerebrospinal fluid of autistic children. Our preliminary results support the idea that cytokines released during an immune response could alter cortical connectivity through regulating sMHCI levels on neurons. If substantiated in future experiments, our work may provide an exciting link between a strong or abnormal immune response during early brain development and changes in cortical connectivity that could underlie the pathogenesis of autism.

Characterization of Animals that Lack MeCP2 Function in Tyrosine Hydroxylase-Expressing Neurons

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Rett Syndrome (RTT, MIM312750) is a dominant X-linked neurodevelopmental disorder characterized by language regression, loss of volitional hand use, acquired microcephaly, gait apraxia/ataxia, autonomic irregularities, and unusual hand stereotypies. After a period of apparently normal development, girls with RTT undergo a period of regression in which they lose skills and develop autistic features. Over 95% of individuals with typical RTT have mutations in Methyl-CpG-binding Protein 2 (*MECP2*), a transcriptional repressor. *MECP2* mutations have been identified in a broad spectrum of phenotypes including isolated mental retardation, autistic spectrum disorders (ASD), bipolar disease with X-linked mental retardation (XLMR), or an Angelman-like syndrome. A variety of RTT mouse models have been generated and these animals display a variety of phenotypes that are reminiscent of both RTT and other ASD such as anxiety, stereotyped behavior, and social interaction deficits. The spectrum of phenotypes present within both people with *MECP2* mutations and animal models with alterations in MeCP2 function leads us to propose that specific phenotypes result from MeCP2 dysfunction within specific neuronal populations. This hypothesis posits that loss of MeCP2 function in one neuronal subtype might reproduce one or more of the features of RTT. To test this hypothesis we are deleting *Mecp2* in specific neuronal populations using Cre/LoxP technology and analyzing the phenotypes of the resulting mice to allow comparisons between the different neuronal specific knock-outs. In this study, we analyze the behavioral consequences of removing MeCP2 function from neurons that express tyrosine hydroxylase (TH), an enzyme required for the biosynthesis of dopamine and norepinephrine.

Methods: Experimental animals were generated from a cross of heterozygous $Mecp2^{FLOX}$ females ($Mecp2^{FLOX/+}$) to heterozygous TH-Cre animals ($TH\text{-}Cre^{Tg/+}$) to generate a cohort of sixty-four male animals (total n=64). Within this cohort are sixteen animals (n=16 for each genotype) of the four expected genotypes ($Mecp2^{+/Y}$;$TH\text{-}Cre^{+/+}$ | $Mecp2^{+/Y}$;$TH\text{-}Cre^{Tg/+}$ | $Mecp2^{FLOX/Y}$;$TH\text{-}Cre^{+/+}$ | $Mecp2^{FLOX/Y}$;$TH\text{-}Cre^{Tg/+}$). A predefined battery of behavior tests was performed on all animals. All testing was performed by experimenters who were blind to the genotype.

Results: All animals have survived to date up to five months of life except one animal that lacked Mecp2 in the TH-expressing neurons ($Mecp2^{FLOX/Y}$;$TH\text{-}Cre^{Tg/+}$) who died unexpectedly at 14 weeks of life. No overt abnormalities, such as hindlimb-clasping, forepaw stereotypy, obvious tremor, or dystonia, have been observed to date in any of the animals. There is no difference in the growth curve of any of the genotypes. When subjected to behavior tests to assess anxiety (Light-Dark Box and Open Field) at 14-16 weeks, there were no observable differences in anxiety between the genotype groups. However, in the open field, there was a significant 2-way interaction ($p=0.006$) for total distance traveled, with $Mecp2^{FLOX/Y}$;$TH\text{-}Cre^{Tg/+}$ animals traveling less distance than $Mecp2^{FLOX/Y}$;$TH\text{-}Cre^{+/+}$ ($p<0.05$). Furthermore, there was a difference in vertical activity (2-way ANOVA $p=0.002$), again with $Mecp2^{FLOX/Y}$;$TH\text{-}Cre^{Tg/+}$ showing less vertical activity ($p<0.05$). The $Mecp2^{FLOX/Y}$;$TH\text{-}Cre^{Tg/+}$ animals also showed motor impairment during the dowel walking task performed at 12 weeks of life in which they had less side touches than the other genotypes (Kruskal-Wallis $p<0.001$). On a test of social interaction performed at 20 weeks of life, the partition test, $Mecp2^{FLOX/Y}$;$TH\text{-}Cre^{Tg/+}$ animals interacted less on the second exposure to a familiar animal compared with $Mecp2^{FLOX/Y}$;$TH\text{-}Cre^{+/+}$ animals (2 way ANOVA $p=0.03$). There was no difference between the genotype groups on a test of hippocampal and amygdala dependent learning (conditioned fear) at 21 weeks of life. At 24 weeks of life, preliminary results on a subset of animals (n=9 per genotype) show that $Mecp2^{FLOX/Y}$;$TH\text{-}Cre^{Tg/+}$ animals have decreased pre-pulse inhibition compared with $Mecp2^{FLOX/Y}$;$TH\text{-}Cre^{+/+}$ animals at prepulse level 74dB (2 way ANOVA $p=0.01$, post-hoc $p<0.05$), and prepulse level 78dB (2way ANOVA $p=0.017$, post hoc $p<0.05$).

Conclusions: These results demonstrate that MeCP2 function within TH-expressing neurons is required for proper motor function and normal pre-pulse inhibition. Both of these features are suggestive of deficits within the dopamine system. These animals also have alteration on a social interaction task, which might suggest alteration in social recognition. The motor deficit observed makes the interpretation of these social interaction results difficult. Future social interaction experiments are planned to clarify this issue. Importantly, the spectrum of phenotypes we observed when MeCP2 function was removed from TH-expressing neurons is distinct from those phenotypes observed in separate identical experiments in which MeCP2 function is removed from other neuron populations. This demonstrates specific phenotypes result from Mecp2 dysfunction within distinct neuronal populations and that this approach can determine the specific neuronal populations that are important for such phenotypes as anxiety, growth abnormalities, and alterations in social behavior.

Young Investigator Program
POSTER SESSION B
Posters 9-15

Andrus Gerontology Center
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The mGluR Theory of Fragile X

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Autism is a disorder with no known etiology and no suitable biomarkers for diagnosis. Although many studies have identified genes linked to autism, there is no clear picture of how autism occurs, nor what molecular mechanisms are involved. Given this, the study of Fragile X syndrome (FXS), a single-gene disorder with the largest genetic overlap with autism, could yield important clues about the what exactly goes wrong in the autistic brain. FXS is the most common inherited form of mental retardation, and is caused by the loss of the *FMR1* gene product, FMRP. Approximately 30% of FXS patients meet the strict DSM criteria for an autism diagnosis, and as high as 60% fall within the autism spectrum. Interestingly, it has recently been shown that autistic behaviors directly correlate with the level of FMRP expression in patients with pre- and full mutations of *FMR1* (Loesch et al., 2007). Given this, the study of FXS is a highly promising approach to understanding the etiology of autism.

FMRP is an mRNA binding protein that has been shown to function as a repressor of translation at synapses (Jin et al., 2004; Vanderklish and Edelman, 2005). Interestingly, many lasting consequences of group I metabotropic glutamate receptor (Gp I mGluR) activation require protein synthesis, and at least one of these, synaptic long-term depression (LTD), is exacerbated in the absence of FMRP (Huber et al., 2000). These and other findings led to the mGluR Theory of Fragile X, which suggests that FXS symptoms are the result of the exaggeration of mGluR-mediated protein synthesis (Bear et al., 2004). Since its inception, the popularity of this theory has grown tremendously, and our results suggest that several phenotypes in the FMR1 knockout (Fmr1-KO) mouse are rescued by genetic and pharmacological antagonism of mGluR5. These phenotypes include excessive protein synthesis and exaggerated inhibitory avoidance extinction (IAE).

Consistent with a previous study (Qin et al., 2005), we find that basal protein synthesis is elevated in adult Fmr1-KO hippocampal slices. This elevation is significant (~20%), and exists in both adult and juvenile animals. To investigate the role of mGluR5, heterozygous (mGluR5-HET) mice with a 50% reduction in mGluR5 were bred with Fmr1-KO mice. Remarkably, our results show that the genetic reduction of mGluR5 is sufficient to rescue the excessive protein synthesis seen in Fmr1-KO mice. To see whether an acute pharmacological treatment could also rescue the excessive protein synthesis, we applied the selective mGluR5 antagonist, MPEP to WT and Fmr1-KO slices. Our results show that a 5 min exposure is sufficient to reduce Fmr1-KO protein synthesis to WT levels. Consistent with this, application of the Gp I mGluR agonist DHPG increases protein synthesis in WT, but not Fmr1-KO hippocampus. These results confirm our hypothesis that mGluR5 and FMRP are functional opponents in the regulation of protein synthesis, and provide strong support for mGluR antagonists as a treatment for FXS.

Further experimentation using Fmr1-KO/mGluR5 mice has revealed that several other FXS phenotypes are rescued by the genetic reduction of mGluR5 (Dolen, Osterweil & Bear, submitted). Interestingly, the extinction of fear learning (IAE) has recently been found to be protein synthesis dependent (Power et al., 2006). Given this, IAE was tested in WT, Fmr1-KO, mGluR5-HET and Fmr1-KO/mGluR5-HET mice. Our results show that although Fmr1-KO mice do not differ from WT in IA training, they show a significant deficit in extinction. This deficit is rescued in Fmr1-KO/mGluR5-HET mice, which show no difference from WT mice. Thus, reduction of mGluR5 is sufficient to rescue the learning deficit seen in Fmr1-KO mice. We strongly believe that antagonism of Gp I mGluRs is a valid treatment for FXS, and possibly autism.

Consequence of Mecp2 Mutation on Neurodevelopment

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Autism is a heterogeneous neurodevelopmental disorder that manifests disturbances in social, vocal, and behavioral capabilities. Autism Spectrum Disorders (ASD) is a broad category of pervasive developmental disorders that includes Autism, Fragile X, Angelman's Syndrome, Asperger, and, Rett Syndrome (RTT). Due to the myriad of symptoms and the presentation of different severities, the pathogenesis of ASD is quite complex and often their etiologies unknown. RTT is one exception where mutations in MECP2, a gene encoding methyl-CpG binding protein 2, have been identified in more than 80% of cases. RTT is clinically characterized by mental and growth retardation, seizures, hand stereotypies and loss of language. Mecp2, a transcription repression factor, is thought to be associated with the regulation of other ASD. These observations indicate that RTT may serve as a model for understanding common concepts in the molecular pathogenesis of Autism, for which few models exist. To begin to understand the consequences of transcription factor mutations such as that of Mecp2, we utilize the olfactory system as a tractable neurodevelopmental model.

We have previously described defects in olfactory sensory neurons (OSNs) of nasal biopsies taken from RTT patients, which show similar neuronal defects to those found in RTT postmortem brains. Specifically, OSNs taken from nasal biopsies of RTT patients show maturation disruptions and dendritic abnormalities. This study also validates the olfactory system as a good model of neurogenesis and neurodevelopment, which is particularly useful for RTT studies, where studies using brain tissue are often complex and the results confounding. With the availability of mouse models of RTT, such as Mecp2 null animals, we have identified defects in olfactory neurodevelopment that occur as a consequence of Mecp2 deficiency, which include a delay in maturation at the time when synaptogenesis initially peaks. We have also identified the dynamic temporal and regional changes in protein expression that occur with Mecp2 deficiency that may underlie the biological defects seen in RTT. Collectively we have used the results of these studies as the foundation for understanding the basis of functional compromise in Mecp2 dysfunction and RTT. Our current study examines the long-term consequence of Mecp2 mutation on neuronal maturation and maintenance. We use a Mecp2 mouse model expressing a truncating Mecp2 mutation, $Mecp2^{y/308}$. $Mecp2^{y/308}$ male mice exhibit a phenotype similar to RTT, and survive approximately one year. We have taken advantage of the longevity of this mouse model to assess the implication of Mecp2 mutation during pre- and late-stage-symptomatic ages. Using a previously described labeling paradigm, we look at the maturation of OSNs in these mice. Our data show that terminal differentiation in the $Mecp2^{y/308}$ mutant models show a similar defect to the Mecp2 null mouse model; however, this neuronal defect is transient and does not persist into adulthood. Additionally, using immunohistochemical procedures, we have assessed OSNs and their targets and found that disruptions in neuronal homeostasis occur throughout adulthood and are consistent with neuronal compensation and not cellular demise. These data suggest that Mecp2 truncation or deficiency disrupts synaptogenesis and neuronal maturation. Our working hypothesis is that this truncating mutation results in a neuronal defect early during development that does not alter the olfactory system's intrinsic ability to compensate for disruptions in neurodevelopment.

Evidence for Implicit Learning Ability and Impairments in Individuals with Autism

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Background: Implicit learning, or the unconscious acquisition of information from the environment, may play an important role in typical development. The social and communication deficits of autism suggest a possible impairment in the neurological mechanism that supports implicit learning. Subjects with autism have previously demonstrated an impaired ability to implicitly learn a complex sequence and cerebellar abnormalities were implicated. However, the impairment may have been the result of specific features of the testing paradigm.

Methods: In the present study, two variations of the sequence reaction time task were designed to test the effect of different stimuli (neutral v. social) on implicit sequence learning in three subject groups. The autism proband group consisted of 17 subjects (16 males and one female, mean age = 18.59, SD = 6.53); the relative group consisted of 33 subjects (mean age = 40.70, SD = 13.37) or 28 parents (11 fathers and 17 mothers), and five unaffected siblings (two male, three female); and the control group consisted of 21 subjects (nine males, 12 females, mean age = 24, SD = 8.32). Within group performance on each task was examined with t tests for significant changes in RT across seven testing blocks.

Results: All three groups demonstrated implicit sequence learning on the task that used neutral stimuli to convey the sequence as indicated by significant changes in RT (all $p < .05$). However, the autism group took longer to demonstrate significant changes in RT than the other two groups (no significant RT decrease until blocks four to five, while relatives and controls had a significant RT reduction from blocks one to two), and had greater difficulty recovering from disruption. In the task that used socially relevant stimuli, the autism group did not demonstrate implicit sequence learning, but the relatives and control groups had significant reductions in RT (all $p < .01$). Additionally, the autism and relative groups were slower at both tasks than controls, which may be indicative of familial fine motor impairments.

Conclusions: Thus, IL can occur in subjects with autism, but it may take longer and it is more sensitive to disruptions to learning than in subjects without autism. The difficulties with disruption may be associated with previously identified executive functioning deficits in tasks that require rapid shifts in attention. Follow up studies need to examine the neurophysiological response to learning disruptions (e.g. changes or shifts in stimuli), which may contribute to the development of autism related social and communication impairments.

Evidence for Paternal Allele-Specific Chromatin Extension and Looping of the *SNRPN* to *UBE3A* Locus in Mature Neurons by Fluorescence in situ Hybridization

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The imprinted region 15q11-13 contains a cluster of both paternally and maternally imprinted genes whose complex developmental and tissue specific expression is regulated by small imprinting control regions (ICR) within the cluster. Several neurodevelopmental disorders are associated with deletions, mutations or aberrant gene expression in this region including Angelman syndrome, Prader-Willi syndrome, autism, and Rett syndrome. Maternal duplication of 15q11-13 is the most common cytogenetic abnormality found in Autism patients. It has been proposed that chromatin looping in this region may allow the paternal ICR to bi-directionally regulate gene expression over several Mb. *SNRPN* is a paternally expressed gene within the cluster whose gene products are involved in splicing and are highly expressed in brain. The brain-specific *SNRPN* transcription unit also includes multiple small nucleolar RNAs and the antisense to *UBE3A*. *UBE3A* is oppositely oriented to *SNRPN* and is paternally silenced in neurons, presumably by the *UBE3A* antisense. Utilizing DNA FISH we have found visual evidence for allele-specific chromatin extension and looping of *SNRPN* and *UBE3A* in human post-mortem brain. In mature neurons, the paternal allele was highly extended and looped into the euchromatin while the maternal allele was observed as a single spot found primarily in the heterochromatin. A similar pattern was found using a probe to the *Snrpn* locus in mouse brain samples of multiple ages. The paternal allele-specific chromatin extension increased with brain age and neuronal maturity (~0.5μ at 1d to ~4μ at 10wk), while the maternal allele remained small and compact (~0.5μ at all ages). High concentrations of RNAse A used pre- or post-hybridization did not affect the size of the chromatin structures, demonstrating that the extended signal was due to DNA not RNA. Extended paternal chromatin loops were specific to neurons as they were not found in glial cells, thymus, kidney, liver or spleen. These results suggest dynamic changes in chromatin conformation of the paternal *SNRPN* to *UBE3A* locus during neuronal maturation that may serve to regulate neuronal specific expression and imprinting in this region.

Developmental Serotonin Reduction Alters Several Behavioral Phenotypes in the Laboratory Rat

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It has been well established that a functioning serotonin (5-HT) system is essential for proper brain development, and abnormalities in the developmental program of 5-HT have been implicated in brain alterations of those inflicted with neurodevelopmental disorders. Specifically, alterations in 5-HT neurotransmission during critical development periods for people with autism may contribute to the constellation of core behavioral features characteristic of the disorder such as social dysfunction and behavioral perseveration. Since the relationship between when 5-HT alterations occur and the types of behaviors affected has not been determined, we examined how developmental reductions in 5-HT affect rodent autism-like behavior. To this end, groups of rats were exposed to one of two methods shown to reduce brain 5-HT. Two groups of rats received 15 mg/kg p-chloroamphetamine (PCA) or saline on postnatal days (PD) 22 and 24, time-points that correlate to preadolescence in humans, while another two groups were either socially isolated for 1 hour/day for 8 days starting on PD 2 or remained socially housed. Behavioral analysis for each group began on PD 35. To test the role of 5-HT on autism-like behaviors, we examined how developmental 5-HT reduction affected social preference, rotarod motor learning, and perseveration behavior. Our results show that rats with chemical 5-HT lesions showed reductions in social preference, increased early motor learning, and increased perseveration behavior. Similarly, socially isolated animals also showed decreased social preference and increased perseveration behavior. However, socially isolated rats showed significant decreases in early motor learning, as compared to controls. These results demonstrate that developmental reductions in 5-HT neurotransmission can affect select behaviors when tested at later time-points, and suggests that some of the core behavioral features seen in autism may be due to early serotonergic deficits. Additionally, the time-period when changes in 5-HT occur in clinical cases may indicate the range of behavioral deficits observed in neurodevelopmental disorders.

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Rett syndrome is an X-linked neurological disease that primarily affects females and has an estimated incidence of 1;10,000. Affected children lose acquired skills and develop seizures, autistic features and hand stereotypies. MeCP2, the product of the gene mutated in most cases of Rett syndrome, has been shown to be a regulator of gene expression. We have proposed that part of this regulation is exerted at the level of RNA processing. Support from this hypothesis comes from the fact that MeCP2 forms an RNA dependent complex that includes the splicing modulator Y box-binding protein 1 and regulates splicing of reporter minigenes. In addition, splicing patterns were aberrant in brains of a mouse model of Rett. We are attempting to delineate the mechanisms by which MeCP2 carries its splicing-related function. In addition, we are analyzing the effects of mis-spliced cell adhesion molecules in the regulation of *ex-vivo* synapse formation and its possible contribution to Rett syndrome phenotypes.

Brain-Specific Phosphorylation of MeCP2 Regulates Activity-Dependent Bdnf Transcription, Dendritic Growth, and Spine Maturation

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Rett Syndrome (RTT) is one of few autism spectrum disorders with a known genetic basis. Mutations or duplications in the X-linked gene encoding methyl-CpG-binding protein 2 (MeCP2) are the primary cause of Rett and Rett-like syndromes. RTT is characterized by normal development for the first 6-18 months of life, followed by developmental regression and cognitive decline. MeCP2 is expressed in many mammalian tissues and functions as a global repressor of gene transcription; however, the molecular mechanisms by which MeCP2 dysfunction leads to the neural-specific phenotypes of RTT remain poorly understood. Here we show that neuronal activity and subsequent calcium influx trigger the de novo phosphorylation of MeCP2 at serine 421 (S421) by a CaMKII-dependent mechanism. MeCP2 S421 phosphorylation is induced in response to physiological stimuli in the intact brain and occurs selectively in the nervous system. Significantly, we find that S421 phosphorylation controls the ability of MeCP2 to regulate dendritic patterning, spine morphogenesis, and the activity-dependent induction of Bdnf transcription. These findings suggest that, by triggering MeCP2 phosphorylation, neuronal activity regulates a program of gene expression that mediates synaptic maturation in the brain and that disruption of this process in individuals with mutations in MeCP2 may underlie the neural-specific pathology of RTT.