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EFND
Environmental Factors *in* Neurodevelopmental Disorders
SYMPOSIUM

August 25 & 26th, 2005
Hyatt Regency in Bethesda, Maryland

Sponsored by The Coalition for Safe Minds and the National Autism Association
Through a Generous Contribution from NIEHS.

safeMinds
Sensible Action for Ending Mercury-Induced Neurological Disorders

NATIONAL AUTISM ASSOCIATION

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Environmental Factors in Neurodevelopmental Disorders. Proceedings of an National Institute of Environmental Health Sciences (NIEHS)-Sponsored Symposium. Bethesda, Maryland, 25-26 August 2005.

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The Environmental Factors in Neurodevelopmental Disorders Symposium, an NIEHS-Sponsored Conference, August 25-26, 2005, Bethesda, Maryland

Introduction

In August 2005, a two-day symposium was held in Bethesda, Maryland on the role of environmental factors in the pathogenesis of autism spectrum disorders and related neurodevelopmental conditions. Dr. Kenneth Olden – then the director of NIEHS – conceived the idea for the conference, and the meeting was sponsored by NIEHS. Two autism research groups, the Coalition for SafeMinds and the National Autism Association, co-organized the meeting with a team of researchers they recruited to the effort.

Section 1. Overview of the symposium

Section 1A. Background

The causes of most neurodevelopmental disorders, including autism spectrum disorders, remain unknown. Despite compelling data to suggest heritable contributions to these conditions, exclusively genetic models are inadequate to explain variation in linkage of candidate genes to autism within different populations (Risch *et al*., 1999; D'Amelio *et al*., 2005), disparity in both the prevelance and the phenotypic expression of autism among siblings in families with more than one affected member and in monozygotic twin pairs (Spiker *et al*., 1994; Le Couteur *at al*., 1996; Persico and Bourgeron, 2006), and the reported increases in autism prevalence across time. Whether the full extent of this apparent increase in prevalence is an artifact of changing patterns of clinical recognition, as opposed to a reflection of true disease rates, is unclear; some recent reports indicate that changes in diagnostic patterns alone are insufficient to account for the increases in reported prevalence (Newschaffer 2005; Rutter 2005). Independent of whether the reported increases in autism rates are real, however, environmental agents may be important to consider in autism pathogenesis because they may modulate disease risk by acting in concert with environmental susceptibility genes during periods of pre- or postnatal maturational vulnerability. Concerned that the absence of a well-developed, prioritized environmental agenda from autism research programs would impede discovery of the causes of autism as well as the ability to translate an understanding of causal pathways into new treatment avenues, a group of parents representing the National Autism Association and the Coalition for SafeMinds and physicians began to review federal initiatives for development of strategic plans for the short- and long-term funding of autism research.

One such initiative began in 2003 with the Consolidated Appropriations Bill for the Department of Labor, Health and Human Services and Education and Related Agencies. In the Conference Report associated with the bill the Interagency Autism Coordinating Committee (IACC) was asked to convene a panel of experts to evaluate the field of autism research and develop a matrix of action items to be used in planning future research. The purpose of this research matrix was to "help guide further research planning at NIH and as a tool for the entire autism community" and was to be utilized as a "living document that can [be] revised and expanded as current goals are achieved and new goals are identified" (Conference Report No. 108-10).

The panel convened in July 2003 and their recommendations are documented in the U.S. Department of Health and Human Services (DHHS) publication, *Congressional Appropriations Committee Report on the State of Autism Research, April 2004* (DHHS,

2004). The IACC provided input and approved the final draft of the matrix at the November 21, 2003 IACC meeting. Goals and activities comprising the autism matrix fall into eight categories, including: 1) characterization of autism, 2) screening, 3) early intervention, 4) school and community interventions, 5) specific treatments, 6) neuroscience, 7) environmental factors, and 8) epidemiology. As requested by the conferees, the matrix was understood to be subject to ongoing revision, a "*living document"* tracking the achievement of goals and the continuous acquisition of new knowledge and insights.

On March 21st, 2005, parents representing the National Autism Association and the Coalition for SafeMinds and physicians met with representatives from the National Institute of Environmental Health Sciences (NIEHS) to discuss concerns regarding reports of increasing rates of autism and neurodevelopmental disorders in American children. In 2004, the CDC issued an "Autism Alert" announcing that the reported prevalence of autism spectrum disorders had risen to alarming levels, and was currently affecting approximately one in every 166 American children. A number of investigations had also revealed intriguing data suggesting that environmental factors combined with genetic vulnerability may be responsible for this otherwise unexplained epidemic. The purpose of the NIEHS meeting was two-fold: first, to share information regarding advances in the understanding of some of the biologic mechanisms involved in autism; and second, to ask NIEHS to take the lead in helping to advance the science in these areas, thereby assisting the National Autism Association and the Coalition for SafeMinds in efforts on behalf of children suffering from autism and other neurodevelopmental disorders.

As a result of this meeting, Dr. Kenneth Olden, then the director of NIEHS, asked the National Autism Association and SafeMinds to bring a group of experts in the fields of toxicology, environmental health science, neuroscience, and clinical science together with the researchers who had presented their data at the meeting in order to review the new findings, make recommendations regarding future research initiatives and design a roadmap for future research into the role of environmental factors in the pathogenesis of autism.

Following Dr. Olden's suggestion, the Coalition for Safe Minds and the National Autism Association, with generous support from NIEHS, convened the Environmental Factors in Neurodevelopmental Disorders Symposium on August 25 and 26, 2005 in Bethesda, Maryland. A major goal of the symposium was, through a review of the state of the science, to define a research agenda that would elucidate the mechanisms by which environmental toxicants may induce damage in the developing infant and child, leading to a decline in neurological function and an increase in pervasive developmental disorders.

Section 1B. Participants

Kenneth Olden, Director Emeritus of NIEHS, moderated the meeting. Experts presenting research included:

Thomas Burbacher, PhD, University of Washington
Jay Charleston, PhD, StereoTomeNW
Mady Hornig, MD, MA, Mailman School of Public Health, Columbia University
S. Jill James, PhD, Arkansas Children's Hospital
Richard Deth, PhD, Northeastern University
Martha Herbert, PhD, MD, Harvard Medical School
Ellen Silbergeld, PhD, John Hopkins School of Public Health

David Baskin, MD, Texas Medical Center
Anna Choi, DSc, Harvard School of Public Health
Wolfgang Streit, PhD, McKnight Brain Institute at the University of Florida
Boyd Haley, PhD, University of Kentucky
Diana Vargas, MD, Johns Hopkins School of Medicine
Ray Palmer, PhD, University of Texas Health Science Center San Antonio
Jeff Bradstreet, MD, International Child Development Research Center
Elizabeth Mumper, MD, Advocates for Children
Mark Blaxill, SafeMinds

National Institutes of Health representatives in attendance included:
David Schwartz, MD, Director, NIEHS
Sue Swedo, MD, Senior Investigator, Pediatrics and Developmental Neuropsychiatry Branch, National Institute of Mental Health (NIMH)
Ann Wagner,PhD, Autism Interventions Research Program, NIMH
Cindy Lawler, PhD, Scientific Program Director, NIEHS
Molly Oliveri, PhD, NIMH
Audrey Thurm, PhD, NIMH
Deborah Hirtz, National Institute of Neurological Disorders and Stroke (NINDS)
William Raub, PhD, Deputy Assistant Secretary for Science Policy, Department of Health and Human Services

Capitol Hill staff in attendance represented the offices of:
Senator Joseph Lieberman
Senator Deborah Stabenow
Senator Michael Enzi
Senator John McCain
Congressmen Dan Burton
Congressman Dave Weldon
Congressman Chris Smith

Autism groups in attendance:
The Coalition for SafeMinds
The National Autism Association
Cure Autism Now
Autism Research Institute
Autism Speaks
Autism Society of America
Unlocking Autism
Autism One
A-Champ
Generation Rescue
Dads Against Mercury

Section 1C. Themes

The symposium was organized around several key themes pertaining to the reported rise in the prevalence of neurodevelopmental disorders, specifically autism; the potential mechanisms involved in the development of autism spectrum disorders; and identification of clinical and biochemical features that might provide clues to causal pathways and potential treatment strategies. These themes included:

i. *An appraisal of epidemiologic studies on the role of environmental factors in autism, including their potential contribution to increased prevalence rates*
   What findings are most robust? What are the evidentiary gaps?

ii. *General properties of toxins and their relevance to mechanisms of developmental neurotoxicology*
   What aspects of toxins are relevant to the development of neurotoxicity? What pathways may lead to neurodevelopmental damage after exposures to toxins?

iii. *Structural and cellular responses to toxins in the central nervous system*
   How do neurons, microglia, astrocytes and oligodendrocytes respond to toxic exposures?

iv. *Interactions of genes, environment, and timing in neurodevelopmental disorders*
   What genes exacerbate central nervous system damage after exposure to environmental agents? How might maturation influence that gene expression?

v. *Metabolic pathways influencing host response to toxins*
   What are the critical mechanisms involved in the response to environmental neurotoxicants? Is there evidence to suggest that some of these same mechanisms are involved in the pathogenesis of autism and related neurodevelopmental disorders?

vi. *Clinical findings in autism spectrum disorders*
   What clinical features are most frequently found to be disturbed in children with autism? What biologic parameters and pathways are disrupted? Are specific clinical disturbances associated with specific biologic ones (possibly defining a tractable endophenotype)? Are these patterns of abnormality consistent with those that would be predicted if a neuropsychiatric condition were environmentally-induced?

vii. *Research approaches in developmental neurotoxicology*
   What strategies are currently being implemented? What new approaches should be considered? How should these varied research strategies be prioritized?

Section 2. Symposium proceedings

Section 2A. Presentation summaries

i. An appraisal of epidemiologic studies on the role of environmental factors in autism

What's going on? The question of time trends in autism
Mark Blaxill

Blaxill opened the first session of the symposium with a review of existing epidemiological studies regarding time trends in the rate of autism. He showed data indicating that the reported frequency of autism has risen roughly fifteen-fold in the U.S. within the selected states where early surveys had been conducted, comparing rates from surveys spanning the birth years 1950-1990 to rates from surveys based on birth years in the mid-1990s. He argued that the hypothesis that these increases were a result of increased awareness and a broadening of the diagnostic concept of autism could be segmented into three distinct and testable propositions: diagnostic gap or oversight, diagnostic substitution, and diagnostic expansion. He reviewed the available evidence on all three propositions and demonstrated that in all cases where these could be tested, they had been falsified. He reviewed a rough calculation on the annual cost of the disease and argued that the increases in disease frequency required a shift to a new model of the disease. A new model would define autism pathogenesis as being clearly driven by specific environmental exposures.

The association between environmental mercury and increasing autism rates
Raymond F. Palmer, PhD

Over the last 15 years, data from various sources indicate that autism trends in the US have increased exponentially. Texas reflects similar temporal trends. In Texas, community resources and other socio-demographic factors are reported to account for approximately 18% of the variability in the temporal rate of change in autism (Palmer et al., 2005). Results of a similar study using national data are consistent with the Texas results (Mandel and Palmer, 2005). However, the great majority of the variation in the rate of change in autism has not yet been explained.

Using Graphical Information Systems (GIS), Palmer and colleagues have shown considerable regional variability in the pattern of change in prevalence rates within the state of Texas. They show that the areas with the greatest increase in autism prevalence correspond to locations in which high levels of environmentally-released toxicants are found. Using advanced statistical methods and data from the Environmental Protection Agencies Toxic Release Inventory (EPA-TRI) and the Texas Educational Administration (TEA), these researchers found that for every 1000 pounds of environmentally released mercury reported at the county level, there was a 17% increase in autism rates, after adjusting for relevant covariates such as urbanicity and community resources. They also report a 43% increase in special education rates; this increase is fully accounted for by increases in autism alone, as compared to any other special education categories (Palmer et al, 2006). Because this study was cross-sectional and used county level exposure estimates, however, interpretation is highly limited.

In a recent analysis, Palmer *et al*. used distance to actual mercury pollution sources to determine its relationship to the rate of change in autism over time. By avoiding assumptions that exposure risk followed county boundaries instead of actual geographic proximity, this procedure served to increase precision in deriving estimates of potential exposure. Using these methods, these researchers found that the distance from industries using mercury compounds was inversely related to the exponential rate of change in autism prevalence (e.g., shorter distances to potential mercury exposure sites were related to more rapid increases in autism rates). Further, the number of coal-burning power plants within a defined geographic area (within a 32-mile school district radius) was found to be related to higher rates of change in autism over time.

This work demonstrates the utility of GIS and advanced statistical methods in the epidemiologic investigation of environmental exposures and their relationship to the temporal increase in autism. This work needs to be corroborated in other geographic areas. Ultimately this work should inform studies of gene-environment interactions relevant to mercury and other neuro-toxicant exposures.

ii. General properties of toxins and their relevance to mechanisms of developmental neurotoxicity

A cloud of unknowing: why don't we know more about environmental risk factors and autism spectrum disorders (ASD)?
Ellen K. Silbergeld, PhD

Understanding the role of mercury as a cause of ASD has been difficult and controversial for many reasons. Unraveling the contribution of mercury to risks for clinical disease in children will require carefully designed epidemiological studies. For purposes of understanding mechanisms by which environmental risks and genetic susceptibility may interact in ASD, mercury may have additional value for research, by providing useful biological models (both *in vivo* and *in vitro*) for understanding interactions of environmental risk factors, genetics, and infections. Animal models of complex human diseases may be valuable either because they explicate mechanisms for exposures of importance to human populations (such as lead poisoning), or because they represent the mechanisms by which unknown risks induce diseases (such as the 6-hydroxydopamine model of Parkinsonism). For this purpose, the mechanisms of mercury toxicity are highly relevant to some of the mechanistic processes that have been proposed for ASD. First, mercury affects the developing brain through many of the biological mechanisms that have been identified in ASD; second, mercury interacts with mechanisms contributing to autoimmune disease and infections; and third, mercury-induced immunotoxicity is genetically modulated in animal models. Mercury induces glial activation in many of the brain regions recently reported to be affected in brains from persons with ASD. In the developing brain of mice, mercury disrupts neuronal migration through inhibition of immunological signaling pathways between glia and neurons. Developmental exposure to mercury affects the development of the immune system and results in persistent alterations in immune function at maturity, in mice. Finally, the immunotoxic effects of mercury are highly dependent upon genotype in experimental animals, with both the nature and severity of toxic effects modulated by mouse strain. These fundamental mechanisms of mercury-induced neurotoxicity and immunotoxicity may provide opportunities for modeling basic mechanisms of pathophysiology involved in progressive developmental neurotoxicity.

Comparison of blood and brain mercury levels in infant monkeys exposed to methylmercury or vaccines containing thimerosal

Thomas Burbacher, PhD

Public perception of the safety and efficacy of childhood vaccines has a direct impact on immunization rates (Biroscak *et a*l., 2003, Thomas *et al*., 2004). The current debate linking the use of thimerosal in vaccines to autism and other developmental disorders (IOM 2001, 2004) has led many families to question whether the potential risks associated with early childhood immunizations may outweigh the benefits (Blaxill *et al*., 2004; http://www.SafeMinds.org).

Recent reports have indicated that some infants receive ethylmercury (in the form of thimerosal) at or above the Environmental Protection Agency (EPA) guidelines for methylmercury (MeHg) exposure (0.1 μg/kg/day), depending on the exact vaccinations, schedule, and size of the infant (Ball *et al*., 2001). Other estimates (Halsey, 1999) have indicated that the schedule could provide repeated doses of ethylmercury from approximately 5 to 20 μg/kg over the first 6 months of life. Studies in preterm infants indicate that blood levels of mercury following just one vaccination (hepatitis B) increase by over 10-fold to levels above the EPA guidelines (Stajich *et al*., 2000). EPA guidelines for MeHg are based on several decades of studies of humans and animal models of developmental toxicity (Burbacher *et al*., 1990; National Research Council, 2000). As little data exist for ethylmercury, the use of the MeHg guidelines would seem appropriate if the two compounds have similar toxicokinetic profiles and neurodevelopmental effects. The current study was initiated to provide a direct comparison of the blood and brain levels of mercury in infant nonhuman primates exposed orally to MeHg or via intramuscular (i.m.) injections of vaccines containing thimerosal. The routes of administration (oral for MeHg and i.m. injection for thimerosal-containing vaccines) were chosen to mimic the two routes of mercury exposure for humans. The dosages and schedule of administration of mercury were chosen to be comparable to the current immunization schedule for human newborns, taking into consideration the faster growth (approximately 4 to 1) of the macaque infant (Gunderson and Sackett ,1984).

The key findings of the study indicate that the toxicokinetic profiles of thimerosal and MeHg are not similar; thus MeHg is not a suitable reference for risk assessment from exposure to thimerosal-derived Hg. Whereas total mercury levels were lower in both the blood and brain of thimerosal-exposed infant monkeys when compared to monkeys exposed to MeHg, the levels of inorganic mercury in the brains of these animals were nearly twice as high. Results from previous studies of nonhuman primates (Vahter *et al*., 1994, 1995) have indicated that inorganic Hg has a very long half-life once in the brain (over 1 year) compared to MeHg (approximately 37 days). In the same studies, inorganic Hg was associated with a significant increase in the number of microglia in the brain, representing a neuroinflammatory response (Charleston *et al*., 1994, 1995, 1996). It is important to note that a recent publication has demonstrated “an active neuroinflammatory process” in brains of autistic patients, including a marked activation of microglia (Vargas *et al*., 2005). Further research on the toxicokinetics and possible developmental effects of thimerosal exposure during infancy is needed to afford a meaningful interpretation of the potential developmental effects of immunization with thimerosal-containing vaccines in newborns and infants. This information is critical if we are to respond to public concerns regarding the safety of childhood immunizations.

Methylmercury neurotoxicity in a Faroese birth cohort

Philippe Grandjean, MD; Anna Choi, ScD

Methylmercury (MeHg) is a worldwide contaminant found in seafood and freshwater fish. It is a well-established neurotoxicant that can have serious adverse effects on the developing nervous system. The toxicity of MeHg was known from occupational exposures over 100 years ago. In Minamata, infants were born with serious neurological damage, even if their exposed mothers were virtually unaffected. Recent epidemiological studies have found more subtle adverse effects on brain functions at lower levels of MeHg. Efforts to develop preventive measures to MeHg exposures are deemed critical and necessary.

To study the adverse effects from MeHg-contaminated seafood, a Faroese cohort of 1,022 singleton births was assembled during 1986-1987. The Faroe Islands are located in the North Atlantic between Iceland and Scotland. Approximately 45,000 people reside on 18 islands covering an area of 1,400 km^2. Traditional food includes pilot whale meat (main source of MeHg) and blubber (source of polychlorinated biphenyls, PCBs). People exposed to MeHg are not necessarily exposed to PCBs, as intakes of whale meat and blubber correlate only weakly.

The cohort children completed the detailed clinical examinations at ages 7 years and 14 years. Exposure biomarkers included mercury concentration in cord blood, maternal hair, and child's hair and blood at 7 years and 14 years. At 7 years, these researchers found that mercury-related neuropsychological dysfunctions were most pronounced in the domains of language, attention, and memory, and to a lesser extent, in visuospatial and motor functions. A doubling in prenatal mercury exposure was associated with a decrease in these functions corresponding to about 10% of the standard deviation, or a developmental delay of about 1.5 months. Using neurophysiological methods, we found that a decrease in heart rate variability was associated with increased prenatal exposure. The greatest mercury-associated change in blood pressure at age 7 years occurred below cord blood mercury concentrations of 10 μg/l. Brainstem auditory evoked potentials (BAEP) were also used as an objective outcome measure of neurobehavioral toxicity. Delayed peak latencies were found on both examinations occasions, especially for the I-III interpeak interval. We also found that the child's hair mercury level at age 14 years was associated with prolonged III-V interpeak latencies. The apparent sensitivity of the peak III-V component to recent MeHg exposure suggested a possible change in vulnerability to MeHg toxicity.

The children were vaccinated according to governmental recommendations. Thimerosal was still in use in the mid-1980s, but was phased out shortly thereafter. Hair samples had been collected from 5 children at age 14-15 months, i.e., after their 12-month vaccination. The first 2-cm hair segment was analyzed by gas chromatography, but no ethylmercury was detected (<0.1 μg/l). In this population-based cohort, specific diagnoses were not the main consideration; however, autism was not found among the 90% of the children who participated in the examinations.

iii. Structural and cellular responses to toxins in the central nervous system

Microglial activation
Wolfgang J. Streit, PhD

Microglia are representatives of the immune system specially evolved to meet the unique demands of the central nervous system. They are distributed ubiquitously throughout the brain and spinal cord and stand ready as immediate responders to any disturbance in tissue homeostasis. They become activated very rapidly after an injury has occurred regardless of its nature. The ultimate purpose of this microglial activation is to provide instant neuroprotection, prevent further aggravation of damage, and to restore homeostasis as much as possible. Injury to neurons triggers a localized microglial reaction that is characterized by microglial cell division, demonstrating that microglia have retained the ability to expand their numbers whenever necessary. Their mitotic potential sets them apart from most other cells in the brain, which is usually thought of as a post-mitotic organ with little renewal capability. Thus, microglia constitute a very responsive force within the CNS that can replenish itself.

A number of specific examples illustrate the response of microglia to injury. When motoneurons undergo axotomy of their peripheral axons, microglia accumulate around the nerve cell bodies and protect them from further deterioration. This prevents motoneurons from dying and allows them to regenerate their axons, which eventually reconnect with their target muscles. On the other hand, when motoneurons are killed experimentally by injecting a retrogradely transported, toxic lectin into the nerve, microglia transform into brain macrophages and remove dead motoneurons. The ability of microglia to assume distinct functional states, such as activated microglia or brain macrophages, has been termed “functional plasticity.” These functional states of microglia can be recognized after administration of the neurotoxin, trimethyltin, and used to identify areas undergoing neurodegeneration. Overall, acute microglial activation is seen as a beneficial neuroinflammatory process that promotes clearance of debris and wound healing after a brain injury has occurred.

A number of observations in the normally developing CNS also support beneficial roles of microglia in the removal of apoptotic cells, angiogenesis, and the promotion of axonal guidance. It is conceivable that prenatal exposure to agents that adversely impact microglial cell function during CNS development could result in severe developmental deficits.

Methylmercury exposure in the primate brain
Jay S. Charleston, PhD

Charleston presented a review of stereological assessments (cell counts) of changes in specific cell populations within the central nervous system of non-human primates following long-term sub-clinical methylmercury (MeHg) exposure. Groups of cynomologous monkeys were exposed via the oral route to 50 μg MeHg per kg body weight per day for 6, 12, or 18 months, or for 12 months followed by 6 months of clearance. In addition, one group of animals received inorganic (iHg) mercury via an indwelling catheter for three months. Pharmacokinetic analysis of blood and brain levels indicated that iHg exposure resulted in relatively low levels of mercury within the brain, whereas MeHg exposure resulted in increasing levels of iHg within the brains as a function of exposure duration. MeHg cleared

from the brain rapidly, whereas iHg levels did not. These pharmacokinetic results provide strong support for the hypothesis that MeHg is demethylated in the brain, and, once demethylated, it becomes sequestered in the brain as iHg (Vahter *et al*., 1995).

The stereology results indicated that the neurons did not decrease in cell number, suggesting that these exposure levels were not overtly toxic to neurons. This lack of any loss of neurons correlated with the failure to find any clinical behavioral changes in association with the mercury exposure. However, microglia were found to increase in number within the thalamus and occipital pole (visual center), and astrocytes decreased within the thalamus (Charleston *et al*., 1994, 1996). Autometallography techniques, which result in the deposition of visible silver grains over iHg deposits within individual cells, were used to determine the location of the sequestered iHg. The majority of mercury deposits were localized to astrocytes and microglia, and neurons were relatively unlabeled (Charleston *et al*., 1995, 1996). Immunohistochemical techniques were employed to assess apparent changes in the physiological status of the glial cells. These studies demonstrated that mercury deposits were associated with increased expression levels of metallothionein (a metal-binding protein) and glial fibrillary acid protein (GFAP) within Bergmann glial astrocytes of the cerebellum and provide convincing evidence that sequestered iHg may represent the proximate toxic form of mercury within the CNS following MeHg exposure. Furthermore, the changes in astrocyte physiology and in astrocyte and microglial cell numbers suggest that the immune function of the CNS, as represented by microglial cells, has likely been perturbed by the MeHg exposure.

The above results support the concept that a similar mechanism may be possible following ethylmercury exposure, namely, that ethylmercury may be de-ethylated in the brain and that iHg may become sequestered in the brain as a result. Recent results published by Burbacher *et al*. (2005), featuring a non-human primate model of human pediatric exposure to thimerosal (ethylmercury used as a preservative in vaccines), indicated that iHg levels in the brains were elevated to a greater extent than would be expected based on predictions from MeHg studies. Charleston and colleagues are initiating a stereologic and histochemical study of these same brains to determine whether a similar response is observed after thimerosal exposure as was observed in the brains of MeHg-exposed monkeys. As in the above-described studies, stereologic methods will be employed to determine changes in neuron, astrocyte and microglia populations within six brain sites. Autometallography techniques will be used to localize the iHg observed in these brains. Finally, immunohistochemical techniques will be used to assess the immunological status of the brains in a study modeled after the survey recently completed by Vargas and colleagues (2005). This series of studies will test the hypothesis that de-ethylation of ethylmercury within the brain leads to compromise of the normal immune monitoring function of the microglia within the CNS.

Studies of thimerosal toxicity in human neurons, fibroblasts, and immortalized B lymphocytes
David S. Baskin, MD

Baskin reported on his investigations of the short-term toxicity of thimerosal in cultured human cerebral cortical neurons and in normal human fibroblasts. Cells were incubated with thimerosal at concentrations of 125 nM-250 µM for 45 minutes to 24 hours. A 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) dye exclusion test was used to identify nonviable cells, and TUNEL assay was used to label DNA damage. Detection of active caspase-3 was performed in live cell cultures using a cell-permeable, fluorescent caspase inhibitor.

The morphologic characteristics of fluorescently-labeled nuclei were analyzed. After 6 hours of incubation, thimerosal toxicity was observed at 2 µM, based on manual detection of fluorescent, attached cells; using the more sensitive GENios Plus Multi-Detection Microplate Reader with Enhanced Fluorescence, toxicity could also be observed at a thimerosal concentration of 1 µM. The lower limit of toxicity did not change after 24-hour incubation. Cortical neurons demonstrated higher sensitivity to thimerosal compared to fibroblasts. The first sign of toxicity was an increase in membrane permeability to DAPI after 2 hours of incubation with 250 µM thimerosal. A 6-hour incubation resulted in failure to exclude DAPI, generation of DNA breaks, caspase-3 activation, and development of morphological signs of apoptosis.

The results of these studies demonstrate that thimerosal in micromolar concentrations rapidly induces membrane and DNA damage, and initiates caspase-3-dependent apoptosis in human neurons and fibroblasts.

Effects of thimerosal on apoptosis and cell division were then investigated in cultures of immortalized human B lymphocytes. Cells at three different concentrations (10,000; 100,000 and 1,000,000 cells/mL) were exposed to 100 nM-50 µM thimerosal for 24 hours.

Nanomolar and low micromolar thimerosal induced caspase-3 dependent apoptosis in human B cells. Cell culture density significantly influenced B cell sensitivity to thimerosal. At 10,000 cells/mL, the lowest thimerosal concentration inducing caspase-3 activation was 250 nM; the peak apoptotic response was seen at 1 µM. A 10-fold increase in cell concentration raised the lowest toxic concentration to 500 nM; the peak apoptotic response was seen at 2 µM. An additional 10-fold increase further shifted the lowest toxic thimerosal concentration to 2 µM; the peak apoptotic response was 10µM.

In cell division experiments, B cell cultures were monitored for 168 hours with two concentrations of thimerosal: 100 nM (not inducing apoptosis), and 1 µM (pro-apoptotic). 24 hrs of incubation with 1 µM thimerosal completely suppressed B cell division, and was accompanied by cell death, whereas 100 nM thimerosal significantly inhibited cellular proliferation without inducing cell death. This indicates that thimerosal can suppress division of cultured human B-cells at concentrations which are nontoxic, if assessed by techniques that detect apoptotic and necrotic cell death.

The results of this portion of Baskin's study demonstrate that thimerosal toxicity measured *in vitro* is inversely proportional to the cellular concentration in the culture media. Considering the wide range of cellular concentrations routinely used in toxicological experiments, this effect should be considered when analyzing thimerosal toxicity in cell culture conditions. He also found that nanomolar concentrations of thimerosal induce cell death at cell culture concentrations most closely approximating the white blood cell concentrations that are likely to be achieved in blood after exposure to thimerosal in vaccines, and that thimerosal doses equal to doses measured and reported in preterm infants after a single vaccination can profoundly inhibit B cell proliferation without producing cell death.

iv. Interactions of genes, environment, and timing in neurodevelopmental disorders

Of mercury, microbes, mice and men: animal models of neurodevelopmental damage and implications for translational research
Mady Hornig, MD, MA

Autism is one of the most highly heritable neuropsychiatric disorders, yet its genetics remains elusive. Reports of a more than ten-fold increase in the apparent prevalence of the disorder over the past few decades, suggest – but do not confirm – potential risk factors from the environment. Infections, toxins, or dietary antigens, or common host responses to these environmental factors (immune or oxidative stress responses), may act as triggers or amplifying factors in autism pathogenesis. Hornig proposed a "three strikes" hypothesis, wherein environmental factors (first dimension) interact with susceptibility genes (second dimension) during vulnerable periods of brain development (the third dimension of timing) to produce damage and dysfunction. As an example of the potential utility of this "three strikes" approach for understanding the pathogenesis of neuropsychiatric conditions, Hornig described studies in schizophreniform disorder, wherein the coalescence of a genetic factor (a functional polymorphism in the catechol-O-methyltransferase gene) with a common environmental factor (use of cannabis) during adolescence (the element of timing) was associated with a significant increase in development of the psychotic disorder, yet none of these same factors individually compounded the risk (Caspi *et al*., 2005).

Using findings across several rodent models of neurodevelopmental damage that are based on exposure to pre- or postnatal environmental stimuli, Hornig noted similarity across the models in several brain and behavioral outcomes, and cited evidence to suggest convergence amongst host inflammatory, immune, apoptotic, and oxidative stress responses activated by the different environmental stressors. Extending the "three strikes" concept, the wide spectrum of CNS and systemic abnormalities observed in autism might best be understood by focusing not only on the specific effects that may be associated with each of the three factors (the gene, the specific environmental factor, and the precise timing) but also by exploring the modifying effects of potential interactions amongst these individual factors. It is at low, background exposure levels that the effects of genes and maturational factors are most likely to serve as critical modifiers of the neurodevelopmental equation.

In the mouse strain-dependent model of postnatal thimerosal neurotoxicity, for example, low dose exposures were administered to immature mice varying in an immune response allele (Hornig *et al*., 2004). Although previous work demonstrated that the effects of mercury on brain development and function were more dramatic when administered during prenatal or early postnatal life (Rice and Barone, 2000), dosages employed and cumulative exposures attained in these earlier studies were far higher than those used in the current study, which attempted to mimic the mercury exposure in the prior US childhood immunization schedule. Also, although earlier work identified the immune response genes contributing to mercury susceptibility (Monestier et al., 1994; Hultman et al., 1996), these studies were in adult animals instead of immature ones, and focused predominantly on autoimmune effects in extraneural organ systems such as the kidney without consideration of effects on brain or behavior. The coincidence of the environmental exposure and the gene at a specific window of vulnerability in the thimerosal mouse model resulted in interactive effects not observed in mice exposed at the same age but without the genetic susceptibility. Although the relevance to human populations, if any, is as yet unclear, the findings of enlarged brains, abnormal

behavior, and autoimmune disturbances in this experimental animal model suggest that dissection of the mechanisms at play using this multifactorial “three strikes” approach may help to elucidate the pathogenesis of autism. Other environmental agents may also provoke CNS damage, depending on the genetic and temporal context.

These animal model studies can critically inform the design of more powerful epidemiologic studies capable of assessing the combined influences of genes, environment, and timing on neurodevelopmental outcomes. Investigations in prospective birth cohorts make such analyses feasible and are poised to lead to discovery of potential new biomarkers and interventional strategies for ASD.

Implications of an environmental model of autism for research and treatment: the case of neuroimaging
Martha Herbert, PhD, MD

Neuroimaging primarily provides indirect rather than direct measures of environmental impact upon the brain. A number of findings in autism neuroimaging are consistent with the possibility of environmental influences, although they do not provide direct proof. Whereas high dose exposures can lead to cell death (and thus tissue loss, often focal), low-dose exposures are likely to lead to a different set of brain changes. In studies of autism, measures of brain volume indicate an increase in overall brain size and widespread, non-uniform alterations of scaling (or proportion), including disproportionately enlarged white matter or altered asymmetry. These changes may reflect mechanisms such as altered signaling and growth regulation consequent to low-dose toxic exposures (Herbert and Ziegler, 2005). The finding of atypically rapid increases in postnatal brain size could also be due to environmental factors, as could the observation that later-developing areas of the brain are sometimes more strikingly affected than earlier-developing areas (Carper *et al.*, 2002; Carper and Courchesne, 2005; Herbert *et al.*, 2004; Herbert *et al.*, 2005). Widespread impairments in brain connectivity (Just *et al.*, 2004; Horwitz *et al.*, 1988) could also be a consequence of the impact of environmentally-mediated brain metabolic alterations on brain function (Herbert, 2005). Although brain imaging research has focused on identifying brain changes that underlie behavior, the expanding repertoire of imaging techniques creates unprecedented opportunities for exploring properties of brain tissue in living individuals and searching for indications of environmental influence. Because environmental injury to the brain may occur through mechanisms that are also particularly amenable to treatment (Herbert, 2005) brain imaging measures may also prove useful for tracking treatment efficacy.

v. Metabolic pathways influencing host responses to toxins

Environmental factors in neurodevelopmental disorders
Boyd Haley, PhD

Data were presented that established that a major brain protein responsible for maintaining the structural integrity of axons was exceptionally sensitive to both inorganic mercury and thimerosal (ethylmercury). This was followed by data which strongly support the concept that autistic children represent a subset of the human population that cannot effectively excrete mercury. They are therefore very susceptible to the toxic effects of mercury. Considering the toxic effects of mercury, biochemical pathways were discussed pointing out known points of major mercury inhibition (called cross-over points) and showing how mercury toxicity can

inhibit several enzymes causing multiple aberrancies in metabolism. Information was then presented showing how mercury interacts with both the sulfhydryls and disulfides of enzymes, describing the molecular level chemistry that causes enzyme inhibition. Such enzyme inhibition is known to occur in specific toxicities; the inhibition of methionine synthetase and the well known 'porphyrin profile' are examples not only of mercury toxicity but also of autism. Problems were presented regarding the use of chelators to reverse this inhibition. It was pointed out that DMPS and DMSA are not true mercury chelators. New and more effective chelators can and should be synthesized. Several possible chelator structure examples were presented in which a moiety that binds mercury exceptionally tightly was coupled to a moiety which is a natural compound. The resulting chemical property of the proposed new chelator allows for the solubilization of the mercury chelator, promoting effective distribution throughout the body. Finally, a discussion of the potential applications of vitamin C and melatonin to enhance the cellular production of the reduced form of glutathione was presented. Once appropriately tested, the use of these non-toxic, natural compounds may provide nutritional aid to help children with autism excrete mercury.

How environmental factors can synergize with genetic factors and inflammation to impair methylation and cause developmental disorders
C. Richard Deth, PhD

Methylation reactions provide a critical mechanism by which many cellular processes, including gene expression, are regulated. The folate- and vitamin B12-dependent enzyme methionine synthase exerts control over methylation. Methionine synthase is also essential for dopamine-stimulated methylation of membrane phospholipids, a process which appears to be central to the synchronization of neuronal networks during tasks requiring attention. Impaired folate-dependent methylation, impaired attention and impaired neuronal synchronization are all features of autism and related neurodevelopmental disorders. Dr. Deth's lab showed that thimerosal and other neurodevelopmental toxins inhibit methionine synthase activity, and demonstrated subsequent effects of such enzyme inhibition on dopamine signaling. The researchers suggest that the reduced activity of this enzyme, triggered by thimerosal and other neurotoxicants may make an important contribution to autism.

Under conditions of cellular oxidative stress, methionine synthase activity is reduced, and an increased proportion of homocysteine is diverted toward synthesis of glutathione. Glutathione serves to counteract cellular oxidative stress. Deth's studies indicate that oxidative stress converts methionine synthase to a form that has an absolute dependence on methylB12 as a co-factor. In turn, increased demand for methylB12 synthesis requires consumption of glutathione stores. Thimerosal and other heavy metals and developmental toxins cause oxidative stress and lower cellular glutathione levels, impairing the synthesis of methylB12 and the activity of methionine synthase. Further, recent results indicate that the methylB12-dependent form of methionine synthase is the predominant form in brain. Genetic polymorphisms with adversely effects on methionine synthase and methylation efficiency are present at higher frequency in autistic children (James *et al*., 2004).

Based upon the above findings, Deth proposes a "Redox/Methylation Hypothesis of Autism," in which normally latent genetic vulnerabilities are revealed by exposure to thimerosal, heavy metals and/or other environmental agents that promote oxidative stress. Oxidative stress leads to reduced activity of methionine synthase, including reduced synthesis of methylB12.

As a consequence, the ability of dopamine to promote synchronization of neural networks is impaired, accounting for the loss in attention capacity and other higher-order cognitive abilities that are hallmarks of autism. The Redox/Methylation Hypothesis is strongly supported by the benefits reportedly experienced by a significant number of children when treated with anti-oxidant or pro-methylation therapies. He concluded that further investigations of this explicit molecular hypothesis are warranted, which may aid in identifying additional treatment options.

Oxidative stress and abnormal methionine metabolism in autism: metabolic biomarkers and genetic polymorphisms
S. Jill James, PhD

The metabolic phenotype of an individual reflects the influence of endogenous and exogenous factors on genotype. As such, it provides a window through which the interactive impact of genes and environment may be viewed and relevant susceptibility factors identified. Although abnormal methionine metabolism has been associated with other neurologic disorders, these pathways and related polymorphisms have not been evaluated in autistic children. James presented results from her recently published metabolic study in autistic children showing evidence for impaired cellular methylation capacity, increased plasma levels of oxidized (inactive) glutathione, and decreased levels of the reduced (active) form of glutathione. A decrease in redox capacity could render these children less able to detoxify environmental toxins and more vulnerable to oxidative damage. Targeted nutritional intervention with supplements of methylB12 and folinic acid was found to normalize the metabolic imbalance in many of the children. Additional work is needed to determine the relationship of the metabolic imbalance to both the origins of the disorder and the potential clinical impact of interventions correcting the imbalance. Such studies, including controlled trials of nutritional strategies that help to restore metabolic balance, may assist in identifying those children most likely to benefit from such treatment, any associated risks of the therapy, and the scope and degree of improvements that may be expected.

Using the abnormal metabolic phenotype as a map for the selection of functional candidate genes, James evaluated several genetic polymorphisms that have been shown to modulate these metabolic pathways. In children with the abnormal metabolic phenotype, significant increases in frequency were found for the reduced folate carrier (RFC1), transcobalamin II (TCN2), and the catechol-O-methyltransferase (COMT) genes as well as evidence for several gene-gene interactions. Subtle alterations in gene expression due to multiple polymorphisms and environmental factors that interact to affect the same metabolic pathway could induce a metabolic imbalance that would negatively affect brain cell function and ontogeny during critical periods of pre- and postnatal brain development. James hypothesizes that *part* of the genetic basis of autism may involve multiple susceptibility alleles that interact to create a fragile condition consistent with autism. She concludes that the inability to maintain glutathione redox status and to control oxidative stress may contribute to the development of neurologic, immunologic and gastrointestinal dysfunction that occur frequently with autism.

These new results support the possibility that some forms of autism could be a *manifestation* of a genetic predisposition to abnormal methionine/glutathione metabolism and oxidative stress. Further, the abnormal metabolic profile observed in a significant proportion of autistic children suggests the possibility that some autistic behaviors could be a neurologic manifestation of a genetically-based *systemic* metabolic derangement. Such a paradigm shift

from a purely *neurodevelopmental* disorder to a broader *systemic* disorder would widen the biologic basis of autism to encompass not only the neurologic manifestations but also the pathologic gastrointestinal and immunologic findings that have received increasing attention in recent years.

vi. Clinical findings in autism spectrum disorders

Is there brain inflammation in autism?
Diane Vargas, MD

Autism is a heterogeneous neurodevelopmental disorder characterized by impaired communication and social interaction. It may be accompanied by mental retardation and epilepsy. Its cause remains unknown, despite evidence that genetic, environmental, and immunological factors may play a role in its pathogenesis. To investigate whether immune-mediated mechanisms are involved in the pathogenesis of autism, we used immunocytochemical profiling of microglia and neuroglia expression (HLA-DR and GFAP staining of fixed brain tissue), cytokine protein arrays, and enzyme-linked immunosorbent assays to study brain tissues and cerebrospinal fluid (CSF) from patients with autism and determine the magnitude of neuroglial and inflammatory reactions and their cytokine expression profiles. Brain tissues from cerebellum, midfrontal, and cingulate gyrus obtained at autopsy from 11 patients with autism were used for morphological studies. Fresh-frozen tissues from seven patients and CSF from six living patients, all diagnosed with autism, were used for cytokine protein profiling. We demonstrated an active neuroinflammatory process in the cerebral cortex, white matter, and, notably, in cerebellum of patients. Immunocytochemical studies showed marked activation of microglia and astroglia. Cytokine profiling indicated that macrophage chemoattractant protein (MCP)–1 and tumor growth factor–1, immunomodulatory molecules derived from neuroglia, were the most prevalent cytokines in brain tissues. CSF showed a unique proinflammatory profile of cytokines, including a marked increase in MCP-1. Our findings indicate that innate neuroimmune reactions play a pathogenic role in an undefined proportion of patients with autism, suggesting that future therapies might modify neuroglial responses in the brain.

Recent advances in the biology of autism
Jeff Bradstreet, MD

Bradstreet began his presentation by noting that the formal positions of the American Academy of Pediatrics and the American Academy of Neurology on the biology of autism have not been updated in the last 6 years. He commented that updates are warranted due to the volume of published works that define a range of specific immunologic, gastrointestinal and biochemical abnormalities in some children with ASD. He also voiced concern regarding the catastrophic rise in the reported prevalence of the disorder.

Bradstreet reviewed some of the recently published, critical findings, including associations of autism with:

1. An inflammatory bowel disease condition that appears to be unique, in conjunction with abnormalities in intestinal, cerebrospinal fluid, brain and serum immune/autoimmune parameters
2. Microglial activation, most notably in cerebellum
3. Methylation disturbances; specifically, methionine cycle and COMT

4. Thiol deficiencies (cysteine, glutathione), metallothionein abnormalities, and associated increases in oxidative stress
5. Increased relative body burden of metals, specifically mercury, with corresponding low relative excretion
6. Increased risk of seizures
7. Evidence of a relative mitochondrial deficiency
8. Male-dominated pattern of 4:1 (males:females) in individuals with autism and mental retardation, with estimates of 8:1 in higher-functioning individuals with ASD

Bradstreet voiced concern that many of these biological conditions are not addressed by practitioners, leaving families with little to no resources for assistance with medically-related issues. In his opinion, ignoring the signs of inflammatory bowel disease was the most illogical. He pointed out that in his practice, children often present with protracted periods of diarrhea and/or constipation which parents have frequently been told are attributed to autism alone; efforts are almost never made to address any underlying pathophysiologic processes. Many of the conditions referenced above might be palliated or corrected through application of a range of treatments currently available to clinicians.

Bradstreet utilized mercury exposure as a model of the coincidence of genetic vulnerability with environment risk factors. The issue regarding the increasing relative body burden of mercury has been brushed aside despite several published studies to this effect. In addition, anecdotal observations suggest that chelation of heavy metals results in improvement in a large percentage of children with ASD. Even when exposure of infants to organic mercury is limited to the postnatal period through breast milk, as in a subset of children in the Iraqi methylmercury grain exposure of 1972 (Amin-Zaki *et al*., 1981), ASD outcomes very similar to both autistic disorder and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS) are described.

Together with the findings from Burbacher and colleagues (2005), these studies raise serious concerns that exposure to mercury from the medical product thimerosal deserves further investigation. Burbacher's nonhuman primate data show that once mercury has entered the brain, there is much higher retention of mercury in monkeys exposed to thimerosal than in the brains of those exposed to methylmercury. Previous work demonstrates that mercury is capable of inducing microglial cell responses similar to those observed by Vargas and colleagues (2005).

Bradstreet noted that according to a report in the MMWR (2005) on the development of criteria for safety with respect to mercury in adults, the threshold for the appearance of acute toxicity of mercury was defined as a mercury level of 10 μg/L. It is accepted that mercury is more toxic to children than to adults (by a factor of 10-fold); therefore, a reasonable threshold level for toxicity in a child on this basis might be estimated to be 1 μg/L. If so, this would mean that exposure to any single vaccine containing thimerosal would lead to a blood level that would exceed this threshold. Such concerns warrant additional research and analysis.

Glutathione and cysteine are critical to the defense from and elimination of heavy metals. It is documented in some studies that children with ASD have lower levels of cysteine and glutathione than control children. This adds to concerns about administration of mercury to such children. Some children with lower levels of these antioxidants may represent a subset

with a pre-existing vulnerability to this form of mercury. Further, thiol chelators are excellent antioxidants, so the anecdotal accounts of improvement in children with ASD following chelation with thiol compounds might relate more generally to restoration of antioxidant defenses than solely to the potential effects of these agents on heavy metal body burden.

Finally, the pathways involved in methylation and oxidative stress responses are prime candidates for further research, along with immunologic regulators of glial activation.

Bradstreet concluded with the following key points:

1. Environmental factors such as mercury generate oxidative stress and disrupt the immune system.
2. Genomic polymorphisms in COMT; TCN2; 5,10-methylenetetrahydrofolate reductase (MTHFR); glutathione-S-transferase (GST); and superoxide dismutase (SOD); along with epigenetic factors may contribute to mechanisms underlying vulnerability (intrinsic oxidative stress).
3. Hormones and xenobiotics may serve as modulating or risk factors contributing to the male predominance in this condition.
4. Glial activation may be related to oxidative stress as well as to the downstream effects of other immune activators.
5. Anti-inflammatory and antioxidant agents may have a therapeutic role in ASD through restoration of supplies of reduced glutathione, n-acetylcysteine, and other protective compounds.
6. Mercury chelators may work through alteration in redox balance rather than merely through direct effects on mercury burden.

Integrating the science: case studies of children with ASD
Elizabeth Mumper, MD

According to Mumper, clinical impressions of increased numbers of children with neurodevelopmental disorders are corroborated by growing epidemiologic evidence. In Virginia, Department of Education data show an eleven-fold increase in autism and a sixty-six-fold increase in attention-deficit/hyperactivity disorder (AD/HD) during a 15-year period. Recent research lends credence to the possibility that genetic susceptibilities predispose certain children to metabolic problems when challenged with environmental toxins. Mumper's practice includes over 1,000 children with neurodevelopmental disabilities (out of 2,000 patients). The vast majority of the developmentally disabled children show laboratory evidence of oxidative stress, problems with methylation biochemistry, and decreased function of transsulfuration pathways. Many children diagnosed with autism in her practice show improvements in language acquisition rates and decreases in behaviors associated with autism after treatment for these underlying metabolic problems.

Mumper voiced concern regarding a number of environmental toxins, but her most recent focus has been on mercury. Known consequences of mercury toxicity are compatible with pathology reported in children with autism, including low glutathione, low sulfate, and mitochondrial dysfunction. Clinical symptoms of heavy metal toxicity—including apathy, hypotonia, clumsiness, and sensitivity to sound, light and noise—are observed in many patients. Gastrointestinal effects of mercury poisoning are consistent with symptoms and pathology observed in children with autism. Laboratory evidence of immune dysregulation, consistent with heavy metal toxicity, is found in the majority of her autistic patients. Areas of

the brain known to be adversely affected in mercury poisoning, including Purkinje cells, basal ganglia, visual cortex, and cerebral cortex, are abnormal in children with autism. Known biochemical effects of mercury poisoning, including disruptions in COMT, increased glutamate, and abnormal serotonin and dopamine metabolism are also observed in autism. Genetic testing in these patients reveals patterns consistent with susceptibility to increased risk upon exposure to heavy metals.

Mumper presented case histories to illustrate clinical improvements upon identification and treatment of metabolic problems in methylation pathways and correction of oxidative stress. Specific abnormalities documented include altered immune function, impaired intestinal integrity and methylation, decreased natural killer cell function (which correlates with glutathione depletion) and a new variant of inflammatory bowel disease and oxidative stress.

Mumper's clinical experience gave credence to the concerns raised by Hornig, Burbacher, James, Deth, Herbert, Haley, and Bradstreet. Her desire is to continue initiatives to help other professionals recognize that many children with autism have an acquired illness, encourage care-providers to search for treatable causes of pain, self-abusive behaviors, and cognitive impairments, and develop individualized treatment plans based on each child's unique biochemistry and pathology. She strongly urges NIEHS to support initiatives that remove ongoing environmental toxins and to evaluate novel treatment strategies for children with neurodevelopmental disorders targeted toward identified biomedical abnormalities.

vii. Research approaches in developmental neurotoxicology

Research approaches in developmental neurotoxicology: current programs for funding and funded investigations
Cindy Lawler, PhD

Lawler began her presentation with a brief overview of autism research activities among various federal agencies, including NIH, USEPA and the CDC. Involvement of numerous agencies creates a need for coordination at multiple levels. Within the NIH structure there are five institutes participating in autism research and those institutes are brought together under the umbrella of the Interagency Autism Coordinating Committee (IACC). The IACC consists of the National Institute of Mental Health (NIMH), which is designated as the lead agency; The National Institute of Environmental Health Sciences (NIEHS); The National Institute of Child Health and Human Development (NICHD); the National Institute of Neurological Disorders and Stroke (NINDS); and the National Institute of Deafness and Other Communication Disorders (NIDCD). These agencies brought together a panel of experts in 2003 to develop a research matrix that would be used to guide autism research activities among the five agencies.

Lawler stated that autism is a priority area at NIEHS and reviewed the recent activities and initiatives at the Institute with regard to autism research. These include joint funding with the EPA of two child health centers, located at the University of California at Davis and the University of Medicine and Dentistry of New Jersey, which focus on possible environmental aspects of autism and related neurodevelopmental disorders. Lawler also described the development of the Environmental Genome Project at NIEHS and how this initiative is being utilized in an effort to identify and define susceptibility genes for autism.

Creating a roadmap for investigations into environmental factors and neurodevelopmental disorders: a brainstorming session
Ken Olden, PhD, ScD, LHD

A discussion, led by Olden, ensued among both panel participants and representatives from the autism community with regard to the best way to assimilate the current data with regard to environmental factors in neurodevelopmental disorders and prioritize future research goals. A hierarchy was suggested that included the four principles outlined below.

Helping the most children most rapidly. This requires a crisis-sensitive translational research program, biased toward producing short-term deliverables that will serve the needs of today's affected population, and not just the development of longer-term insights. Many researchers stand to benefit from exposure to the rich experience of the community-based clinicians, many of whom have embraced a multisystem model of autism and are working towards systematic validation and optimization of diagnostic and treatment approaches for these complex disorders.

Specifying ASD as the result of a biologic process. The biologic bases of autism need to be understood as primary. In some cases, behavioral and brain alterations may be only one of a set of multi-system consequences occuring downstream of the site(s) of initial biologic disruption. Alternatively, insults sustained by the brain may be coterminous with those that occur in peripheral organ systems. In still other circumstances, the biologic disruption linked to ASD may be largely restricted to the CNS – but systemic effects may be observed due to comorbidity. Exclusion of extra-CNS manifestations from the analysis due to assumptions that such features are most likely irrelevant to our understanding of ASD pathogenesis may hinder progress toward discovery of causal pathways. Achieving a flexible integration of an empirical framework – one that builds upon data derived from a thorough characterization of CNS and extra-CNS effects to discern new patterns – with *a priori* models that impose pre-defined frameworks upon the data is most likely to yield positive results. Leaving room for the possibility that the brain and behavioral abnormalities in ASD may be part of a more encompassing syndrome rather than an isolated brain problem may help lead to novel ideas about pathogenesis.

Assessing the potential contribution of environmental factors to autism as causal or exacerbatory agents. Environmental factors may trigger adverse outcomes by acting upon a substrate of genetic vulnerability. Alternatively, environmental factors may be employed as sensitive probes with the capacity to trigger definable changes in vulnerable components of developing neural circuitry, or identifying the biological mechanisms that are the most responsive to environmental stressors – and the most plastic. By identifying pathways with both vulnerability and the potential for plasticity, the investigation of the effects of environmental exposures may provide clues to developmental regulatory systems and uncover pathways capable of adaptation or recovery and ripe for the application of novel treatment strategies. If there are environmental factors that contribute disproportionately to autism, then study of the mechanisms by which they contribute to disease should be prioritized.

Framing autism as a treatable disorder. Placing the cause of ASD squarely in either the nurture (environmental) or the nature (genetic) camp has little impact on the potential for

treatment response. A wide range of causal mechanisms – genetic, environmental, or an interaction of the two – may target pathways that are modifiable by therapeutic interventions. Even where brain architecture has been disrupted, knowledge of the mechanisms that are impaired and the connections that may be severed may help to identify strategies for treatment.

Section 2B. Participant recommendations for an environmental research agenda for ASD: broad themes

Both as a prelude to and during discussions at the symposium, participants were asked to identify areas they believed to be the top research priorities for the development of an environmental research agenda for ASD and related neurodevelopmental disorders. Several broad themes emerged within the categories of epidemiologic, toxicologic (*in vitro*, animal model), genetic, and clinical research, and are outlined below. More specific recommendations, directed toward expansion of existing research infrastructure and development of new research methods and approaches, are described in Section 2C.

Epidemiologic research

Research need: *Prospective, large-scale, population-based studies of the prevalence of ASD and other neurodevelopmental disorders (NDD)*

Rationale: Studies in geographically distinct, genetically diverse, unselected populations across time will permit a wide range of hypotheses to be tested regarding the association of ASD and related disorders with environmental factors, gene:exposure relationships, and the timing of exposures relative to fetal or postnatal maturation.

Recommendations and enhancements:
Involve existing international consortia, or foster the establishment of such consortia, in order to facilitate examination of the influence of geographic, genetic, and exposure variables on ASD prevalence

Bank appropriate biological specimens to support future research

Enhance the value of biological specimens in prospective, population-based studies by:

- acquiring multiple samples over time, preferably before birth or even before conception, and continuing at regular intervals

  this approach enhances the probability of being able to rigorously test the hypothesis that an adverse outcome is related to an exposure

- adding samples from mothers and fathers to those from children born into birth cohorts

  parent-child trio designs strengthen genetic and epigenetic investigations and may increase statistical power for certain analyses

- serial acquisition of samples appropriate for gene expression studies (RNA)

  a clearer understanding of which genes are dysregulated at different time points in ASD, and of their relationship, if any, to different exposures and/or genetic polymorphisms may help elucidate pathogenesis and lay the foundation for biomarker discovery

- serial acquisition of samples appropriate for more direct measurements of environmental exposures, including toxicants and microbes

  reducing exposure misclassification enhances the capacity to detect exposure:outcome relationships, should they exist

Prospective, large-scale, population-based studies on the prevalence of ASD and other NDD, cont'd.

minimizing reliance on estimates of exposure as opposed to values derived from serial biologic measurements may enhance the capacity to detect gene-environment or gene-environment-timing interactions, should they exist

- collecting exposure-related and clinical data in a time frame parallel to the serial collection of biological samples

link registry data with that from screening and direct clinical characterization

use rigorous and well-validated diagnostic ascertainment procedures

include standardized instruments for diagnosis to foster capacity to make comparisons across population-based studies

extend clinical assessments beyond the core behavioral domains of an ASD diagnosis to evaluate potential dysfunction in other CNS domains (cognitive, sensory, fine and gross motor, affective, anxiety) and in extra-CNS organ systems and biochemical pathways (peripheral nervous system, gastrointestinal tract, immune system, metabolic and oxidative stress mechanisms) that are frequently but inconsistently affected in children with ASD

utilize multiple informants from preschool, educational, and other community settings

define the developmental trajectory of clinical and biological parameters in children with later ASD and non-ASD outcomes to help identify the brain regions, neural circuitry, and biochemical pathways that are must vulnerable to disruption and to facilitate discovery of early biomarkers and behavioral predictors that may prove useful for prompt recognition of children at risk for ASD

incorporate serial structural and functional brain imaging into study design

Leverage the large investment of prospective cohort studies and increase the probability of a high yield for obtaining unique ASD-related findings by examining clinical, brain imaging, and laboratory parameters that elucidate the function of a wide range of CNS and extra-CNS domains, and that are not restricted to a narrow set of causal hypotheses

Collaborate with investigators working on ongoing, population-based studies to examine the relationships of candidate exposures to outcomes

- assess age-specific prevalence of ASD as a function of estimated or documented exposures

- evaluate time trends in prevalence using age-period-cohort analytic approaches in an effort to isolate variance due to secular trends

Compare rates of ASD/NDD in populations with low and high levels of exposure to candidate environmental agents

- e.g., compare ASD/NDD risk in counties documented to have high levels of environmental contaminants (e.g., near superfund sites, coal burning plants) in air, soil, and water with risk in counties documented to be relatively free of environmental contaminants

build upon ecologic study designs by directly measuring exposures and rigorously diagnosing subjects in a subsample

Research need: *Specific investigations into candidate environmental exposures and their relationship to ASD/NDD in population-based studies*

Rationale: Studies in populations that differ in timing, dose, or route of exposure to a candidate exposure agent as well as in genetic factors will help to delineate the role of such influences on the risk for ASD/NDD. Well-designed and controlled studies that include vaccine-based mercury as one of many potential candidate exposures to be investigated would add science to the debate regarding the temporal association between vaccination and the development of autism reported by some parents, and the time-trend prevalence data that suggest changes in autism rates in parallel with changes in the amount of mercury present in required childhood vaccines.

Recommendations and enhancements:
Examine the influence on ASD risk of exposures that are both common, and commonly implicated, such as mercury

- determine cumulative mercury burden from pre- and/or postnatal exposure(s) to methyl- and ethylmercury

  include all potential sources – environmental, dietary, pharmaceutical

- use immunologic, genetic, genomic, and metabolic profiling to try to identify factors associated with ASD susceptibility in cases or, alternatively, ASD resistance in controls

Determine whether age-specific prevalence rates of ASD/NDD within relatively unvaccinated (or less vaccinated) populations differ from those currently reported for the general US population

- examine rates of ASD/NDD, including ASD, in populations likely to have larger numbers of unvaccinated members than in the general US population (i.e., Amish, Christian Scientist, chiropractic and other alternative health care consumers)

  specifically gather information regarding vaccination, especially the type and timing, rather than assuming the degree of exposure (e.g., Amish groups vary greatly in their acceptance of and access to vaccines and other modern health measures)

  strive to control for potential sources of bias, and exercise caution in interpreting results given the likelihood of multiple sources of population differences

- where feasible, compare age-, period- and cohort-specific rates of ASD/NDD in vaccinated and unvaccinated individuals within the same population

- where feasible, compare age-, period- and cohort-specific rates of ASD/NDD in children attending private clinics that utilized single dose vaccine formats (ideally verified to be free of thimerosal through use of lot numbers) with rates in children attending private or public clinics

Compare the age-specific prevalence rates of ASD/NDD before and after removal of thimerosal from infant vaccines in defined US subpopulations (i.e., Brick Township, NJ, or using the California developmental disabilities services database) or in countries with vaccination protocols different than those in the US, including those still using thimerosal-preserved products

Specific investigations into candidate environmental exposures, cont'd.

- acquire detailed information on immunization, including dates and lot numbers, to increase the accuracy of exposure classifications

  include all potential sources – environmental, dietary, pharmaceutical

  where individual level data are unavailable or incomplete, enhance the value of time-trend studies by determining the number of thimerosal-preserved and thimerosal-free pediatric vaccine doses sold each year and the shelf-life of each product, and derive estimates of per capita thimerosal exposures over time

- use rigorous procedures for diagnosis

- include measures with the sensitivity to detect subtle alterations in CNS function and neural development

Compare rates of ASD/NDD among the offspring of age-matched women exposed prenatally (or postnatally through breask milk) to thimerosal-preserved or thimerosal-free forms of Rho D immune globulin, influenza vaccine, both, or neither of these

- specifically gather information regarding the level of exposure of offspring to thimerosal through maternal Rho D immune globulin and/or influenza vaccine administration (lot numbers, other documentation)

  include all other potential sources of mercury – environmental, dietary, pharmaceutical (e.g., maternal influenza vaccination)

  Rh-negative women began to routinely receive Rho D immune globulin prophylactically during pregnancy in 1991, when most, but not all, immune globulin products contained thimerosal; due to extended shelf-life, some women may have received thimerosal-preserved products during pregnancy well into 2003.

- distinguish between risk due to gestational exposure to mercury vs. that due to immune globulin

  compare ASD outcomes in offspring of age-matched women who are or are not Rh-negative and who were exposed or not exposed gestationally to thimerosal (Rho D immune globulin, influenza vaccine, both, or neither)

- distinguish between risk due to gestational exposure to mercury and/or immune globulin and risk linked to Rh factor alleles, or to interaction of Rh alleles with exposure

  a link between Rh status and ASD risk is previously reported (Juul-Dam et al., 2001; Holmes et al., 2003) but a recent study reports no association of ASD risk with Rh-negative status or maternal-fetal incompatibility at the Rh locus (Zandi et al., 2006); however, little data exist on maternal gestational exposures to mercury through Rho D or other sources in conjunction with outcomes

  compare rates of ASD in offspring of Rh-negative women who received mercury-preserved agents with rates in those who received mercury-free versions

  incorporate controls for effects of gestational immune globulin alone – offspring of mothers with systemic lupus erythematosus and other autoimmune diseases have been reported to have increased rates of learning and other behavioral abnormalities, suggesting that gestational administration of immune globulins may serve on its own to disrupt brain development

Research need: *Specific investigations into candidate environmental exposures and their relationship to ASD/NDD in high-risk subgroups (siblings of children with ASD/NDD)*

Rationale: Autism is described as one of the most highly heritable neuropsychiatric disorders. An investigation into the rates of autism in "high risk" children following the diagnosis of autism in their sibling as a function of defined environmental exposures may assist in delineating the relative contributions of environmental factors and genes to autism pathogenesis. Due to parental concerns regarding a potential link between vaccination and autism, many families who believe their child's regression was related to vaccination have chosen not to vaccinate subsequent siblings; this has created a natural control population of children enriched for many of the same genetic influences as their sibs diagnosed with ASD as well as for many environmental factors other than vaccines.

Recommendations and enhancements:
Compare rates of ASD/NDD in "high risk" baby sibs of children with ASD/NDD as a function of their degree of exposure to mercury, including but not limited to mercury-based vaccines

- specifically gather information regarding vaccination, especially the type and timing (and lot numbers where possible), rather than assuming the degree of exposure

  include all potential sources of mercury – environmental, dietary, pharmaceutical

  use immunologic, genetic, genomic, and metabolic profiling to try to identify factors associated with ASD susceptibility in cases or, alternatively, ASD resistance in controls

- specifically assess levels of mercury and related toxicants in biologic samples to avoid possible confounding

  if sibs of ASD children have a genetic susceptibility to respond adversely to mercury, they are likely to be vulnerable not only to mercury present in vaccines but also to mercury from other sources and to other related or disparate toxicants, including PCBs and PBDEs (flame retardants present in computer keyboards); thus, removal of mercury from vaccines may not be sufficient to eliminate risk for sibs of ASD children

- evaluate exposed and unexposed "high risk" sibs for genetic polymorphisms that are thought to enhance vulnerability to environmental agents

Toxicologic research

Research need: *Studies of the developmental toxicity of exposures to environmental agents at low levels*

Rationale: Genetic and maturational determinants are likely to exert a critical influence on outcomes after low level exposures. Understanding the mechanisms by which low level exposures lead to CNS disturbance in vulnerable individuals – which may be different than the mechanisms triggered by high level exposures to the same agent – will provide the basis for identification of biomarkers and treatment strategies.

Recommendations and enhancements:
Establish animal models of exposure-related neurodevelopmental damage based on aberrant gene x environment x timing interactions

- dissect the mechanisms underlying gene x environment x timing interactions using animal models

  examine the effect of timing of exposure on outcome

  study alterations of key signaling pathways in brain development that are modulated by gene:environment interactions

- elucidate at the cellular level the effect of timing of exposures on progressive changes in brain development

  include investigations of changes in regional grey and white matter volumes, programmed cell death, pruning mechanisms and cellular metabolism

  conduct long-term follow-up studies to define the longitudinal trajectory of change in numbers and responses of specific resident cells of CNS as a function of exposure type, dose, and timing; co-exposure; and genetics

- develop mechanism-based biomarkers to facilitate assessment of exposures

  utilize the clues derived from these models to derive candidate biomarkers for diagnosis and identification of ASD subgroups that are most likely to benefit from specific treatment approaches

Develop and validate animal models and *in vitro* systems that model neurodevelopmental and neuroimmunological processes implicated in ASD/NDD

- test hypotheses regarding possible environmental influences in autism using these *in vivo* and *in vitro* models

- incorporate multiple, convergent approaches for assessment of outcomes into these investigations (e.g., biochemical, structural and functional imaging, behavioral, physiologic, genomic)

Studies of the developmental toxicity of exposures to environmental agents at low levels, cont'd.

Expand understanding of the differential neurotoxic effects of mercury compounds

- clarify differences across mercury compounds with respect to the timing of exposure, route of administration, specific mercury species administered, genetic susceptibility, neuroprotective mechanisms, and clearance and detoxification

- clarify effects of different mercury compounds after low dose exposures using mouse strains that are sensitive or not sensitive to low dose toxicity

  examine alterations in neuronal and glial subsets in the brains of mice from strains differing in sensitivity to mercury

- compare neurotoxicity and excretion rates of mercury in mice before and after genetic or nutritional depletion of glutathione, and compare to mice of the same strain with normal glutathione reserves; evaluate the degree of protection after restoring glutathione levels in depleted mice

  the intracellular tripeptide, glutathione, is the major mechanism for mercury detoxification (conjugation) and excretion; clinical evidence suggests glutathione stores may be low in autistic children

- evaluate effects on exposure-induced biochemical profiles of pre-treatment with substances that increase oxidative stress or deplete antioxidant reserves

Evaluate the neurotoxicity, immunotoxicity, and gastrointestinal toxicity of the standard infant vaccination protocol (including thimerosal) in non-human primates

- include an investigation of the integrity of the blood-brain barrier following exposure to thimerosal-preserved vaccines

  thimerosal is highly immunotoxic and may be a necessary prerequisite for vaccine-induced autoimmunity in sensitive individuals

  the blood-brain barrier may be adversely impacted during vaccination due to the presence of thimerosal

Undertake dose escalation studies with inorganic and organic mercury in developing non-human primates to evaluate impairments in attention and neural synchronization

- measure attention and cognition with behavioral tests and correlate with assessments using electrophysiologic techniques (e.g., MEG, ERP)

- address whether mercury or its organic derivatives produce impairments of behavior reminiscent of autism or alter electrophysiological parameters

  determine the nature of the relationship, if any, between the exposure level and the observed deficits (linear dose-response curve, threshold effect, or biphasic curve)

  compare the effects of mercury in the presence of induced oxidative stress (e.g., methionine and/or folate restriction)

Studies of the developmental toxicity of exposures to environmental agents at low levels, cont'd.

Conduct *in vitro*, dose-response studies to investigate the metabolic responses and toxicity of thimerosal in cultured, freshly-obtained lymphocytes (or in Epstein Barr Virus-transformed lymphoblastoid cell lines) obtained from children with autism and unrelated controls

Characterize the mechanisms underlying the observed differences in neurotoxicity in the strain-dependent mercury mouse model

- include studies of the distribution and accumulation of mercury in different cell populations, brain regions, and in extraneural organ systems (e.g., kidney, adrenal, gastrointestinal tract, immune organs)

  these studies would help to establish whether differences in levels of mercury accumulation/excretion contribute to or explain differences in toxicity in different mouse strains

  methods to alter accumulation/excretion might be examined for safety and efficacy in the mouse model if difference in distribution and/or accumulation are observed

- identify targets of immunoreactivity of autoantibodies reactive with brain through immunoprecipitation and mass spectroscopy

- clarify the genes associated with susceptibility in the mouse model through studies in transgenic mice

  identify the human homologues of the candidate genes associated with susceptibility in the mouse model and review existing human genetic data to determine whether there is evidence of previously reported linkage to ASD; if promising, design appropriate studies in humans to evaluate prevalence of genetic markers in ASD children with documented exposure histories

- challenge thimerosal-exposed susceptible mice with viruses or other infectious agents and evaluate disease severity, including induction of autoimmunity

  determination of infectious or immune sequelae in the mouse model will assist in clarifying the mechanisms that contribute to CNS damage

- study peripheral blood cytokine and chemokine responses

  determination of peripheral blood biomarkers will create a foundation for development of diagnostic biochemical profiles that may be more readily translated into humans

Investigate effects of pre- or postnatal neurotoxicant exposure in experimental animal studies, focusing on combined, low dose exposures to agents implicated in neurodevelopmental damage

- determine patterns of behavioral and neuropathological signs in these models and compare to those found in patients with ASD/NDD

- assess whether prior exposures to toxicants in early life lead to synergistic, potentiating, or cross-sensitizing effects upon repeat exposure to the same or different agents

Studies of the developmental toxicity of exposures to environmental agents at low levels, cont'd.

Establish more systematic connections among changes at the tissue, distributed processing, physiologic, and behavioral levels, using temporally-sensitive neuroimaging methods such as EEG, MEG, fMRI or perfusion/metabolism scans

- perform animal model and human studies in parallel

- define the brain-based parameters that are key correlates of dysfunction to build a platform for tracking the course of illness and evaluating changes as a result of intervention

  evaluate changes in these key parameters after instituting specific interventions and determine the correlation of these changes in brain-based parameters with standardized measurements of treatment efficacy, including improvement of behaviors in specific domains (expressive or pragmatic language, social interaction, repetitive behaviors, cognition, attention, fine or gross motor coordination or planning, sensory), or other clinical parameters (gastrointestinal, immune)

Develop and validate protocols for establishing cumulative mercury load in individual children with neurodevelopmental disorders as a means for determining the need for chelation

Design more effective, safer chelating agents and ensure safety of protocols to be implemented through rigorous work in animal models, including non-human primates

- address capacity of chelating agents to specifically target toxic heavy metals without disrupting balance of metals essential to cell signaling and enzyme function (e.g., zinc, calcium)

  assess the ability of the agent to cross the blood-brain barrier, chelate mercury from brain tissues, exit the brain, and promote the safe excretion of chelated mercury without damage to renal or gastrointestinal tissues

Conduct prioritized studies to examine environmentally-sensitive pathways in animal models of exposure and in children with ASD

- include methylation, methionine synthase, transsulfuration, oxidative stress, immune, neuroendocrine, among many others

Genetic, epigenetic, and genomic research

Research need: *Genetic, epigenetic, and genomic analyses based in prospective birth cohort studies of ASD and other NDD with data on exposures, timing of exposures, diagnostic classification, and endophenotypic expression, and in animal models*

Rationale: Comparing genetic, epigenetic and genomic findings across ASD subsets that differ in their endophenotypic expression may uncover potent associations that are otherwise obscured by the clinical heterogeneity of the population. In addition to clinical parameters, altered reactions of an individual to certain types of environmental exposures, as detected through analysis of their biological samples, may serve as a proxy for earlier or future adverse reactions to exposures and may be important as a tool for endophenotyping. This role in endophenotyping and the possible enhancement of the odds of detecting genetic

associations important in ASD pathogenesis is maintained even if the exposure under consideration is not the “true” pathogenic agent. Alternatively, if certain candidate gene polymorphisms only present a risk in the context of exposure to certain types of environmental agents, and that risk is further tempered by the requirement for the exposure to occur during a certain period in pre- or postnatal life, or in the presence of other genes or environmental exposures, then some of the inability to consistently replicate the association of candidate genes with ASD across populations may reflect insufficient parsing of the population into the appropriate endophenotypic subsets. Basing genetic analyses within a prospective birth cohort that provides such data may permit important associations to be elucidated that would otherwise go undetected.

Understanding the nature and timing of specific exposures among those at greatest risk is an essential step if we are to close the gap between environmental exposure and genetic susceptibility. Identifying genetic polymorphisms and other metabolic susceptibilities and differences present at birth would accelerate mounting prevention efforts. Early identification of those at risk may make it feasible to intervene early and/or to eliminate exposure to agents that may serve as toxins during critical periods of vulnerability–following the success of programs that identify phenylketonuria (PKU) at birth and determine which children require a phenylalanine-free diet to avert cognitive impairment.

Recommendations and enhancements:
Identify and study candidate genes that modulate response to specific environmental toxicants

- acquire detailed information on candidate exposures to permit studies of gene:exposure relationships

Review linkage studies to determine whether candidate genes frequently associated with ASD are implicated in host responses to toxins

Study effect of different gene x exposure x timing interactions in animal models

Explore whether birth years of samples in human genetic studies noting associations or lack of associations with candidate environmentally-sensitive genes suggest a secular trend

Develop specific strategies to test hypotheses of gene:environment and gene:infection interactions

Clinical research

Research need: *Rigorous investigations into the predictive value of biomarkers and endophenotypic characteristics in ASD/NDD for the identification of causal pathways and the selection of the safest, most effective treatment options*

Rationale: Heterogeneity in the etiologic factors linked to ASD, and their diverse phenotypic expression, have hindered the search for biomarkers and effective treatments. Changes outside the CNS – indeed any aberrations outside of the core domains of dysfunction described for autistic disorder and other ASD in DSM-IV-TR – are rarely documented, let alone queried in a standardized fashion. Increased attention to subtyping in ASD/NDD may increase the power of genetic, genomic, biochemical and immune analyses, and may also improve treatment outcomes by identifying children most likely to benefit from selected interventions.

Recommendations and enhancements:
Develop reliable biomarkers for identification of children at risk for development of an ASD/NDD

- as causal contributors to ASD/NDD are identified, implement strategies for prevention where feasible

Determine the most effective pathways in which to target interventions, and the best timing for institution of treatment
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Develop *in vivo* biomarkers to screen individuals for the occurrence of exposure, determine the level of exposure, and track response to treatment

Develop better methods for identifying individuals in need of treatment, along with safer and more effective treatment protocols for toxicant exposures

Develop animal models of exposure and vulnerability for the purpose of testing treatment strategies

Utilize strategies to identify whether common substances may act as environmental triggers in children with autism

- enhance the possibility of identifying substances that may trigger an immune response in individuals with ASD by employing an “Environmental Medical Unit” (EMU) approach

  the EMU – a “clean room” for evaluation and testing of individual sensitivities to environmental and dietary exposures – is employed after an individual’s exposures have been brought to a baseline level and permits controlled evaluation of the capacity of individually- and serially-introduced substances to trigger allergic or other immune responses, or behavioral deterioration of unknown cause

Rigorous investigations into the predictive value of biomarkers, cont'd.

Identify biomarkers using samples from a multi-site, prospective study of toddlers diagnosed with speech and developmental delays

- predictive biomarkers of autism may serve as an adjunct to behaviorally-based diagnoses and will improve our understanding of the contribution of altered biochemical processes and patterns of DNA methylation (epigenetics) to the pathogenesis of ASD/NDD

  candidate markers currently include immunologic factors (e.g., autoantibodies, cytokines, NK activity, T cell subsets), markers of oxidative stress and detoxification capacity (e.g., oxidized and reduced levels of glutathione, thioredoxin, methionine, cysteine, selenium), mitochondrial parameters (e.g., pyruvate, lactate, carnitine, creatine), and neuromodulator levels (e.g., serotonin, GABA, dopamine, glutamate, melatonin)

  compare premorbid profiles of children who subsequently develop autism with those of children who later appear normal to yield insights into potential candidate genes and causal environmental factors

  attempt to correlate biochemical endophenotypes with relevant candidate SNPs known to affect the identified biochemical pathways

Profile cerebrospinal fluid in children with autism, evaluating whether cytokines, chemokines, lipid peroxidation, and other measures correlate with neurological and behavioral characteristics of autism

Evaluate kidney function and gut flora in children with autism compared to age-matched controls

Compare banked tissue samples from well-characterized children with autism (lymphocytes, GI tissue, others) with samples from similarly well-characterized controls using microarray approaches

Evaluate children who develop autism for immunologic, genetic, and metabolic evidence of impaired detoxification capacity (relevant SNPs and metabolic biomarkers)

- compare the allelic frequency of common polymorphic variants in key metabolic pathways in children with autism and in unaffected controls, and determine whether these are correlated with the observed metabolic phenotype

  determination of a genetic basis for observed metabolic phenotypes may reflect genetic vulnerability to oxidative stress induced by toxicants and assist in identifying groups of children at highest risk of exposure-related injury

  current candidate pathways include those involving precursors of glutathione synthesis and regulators of responses to oxidative stress

  genetic evidence suggests that autism involves vulnerability genes; metabolic evidence suggests that some children with autism may have lower plasma cysteine and glutathione levels; identification of genetic risk factors in the context of abnormal expression at the genomic or protein level will assist in establishment of targeted interventions to correct identified abnormalities and of biomarkers to facilitate selection of treatments with the highest probability of helping individual children

Research need: *Rigorous investigations into treatment approaches with documentation of pre-treatment and post-treatment changes in behavioral, biochemical, physiologic, and/or brain parameters as well as in extra-CNS features*

Rationale: Reducing heterogeneity in ASD study populations undergoing various treatments by deriving subgroups that share phenotypic or genotypic characteristics will lead to better comparability across studies of the same treatment and improve our understanding of which treatments are most effective and safe for different subgroups. Caareful attention to standardized documentation of CNS and extra-CNS (e.g, GI, immune) signs in ASD populations undergoing treatment will enhance the value of clinical trials and lead to clues about etiologic factors and mechanisms involved in ASD pathogenesis.

Recommendations and enhancements:
Document status of ASD individuals prior to initiating treatments, and carefully investigate and document successful outcomes

- enhance the scientific value and improve comparability of treatment studies by employing standardized instruments for diagnosis, assessment of overall improvement in core signs of ASD, as well as for ratings of specific gains that may be restricted to a single domain (including both CNS and extra-CNS domains such as GI, immune)

  gains in extra-CNS domains may lead to improvements in overall function; e.g., interventions targeting GI function may lead to general gains in core ASD features due to reduced pain and increased capacity to attend to ongoing behavioral interventions

  strive to follow good principles of experimental study design, by mapping outcomes after changing only one aspect of treatment where feasible; when multiple aspects of a comprehensive treatment program are introduced at the same time, document timing of introduction as well as timing of the discontinuation of any components that may be necessary

As underlying mechanisms (e.g., oxidative stress, infection, inflammation) and their role in specific individuals are identified, develop leveraged treatment algorithms focused on treating underlying mechanisms and not just the observable symptoms

Initiate controlled intervention trials to document the safety and validity of alternative biomedical treatments for autism

- efforts should be made to identify children most likely to improve with selected interventions by establishing parameters of immune, antioxidant, or metabolic disturbance that are predictive of improvement with intervention

  carefully document deficiencies prior to initiating treatment, and record additional treatments - behavioral and/or biochemical – that may be ongoing or newly implemented

  assess the capacity of interventions to restore immunologic and detoxification capacity in children with ASD, as well as their potential effect on behaviors such as speech, social interaction, and cognitive function

  the experience of physicians in private practice that currently treat large numbers of ASD children suggests that double-blind, placebo-controlled interventions using the following types of agents would be among the most promising: a) methylB12, folinic acid, NAC, zinc and selenium supplements to support glutathione, antioxidant, and detoxification function in lymphocytes, CNS, and gut; b) essential fatty acid supplements, Co-Q10, and carnitine supplements to

support mitochondrial function; c) carefully controlled trials of mild chelation therapy for environmental heavy metal exposures, including transdermal and oral routes (DMSA, DMPS)

Controlled, blinded clinical trials should be undertaken to assess the effectiveness of agents that address relevant pathways

- include agents already being employed in the treatment of autism: 1. Heavy metal chelation. 2. MethyB12. 3. Folinic Acid. 4. Trimethylglycine. 4. Omega-3 fatty acids. 5. PPAR agonists. 6. Nemantine. 7. Valcylovir. 8. Aripiprazole.

  In addition to standard endpoints on autism rating scales (e.g., ADOS), blood metabolite and MEG changes should be assessed and compared with in vitro and in vivo studies described above

Evaluate predictors of improvement as well as the safety and efficacy of immune modulation therapies as a treatment of autism

Conduct placebo-controlled double blind studies of nutritional interventions that have been reported to improve immunological, gastrointestinal function, cognitive function, and speech in autistic children

- a multidisciplinary approach should be employed for the appropriate assessments and endpoint comparisons

  include monitoring by longitudinal EEG coherence and MRI studies to track response of brain function with treatment

  This line of investigation would help to ascertain if targeted nutritional interventions result in documented and measurable improvement

Systematically investigate the possibility of using both natural and pharmacological means of inhibiting microglial activation in children, given reports of chronic microglial activation (Vargas *et al*., 2005)

- Investigate proximate triggers of microglial activation (toxic, viral, and immune)

  Vargas et al. (2005) observed very high levels of IFN-gamma, compatible with an infectious disease state

Employ a data mining approach to gather information about clinical experience of physicians treating children with autism who have gathered a significant amount of laboratory information on these children

- Areas that clinicians have identified that deserve additional investigation and documentation include: serum transaminases, mitochondrial parameters, purine metabolism, oxidative stress markers, impaired detoxification pathways, immune function / markers, kidney function, gut flora

Section 2C. Participant recommendations for an environmental research agenda for ASD: specific strategies to address research gaps

Infrastructure needs

Develop a tissue repository and database of ASD cases to support comparative studies with animal models and findings from clinical research

- Examination of tissues from this tissue bank would also generate new hypotheses, leading to the development and refinement of additional animal models

Establish an international registry of ASD, including a biological sample repository sufficient to test hypotheses relating to both genetic susceptibility and exposures to acquired risk factors (dietary, environmental, drug, microbial, others)

- sample repository should include a range of sample types to ensure capability of addressing multiple causal hypotheses

  develop and distribute guidelines to ensure appropriate sample collection methods and encourage inclusion of standardized clinical information to leverage value of samples for a wide range of research pursuits

  include sample types suitable for analysis of RNA as well as DNA and protein to support research that will enhance our understanding of the relationship of genetic to genomic and proteomic mechanisms, as well as epigenetic processes

Strengthen the leadership, scientific direction, and resources of existing autism tissue banks, and establish additional tissue banks

- under the guidance of a scientific advisory board, efforts should be directed toward enhancing the available clinical data, and coordinating access of researchers to the most appropriate tissues

- Intensify efforts to obtain brain specimens from autism patients of different ages to facilitate detailed histopathologic studies

  comparison of histopathologic findings across the developmental trajectory will assist efforts to reconstruct the sequence of cellular/molecular events that culminate in the derangements of cerebral cytoarchitecture that are frequently reported in ASD, primarily in the limbic system and cerebellum

- record and link an increased range of clinical data to autopsy material to facilitate the capacity to understand the relationship of structural brain changes to functional disturbances

- facilitate analysis of secular trends in the neuropathologic findings of ASD by including actual month and year of birth in addition to age at time of death

Model systems needed for research into mechanisms

Develop and refine animal and tissue culture models required for research into mechanisms

Carefully define the expected changes in brain tissues after exposures to toxins such as mercury, using postmortem studies and animal models

- utilize metabolic neuroimaging to capture the effects of metabolites, alterations in blood-brain barrier integrity, activation and proliferation of microglia, and changes in perfusion

  Based upon the known role of microglial cells during CNS injury, and recent findings of chronic microglial activation (Vargas et al., 2005), investigate the role of microglial cells during neural development in vivo and in vitro to establish a foundation for studies of exposure-induced CNS toxicity

- focus on their potential roles in angiogenesis, axonal guidance and neurogenesis

Database design and development

Develop autism-related databases that include data reflecting exposure, pathophysiologic and biomarker findings, clinical signs, and treatment responses

Integrate clinical data with that of basic research in the database design

Organize and support data mining by the existing physician community involved in the treatment of children with ASD, utilizing interactive, web-based interfaces to access existing databases

Support the development of databases that provide detailed documentation of diagnostic and treatment algorithms being employed by clinicians in the community

Prospectively track treatment safety and efficacy with an interactive interface

Clinical trials

Support and encourage double-blind, placebo-controlled and crossover studies of current peer-supported clinical interventions

Develop methods for operationalizing and evaluating complex, individually-tailored interventions

Reference ranges and endpoints

Generate meaningful age-specific reference ranges for exposures and secondary metabolic consequences

Reevaluate existing clinical reference ranges to increase sensitivity to metabolic consequences of exposures

Develop biomarkers panels with age-specific reference ranges that are:

- sensitive to metabolic and cellular impacts of low level and combined environmental exposures

- predictive of subgroups and differential treatment response

Education and training

Fund and support training fellowships for clinical and basic researchers

Develop curriculum for CME credits for community physicians treating children with ASD, focusing on research into the biologic bases of the disorder; standardized methods for establishing diagnosis and important differential diagnosis; value, interpretation, and limitations of laboratory assays and diagnostic imaging; treatment options and rational treatment selection process; standardized methods for establishing diagnosis evaluating clinical status and ongoing management of multifaceted treatment programs.

Section 3. Building a road map for environmental research in autism

The current autism research matrix includes the following content areas: communication and collaboration, characterization of autism, school and community interventions, early interventions, epidemiological studies, specific treatments, neuroscience, screening, and the role of the environment. In this last content area, environmental factors, research needs are particularly large. Recommendations are made herein with respect to research priorities, based on the consensus derived through this symposium of participants with expertise in toxicology, environmental health science, neuroscience, and clinical science.

Based on the translational medicine model that Dr. Olden introduced at the symposium, we propose that research proposals be evaluated according to the following four principles:

1. Preference for treatment concepts and modalities that have substantial support from clinicians and parent groups, notably, approaches that recognize the diversity of clinical profiles in ASD/NDD populations and may be individualized

   Support for various treatment strategies is expected to come from service outcome research as well as from individualized, evidence-based medicine

   Additional support for novel treatment avenues is anticipated to be drawn largely from case studies or series that rigorously document improvement and/or recovery from an objectively recorded baseline; the degree of improvement may vary for subsets of ASD/NDD with different clinical characteristics, as well as within different CNS and extra-CNS (e.g., GI, immune) functional domains

2. Preference for investigations with a focus on multifaceted treatment modalities (as opposed to single molecule clinical trials) that address the often complex, frequently systemic disturbances of ASD/NDD individuals, including dietary strategies, methods for limiting oxidative stress, immune system support, anti-inflammatory regimens, and detoxification protocols

   Complementary treatment strategies have drawn the largest base of "bedside" support, but deserve special attention as they pose practical challenges for implementation within a paradigm of evidence-based medicine

3. Preference for research into environmental hypotheses that have been built from a foundation of clinical science, and extended and refined through basic research pursuits

   Clinical and basic research should continue to identify factors that may influence risk for adverse events after environmental exposures and delineate the mechanisms by which neurodevelopmental damage may occur

4. Preference for basic research that effectively tests hypotheses consistent with "final common pathway" models of neurodevelopmental disturbance

   Bench-to-bed research will enable insights about potential new treatments to arise more rapidly when the design of such studies is built upon a clearly-defined but broadly-conceived model of neurodevelopmental damage

Together, the application of these principles should focus efforts across disciplines, facilitating convergence toward a more comprehensive understanding of the disease process and promote identification of strategic treatment targets.

A recommendation was made for designation of a special study section that would assist in implementation of these suggested additions to the current autism research matrix. The resolution to complex disorders of gene-environment-timing interactions such as ASD necessitates coordinated, interdisciplinary approaches. The expertise across the range of approaches required is spread widely across different institutions; thus, a program project-like grant mechanism that encouraged applications from groups of principal investigators, each with their own group of co-investigators, with thematic linkage across these different interactive modules may be required. Such interdisciplinary applications would pose special demands on scientific review that would ideally be met by the proposed special study section. In addition, it is recommended that all research initiatives be conducted by independent investigators and institutions without perceived or documented conflicts of interest or influence from the pharmaceutical industry or regulatory agencies (i.e., CDC, FDA). Members from the autism community should be invited to actively participate in the design and implementation of the investigations, thus increasing acceptance of the findings when investigations are complete.

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APPENDIX

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safeMinds
Sensible Action for Ending Mercury-Induced Neurological Disorders

NATIONAL AUTISM ASSOCIATION
Think Autism. Think Cure.

ENVIRONMENTAL FACTORS in NEURODEVELOPMENTAL DISORDERS
AN NIEHS-SPONSORED SYMPOSIUM

August 25 and 26, 2005
Hyatt Regency, Bethesda, Maryland

DAY ONE: Continental Breakfast 7:30 to 8 am

8:00 **INTRODUCTION:**
►Ken Olden (Introduction and charge to participants)
►Jeff Segal (Moderator)

8:15 **APPRAISAL OF EPIDEMIOLOGIC STUDIES ON THE ROLE OF ENVIRONMENTAL FACTORS IN AUTISM**
What findings are most robust? What are the evidentiary gaps?
►Mark Blaxill (Time trends)
►Ray Palmer (GIS studies)

9:00 **GENERAL PROPERTIES OF TOXINS AND THEIR RELEVANCE TO MECHANISMS OF DEVELOPMENTAL NEUROTOXICITY**
What aspects of toxins are relevant to the development of neurotoxicity? What pathways may lead to neurodevelopmental damage after exposures to toxins?
►Ellen Silbergeld (Mechanisms)
►Tom Burbacher (Non human primates)
►Anna Choi (humans)

9:45 **MORNING BREAK**

10:00 **STRUCTURAL AND CELLULAR RESPONSES TO TOXINS IN THE CNS:**
How do neurons, microglia, astrocytes and oligodendrocytes respond to toxic exposures?
►Wolfgang Streit (Microglial activation)
►Jay Charleston (General CNS pathology)
►David Baskin (Apoptosis)

11:15 **INTERACTIONS OF GENES, ENVIRONMENT, AND TIMING IN NEURODEVELOPMENTAL DISORDERS**
What genes exacerbate CNS damage after exposure to environmental agents? How might maturation influence gene expression?
►Mady Hornig (Animal models)
►Ian Lipkin (Cohort investigations)
►Martha Herbert (Structural brain imaging)

12:00 **WORKING BAG-LUNCH PANEL DISCUSSION WITH MORNING PRESENTERS**

1:00 METABOLIC PATHWAYS INFLUENCING HOST RESPONSES TO TOXINS:
What are the critical mechanisms involved in the response to environmental neurotoxicants? Is there evidence to suggest that some of these same mechanisms are involved in the pathogenesis of autism and related neurodevelopmental disorders?
►Boyd Haley (Heavy metals)
►Richard Deth (Methylation)
►Jill James (Antioxidant/transsulfuration, methylation)

2:00 CLINICAL FINDINGS IN AUTISM SPECTRUM DISORDERS
What clinical features are most frequently found to be disturbed in children with autism? What biologic parameters and pathways are disrupted? Are specific clinical disturbances associated with specific biologic ones (possibly defining a tractable endophenotype)? Are these patterns of abnormality consistent with those that would be predicted if a neuropsychiatric condition were environmentally-induced?
►Diane Vargas (Post-mortem brain analysis)
►Jeff Bradstreet (Heavy metals)
►Elizabeth Mumper (Case studies)

4:00 PANEL-DISCUSSION/QUESTIONS AND ANSWERS

DAY TWO: Continental Breakfast 7:30 to 8:15 am

8:00 RESEARCH APPROACHES IN DEVELOPMENTAL NEUROTOXICOLOGY
►Cindy Lawler (Current programs for funding and funded investigations)

8:30 CREATING A ROADMAP FOR INVESTIGATIONS INTO ENVIRONMENTAL FACTORS AND NEURODEVELOPMENTAL DISORDERS – BRAINSTORMING SESSION
►Ken Olden *With what conceptual framework may we approach the current data and prioritize future research goals?*

11:30 CONCLUSIONS AND NEXT STEPS
Open discussion with NIH Directors, participants and observers regarding strategies for implementation

12:30 ADJOURN

Sponsored by The Coalition for Safe Minds and the National Autism Association
through a generous contribution from NIEHS

II. Participant names and affiliations

David Baskin, MD, Texas Medical Center (presenter)
Mark Blaxill, SafeMinds (presenter)
Jeff Bradstreet, MD, International Child Development Research Center (presenter)
Thomas Burbacher, PhD, University of Washington (presenter)
Jay Charleston, PhD, StereoTomeNW (presenter)
Anna Choi, DSc, Harvard School of Public Health (presenter)
Richard Deth, PhD, Northeastern University (presenter)
Boyd Haley, PhD, University of Kentucky (presenter)
Martha Herbert, PhD, MD, Harvard Medical School (presenter)
Mady Hornig, MD, MA, Mailman School of Public Health, Columbia University (presenter)
S. Jill James, PhD, Arkansas Children's Hospital (presenter)
Elizabeth Mumper, MD, Advocates for Children (presenter)
Kenneth Olden, Director Emeritus, NIEHS (symposium moderator)
Ray Palmer, PhD, University of Texas Health Science Center San Antonio (presenter)
Jeff Segal, MD, Medical Justice Corporation (session moderator)
Ellen Silbergeld, PhD, John Hopkins School of Public Health (presenter)
Wolfgang Streit, PhD, McKnight Brain Institute at the University of Florida (presenter)
Diana Vargas, MD, Johns Hopkins School of Medicine (presenter)

Representatives of DHHS and NIH:

Cindy Lawler, PhD, Scientific Program Director, NIEHS
David Schwartz, MD, Director, NIEHS

Molly Oliveri, PhD, NIMH
Sue Swedo, MD, Senior Investigator, Pediatrics and Developmental Neuropsychiatry Branch, National Institute of Mental Health (NIMH)
Audrey Thurm, PhD, NIMH
Ann Wagner,PhD, Autism Interventions Research Program, NIMH

Deborah Hirtz, National Institute of Neurological Disorders and Stroke (NINDS)

William Raub, PhD, Deputy Assistant Secretary for Science Policy, Department of Health and Human Services (DHHS)

Representatives of the offices of:

Senator Joseph Lieberman
Senator Deborah Stabenow
Senator Michael Enzi
Senator John McCain
Congressmen Dan Burton
Congressman Dave Weldon
Congressman Chris Smith

Autism groups in attendance:

The Coalition for SafeMinds
The National Autism Association
Cure Autism Now
Autism Research Institute
Autism Speaks
Autism Society of America
Unlocking Autism
Autism One
A-Champ
Generation Rescue
Dads Against Mercury