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Immunologic and neurodevelopmental susceptibilities of autism

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ABSTRACT

Symposium 5 focused on research approaches that are aimed at understanding common patterns of immunological and neurological dysfunction contributing to neurodevelopmental disorders such as autism and ADHD. The session focused on genetic, epigenetic, and environmental factors that might act in concert to influence autism risk, severity and co-morbidities, and immunological and neurobiological targets as etiologic contributors. The immune system of children at risk of autism may be therefore especially susceptible to psychological stressors, exposure to chemical triggers, and infectious agents. Identifying early biomarkers of risk provides tangible approaches toward designing studies in animals and humans that yield a better understanding of environmental risk factors, and can help identify rational intervention strategies to mitigate these risks.

1. How can chemical exposure contribute to autism risk? I.N. Pessah, UC, Davis, CA, USA

Autism is a heterogeneous neurodevelopmental disorder defined by core deficits in social reciprocity, communication, and restrictive/repetitive patterns of interest and behavior (American Psychiatric Assoc, 2000). Although autism may be one of the most heritable complex disorders, the genes linked to autism risk do not segregate in a simple Mendelian manner (Folstein and Rosen-Sheidley, 2001; Trikalinos et al., 2005). There is increasing scientific interest in identifying complex interactions among multiple genes that contribute to autism risk, and non-heritable environmental exposures may significantly influence susceptibility and variable expression of autism and autism-related traits. A major challenge in the field is to identify environmental factors of relevance to autism. From a toxicologist's perspective, the identity of "defective" genes and signaling pathways linked to autism provide important clues about exposures to environmental chemicals that influence autism susceptibility, severity, and/or treatment outcomes. One fundamental way by which heritable genetic vulnerabilities can amplify the adverse effects triggered by environmental exposures is if both factors (genes × environment) converge to dysregulate the same neurotransmitter and/or signaling systems at critical times of development. Current research is being directed at defining distinct neurological endophenotypes in autism that may possess overlapping sets of susceptibility genes (Muller, 2007). It is therefore reasonable to expect that as subgroups of autistic children are better defined based on common biological abnormalities, distinct sensitivity to environmental modifiers of autism risk, severity and/or treatment outcomes will be identified. Current efforts to identify clinical endophenotypes within the autism spectrum are therefore likely to help our understanding of the constellations of genes that confer differential sensitivity to distinct environmental exposures during gestational and neonatal development. Such approaches will likely prove useful in defining subgroups of children that differ in susceptibility to environmental exposures that promote autism risk and severity.

Understanding how low-level chemical exposure influences molecular, cellular and behavioral outcomes relevant to autism

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will better inform geneticists, neuroscientists and immunologists about autism's complex etiologies. Recent genetic findings coupled with emerging histological, electrophysiological and functional imaging studies suggest that autism results from an imbalance in the ratio of excitatory and inhibitory neurons within the developing brain (Rubenstein and Merzenich, 2003) and the neural networks they form (Belmonte and Bourgeron, 2006). Such imbalances are likely to result in the failure of connections to form normally between brain regions involved in higher-order associations (Geschwind and Levitt, 2007). The inherent imbalances in neuronal connectivity present in children at risk for autism are likely to provide the biological substrate for enhanced susceptibility to environmental triggers that are known to target signaling systems that establish the basic patterns of connectivity, from early neuronal migration and axonal pathfinding to disruption of later postnatal events that function to refine neuronal connections, such as dendritic growth, synapse formation and pruning (Belmonte and Bourgeron, 2006; DiCicco-Bloom et al., 2006; Geschwind and Levitt, 2007). The toxicological literature includes ample evidence that many of the environmental chemicals of concern to human health either directly or indirectly affect the signaling systems that are impaired in autism. For example, several pesticides of historical and current importance are in fact known to interfere with acetylcholine (Ach) and γ-aminobutyric acid (GABA) neurotransmission, which are neurotransmitter systems altered in subsets of autistic individuals. Another example is the possible role of persistent organic pollutants that directly alter Ca^{2+} signaling pathways and Ca^{2+}-dependent effectors and are thus likely to impact a broad range of transmitter systems, including cholinergic, GABAergic, and dopaminergic.

1.1. Chemicals that interfere with cholinergic neurotransmission

Organophosphates (OPs) are potent inhibitors of the enzyme acetylcholinesterase (AChE) that is responsible for rapid hydrolysis of acetylcholine at nicotinic and muscarinic synapses. Inhibition of AChE by OPs has been amply documented as causing a plethora of toxicological effects that result from initially overstimulating, and consequently desensitizing, cholinergic transmission. More recent research suggests that some OPs cause developmental neurotoxicity by mechanisms independent of AChE inhibition (Jameson et al., 2007; Pope, 1999; Ricceri et al., 2006; Schuh et al., 2002; Slotkin et al., 2007a,b). For example, the OP chlorpyrifos has been shown to enhance the phosphorylation of the Ca^{2+}/cAMP response element binding protein CREB in neuronal cultures at concentrations well below those that inhibit AChE (Schuh et al., 2002). Tight regulation of CREB activity by Ca^{2+}-dependent phosphorylation is critical for normal neural progenitor proliferation and differentiation, dendritic development and cognitive function (Peltier et al., 2007; Redmond and Ghosh, 2005). CREB activity in turn regulates the expression of many nerve-specific genes including NF-l, TH, chromogranin, pEJ, and a number of immediate early genes that encode transcription factors (e.g., c-Jun). Slotkin and co-workers recently showed that neonatal exposure of rats to two widely used OPs, chlorpyrifos and diazinon, produced marked changes in the pattern of expression of specific isoforms of fibroblast growth factor genes (*fgf*) and their receptor (*fgfr1*) at doses below those that cause systemic toxicity or growth impairment resulting from AChE inhibition (Slotkin et al., 2007a,b). Although the molecular mechanisms mediating these developmental effects are unknown, they may have a common origin since the nuclear accumulation of FGFR1 protein is activated by changes in cell contacts and by stimulation of cells with growth factors, neurotransmitters and hormones. Stachowiak and co-workers proposed that FGFR1 represents a key convergence point for integrating signaling cascades initiated by specific membrane receptors that transmit signals to sequence specific transcription factors through co-activation of CREB (Stachowiak et al., 2003). Investigation into the adverse toxicological consequences of OPs via AChE-dependent and independent mechanisms and how they can be synergized by inborn errors in Ca^{2+} signaling such as Timothy syndrome (see below), which has a 60% autism rate, are needed to permit more meaningful risk assessments of susceptible populations.

Neurotransmission at cholinergic synapses is essential for functional aspects of cognition, reward, motor activity and analgesia. In addition, dysregulation of cholinergic neurotransmission has been associated with several pathological conditions including autism. Regional differences in the expression of specific nAChR subunits have been documented in postmortem adult brains obtained from autistic and non-autistic individuals. A decrease in the density of [^{3}H]epibatidine binding sites within the parietal cortex, cerebellum and thalamus of autistic brains was correlated to decreases in the levels of α3, α4 and β2 subunits by Western blotting (Lee et al., 2002; Martin-Ruiz et al., 2004; Perry et al., 2001). In the cerebellum, the [^{3}H]epibatidine binding density to α3 and α4 subunits was reduced by 40–50% in the granule cell, Purkinje and molecular layers in the autistic group compared with the non-autistic group. Cerebella from autistic patients possessed approximately 3-fold higher binding of [^{125}I]α-bungarotoxin to the α7 subunit in the granule cell layer compared to the general population and developmental delay comparison groups (Perry et al., 2001). How these differences in nAChR expression arise is unclear. However, these findings raise important questions as to whether deficits in nAChR neurotransmission identified in at least a subset of autistic children might confer a particularly high sensitivity to the adverse effects of OPs and/or neonicotinoid pesticide exposures. Allelic variants of the gene that encodes for paraoxonase (PON), a metabolic enzyme that is critical to the detoxification of OPs, have been associated with autism in North America and these variants appear to have lower metabolic activity *in vitro* (D'Amelio et al., 2005). Lower PON activity was also recently associated with autism in a Romanian study (Pasca et al., 2006). Thus, reduced metabolic capacity to detoxify and excrete OPs, in conjunction with deficits in nAChR neurotransmission could provide a framework for future studies of gene × environment interactions relevant to autism risk. Environmental epidemiological studies of possible associations between pesticide exposures and the major triad of deficits seen in autism (social reciprocity, language, and stereotyped behavior) have only begun to receive attention (Hertz-Picciotto et al., 2006). Eskenazi and co-workers recently reported evidence of a positive association between levels of dialkylphosphate metabolites (biomarkers of OP exposure) in maternal and child urine with risk of pervasive developmental disorder at 24 months of age in a longitudinal birth cohort of primarily Latino farm worker families in California (Eskenazi et al., 2007). Although these new associations between OP exposure and pervasive developmental disorders should not be interpreted as evidence of causation, clearly more research is needed to understand how long-term low level exposure to OPs, neonicotinoids and other chemicals that interfere with cholinergic signaling influence adverse developmental outcomes, especially in children in whom these signaling systems are genetically impaired.

1.2. Chemicals that interfere with GABA neurotransmission

Several classes of pesticides are known to interfere with GABA-mediated neurotransmission because they bind to the type A family of GABA receptors (GABR) and block their ability to mediate chloride fluxes. Along with nAChRs discussed above, GABR channels are members of the ligand-gated ion channel superfamily

of genes that code for proteins having high sequence homology, but divergent spatiotemporal expression patterns and functions. In immature neurons, GABR primarily mediate excitatory responses, but during maturation and in mature neurons they mediate rapid inhibition of transmission (Michels and Moss, 2007). This "switch" in function is the result of a reversal in direction of the chloride gradient across the neuronal membrane with the maturation-dependent expression of the potassium–chloride co-transporter KCC2 that lowers the internal Cl^- concentration below that found in the extracellular fluid (Lu et al., 1999). The physiological relevance of these different functional GABR responses during neuronal maturation remains unclear. What is clear is that altered expression and function of these chloride channels have been implicated in autism and associated co-morbidities including sleep disturbances, anxiety, and epilepsy.

Organochlorine (OC) insecticides were widely used for both large-scale agriculture and domestic pest control beginning in the early 1940s. The vast majority of OC insecticides were banned from use in the United States beginning in the late 1970s due to their persistence in the environment, adverse effects on avian reproduction, and their ability to promote hepatic tumors in rodent studies. OC insecticides that possess polychloroalkane structures are known to bind to GABR in the mammalian brain and potently block their ability to conduct Cl^-, with many having nanomolar affinity for their receptor binding site (Cole and Casida, 1986; Lawrence and Casida, 1984). In the United States, toxaphene represents one of the most heavily used pesticides and was banned in 1990, whereas heptachlor and dieldrin were banned in the late 1980s. Examples of polychloroalkane insecticides that are currently being used in the United States include endosulfan and lindane. Dicofol, an OC structure similar to DDT, is also currently registered for use on agricultural crops. Because of their chemical stability, global distribution from countries that continue to use these compounds, and their propensity to bioaccumulate, exposures to OC insecticides continue to be a significant concern to human health worldwide. Yet relatively little is known about their developmental neurotoxicity, and the long-term consequences of low-dose exposures have not been adequately evaluated (Slotkin et al., 2007a,b).

Another class of insecticide that interferes with GABA neurotransmission and has attained broad domestic and commercial use is the 4-alkyl-1-phenylpyrazoles. Although these compounds do not persist in the environment to the extent observed with OCs, they are heavily used within the home and for commercial pest control. For example fipronil is formulated as a topical for control of fleas and ticks on pets. Fipronil's insecticidal activity is mediated primarily through its actions as a non-competitive antagonist of GABR. In this regard, fipronil was initially developed as an insecticide because of its higher selectivity for insect forms of GABR relative to mammalian GABR (Bloomquist, 2003). However results from recent studies indicate that fipronil, like endosulfan and lindane, is indeed a high affinity noncompetitive antagonist for the mammalian β_3-homopentameric GABR ($GABR\beta_3$) (Chen et al., 2006; Sammelson et al., 2004). In fact, the $GABR\beta_3$ recognizes fipronil with nanomolar affinity in a manner indistinguishable from its interaction with insect GABA receptors. These new findings raise questions about the high degree of selectivity once attributed to 4-alkyl-1-phenylpyrazoles. More importantly, the finding that a diverse group of widely used insecticides converge on a common molecular target within mammalian $GABR\beta_3$ has important implications for human risk, especially in those individuals with heritable impairments in $GABA_AR$ signaling pathways.

The hypothesis of an imbalance between excitation and inhibition within developing CNS circuits as a common etiological factor contributing to autism susceptibility is supported by several reports of GABR abnormalities in autism. Of particular relevance to the environmental exposures to OC insecticides and 4-alkyl-1-phenylpyrazoles discussed above, is the significantly lower level of $GABR\beta_3$ expression measured in postmortem brain samples obtained from children diagnosed with autism, Rett syndrome and Angelman syndrome that may stem from abnormal regulation of DNA methylation by the methyl CpG binding protein MeCP2 (reviewed below by Dr. LaSalle). Autism has been linked to polymorphisms within additional genes that encode GABR, including $GABR\gamma_1$ located on 15q11-13 (Ashley-Koch et al., 2006; Vincent et al., 2006). Complex epistatic interactions between genes that encode $GABR\alpha_4$ and $GABR\beta_1$ within 4q12 have also been reported in autistic probands (Ma et al., 2005). Collectively these data implicate GABR dysregulation as a major contributor to imbalances between excitation and inhibition in the autistic brain and may provide the best lead for studying gene × environment interactions that enhance autism susceptibility.

Roberts and co-workers recently published the results of a study that examined possible association between maternal residence near agricultural pesticide applications during key periods of gestation and development of autism (Roberts et al., 2007). Of nearly 250 hypotheses tested, children of mothers living within 500 m of field sites with the highest non-zero quartile of organochlorine (OC) (primarily endosulfan and dicofol) poundage had a risk factor for autism that was 6.1 times higher than that of mothers not living near agricultural fields. Autism risk increased with poundage of OC applied and decreased with distance from field sites (Roberts et al., 2007). Although small in sample size, these new findings underscore the critical need to understand how heritable imbalances in GABR signaling and their associated pathways influence susceptibility to chemicals that are known to directly target GABA receptors, especially $GABR\beta_3$, during gestation and postnatal development. However, predicting the functional consequences of exposure to these chemicals is difficult because we do not have sufficient scientific data regarding the specificity and functional outcome of interactions between toxicants that target GABR and the sixteen known GABR subunits that oligomerize as heteropentamers or homotetramers to give rise to the diverse functional roles of these receptors in regulating CNS excitability. Development of relevant models in mice will be essential to understanding the mechanisms by which complex gene × environment interaction at the level of GABR signaling influence autism risk and to better predict susceptible endophenotypes.

1.3. Chemicals that interfere with calcium (Ca^{2+}) signaling

Changes in localized and global intracellular Ca^{2+} concentration represent one of the most common ways in which cells regulate cell cycle, terminal differentiation, migration, and death. Moreover intracellular Ca^{2+} orchestrates hundreds, if not thousands, of biochemical processes essential for metabolism, transport, secretion, and regulation of gene transcription and translation in most mammalian cell types. Thus, Ca^{2+} is a fundamental regulator of most biological processes. Although Ca^{2+}-regulated processes vary according to cell type and the chemical and physical environment in which the cell finds itself, significant progress has been made toward understanding the mechanisms by which cells generate spatially and temporally segregated Ca^{2+} signals that are context-specific (Berridge, 2006). Both the nAChR and GABR that are implicated in autism contribute directly or indirectly to the regulation of Ca^{2+}-dependent pathways and are themselves regulated by the Ca^{2+}-dependent process. For example, GABR activation in neurons initiates at least two distinct signal transduction pathways, one in which the Cl^- current is activated and another which involves the elevation of intracellular Ca^{2+}

through functional coupling with voltage activated Ca^{2+} channels (Lyons et al., 2001). Moreover, several of the candidate genes for autism encode proteins whose primary role is to generate intracellular Ca^{2+} signals or are themselves tightly regulated by local fluctuations in Ca^{2+} concentrations (Pessah and Lein, 2008).

A large number of priority chemicals of concern to human environmental health have been shown to affect the integrity of cellular Ca^{2+} signals. Prominent examples are the polyaromatic hydrocarbons that mediate their toxicity through the arylhydrocarbon receptor (AhR), such as dioxin, or through selective interactions with ryanodine receptors (RyR) in the brain (Zimanyi and Pessah, 1991), such as non-coplanar polychlorinated biphenyls (PCBs). Interactions of non-coplanar PCBs with RyR greatly sensitize the release of Ca^{2+} from the microsomal intracellular stores (Pessah et al., 2006; Wong et al., 1997), and are likely to contribute to imbalances in excitatory and inhibitory neurotransmission and the abnormal development of brain circuitry (Kenet et al., 2007; and review below by Lein). Sensitization of RyRs by PCBs has been shown to be responsible for a host of toxicological outcomes *in vitro* including amplification of NMDA-mediated excitation and Ca^{2+}/caspase mediated apoptosis (Gafni et al., 2004; Howard et al., 2003; Wong et al., 2001). RyRs are physically and functionally linked to voltage gated Ca^{2+} channels at the surface of the neuron where they form Ca^{2+} release units responsible for generating microdomains of signaling (Ca^{2+} microdomains) (Berridge, 2006). Of relevance to autism susceptibility, a gain-of-function missense mutation in the L-type Ca^{2+} channel CaV1.2 causes Timothy syndrome which has a 60% autism rate (Splawski et al., 2004). Could inherent abnormalities in Ca^{2+} signaling pathways represent a major point of convergence in the pathobiology of autism? Could environmental factors that dysregulate the spatial and temporal fidelity of Ca^{2+} microdomains amplify inborn weaknesses in generating and decoding Ca^{2+}-dependent processes leading to heightened susceptibilities to autism risk and severity and its associated co-morbidities? Considering that defective neuronal connectivity is now being considered a common etiological feature in most forms of autism, and its phenotypic manifestation is closely linked to imbalances in brain circuitry, then the hypothesis of gene × environment as it relates to Ca^{2+} signals requires more attention.

2. Developmental neuroendocrine effects of polychlorinated biphenyls (PCBs): parallels with attention deficit hyperactivity disorder (ADHD). R.F. Seegal, Wadsworth Center, Albany, NY, USA

ADHD is the most commonly diagnosed neuropsychiatric/neurological disorder in children (McGough, 2005) and is characterized by behavioral hallmarks including attentional deficits, impulsivity and motor over-activity (Biederman, 2005; Himelstein et al., 2000). The incidence of this disorder ranges from 2 to 16% (Goldman et al., 1998), indicating the difficulties in diagnosis, the potential co-morbidity with other neuropsychiatric/neurological disorders and the likelihood that ADHD is a disorder that includes multiple and perhaps different core deficits (Pennington, 2005). This diversity of diagnoses and symptoms of ADHD complicate studies whose aims are to determine the underlying causes and mechanisms responsible for this common disorder.

We will first briefly describe the neurochemical and neuroendocrine factors shown to be either associated with or acting as etiologic factors in ADHD and then describe the rationale for examining the potential roles that polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) play in this disorder.

2.1. The role of central catecholamines in ADHD

There is considerable evidence that dysfunctions in central catecholamines are intimately associated with ADHD with the most direct evidence based on the knowledge that medications most successful in ameliorating ADHD symptoms increase the release of dopamine (DA) and norepinephrine (NE) (Madras et al., 2005; Solanto, 2002). These findings suggests that hypo-functionality of central catecholamines is a major factor contributing to the behavioral phenotype (Russell, 2003). A likely explanation for this catecholamine hypo-functionality is derived from imaging studies conducted in children and adults diagnosed with ADHD. Thus, Cheon et al. (2003) report increased dopamine transporter (DAT) densities in basal ganglia. Such an increase in DAT densities, assuming no change in transporter function, would result in a decrease in synaptic concentrations of DA and subsequent alterations in postsynaptic DA receptor regulation in the basal ganglia and prefrontal cortex (PFC). Alternatively, the increases in DAT densities may represent an up-regulation of DAT induced by reductions in synaptic concentrations of DA and NE due to processes not yet completely understood.

Laboratory studies also support the hypothesis that alterations in central catecholamines are associated with behavioral changes reminiscent of those seen in ADHD. The most commonly studied rodent model of ADHD is the spontaneous hypertensive rat (SHR) which demonstrates many of the behavioral deficits seen in ADHD (Sagvolden et al., 2005). Most importantly, there are also dysfunctions in central catecholamines, including impaired release of catecholamines in PFC and striatum (Davids et al., 2003), increased tissue concentrations of DA receptors, decreased NE function in PFC and striatum (Viggiano et al., 2004) and increased DAT densities (Watanabe et al., 1997).

The DAT knockout mouse has also been proposed as an animal model of ADHD (Gainetdinov and Caron, 2003). This knockout exhibits hyperactivity, the inability to inhibit on-going behaviors and cognitive and sensorimotor gating deficits (Madras et al., 2005) that mimic some of the deficits seen in ADHD. Because DAT function is virtually absent, it has been suggested that DA *hyper*-functionality, rather than *hypo*-functionality, may contribute to ADHD (Gainetdinov and Caron, 2003). However, these authors, who developed the DAT knockout model, show that these mice adapt to reductions in DAT by reducing tyrosine hydroxylase (the rate-limiting enzyme responsible for the synthesis of DA), the number of vesicular DA storage sites and postsynaptic DA receptor densities. Thus, it is likely that the DAT knockout mouse also demonstrates functional hypo-dopaminergic tone, perhaps simplifying the task of understanding the role that altered central DA and NE function plays in the etiology of ADHD.

2.2. The role of thyroid hormone deficiencies in ADHD

The impetus for study of possible relationships between thyroid hormone dysfunctions and ADHD was provided by Hauser et al. (1993) who demonstrated that a majority of children with resistance to thyroid hormone (RTh) exhibited ADHD-like symptoms. Recent studies, however, have shown that children with RTh exhibit more profound deficits, including developmental delays, than seen in children diagnosed with ADHD (Stein et al., 1995). Nevertheless, examination of the relationships between mild hypothyroidism and ADHD has proven fruitful. Stein and Weiss (2003) have shown that thyroid hormone concentrations, in children referred to a clinic specializing in learning and behavioral problems, are associated with attentional deficits and hyperactivity and that the ADHD subtype, primarily inattentive, was associated with reductions in free thyroid hormone concentrations.

The importance of alterations in thyroid hormone function during development in the etiology of ADHD extends beyond evidence of alterations in behavior. Ausó et al. (2004) and Dulce Madeira et al. (1990) demonstrate that reductions in thyroid hormone function during development, induced respectively by either methimazol (MMI) or propylthiouracil (PTU), reduce cortical neogenesis and alter its cytoarchitecture, including reductions in PFC volume. These findings are particularly relevant to ADHD since imaging studies carried out in children and adults diagnosed with ADHD demonstrate significant reductions in cortical, basal ganglia and callossal volumes (Seidman et al., 2005). Finally, Evans et al. (1999) have shown that experimentally induced hypothyroidism results in reductions in central catecholamines and neurotransmitter metabolic enzymes, raising the important, but as yet, incompletely addressed question of the relationships between developmental exposure to PCBs and PBDEs, fetal and maternal hypothyroidism and reductions in fetal and offspring central catecholamines.

Taken together, the above results suggest a surprisingly consistent image of the neurochemical and endocrine changes associated with ADHD: a positive response to psychostimulants that increase neuronal release of DA and NE; imaging studies that indicate significant increases in basal ganglia DAT densities that are likely to result in a decrease in synaptic concentrations of DA; genetic studies demonstrating associations between the DAT and postsynaptic DA receptors including the DA D4 receptor, present at high concentrations in the PFC (Heiser et al., 2004); and evidence of associations between thyroid hormone reductions and ADHD.

2.3. Why PCBs?

PCBs are widespread persistent environmental contaminants shown to affect cognitive function in infants and children exposed during development (Jacobson and Jacobson, 1996). Most germane to this discussion are recent findings from Jacobson and Jacobson (2003) and Stewart et al. (2005) demonstrating that such exposures lead to deficits in attentional domains, including hyperactivity and impulsivity.

The extensive literature demonstrates that PCBs reduce dopamine and norepinephrine concentrations in a variety of experimental paradigms, including cells in culture (Shain et al., 1991), the brains of adult rodents and non-human primates (Seegal et al., 1991), and the brains of mice and rats exposed during development, including the PFC and basal ganglia (Seegal et al., 1997). In addition to reductions in concentrations of central catecholamines there is also convincing evidence that PCBs inhibit both the dopamine transporter and the vesicular monoamine transporter (Bemis and Seegal, 2004; Mariussen and Fonnum, 2001)—transporters also shown to be altered in ADHD and experimental models of ADHD (Gainetdinov and Caron, 2003; Madras et al., 2005). Although it may seem counter-intuitive that DAT inhibition could result in catecholaminergic hypofunction, there is evidence that such inhibition results in a reduction in synaptic catecholamine levels. Thus, Seegal et al. (2002) have shown, using *in vivo* microdialysis, that exposure of the adult rat to PCBs for periods longer than 3 days results in significant reductions in extracellular concentrations of striatal DA.

In summary, there appears to be convincing evidence that PCBs and PBDEs induce changes in neuroendocrine function in laboratory animals reminiscent of those seen in humans diagnosed with ADHD. Understanding the mechanisms by which PCBs and PBDEs induce these changes following developmental exposure will aid in understanding the etiology of this widely diagnosed disorder.

3. Polychlorinated biphenyls (PCBs) modulate the development of neuronal connectivity. Pamela J. Lein, CROET, Oregon Health & Science University, Portland, OR 97239, USA

PCBs are persistent widespread environmental contaminants, and high residue levels are still detected in human tissues (DeCaprio et al., 2005; Humphrey et al., 2000; Park et al., 2007). Epidemiological data indicate that PCBs negatively impact neuropsychological function in exposed children (Carpenter, 2006; Schantz et al., 2003), and experimental animal studies confirm that developmental PCB exposure causes cognitive and psychomotor deficits (Mariussen and Fonnum, 2006). PCBs disrupt thyroid hormone function (Hagmar, 2003; Zoeller, 2007), perturb dopaminergic neurotransmission (Mariussen and Fonnum, 2006; Seegal, 1996) and increase intracellular Ca^{2+} levels in neurons via several mechanisms (Kodavanti, 2005; Mariussen and Fonnum, 2006), including ryanodine receptor (RyR) activation (Pessah and Wong, 2001). The relevance of these molecular effects to PCB-induced neurobehavioral deficits has been difficult to establish, in part because neurodevelopmental events targeted by PCBs have yet to be identified. We are investigating the hypothesis that PCBs disrupt normal patterns of neuronal connectivity via effects on dendritogenesis. This hypothesis is derived from observations that (1) subtle perturbations of temporal or spatial aspects of dendritic growth are associated with altered behavior in experimental models (Barone et al., 2000; Berger-Sweeney and Hohmann, 1997); (2) in humans, aberrations in dendritic structure are thought to contribute to functional deficits observed in a variety of neurodevelopmental disorders (Huttenlocher, 1991; Jagadha and Becker, 1989; Rubenstein and Merzenich, 2003; Zoghbi, 2003); and (3) both thyroid hormone (Kapfhammer, 2004) and intracellular Ca^{2+} (Lohmann et al., 2005; Redmond and Ghosh, 2005) influence neuronal connectivity via dynamic control of dendritic structure.

To test this hypothesis, we examined spatial learning and memory in parallel with dendritic growth and plasticity of cerebellar Purkinje cells and neocortical pyramidal neurons in weanling rats exposed to the PCB mixture Aroclor 1254 (A1254) in the maternal diet at 1- or 6-mg/(kg d) throughout gestation and lactation. These doses and routes of exposure are similar to those reported to generate PCB levels in rat adipose tissue and brains within the range of levels observed in humans (Brunn et al., 1990; DeCaprio et al., 2005; Hany et al., 1999; Stellman et al., 1998). These doses did not cause maternal or fetal toxicity as assessed by body weight of pregnant and lactating dams, litter size, and weight gain in pups. However, developmental A1254 exposure impaired performance in the Morris water maze and altered dendritic morphology of both cerebellar Purkinje cells and neocortical pyramidal neurons, enhancing dendritic growth in untrained animals but attenuating or reversing experience-dependent dendritic growth in maze-trained littermates. Both behavioral and structural deficits were more pronounced in the 1 versus 6-mg/(kg d) A1254 treatment group. Consistent with previous reports (Crofton et al., 2000; Roegge et al., 2004; Zoeller et al., 2000), dose-dependent decreases in serum thyroid hormone were observed in weanlings exposed developmentally to A1254. However, it seems unlikely that the PCB effects on dendritic growth were due to hypothyroxinemia because neonatal hypothyroidism would be expected to decrease basal dendritic growth in cerebellar and cortical neurons (Nicholson and Altman, 1972; Rami et al., 1986; Ruiz-Marcos et al., 1994; Uylings et al., 1994). In contrast, radioligand binding and western blot analyses indicated that developmental A1254 exposure increased basal cerebellar [^{3}H]ryanodine binding sites and RyR expression, but inhibited training-induced RyR upregulation. Interestingly, the relationship of A1254 dose to A1254 effects on basal and training-

induced RyR expression closely paralleled the inverted-dose related effects of developmental A1254 exposure on dendritic morphology. Given the fundamental role of RyR in Ca^{2+} signaling and the critical influence of Ca^{2+} signaling on basal and activity-dependent dendritic growth, these data suggest that PCB effects on RyR could be largely responsible for the effects of developmental PCB exposure on behavior and dendritic morphology that we observed in weanling rats. In support of that conclusion, *in vitro* studies demonstrated that dendritic growth of neocortical pyramidal neurons was influenced by PCB95, a non-coplanar congener that sensitizes ryanodine receptors (RyR), but not by PCB66, a coplanar congener with negligible RyR activity (Pessah et al., 2006).

These data demonstrate that developmental exposure to environmentally relevant levels of PCBs interferes with normal patterns of dendritic growth and plasticity, and suggest that PCB effects on RyR expression and function may mediate this response. The relevance of these findings to autism spectrum disorders is suggested by recent genetic studies implicating genes that encode Ca^{2+}-regulated signaling proteins involved in synapse formation and dendritic growth in autism (Krey and Dolmetsch, 2007) and emerging histological, electrophysiological and functional imaging studies suggesting that autism results from alterations in neuronal connectivity (Belmonte and Bourgeron, 2006; Geschwind and Levitt, 2007; Zoghbi, 2003). Collectively, these observations suggest that PCBs, and in particular non-coplanar PCBs, may be environmental risk factors in autism spectrum disorders and provide important new clues about the possible role of RyR in contributing to environmentally triggered neurodevelopmental deficits. Moreover, they suggest the possibility that exposure to even very low PCB levels could amplify adverse effects in genetically susceptible individuals (Campbell et al., 2006) such as those with heritable deficits in Ca^{2+} signaling.

4. Epigenetic influences on autism risk: a role for $GABA_A$ receptor dysregulation. Janine M. LaSalle, University of California, Davis, CA 95616, USA

Epigenetics is the study of inheritable and reversible modifications to nucleotides or chromosomes that do not change the genetic sequence but can modify gene expression and phenotype. The importance of epigenetic mechanisms in regulating human brain development has been recently revealed by the discovery of the genetic bases of several human neurodevelopmental disorders (Egger et al., 2004; Hendrich and Bickmore, 2001; Zoghbi, 2003). Genetic syndromic causes of autism that involve epigenetic mechanisms include Rett syndrome, an X-linked disorder caused by mutations in *MECP2* and encoding methyl CpG binding protein 2 (Amir et al., 1999). Although originally described as a transcriptional repressor of methylated genes, new roles for MeCP2 in chromatin organization and alternative splicing have recently emerged (Horike et al., 2005; Yasui et al., 2007; Young et al., 2005). Angelman syndrome is an imprinted disorder caused by maternal 15q11-13 deficiency or mutation of the ubiquitin protein ligase E3 gene, *UBE3A,* that also shares features with both Rett syndrome (RTT) and autism (Hitchins et al., 2004; Lalande and Calciano, 2007; Philippart, 2001; Watson et al., 2001). The demonstration of MeCP2 as a regulator of both *UBE3A* (the Angelman gene) and *GABRB3* (encoding a $GABA_A$ receptor subunit) expression within 15q11-13 has revealed some interesting insights into the genetic and epigenetic pathways common to all three disorders (Hogart et al., 2007; Samaco et al., 2005). More common neurodevelopmental disorders of unknown genetic etiologies such as autism and developmental delays are likely to be caused by a combination of genetic and environmental factors (Lamb et al., 2000; Muhle et al., 2004; Volkmar and Pauls, 2003). Since epigenetic pathways act at the interface between genes and the environment, environmental factors can alter epigenetic patterns and downstream gene expression pathways.

Pervasive developmental disorders classified under the DSM IV criteria include autistic disorder, Asperger's syndrome, RTT, childhood disintegretative disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS) (Volkmar and Pauls, 2003). The onset of autistic symptoms in these disorders generally occurs around 1–3 years of age and is characterized by stereotyped mannerisms, abnormal preoccupations, lack of pragmatic language and imaginative play, impaired eye gaze, and impaired joint attention (16). Males with autistic disorder outnumber females by around 4:1, while a 12:1 sex bias is observed for Asperger's syndrome (AS) (Muhle et al., 2004). In contrast, RTT is almost exclusively in females because of the X-linked dominant inheritance pattern (Zoghbi, 2003). While twin studies have demonstrated high heritability of autism-spectrum disorders, monozygotic twins are not always concordant for autistic disorder (Bailey et al., 1995). Epigenetic changes are predicted to be a major determinant in differences between monozygotic twins (Fraga et al., 2005).

Maternal duplications of 15q11-13 by interstitial duplications or marker chromosomes are the most common cytogenetic cause of autism, occurring in 1–2% of patients (Schroer et al., 1998). Multiple studies have shown an association of 15q11-13 markers to autism, and $GABA_A$ receptor genes have been identified as strong candidates to explain the occurrence of seizures in 30–35% of autistic patients (Buxbaum et al., 2002; Ma et al., 2005; Martin et al., 2000; Menold et al., 2001). The 15q11-13 locus contains a cluster of imprinted genes, expressed exclusively from one parental chromosome, as well as a cluster of three $GABA_A$ receptor subunit genes that are biallelically expressed (Nicholls et al., 1993). The 15q11-13 $GABA_A$ receptor genes are implicated in AS, as patients with a 15q11-13 deletion have a more severe phenotype, particularly in occurrence of seizures, than do individuals with *UBE3A* point mutations (Minassian et al., 1998). Additionally, the *Gabrb3* knock-out mouse demonstrates the significance of the 15q11-13 gene as 90% of pups die as neonates, and surviving mice exhibit severe neurological manifestations such as seizures, learning deficits, hyperactivity, and disturbed rest-activity cycles (DeLorey et al., 1998; Homanics et al., 1997). Mouse deletion strains of the syntenic 15q11-13 $GABA_A$ receptor cluster have demonstrated that *Gabrb3* deficiency causes the most severe phenotype, as mice with a deletion encompassing *Gabra5* and *Gabrg3* are phenotypically normal (Culiat et al., 1994).

GABRB3 protein expression is reduced in RTT, caused by mutations in *MECP2* on Xq28 (Samaco et al., 2005). Although *Gabrb3* is biallelically expressed in mouse brain, conflicting data exist regarding the imprinting status of the 15q11-13 GABR genes in humans (Bittel et al., 2003, 2005; Gabriel et al., 1998; Meguro et al., 1997). A recent study showed that all three GABR genes were biallelically expressed in 21 control-brain samples, demonstrating the lack of imprinting in normal human cortex (Hogart et al., 2007). Interestingly, four of eight autism and one of five RTT brain samples showed monoallelic or highly skewed allelic expression of one or more GABR genes, suggesting that epigenetic dysregulation of these genes is common to both disorders. Prader-Willi syndrome (PWS) and Autism spectrum (AS) samples with paternal and maternal 15q11-13 deletions revealed a paternal expression bias of *GABRB3*, while RTT brain samples showed a significant reduction in *GABRB3* and *UBE3A*. MeCP2 binds to methylated CpG sites within an intronic sequence of *GABRB3*. Previous studies demonstrated that homologous 15q11-13 pairing in neurons was dependent on MeCP2 and was disrupted in RTT and autism cortexes (Thatcher et al., 2005). Combining these results suggest that MeCP2 acts as a

chromatin organizer for optimal expression of both alleles of *GABRB3* in neurons.

Since epigenetic mechanisms act at the interface between genetic and environmental factors that determine phenotype, investigation of epigenetic mechanisms are essential for understanding the multiple interacting etiologies of complex diseases such as autism. Since human populations have a diverse array of genetic and environmental differences, the major challenge is how to define precise epigenetic changes that occur as a result of a single environmental or genetic change and to understand how such changes affect disease risk. Future studies will investigate potential epigenetic changes to *MECP2* and 15q11-13 genes following perinatal exposure to organic pollutants, specifically polybrominated diphenyl ethers used in flame retardants.

5. The impacts of maternal immune challenge on the fetal brain and the pathological consequences on behavior. Benjamin K. Yee, Urs Meyer, and Joram Feldon, Laboratory of Behavioral Neurobiology, ETH Zurich, Switzerland

Maternal infection in prenatal life is a notable risk factor in the development of severe neuropsychiatric disorders in later life, including schizophrenia and autism (reviewed in Arndt et al., 2005; Brown, 2006; Brown and Susser, 2002; Fatemi, 2005; Patterson, 2007). One prevalent hypothesis suggests that infection-induced disruption of early prenatal brain development may predispose the organism for long-lasting structural and functional brain abnormalities, leading to the emergence of psychopathological behavior in adulthood (Gilmore and Jarskog, 1997; Meyer et al., 2007; Patterson, 2002). The feasibility of this causal link (between maternal infection during pregnancy and higher risk of brain and behavioral pathology in the offspring) has received considerable support from several experimental models established in both rats and mice (for a review, see Meyer et al., 2007).

One class of experimental models favors the use of agents that stimulate infection-like immune response in the maternal host, such as polyriboinosinic–polyribocytidilic acid (Poly-I:C, a synthetic analog of double-stranded RNA) or lipopolysaccharide (LPS, a gram-negative bacteria cell wall product), rather than live pathogens. Administration of Poly-I:C mimics the acute phase response to viral infection, and is highly effective in elevating the levels of pro-inflammatory cytokines and other mediators of inflammation (e.g., Cunningham et al., 2007; Fortier et al., 2004; Meyer et al., 2006b). Systemic Poly-I:C treatment to pregnant mice and rats results in significant and time-limited alterations of pro- and anti-inflammatory cytokine levels in the three relevant compartments of the maternal–fetal interface: the placenta, the amniotic fluid, and the fetus, including the fetal brain (Gilmore et al., 2005; Meyer et al., 2006b, 2008b). The use of prenatal immune challenge by Poly-I:C is thus a valuable experimental approach in studying the long-term consequences of fetal brain inflammation in specific gestation time on subsequent brain and behavioral development.

This approach has been successfully applied by Benjamin Yee, Urs Meyer and Joram Feldon from the Swiss Federal Institute of Technology (ETH) Zurich in mice, providing a comprehensive characterization of the neuropathological and psychopathological consequences of prenatal immune activation. Their efforts also included experiments designed to identify distinct neuroimmunological factors that may be critical in shaping the risk for brain and behavioral dysfunctions emerging after *in utero* exposure to infection. Their results highlighted the importance of (1) the relative prenatal and postnatal maternal contributions to the behavioral and neurochemical dysfunctions after prenatal immune activation, (2) the precise timing of prenatal immune challenge on the specificity of postnatal neuropathology and psychopathology, and (3) the influence of the anti-inflammatory genetic background in the association between prenatal immune activation and adult behavioral pathology.

In order to dissect the relative prenatal and postnatal contributions in the infection model of schizophrenia and related disorders, pregnant mice were exposed to Poly-I:C or vehicle treatment in early/middle pregnancy (gestation day 9), and offspring born to Poly-I:C- and vehicle-treated dams were then systematically cross-fostered to surrogate rearing mothers, which had experienced either inflammatory or vehicle treatment during pregnancy. The findings from these experiments demonstrate that neuropathological and psychopathological abnormalities emerging after prenatal PolyI:C-induced immune challenge are largely attributable to prenatal rather than postnatal maternal effects on the offspring (Meyer et al., 2006c, 2008a). Regardless of whether the neonates were reared by immune-challenged or control surrogate mothers, multiple brain and behavioral dysfunctions emerged following prenatal Poly-I:C exposure. This strongly indicates that infection-induced disruption of fetal brain development predisposed the offspring to the emergence of psychopathology in later life, and the normalization of neonatal rearing condition provided no protection. Notwithstanding, these cross-fostering experiments further showed that being reared by an immune-challenged surrogate mother was sufficient to induce distinct forms of adult psychopathology and neuropathology (Meyer et al., 2006c, 2008a). The responsible postnatal factors, including post-partum maternal behavior, critical for the latter effects clearly warrant further specification. This would reveal additional environmental factors in early life contributing to later life's vulnerability to psychiatric disorders.

Another series of experiments was designed to evaluate the influence of the timing of maternal immune challenge on the emergence of brain and behavioral dysfunctions in the resulting offspring (Meyer et al., 2006a,b, in press). These experiments demonstrated that the precise timing of prenatal immune activation is a crucial determinant of the specificity of both the structural and functional brain abnormalities in later life (see Meyer et al., 2007). Specifically, prenatal Poly-I:C-induced immune challenge in early/middle gestation (gestation day 9 in mice) led to a pathological profile characterized by suppressed exploratory behavior, abnormalities in selective associative learning in the form of latent inhibition disruption and loss of the US-pre-exposure effect, deficiency in sensorimotor gating in the form of impaired prepulse inhibition, enhanced sensitivity to the indirect dopamine receptor agonist amphetamine and (to a lesser extent) the non-competitive NMDA-receptor blocker, dizocilpine (MK-801), as well as deficient spatial working memory function when the temporal demand on memory retention was high (Meyer et al., 2005, 2006a,b, in press). On the other hand, prenatal immune challenge in late gestation (gestation day 17) led to a partially overlapping symptom profile, which comprised perseverative behavior retarding reversal learning, a severe working memory deficit, potentiated response to acute systemic amphetamine and dizocilpine treatment, and the loss of the US-pre-exposure effect (Meyer et al., 2006a,b, in press). Hence, some of the identified pathological traits are clearly restricted to the symptom cluster associated with prenatal immune activation in early/middle or late gestation (e.g., latent inhibition deficiency and retardation in reversal learning), whereas others are common to both symptom clusters (e.g., potentiation of amphetamine sensitivity). Existing data further indicate that prenatal immune challenge in early/middle gestation is likely to exert a more severe impact on neurodevelopment

at least with respect to the resulting behavioral abnormalities emerging in adulthood, in comparison to identical immune challenge conducted in late gestation (Meyer et al., 2007).

The link between prenatal immune challenge and postnatal brain dysfunctions may appear to be critically modulated by the precise pattern of cytokine reactions taking place at the maternal–fetal interface. To test this hypothesis, Meyer et al. (2008b) compared the psychopathological consequences of prenatal immune activation by Poly-I:C in wild-type mice and transgenic mice constitutively over-expressing interleukin (IL)-10 in macrophages. IL-10 is a cytokine with strong anti-inflammatory and immuno-suppressive functions; it limits production and/or secretion and biological activities of many inflammatory molecules in both acute and chronic conditions (Moore et al., 2001; Murray, 2006). If the emergence of postnatal brain abnormalities after prenatal infection and/or inflammation depends primarily on a shift of increased pro-inflammatory cytokines levels in the fetal brain (Gilmore and Jarskog, 1997), then the impact of prenatal infection ought to be minimal if this is expressed against a background of enhanced IL-10 production.

This prediction has been confirmed by Meyer et al. (2008b) who showed that genetically engineered peripheral over-expression of the anti-inflammatory cytokine IL-10 was sufficient to prevent the emergence of multiple behavioral and psychopharmacological abnormalities in the adult offspring associated with prenatal immune challenge by Poly-I:C (Meyer et al., 2008b). To their surprise, however, in the absence of a discrete prenatal inflammatory stimulus, IL-10 over-expression during prenatal development was in itself sufficient to induce specific behavioral abnormalities in the adult offspring (Meyer et al., 2008b). One startling implication is that the development of normal adult brain functions may be critically influenced by the precise balance between pro- and anti-inflammatory cytokines in prenatal life. Accordingly, shifts of the balance towards either pro-inflammatory or anti-inflammatory cytokine species would precipitate adult behavioral pathology, whereas the concomitant induction of both cytokine classes in the fetal brain may nullify each other's long-term negative influences on brain and behavioral development. This account is consistent with the antagonistic properties of distinct cytokine classes in the peripheral immune system (Moore et al., 2001; Murray, 2006) and suggests that the precise effects of cytokines on early neurodevelopmental processes crucially depend on their identity.

In summary, the findings from experimental models of prenatal immune activation in mice show that early life experience in the form of a discrete immunological manipulation can cumulate into specific brain and behavioral pathology in early adolescence and/or adulthood. The data thus support the biological and neuroimmunological plausibility for a causal link from maternal infection and/or inflammation during pregnancy to higher risks for brain pathology in the offspring. Given that Poly-I:C readily mimics the acute immune response to viral infection, the identified long-term effects of prenatal Poly-I:C-induced immune activation may be of particular relevance for validating the plausibility of the epidemiological association between prenatal infection with viral pathogens and enhanced risk for postnatal brain disorders, including schizophrenia and autism (Arndt et al., 2005; Brown, 2006; Brown and Susser, 2002; Fatemi, 2005; Patterson, 2007). The data from the laboratory of Benjamin Yee, Urs Meyer and Joram Feldon have further identified that this relationship is (1) attributable to prenatal rather than postnatal maternal effects on the offspring, (2) critically influenced by the precise timing of maternal immune response, and (3) dependent on the immunological background (genetically determined or otherwise) of the infected maternal host.

6. Maternal autoantibodies: developmental neurotoxicants of autism risk? Judy Van de Water, University of California, Davis, CA 95616, USA

Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by impairments in social interaction, verbal and nonverbal communication, and stereotyped behaviors and interests (Lord et al., 2000a). ASD encompasses a broad range of phenotypes and diagnosis is currently based solely on behavioral criteria (Lord et al., 2000b). The etiology of ASD is not well understood, though it likely involves both genetic and environmental factors (Volkmar and Pauls, 2003). Although the age of symptom onset would indicate a pre-natal or early post-natal etiology, to date, no early biological markers have been identified for this spectrum of disorders. Such markers would allow for earlier identification and therapeutic intervention, which could significantly improve the prognosis for ASD patients (Aman and Langworthy, 2000) and contribute to the development of prevention strategies.

Immune system dysregulation has been reported in ASD in several studies (Ashwood et al., 2006; Fiumara et al., 1999; Jyonouchi et al., 2005; Molloy et al., 2006; van Gent et al., 1997; Warren et al., 1987, 1990). Systemic immunologic aberrations in individuals with autism have often been associated with autoimmunity; in particular, the generation of antibodies reactive against brain and CNS proteins (Ashwood and Van de Water, 2004; Cabanlit et al., 2007; Cohly and Panja, 2005; Connolly et al., 1999, 2006; Singh and Rivas, 2004; Singh et al., 1993, 1997a,b, 2002; Todd et al., 1988; Wills et al., 2007).

In addition to the presence of autoantibodies in children with autism, a few studies suggest the presence of autoantibodies to brain proteins in the blood of mothers of children with autism. The transplacental passage of maternal IgG isotype antibodies has long been known as a mechanism for fetal immune instruction (Garty et al., 1994) and protection (Harris et al., 2006; Simister, 2003). However, autoantibodies that react to fetal 'self'-proteins can also cross the placenta, and potentially impact fetal development.

For example, Dalton et al. (2003) demonstrated maternal IgG antibody reactivity to adult rat cerebellar Purkinje cells in a mother of multiple children with ASD. These authors also reported the presence of behavioral deficits in the pups of a mouse injected during gestation with her serum (Dalton et al., 2003). Similarly, Zimmerman et al. (2007) recently reported differing patterns of serum immunoreactivity to pre-natal rat brain between mothers of children with autism and mothers of control children. This study also demonstrated that immunoreactivity persisted in maternal circulation for up to 18 years post-delivery.

Our laboratory has recently noted the presence of autoantibodies to human fetal brain proteins at 37 and 73 kDa exclusively in the plasma of mothers of children with autism compared with control mothers (Braunschweig et al., 2008). In this study, maternal plasma was collected on average 3.5 years after the birth of the study child. Fig. 1 illustrates the various band patterns noted in the plasma from mothers of children with autism. Maternal reactivity to a protein at approximately 37 kDa was observed in the plasma of 16/61 mothers of AU children (26.2%) (Fig. 1) compared with 1/40 mothers of developmentally delayed (DD) children (2.5%; $p = 0.0023$), and 5/62 mothers of typically developing (TD) children (8.1%; $p = 0.0086$). Furthermore, the presence of the 37 kDa band yielded a significantly elevated odds ratio of 5.69 (95% confidence interval: 2.09–15.51) when compared with the TD group. Of particular note, reactivity against proteins at both 37 and 73 kDa was observed only in mothers of AU children, yielding highly significant statistical differences between mothers of AU children and mothers of TD children (7/61 *vs.* 0/62; $p = 0.0061$) and mothers of DD children (0/40; $p = 0.0401$).

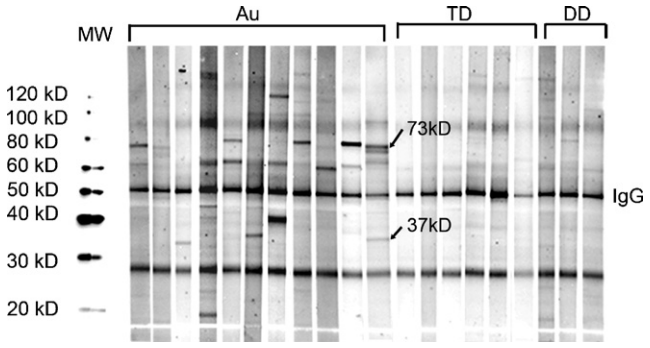

Fig. 1. Typical blot showing reactivity of maternal serum against fetal brain protein. Maternal serum diluted 1:400 was incubated with nitrocellulose strips containing human fetal brain protein and visualized using chemiluminescence and a digital imager. Bands were considered when α > 2-fold densitometry measurement above background was noted.

This pattern was not observed when only the 73 kDa band was present. The presence of these bands did not correlate with maternal age or history of autoimmune disease, nor with birth order or IQ of the index child.

When band prevalence was analyzed based on the pattern of clinical onset of autistic behaviors in children, 6/7 (86%) of the AU mothers that exhibited reactivity to the pair of bands at 37 and 73 kDa had children with the regressive phenotype. Moreover, this association was also evident regarding reactivity to the 37 and 73 kDa bands separately. In contrast, the antibody response of the mothers of early onset children was only significantly different from the TD and DD groups for the 37 kDa band alone. Finally, no association was observed between the presence of autoreactivity to fetal brain antigens and history of autoimmune disease in the maternal populations at the time of blood draw.

Interestingly, no case-control differences were observed in reactivity to kidney, duodenum or adult brain proteins. This finding suggests the possibility that the autoreactivity described herein is targeted towards proteins expressed exclusively, or at substantially higher levels, during fetal brain development. Given the rather small number of comparison children with non-ASD developmental delays included in the present investigation, further study is necessary to determine the specificity of our findings to autism.

These data provide evidence for an association between the presence of maternal immune system biomarkers and a diagnosis of autism in a subset of children. The presence of specific anti-fetal brain antibodies in the circulation of mothers during pregnancy may be a potential trigger that, when paired with genetic susceptibility, is sufficient to induce a downstream effect on neurodevelopment leading to autism. At present, we are investigating maternal plasma reactivity against fetal brain in a prospective cohort to determine the effect of the gestational autoantibody profile as it relates to an outcome of autism. Furthermore, work is currently underway to both replicate these findings in a larger cohort, and to determine the protein targets of these antibodies, the identification of which will allow us to better understand potential pathogenic mechanisms as well as create specific screening assays.

7. Examination of thimerosal effects in neonatal SJL/J mice at vaccination-associated exposure levels. Robert F. Berman, University of California, Davis, CA 95616, USA

Thimerosal (sodium ethyl mercury thiosalicylate) is an antimicrobial preservative used since the 1930s in numerous vaccines and medicinal preparations. Ethylmercury poisoning has occurred in humans, resulting in renal and neurotoxicity in the affected populations (Cinca et al., 1980; Damluji, 1962; Hilmy et al., 1976; Zhang, 1984). However, the possible effects of exposure to lower levels of ethyl mercury, such as those levels associated with immunization with thimerosal-preserved vaccines, are poorly understood. Initial risk assessments for ethylmercury were based on studies of oral methylmercury toxicity (Clarkson et al., 2003; Myers and Davidson, 2000); however, recent data indicate that the kinetics of tissue disposition and metabolism differ substantially for these two forms of organic mercury (Burbacher et al., 2005; Clarkson and Magos, 2006; Harry et al., 2004; Magos, 2003; Magos et al., 1985; Pichichero et al., 2002; Stajich et al., 2000; Suzuki et al., 1973), making the assumptions underlying a risk assessment different for each form of mercury. Concern remains about the possible contribution of exposure to low levels of ethylmercury via childhood vaccinations with thimerosal-preserved vaccines to the etiology of neurodevelopmental disorders, including autism (Bernard et al., 2002).

There is no direct evidence at the present time supporting an association between thimerosal exposure and autism (Davidson et al., 2004; Stratton and McCormick, 2001; Thompson et al., 2007), and the majority of epidemiological studies published to date have not supported such a relationship. However, a study by Hornig et al. (2004) examined the neurotoxicity of thimerosal in SJL/J mice and suggested that toxicity may be dependent on an organism's underlying immune status. Neonatal injections of thimerosal in SJL/J mice produced abnormal brain development, delayed growth, and altered locomotor behavior. SJL/J mice have the mouse major histocompatibility complex H-2^s associated with increased susceptibility to autoimmunity, and develop autoantibodies, including antinucleolar antibodies, to fibrillarin (Havarinasab et al., 2005) when exposed to high levels of methyl and ethylmercury (Havarinasab et al., 2005; Havarinasab and Hultman, 2005; Hultman and Hansson-Georgiadis, 1999). Thimerosal effects were not found in C57BL/6J (H-2^b) or Balb/c (H-2^d) mice that are less susceptible to autoimmunity. Hornig et al. (2004) suggested that the H-2^s haplotype, and altered immune system function in general, might confer heightened susceptibility to neonatal thimerosal exposures in mice. Although antinucleolar antibodies have not been found in autistic children (Singh and Rivas, 2004), the findings in SJL/J mice raised the possibility that children with altered immune system function could be particularly vulnerable to the neurotoxicity of thimerosal, and that the SJL/J mouse strain may provide a sensitive model for thimerosal developmental neurotoxicity studies. These findings are also intriguing because, if confirmed, they would mean that the neurotoxicology of thimerosal, and possibly other forms of mercury, would need to be recast within the framework of immune system status and function. Thus, we reexamined thimerosal neurotoxicity in SJL/J mice. Specifically, we examined the somatic growth, locomotor behavior, and the structure of the hippocampus of SJL/J mice following neonatal thimerosal injections, using experimental procedures that closely followed the original experimental procedures in the Hornig et al. (2004) study. As an extension of this previous study we also included a higher mercury dose (i.e., 10 times higher), measured tissue mercury levels (blood, brain and kidney), used unbiased stereological techniques for assessing the numbers of hippocampal CA1 pyramidal and dentate granule cell numbers, and included tests of social interaction, sensory gating and anxiety. These behaviors were selected because they assess behavioral domains that are considered relevant to core deficits in neurodevelopmental disorders such as autism (Crawley, 2004; Ricceri et al., 2007).

Neonatal SJL/J mice were injected with thimerosal, with and without combined HiB & DTaP vaccines. Injections modeled

childhood vaccination schedules, with mice injected on postnatal days 7, 9, 11 and 15 with 14.2, 10.8, 9.2 and 5.6 μg/kg mercury from thimerosal, respectively, or vehicle. Additional groups received vaccine only or a 10-times higher thimerosal + vaccine dose (Berman et al., 2008). Although low levels of mercury were detected in blood, brain and kidneys 24 h following the last thimerosal injection, survival, body weight, indices of early development (negative geotaxis, righting) and hippocampal morphology were not affected.

Performance was unaffected in behavioral tests selected to assess behavioral domains relevant to core deficits in neurodevelopmental disorders such as autism (i.e., social interaction, sensory gating and anxiety). In an open-field test the majority of behaviors were unaffected by thimerosal injection, although thimerosal-injected female mice showed increased time in the margin of an open field at 4 weeks of age. Considered together our recent results did not indicate pervasive developmental neurotoxicity following vaccine-level thimerosal injections in SJL mice (Berman et al., 2008).

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