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Animal models of autism spectrum disorders: Information for neurotoxicologists

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Abstract

Recent findings derived from large-scale datasets and biobanks link multiple genes to autism spectrum disorders. Consequently, novel rodent mutants with deletions, truncations and in some cases, overexpression of these candidate genes have been developed and studied both behaviorally and biologically. At the Annual Neurotoxicology Meeting in Rochester, NY in October of 2008, a symposium of clinicians and basic scientists gathered to present the behavioral features of autism, as well as strategies to model those behavioral features in mice and primates. The aim of the symposium was to provide researchers with up-to-date information on both the genetics of autism and how they are used in differing in vivo and *in vitro* animal models as well as to provide a background on the environmental exposures being tested on several animal models. In addition, researchers utilizing complementary approaches, presented on cell culture, *in vitro* or more basic models, which target neurobiological mechanisms, including Drosophila. Following the presentation, a panel convened to explore the opportunities and challenges of using model systems to investigate genetic and environment interactions in autism spectrum disorders. The following

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paper represents a summary of each presentation, as well as the discussion that followed at the end of the symposium.

Keywords

Animal models; Autism; Neurotoxicology; Symposium

1. Introduction

Autism is a developmental disorder currently defined through behavioral observation using standardized tools as well as clinical judgment. While epidemiological research is ongoing, the most recent CDC reports, using 14 areas of the US, estimate the prevalence to be 1:150 children (CDC, 2007). Because of the diverse set of symptoms that constitute the autism spectrum, multiple etiologies are thought to play a role, including genetic susceptibility and interactions between genetic and environmental factors. Because of the wide range of potential environmental factors thought to contribute to autism, well-defined animal models that can display core symptoms of the disorder are essential for research into causes and treatments. Identification of biological markers will permit more definitive diagnosis and will allow for early diagnosis before the behavioral symptoms have manifest. Mouse models are the usual standard due to extensive study of their genetics and the availability of detailed behavioral phenotyping data available for many mouse strains. Other models, such as non-human primates, can display behaviors unique to symptoms of autism including changes in eye contact, joint attention, and functional language. On the other hand, non-primate vertebrate species and invertebrates are useful as high-throughput experimental models to study how the nervous system behaves normally, and how perturbations due to environmental exposures affect function.

In October, a symposium was organized in conjunction with the International Neurotoxicology Conference Meeting which included the authors listed above. This paper provides a summary of each presentation so that future discussions can build on what was learned from the interactive nature of the symposium. Each speaker provided a unique and insightful contribution to the discussion, pointing out the opportunities in different animal models in studying autism. Highlighted approaches included using cell culture, Drosophila, and mice to identify and functionally characterize genes of interest for behavior, and primate models housed in naturalistic settings that display consistent and profound social behaviors, which can be targeted by exposures or treatments. First, Susan Hyman started the discussion by explaining the “triad” of autism symptoms, and how research with animal models can isolate and study a particular behavior of interest. David Amaral followed up this presentation by displaying videos of infant macaques whose mothers had been exposed to immune challenges, illustrating how a novel environmental exposure can elicit behaviors with similarities to symptoms of autism seen in children. Both Valerie Bolivar and David Threadgill discussed different methodologies using mouse models, and Flavio Keller described research that illustrated a genetic and environmental interaction using a specific mouse knockout model. The second part of the session was dedicated to *in vitro* research. Emmanuel DiCicco-Bloom and Pamela Lein presented cell culture models in order to illustrate ways to study the mechanism by which changes in cell function occur and how they are similar or different than that seen in autism. Similarities in cell function following environmental toxicant exposure can be enormously helpful in furthering studies, which may lead to interventions, treatments, and possibly prevention of autism. Cells in culture provide researchers with information that cannot be seen in human studies: how do cells behave? How are brain cells different than other cells? Are they similar? What happens when an environmental exposure is applied? Can this be used to better explain the

pathophysiology behind autism spectrum disorder? Finally, Linda Restifo presented new information on the use of Drosophila as a potential high-throughput screening method to test the neurotoxicity and behavioral effects of certain compounds and compound mixtures.

In addition to the presentations that generated discussion, an independent panel convened at the end of the day to answer specific questions regarding how different animal models can determine specific genetic and environmental interactions as they relate to autism. Questions included: (1) what is the "state of the science" with regards to animal/*in vitro* models of neurodevelopmental disorders; (2) what are the challenges to identifying the appropriate behavioral outcomes and neurobiological and neuropathological outcomes; (3) how can alternative models be used for better screening approaches; (4) what are the most promising methods and best strategies for utilizing these models to study gene × environment interactions; and (5) how can modifiers of environmental toxicity be added to the equation, and is the field ready for an additional layer of complexity? These questions were addressed throughout the presentations, and by a panel of stakeholders, parents, and scientists in the field following the expert presentations.

The purpose of the symposium was not to identify and explain every feasible animal model for a disorder which shows heterogeneity in symptom, onset, and severity. However, it did provide a platform by which researchers could present their particular models and demonstrate convergence of "autism like" symptoms in animals that may be induced or exacerbated by a range of environmental agents. The authors hope that the presentation of findings will stimulate further research in the field, especially in genetic susceptibility to environmental factors in developmental disorders. In summary, we hope that this symposium furthered the discussion about genetic and environmental interactions in autism, and how animal models can be used to help determine those leads that should be followed vigorously.

2. The clinical heterogeneity of autism spectrum disorders—Susan Hyman

Autism is a developmental disorder that is currently defined by behavioral symptoms across three general areas: Social Reciprocity, Communication, and Restricted and Repetitive interests and behaviors (DSM IV TR). With publication of the DSM IV in 1994 (American Psychiatric Association, 1994), the diagnostic criteria were expanded to allow for diagnosis of individuals with limited language as well as individuals with higher cognitive abilities. Although there may be a more dimensional approach to diagnosis with publication of the DSM V, currently used diagnostic algorithms include Autism as one of the Pervasive Developmental Disorders—neurodevelopmental disorders with clinical symptoms that pervade across developmental domains. Specific patterns of symptoms allow for classification into diagnoses of:

1. Autism—at least 6 symptoms across all three areas with at least two symptoms in social reciprocity.
2. Asperger Disorder—typical IQ and language with 2 symptoms in social reciprocity and two symptoms in restricted behaviors.
3. Rett Disorder—due to alteration in MeCP2 gene with characteristic regression, hand washing stereotypy, microcephaly, and motor findings.
4. Disintegrative Disorder—late and significant regression.
5. PDD-not otherwise specified—when symptoms are present but no other diagnostic category is fulfilled.

Outcome of individuals with Autism, Asperger Disorder and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS) may not be related to the number of symptoms alone, but is affected by comorbid features of intellectual ability, language and mental health diagnoses. The diagnostic algorithms do not include medical comorbidities such as sleep or gastrointestinal symptoms or symptoms that it would be difficult for an observer to reliably report on such as atypical sensory processing.

The publication of the DSM IV defined behavioral symptoms in such a manner that consistency of diagnosis could occur across clinicians. The ability to have reliability in clinical diagnosis permitted the application of neuroscientific and genetic tools to examine more homogeneous and defined populations. The DSM system uses clinically observable symptoms to characterize neuropsychiatric disorders. These symptoms may not represent symptoms discrete to autism. For example, lack of language development may be due to intellectual disability and impaired development of friendships may be secondary to significant thought disorder. The patterns of symptoms and responses that can be elicited in an object fashion permit the diagnosis to be applied. The symptoms captured in the DSM IV may not reflect the underlying neurobiologic dysfunction that may contribute to the observed clinical phenomena. The diagnostic criteria are inclusive of a range of presentations, so subgrouping is necessary for the investigation of biologic associations. Some groupings may be based on physical findings such as increased head size (Courchesne and Pierce, 2005), while others may be based on medical symptoms such as seizures (Tuchman et al., 2009) or neurobehavioral findings such as cognitive and language ability (Szatmari et al., 2008). What may be increasingly important in the study of environmental and genetic influences on component behaviors of ASDs may be behaviors that are evolutionarily preserved across species. While our current diagnostic characterization of the core social and communication deficits of the ASDs used for diagnosis are defined by symptoms manifest by humans, investigators are using behaviors that serve similar functions in other species to test genetic, environmental and immune hypotheses related to the etiology of ASDs (Moy et al., 2008; Patterson, 2008). Discrete behaviors, such as atypical chemosensory function or locomotor behavior (Radyushkin et al., 2009; Nag et al., 2009a,b), present in at least some individuals with ASDs (Bennetto et al., 2007; Mulligan et al., 2009), represent basic processes that can be seen in other species and possibly allow for testing of specific hypotheses. Known genetic disorders that are associated with an increased rate of ASD, such as fragile X syndrome and Rett's disorder also allow for examination of specific mechanisms for symptoms of ASDs (Wuang and Huber, 2009; Zhao et al., 2007). Animal models for these and other disorders may provide the opportunity for studies related to both understanding the disorder and developing new treatments. Crawley (2007a) has discussed the utility of animal models for autism at length as requiring tasks that fit one of three functions she describes as “(i) face validity (resemblance to the human symptoms), (ii) construct validity (similarity to the underlying causes of the disease) and (iii) predictive validity (expected responses to treatments that are effective in the human disease)”. This review describes examples of animal models representing these varied approaches.

Basic research is intimately tied to clinical understanding and advances in ASD treatment. Early diagnosis would be aided by biologic tests or markers, specific medical treatments require understanding of the neurobiology, and prevention is tied to identification of both genetic and environmental factors that might result in symptoms of ASD. There are two approaches that clinical and basic scientists have taken:

- The Trees Approach examines each feature of autism in isolation. It is particularly useful in the employment of animal models and in examining specific mechanisms.

- The Forest Approach looks at the entire symptom complex of ASD. This may simulate the gestalt and social deficits of the human disorder when animal models are used.

ASDs are common; the population prevalence is now reported to be 1:150 (CDC, 2007). ASDs are costly to families financially (Montes and Halterman, 2008) and personally (Kogan et al., 2008). The symptoms of ASD impact traits that are uniquely human, however there are commonalities on molecular and behavioral levels that make further study of animal models an important avenue of study when investigating the causes and interventions for ASDs. A list of the measurable clinical features of autism is illustrated in Table 1.

3. Using mouse models to recapitulate the behavioral symptoms—Valerie Bolivar

Although ASDs are uniquely human, animal model systems can provide insight into the underlying biology and allow the development and testing of therapeutic agents. As in the case of Huntington's, schizophrenia and Alzheimer's, mouse models may play a critical role in helping us understand the etiology of ASDs and develop more effective therapies. Through careful behavioral analyses, most of the core characteristics of ASDs, i.e., impairments in social interaction, restlessness and distraction, difficulty with language, repetitive and stereotyped motor behaviors (American Psychiatric Association, 1994) can be modeled in mice (Crawley, 2004, 2007a,b). One of these characteristics, impairments in social interaction, has become the focus of many laboratories evaluating mouse models of ASD. Part of the reason for selecting this behavior is its key role in the diagnosis of ASD and the fact that mice display complex social behaviors. They form hierarchies, establish territories, play, mate and rear their young. They engage in both affiliative (e.g., play, huddling) and aggressive (e.g., biting, tail rattling) social behaviors that can be readily measured with a variety of assays (Crawley, 2007c). Social behavior plays a huge role in the life of the mouse. Obviously reproductive related behaviors in adults are necessary to continue the species. However, social behavior plays an important role in animals not yet of reproductive age and appears to be rewarding in its own right. For instance, juvenile mice display socially conditioned place preference, although the effect is strain dependent (Panksepp et al., 2007; Panksepp and Lahvis, 2007). Unlike other common inbred strains, BALB/cJ mice were less responsive to social reward, which is consistent with low levels of social behavior reported in this strain (Brodkin, 2007).

Social behavior is complex and thus a variety of assays are available for those evaluating mouse model systems. As outlined by Crawley (2007a,c) one can evaluate social behavior by measuring social approach to a stranger mouse, reciprocal social interaction, conditioned place preference to conspecifics, preference for social novelty, social recognition, juvenile play and nesting patterns in the home cage. Several of these assays are currently being used to survey inbred strains of mice and will be described in more detail in this brief review. Assays such as reciprocal social interaction (Bolivar et al., 2007; McFarlane et al., 2008), sociability and social novelty (Nadler et al., 2004; Moy et al., 2007), visible burrow system (Arakawa et al., 2007) and social learning of food preference (McFarlane et al., 2008) measure somewhat different aspects of social behavior. It is important when evaluating mice in these assays to remember the characteristics of the specific assay being used. The reciprocal social interaction and social learning of food preference assays allow both mice in of a pair to make physical contact. While they allow measurement of a plethora of behaviors, including sniffing, biting, chasing, mounting, allogrooming and wrestling, it is sometimes difficult to segregate individual performance from the social behavior of the pair. For instance, although pairs of BTBR T+ tf/J (BTBR) mice do not spend much time engaged in social behavior, when a BTBR mouse is paired with a social FVB/NJ mouse, a great deal

of social behavior (often aggressive) occurs (Bolivar et al., 2007). This finding illustrates the importance of the social environment. In mouse model research it is important to manipulate the social milieu of the animal to determine effects on subsequent social exchanges. For example, exposing individuals from less social strains to peers from more social ones may alter later social behaviors, which would have important implications for ASD. However, it is important to note that pairing of BTBR and FVB/NJ adult males resulted in aggression, which may not be desirable in models related to ASDs. Interactions between these two strains at earlier ages or with repeated exposures might lead to more affiliative social behaviors. Perhaps the pairing of BTBR with more moderately social strains might minimize the aggressive responses and maximize more affiliative ones.

In contrast, the sociability and social novelty assays hold the behavior of the stimulus mouse fairly constant, allowing the test mouse the freedom choice to interact with the other animal or not. As a result, fewer types of social behaviors can be measured in these types of assays. In contrast, the visible burrow system allows for a more naturalistic measurement of social behavior. With several animals tested at the same time, it provides a more detailed picture of social behavior than any of the other assays. Thus, the social milieu of the mice can be readily manipulated. However, here too it can be difficult to segregate the performance of individual animals from other members of the group. Each of these assays has advantages and disadvantages that should be considered before use. Furthermore, the level of experience of the experimenter is paramount. For those inexperienced in social behavior research, the sociability and social novelty assays may be implemented more easily. Ultimately, a combination of assays will provide a more complete picture of social behavior. In addition to a combination of social behavior assays, a general battery of behavioral assays can provide more detailed evaluation of any mouse model. Although one cannot expose the same mouse to an unlimited number of assays, general measures of locomotion, anxiety and cognition will provide a more complete picture of the mouse model.

Testing inbred strains of mice is often a first step in the determination of the underlying genetics, as well as a way to evaluate the effects of environmental factors and therapeutic agents. Inbred strains of mice vary in their levels of social behavior, with some strains consistently less social than others (Bolivar et al., 2007; Moy et al., 2007; McFarlane et al., 2008). A/J (A), BALBcBy/J (CBy) and BTBR consistently display low levels of social behavior in reciprocal social interaction and sociability assays. Therefore, these three strains can be useful for ASD related studies of social behavior. However, in the social learning of food preference assay, only A and BTBR display deficits, CBy does not. Also, the A strain displays low activity and high anxiety-like behavior (Moy et al., 2007) and CBy mice display high anxiety-like behavior (Moy et al., 2007), which could influence social performance. In contrast BTBR mice do not display low activity or high anxiety-like behavior (Moy et al., 2007; McFarlane et al., 2008). Therefore, it is necessary to be cognizant of other characteristics (e.g., sensory, emotional or locomotor) that differ among strains that could influence performance in social behavior assays. This does not imply that one or more of these strains should be excluded as possible mouse model systems for ASDs, rather these data illustrate that one must be aware of other factors that may influence performance on social behavior assays. Some of these other factors may be related to ASDs, whereas others may not. When testing mouse models it is important to evaluate behavioral performance with a variety of assays so that informed judgments can be made about the applicability of the model for ASD research.

3.1. Using population-based mouse models in behavioral and genetic research—David Threadgill

The neurodevelopmental and behavioral characteristics comprising autism spectrum disorders show a continuous distribution among normal individuals. Importantly, individuals

with ASDs are typically at the extreme of the normal distribution observed in human populations. Although heritability studies indicate that autism and ASD are highly heritable, few genes contributing to ASDs have been identified and functionally validated. Similarly, little is known about the underlying biological networks and systems of ASDs that are altered by genetic polymorphisms or perturbed by environmental exposures. In order to better understand the genetic and environmental control of ASD, laboratory mice have become a widely used research tool.

In addition to engineered mutants in specific genes, panels of inbred mouse strains with naturally occurring genetic polymorphisms are now recognized as important models in order to understand the variation in ASDs, and to elucidate the genetic characteristics placing individuals at the extremes of ASDs. Major limitations of extant inbred mouse strains that prevent their robust use for genetic analysis of phenotypes with complex etiologies like ASDs, or the elucidation of underlying biological networks, are their small numbers, relatively low and unequal distribution of polymorphisms and complicated historical breeding relationships. A new model called the Collaborative Cross (CC) was conceived (Threadgill et al., 2002), designed (CTC, 2004) and built (Chesler et al., 2008; Iraqi et al., 2008; Morahan et al., 2008) in order to overcome these limitations.

The CC is a novel recombinant inbred panel of close to 1000 independently bred mouse strains derived from eight parental strains and selected to have maximal genetic diversity. Recent genetic analysis of the CC show that the panel captures almost 90% of all genetic variation present in mice and that this variation is uniformly distributed across the genome (Roberts et al., 2007). This characteristic makes the CC the ideal population-level model to elucidate the genetic control of component characteristics of the ASD.

Recent experimental tests using the CC demonstrate the characteristics of this resource. A large pilot experiment based at the University of North Carolina was designed to phenotypically interrogate almost 200 CC strains for a range of physiological and behavioral characteristics. Relevant to ASDs, results from these studies show that the range of quantitative measures of behavioral characteristics, like sociability (ranging from highly anti-social with characteristics of aggression or avoidance to highly social) and voluntary activity (ranging from little exercise to running over 17 km each night on a running wheel), far exceed the range observed in extant mouse resources. More importantly, the measures are uniformly distributed along a continuum and with many strains at the extremes; these strains are candidates for models of individuals with ASD. Further analysis of the CC, with a more in-depth focus on ASD, should support the acquisition of new insights into the genetic causes of the component characteristics of ASD, and the identification of biological networks supporting manifestation of ASD, ultimately leading to new interventions that alter the severity or developmental course of ASD.

3.2. Primate models of autism and determination of immunological mechanisms—David Amaral

Evidence has been accumulating for over 20 years suggesting that immune factors may play a role in the etiology of some forms of autism (Warren et al., 1986, 1996; Ashwood and Van de Water, 2004a, b). Judy Van de Water, Paul Ashwood and colleagues at the M.I.N.D. Institute have conducted comprehensive evaluations of children with autism and their parents to determine what, if any, perturbations of immune function are characteristic of the disorder. Part of this effort has been to evaluate these individuals for evidence of autoimmunity.

Plasma samples from children with autism have been evaluated both with western blot (using adult human cerebellum as protein source) and tissue sections through the cerebellum

of macaque monkeys. Sharifia Wills has found that 21% of children with autism demonstrate antibodies to a 52 kDa band of protein on western blot (Wills et al., 2009). When plasma from the same children is reacted with tissue sections through the cerebellum, specific immunoreactivity is seen for the Golgi neuron and, to a lesser extent, to the basket cells of the molecular layer. Similar labeling is not seen with plasma from typically developing children or from children with developmental delays. Currently, we are evaluating other brain areas to determine the common features of neurons identified by these plasma samples. It currently appears that only GABAergic neurons are labeled, but throughout diverse brain regions. This reveals some of the neurobiological mechanisms behind the antibody reactivity in children with autism.

More germane to the symposium, the plasma of mothers who gave birth to children with autism has also been evaluated. In this case, fetal brain tissue is used as the protein source for the Western blot analyses. Approximately 12% of these samples show IgG immunoreactivity to proteins bands at approximately 37 and 73 kDa (Braunschweig et al., 2008). Since women who gave birth to typically developing children do not have these autoantibodies, it raised the hypothesis that perhaps these unusual IgG cross the placenta, perturb brain development and ultimately lead to autism. To test this, we purified IgG from women who gave birth to children with autism, as well as others who gave birth to typically developing children. These were administered to pregnant rhesus monkeys during the transition from the first to the second trimester of gestation. After birth, the infants were evaluated on a number of neurological and behavioral features (Martin et al., 2008). While there was a subtle decrease in the sociality of the animals treated with IgG from mothers of autistic children, the most striking finding was that these animals engaged in significantly increased levels of whole body stereotypies across several behavioral settings. The following video is taken from the Martin et al. (2008) paper: (http://www.sciencedirect.com/science/MiamiMultiMediaURL/B6WC1-4RSRPYC-1/B6WC1-4RSRPYC-1-F/6725/fe42bc1c9ce2256956c2f63f9c7bf2db/mmc5.mpg). In this clip, there are two young rhesus monkeys in this video. One is a control animal. The other is the offspring of a mother who was exposed during pregnancy to an IgG cocktail obtained from mothers of children with autism. The animals are in a social enclosure that is used for evaluating dyadic social interactions. What is striking in this video is that the IgG treated animal moves into the corner of the enclosure and repeatedly engages in a back flipping behavior. This stereotyped behavior occasionally continued for many minutes at a time. This type of repetitive behavior has not been seen with the control animals or with other rhesus monkeys that were prepared for other experiments with neurosurgical lesions of brain regions such as the amygdala or hippocampus.

Increased stereotypies were not seen in the animals treated with IgG from mothers of typically developing children. We are in the process of replicating and extending these findings. If they are borne out, they would provide strong evidence that manipulations of the maternal environment during pregnancy could have profound effects on the development of the brain, leading to one or more neurodevelopmental disorders.

3.3. Genetic and environmental interactions in the Reeler Mouse–Flavio Keller

A simple model of the respective roles of genes and environment in behavioral disorders, in particular of ASDs, holds that the environment can either facilitate or mask an underlying genetic vulnerability. However, there is increasing evidence that this simple model is insufficient. For example, depending on genetic endowment, normally deleterious environmental exposures can actually be protective. This, and other observations, reveal an unexpected complexity of gene × environment interactions.

For example, Laviola et al. (2006) have examined the behavioral alterations displayed by wild-type, rl/+ and rl/rl mice, expressing, respectively 100%, 50%, or zero levels of reelin, exposed during gestation to the organophosphorous pesticide chlorpyrifos, an acetylcholinesterase inhibitor causing permanent biochemical and behavioral alterations following fetal or neonatal exposure (see, e.g. Slotkin and Seidler, 2007). Reelin is an extracellular matrix protein that plays a key role in guiding the migration of embryonic neurons to their final destinations, especially in layered structures such as the cerebral cortex and the cerebellum. The observed effects do not conform to a simple gene × environment model, since chlorpyrifos-exposed rl/+ and even rl/rl mice showed a better performance than their untreated littermates in some tests (Laviola et al., 2006, 2009; see Table 2). These puzzling results can now be explained by subsequent findings that rl/+ (and also rl/rl) mice are affected by a disarrangement of the basal forebrain cholinergic projection to the cerebral cortex (Sigala et al., 2007). Therefore, chlorpyrifos may partially reverse deficient cholinergic transmission, thus restoring the morphogenetic effect of acetylcholine. In other experiments, we have shown that perinatal estradiol levels in the mouse cerebellum profoundly affect the number of Purkinje cells depending on reelin expression levels and also on the gender of the animal (Biamonte et al., 2009), pointing to important interactions between genetic vulnerability and sex hormones in ASDs. Also, increasing or decreasing the levels of estradiol in the neonatal cerebellum permanently affects emotional and cognitive functioning of mice during early postnatal and adult life (Laviola et al., 2009). These, and other observations, suggest that a new, complex equation involving the genetic makeup with the environmental exposures with the species of organism interaction should be considered in ASDs. There is no doubt that this type of non-deterministic gene × environment × organism will add a new layer of complexity to ASD.

The fetal and neonatal environments contribute to permanent sculpting of neural circuits and to behavioral phenotypes to a much larger degree than previously thought. The mechanisms by which this happens include local random fluctuations of physical and chemical conditions in the fetus, which are then transformed into permanent patterns by genetic mechanisms that alter gene expression. The crucial role of such “developmental noise” in morphogenesis has been argued convincingly by Lewontin (2000). One interesting example of developmental noise is fingerprints in identical twins, which are more similar than those of non-identical twins, but are by far not identical (Jain et al., 2002). The reason is that the pattern of fingerprints is generated by the local shear forces exerted by the flow of amniotic fluid around the finger cusps during the earliest stages of finger development (in a similar way as water flowing on sand creates and undulated pattern in the sand); this unstable pattern of crests and valleys is then translated into a permanent pattern by the genetic mechanisms driving epidermal cell migration and differentiation.

Epigenetic mechanisms that could transform environmental influences into stable gene expression patterns, and therefore permanently affect brain circuits, include histone acetylation, DNA methylation, activity-dependent regulation of transcription factors (including genes related to autism mentioned earlier such as CREB, MECP2), environment-induced variations of neurotransmitters/neuromodulators that influence neurogenesis and neuronal migration, and also variations of hormonal levels induced by different environment stimuli, including social interactions. For example, it has been shown recently that estradiol levels in song nuclei of adult male songbirds vary with a rapid time course following exposure to socially relevant stimuli (Remage-Healey et al., 2008). Such rapid variations of estrogen levels could contribute to shape also the developing brain. In relation to this, endocrine disruptors could be additional environmental factors to be taken into consideration for neurodevelopmental disorders.

3.4. Using in vitro models to study gene-environment interactions in autism—Pamela Lein

The risk, severity and treatment outcome in ASDs is determined not only by complex interactions between genes, but also gene–environment interactions. Therefore, there is significant interest in identifying and characterizing epigenetic and environmental risk factors for ASDs. Using animal models to screen for relevant gene-environment interactions would involve an inordinate investment of time, labor and animals (Lein et al., 2005). Emerging evidence suggests that the behavioral deficits that define ASDs arise from perturbations of structural and functional neuronal connectivity during development (Zoghbi, 2003; Pardo and Eberhart, 2007). In light of this evidence, *in vitro* models that recapitulate the neurodevelopmental events determining neuronal connectivity, specifically the projection of axons to targets, the extension and elaboration of dendritic arbors, and the formation of synapses, may prove to be powerful tools for rapidly identifying candidate environmental risk factors for further evaluation in animal models. A number of well-defined *in vitro* models were developed for assessing these neurodevelopmental endpoints. These models employ neurons derived from brain regions implicated in ASDs, including the neocortex, the hippocampus and the cerebellum, and range from simple models consisting of dissociated neurons grown in the absence of other cell types, to more complex models such as organotypic slice cultures that retain many of the cell–cell interactions observed *in situ*. In addition to their ability to faithfully replicate discrete stages of neurodevelopment of direct relevance to ASDs, major advantages of using *in vitro* models to screen for candidate environmental risk factors for ASDs include: (a) their simplicity relative to animal models, which enables rapid screening and detection even of subtle changes in neurodevelopmental endpoints and (b) the ability to readily manipulate and monitor gene expression, which allows the integration of molecular data with structural and functional changes in neurodevelopment and facilitates the incorporation of relevant genetic polymorphisms into the model. Challenges, or caveats, of using *in vitro* models include the difficulty of incorporating extraneural factors that may influence the effect of environmental risk factors on neurodevelopment and have been implicated in ASDs such as metabolism, hormonal influence and immunological function. Approaches that could be used to mitigate these challenges were discussed.

In ongoing studies, we have identified three different classes of environmental factors that modulate neuronal connectivity in primary cultures of hippocampal neurons. The first class, non-coplanar polychlorinated biphenyls (PCBs), enhance dendritic growth in quiescent cultures but inhibit activity-dependent dendritic growth at nanomolar concentrations. Similar effects are observed *in situ* in animals exposed to environmentally relevant levels of PCBs in the maternal diet throughout gestation and lactation (Yang et al., 2009). Our data indicate that PCB effects on ryanodine receptor (RyR) expression and function contribute to the effects of PCBs on dendritic growth and plasticity (Yang et al., 2009). RyRs function in neurons to regulate calcium-dependent intracellular signaling pathways (Berridge, 2006), and recent genetic studies implicate genes that encode Ca^{2+}-regulated signaling proteins involved in synapse formation and dendritic growth in ASDs (Krey and Dolmetsch, 2007). Therefore, our data identify non-coplanar PCBs as candidate environmental risk factors in ASD and suggest the possibility that exposure even to 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. In contrast, the second class of environmental factors we are studying, the pro-inflammatory cytokines, interferon-γ and interleukin (IL)-6 decrease dendritic arborization and synapse formation in cultured hippocampal neurons (Kim et al., 2002). Our preliminary data suggest that at least interferon-γ modulates dendritic growth and synaptic density similarly *in situ*. Interestingly, these cytokines are elevated in the serum and cerebrospinal fluid of ASD patients (Ciaranello and Ciaranello, 1995). Our data suggest that these increased levels may not be coincidental or consequential

but rather may contribute to the pathogenesis of ASDs, and raise the possibility that conditions promoting the expression of these pro-inflammatory factors interact with genetic susceptibilities that converge on similar neurodevelopmental endpoints. Third, we are investigating organophosphorus pesticides (OPs), which we have shown inhibit axonal growth in developing neurons by interfering with the morphogenic activity of acetylcholinesterase (Yang et al., 2008). The morphogenic domain of acetylcholinesterase shares striking sequence and structural homology with the extracellular domain of neuronal adhesion molecules of the serine esterase family, which includes neuroligin (Graf et al., 2004; Dean and Dresbach, 2006), a gene that is linked to ASD (Dean and Dresbach, 2006). Neuroligins have emerged as potent inducers of synapse formation between central nervous system neurons (Graf et al., 2004; Chubykin et al., 2005; Dean and Dresbach, 2006; Crawley, 2007b; Garber, 2007) and our preliminary data suggest that OPs may inhibit the synaptogenic activity of neuroligins in cultured hippocampal neurons. If these preliminary observations hold up in subsequent testing, it would provide a biological mechanism to support epidemiological evidence linking developmental OP exposures to ASD (D'Amelio et al., 2005). In summary, these data suggest that *in vitro* models of neuronal connectivity in developing neurons are predictive of effects *in situ*, and may prove to be an efficient tool for screening environmental factors in order to identify those with the greatest potential for adverse neurodevelopmental outcomes of relevance to ASD for further testing in animal models.

3.5. Animal and culture models to study the autism-associated patterning gene of the cerebellum, Engrailed 2 (EN2)—Emanuel Dicicco-Bloom

The human cerebellar patterning gene, *EN2*, was shown to be associated with autism spectrum disorders (ASD) by 5 independent laboratories in 8 datasets, making it one of the few susceptibility loci to achieve genetic replication. In mice, genetic deletion, as well as over-expression of *En2*, produces cerebellar abnormalities, including Purkinje neuron deficits and abnormal posterior lobule morphogenesis, that phenocopy some of the human neuropathology and brain imaging. Additional studies demonstrate behavioral deficits in social and motor tasks. *En2* is expressed in multiple cell types in the hindbrain from mid-gestation toward birth in complex patterns of expression that impact cerebellar circuits. Postnatally, the gene is expressed exclusively in cerebellar granule neurons and remains active throughout life. Our studies have focused on the role of *En2* in cerebellar developmental neurogenesis and differentiation, as well as the consequences of gene deletion (knockout, KO) on development of hindbrain monoamine neurotransmitter systems, including norepinephrine (NE) and serotonin (5HT), that project to the forebrain.

Our recent studies have focused initially on the postnatal cerebellum for several reasons including (1) *En2* is expressed specifically in granule neuron precursors allowing us to distinguish cell autonomous effects from those dependent on cell–cell (non-cell autonomous) interactions, (2) the developmental stages of cerebellar granule neurogenesis in vivo are highly well-characterized so that one can draw conclusions about the impact of *En2* on specific cellular events by localizing its expression to specific cerebellar layers, (3) the granule neuron precursors that express *En2* can be isolated in high purity, excluding effects due to contaminating non-neuronal cells, (4) interactions between *En2* and environmental signals can be explored because the developmental effects of extracellular mitogenic and differentiative signals has been well-defined, and (5) techniques for gene overexpression using transfection methods have been fully implemented.

In vivo, the absence of *En2* expression in KO mice leads to increased granule neuron proliferation in postnatal day 7 (P7) cerebellum. In culture, granule neuron precursors from KO mice exhibited enhanced proliferation and increased mitogenic response to IGF1, as well as diminished neurite outgrowth, indicating that the cells remained as precursors in the

absence of the patterning gene. Significantly, IGF1 also elicited increased mitosis in P7 cerebellum in vivo. Conversely, *En2* over-expression reduced precursor proliferation and increased neurite outgrowth, consistent with a role in regulating the transition from proliferation to differentiation in granule neurogenesis. Additional studies indicate specific functional interactions between *En2* and IGF1 that depend on the second messenger, S6 Kinase. Since *En2* is expressed throughout the hindbrain prenatally during embryonic production of NE and 5HT neurons, we examined effects on these transmitters and their growth forward into forebrain targets. In sum, in KO mice, both NE and 5HT are dysregulated, with local increases in the hindbrain and deficits in the forebrain targets, including a 50% reduction in NE in the hippocampus and deficits in amygdala. Monoamine transmitters are critical for normal control of behaviors associated with schizophrenia, depression and ASD. This further supports the role of *En2* mutations in the mouse as a framework to investigate gene × environment interactions in autism.

Based on these findings, we have begun to examine the effects of environmental factors, including methylmercury and TCDD. Methylmercury, a ubiquitous environmental neurotoxicant, is being studied because moderate exposures that exceed dietary levels are teratogenic for hippocampal neurogenesis (Burke et al., 2006; Falluel-Morel et al., 2007). These studies are identifying more sensitive measures of neurogenetic effects at far lower exposures than reported in previous neuropathological findings. The dioxin TCDD, whose putative receptor is expressed in brain regions including cerebellum during neurogenesis, is also being targeted because preliminary studies indicate that En2 KO cells are more sensitive to the neurodevelopmental effects of TCDD than the wild type strain. These results raise the possibility of an important genetic and environmental interaction in cellular development.

3.6. Non-coplanar environmental chemicals that target calcium channels: structure–activity relationships and implications for autism—Isaac Pessah

Ryanodine receptor (RyR) isoforms are expressed in both excitable and non-excitable tissues where they form highly regulated microsomal Ca^{2+} channels. RyR isoforms are broadly involved in shaping cellular signals by coupling the release of Ca^{2+} from ER/SR stores to voltage, ligand and store-operated Ca^{2+} channels of the plasma membrane. A detailed structure–activity relationship (SAR) for polychlorinated biphenyls (PCB) for enhancing RyR activity was presented using [^{3}H]ryanodine ([^{3}H]Ry) binding, Ca^{2+} flux, and single channel gating analyses. The 2,3,6-Cl PCB configuration is most important for optimal activation of RyR, whereas *para*-substitutions sterically hinder or eliminate RyR activity (Pessah et al., 2006). Separation of chiral PCB136 demonstrates stereospecificity toward RyR1 and RyR2 activity (Pessah et al., 2009). The molecular mechanism by which (−)-PCB 136 activates RyR stabilizes the full conductance open state of the channel, prolonging mean open time >8-fold, and decreasing mean close time >2.5-fold, whereas (+)-PCB 136 (≤10 mM) lacks RyR activity (Pessah et al., 2009). Developmental exposure during gestation and lactation to a PCB mixture significantly alters the functional state and level of expression of RyRs within the central nervous system of weanling rats (Yang et al., 2008; Roegge et al., 2006). These effects are associated with deficits in experience-dependent dendritic plasticity (Yang et al., 2008), altered motor activity (Roegge et al., 2006), and altered tonotopy and synaptic plasticity of the primary auditory cortex in exposed rats (Kenet et al., 2007). Results from SAR studies with PCBs led us to investigate if other non-coplanar structures to which humans are highly exposed also influence microsomal Ca^{2+} signaling by sensitizing RyR activation.

Polybrominated diphenyl ethers (PBDEs) are widely used as flame retardants in consumer products. Our recent studies indicate that BDE4 (2,2′-diphenyl ether) is a potent activator of RyR1 and RyR2, whereas *para*-substituted BDE15 (4,4′-diphenyl ether) and unsubstituted

diphenyl ether are inactive. More highly brominated diphenyl ethers indicate there is a stringent SAR toward RyR isoforms that is highly dependent on the composition of the *meta* and *para* substituent (unpublished data).

Finally, the widely used antibacterial triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether), possesses potent activity toward dysregulating basal and evoked Ca^{2+} signaling mediated by RyR activation in excitable cells (Ahn et al., 2008). Also discussed was how triclosan influences cellular Ca^{2+} signaling and its relevance to the overall risks of exposures to non-coplanar persistent organic pollutants.

These results were discussed in light of the high degree of specificity of non-coplanar environmental chemicals toward RyR complexes. RyRs are expressed broadly in both excitable (e.g., skeletal and cardiac muscle, and neurons) as well and non-excitable cells (e.g., dendritic cells), where they produce essential Ca^{2+} signaling microdomains. 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, secretion, and death. Mutations in RyR1 and RyR2 are known to contribute to an increasing number of environmentally triggered susceptibilities, including malignant hyperthermia (Zhou Allen et al., 2008), central core disease (CCD) (Treves et al., 2008), and catecholaminergic ventricular tachycardia (CPVT) (Knollmann and Roden, 2008). Abnormal structural organization and function of the RyR complex within specific regions of the central nervous system have been implicated in a form of familial Alzheimer's disease (Thibault et al., 2007). Although none of the three *RyR* isoforms have been associated with ASDs, 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 (Table 3). This raises several important, unanswered questions regarding the possibility that children at risk for ASD and related developmental disorders represent a genetically diverse group of children that may have heightened susceptibility to chemicals that perturb Ca^{2+} signaling events generated by RyRs and their associated proteins. In this regard, the possible additive effects of non-coplanar chemicals (PCBs, PBDEs, triclosan, etc.) mediating their effects through RyRs and ASD genes may represent a convergence of genetic and environmental interactions that influence risk and severity.

3.7. Use of Drosophila melanogaster (the fruit fly) as a neurogenetic model system for drug and neurotoxicity screening—Linda Restifo

The “fly system” is very powerful for studying genetic disease pathogenesis (Bier, 2005), and has been used increasingly for drug discovery (Tickoo and Russell, 2002; Nichols, 2006). The justification for using fruit flies to study the biology of neurobehavioral disorders comes from the extraordinary phylogenetic conservation of human genes essential for normal cognitive function (Inlow and Restifo, 2004; Restifo, 2005) as well as the availability of well-established tools for behavior genetics (Vosshall, 2007). While the rich history of learning-and-memory mutants in Drosophila (Waddell and Quinn, 2001) makes it easy to conceive of flies with “mental retardation,” can the same conclusion be made regarding autism, for which language delay is a cardinal feature? In a large, ongoing twin study in the U.K., comparison of parents and offspring revealed that the three diagnostic criteria for autism are under independent genetic control, not inherited en-bloc (Ronald et al., 2006). This opens the door to study Drosophila of two autism phenotypes, stereotyped repetitive behavior and impaired social interaction.

One of the biggest contributions of the Drosophila system to autism research will likely be in the area of gene × environment interaction. Consider, for example, fragile X syndrome (FXS; Bardoni et al., 2000). This is a prototypical single-gene disorder, yet, at the phenotypic level, there is a wide range of cognitive disabilities (variable expressivity) and

autism appears in a minority, albeit a substantial one, of affected individuals (incomplete penetrance) (Loesch et al., 2007). This phenotypic variation might be explained by environmental exposures if, for example, the brains of FXS patients are unusually sensitive to developmental neurotoxins. Data from Drosophila are consistent with this view. Mutations in the corresponding Drosophila fragile X gene, dfmr1, cause a specific brain-development defect (Michel et al., 2004) and impaired memory, both of which can be rescued by pharmacological blockade of mGluR during development (McBride et al., 2005). However, the severity of the brain morphological defect is highly associated with the concentration of glutamate in the diet (Chang et al., 2008).

The rationale for using the fly system for drug discovery and testing comes from considering the synergy that emerges from combining neurogenetics with primary cell culture. Restifo and colleagues developed an in vitro cellular bioassay using primary neuron cultures (Kraft et al., 2006) to screen for drugs that either normalize or worsen a mutant neuronal morphogenesis phenotype. In a recently completed proof-of-concept drug screen, several dozen known drugs in each category were identified. Based on the nature of the mutation (causing fascin deficiency) and the design of the screen, each set of drugs could be beneficial to patients with a different medical condition. Drugs that rescue the phenotype are predicted to benefit children with a subset of developmental brain disorders. In addition, a wide variety of dose-dependent neurotoxic drug effects was detected. In some cases, drugs were cytocidal, either before or after a neurite arbor had been elaborated. In other cases, the size or shape of the neurite arbors were altered. Most striking were specific drug-induced changes in neuronal morphology, affecting the cell body and/or the neurites. Software, including NeuronMetrics™ (Narro et al., 2007), can quantify these neurotoxic effects to determine dose–response curves. Using available genetic tools for cell biology research, this system can be used to identify the mechanisms underlying drug-induced developmental neurotoxicity. Furthermore, primary neuron culture can enhance the study of genetic and environmental interaction studies to determine the role of genetic background in controlling susceptibility to neurotoxins, especially in a developmental context.

4. Summary and discussion

A panel discussion ensued on the feasibility of animal or alternative models to provide insight on the etiology, cellular and molecular mechanisms, and/or treatment and prevention of autism. No single biological or clinical marker seems to be predictable for the diagnosis, so the task at hand will continue to present a challenge.

Panel members alluded to several promising candidate loci that contribute to autism susceptibility; however, in general, the critical identity of the genes remains unknown. Furthermore, it was suggested that epistatic interactions of an unknown number of susceptibility genes or gene variants likely lead to the very specific disease phenotypes in humans and animal model systems. Consideration of evolutionary conservation of sequence similarity and gene structure between humans and model species will be required. In the future, studies could be directed profitably at establishing and evaluating animal models with humanized gene variants or mutations based on available data from human autistic patient studies. Such work has begun using material from postmortem tissue in frog oocytes (Limon et al., 2008). However, these manipulations will challenge the evolutionary conservation of sequence similarity, gene structure, as well as physiological differences between humans and animal models.

A discussion also arose on the ability of behaviors in non-primate vertebrates and invertebrate animal models to recapitulate some of the symptoms associated with autism. It was pointed out that some stereotypic behaviors may generally be inherent to multiple

neurocognitive disorders, not contributing solely to the autistic phenotype. Furthermore, it was argued that, in most cases, it has yet to be proven that behaviors in non-primate vertebrate and invertebrate animal models have similar mechanistic underpinnings analogous to those that drive similar behaviors in humans. Given that non-genetic mechanisms are also likely contribute to the autistic syndrome, several panel members expressed some reservation on the ability of invertebrate platforms to recapitulate the human syndrome, contending that modeling autistic disorder in animals will remain a challenging task.

Promising rodent autism models are available, as was abundantly evident throughout the day's presentations. These commonly target a single candidate gene to examine gene function using cellular and morphological endpoints of relevance to neurodevelopment, as well as some behavioral measures. It was mentioned that future studies modeling autism and its behavior in alternative or complimentary animal platforms will require more sophisticated diagnostics to simplify endophenotypes thus allowing the identification of quantitative trait loci and ultimately specific genes in these models. It will also be necessary to better connect behavioral measures in these models to underlying pathological pathway dysfunction, with each of them inheriting several diverse susceptibility genes contributing to major or minor effects along the autism disorder phenotype spectrum. A necessary contribution of animal models will be the development of treatments that show clinical efficacy in humans to demonstrate predictive validity in animal models. Likewise, use of animal models is an important component in developing or adapting pharmacotherapy and behavioral adaptations to improve abnormal behaviors in children with autism. With regards to autism, it would be interesting to see if behavioral interventions in these models produce neurochemical, neuroanatomical, or other neurobiological changes which may help explain improvements in behavior. Such work has been done using environmental enrichment in genetically modified mice (Nag et al., 2009a,b) and those with pharmacological manipulation of stereotyped behavior in mice (Lewis et al., 2007; Schneider et al., 2006). In fact, behavioral manipulations using altered environments in many different settings have been found to be the most productive form of treatment for children and adolescents suffering from autism. These include applied behavioral analysis, sensory-motor interventions, social skills development interactions, and early behavioral intervention (prior to age 3) which focuses on the core symptoms of autism while also individualizing treatments from child to child. In this way, animal models may be somewhat limited in their ability to provide efficacy data on such a heterogeneous disorder, on the other hand, animal models can also shed light on neurobiological processes unable to be seen in the human population. Studies on biological markers *in response to treatment* in children with autism, including but not limited to specific brainwave activity and function, are ongoing and obviously we look forward to the results of such findings.

Another topic for discussion focused on the use of pluripotent stem cells as a possible model for future use. While it was recognized that autism is a behaviorally diagnosed disorder and emphasis should be placed on behavioral endpoints, it was also acknowledged that as biomarkers are discovered, different biomaterials may be used to define the neurobiology of the disease better. Recently, three labs, headed by Shinya Yamanaka (Kyoto Univ.), George Daley (Harvard Med. School), and James Thomson (Univ. of Wisconsin-Madison), independently reported the generation of pluripotent stem cells from adult human dermal cells with the developmental potential seemingly equivalent to human embryonic stem cells. These iPS (induced pluripotent stem) cells were found to be competent enough to generate all three major cell lineages of animals and resemble embryonic stem cells in their pluripotency and other important characteristics. Of relevance to studies of autism, human iPS cells can be differentiated into neurons and glia. The ability to generate iPS cells from human patients is anticipated to open up a new frontier of cell-based research into human

diseases such as autism. In the future, iPS cells from autistic and ASD patients and controls may provide new insights into the contribution of environmental toxicants to autism. For example, the ability to examine neurons and glia that have the complete genetic composition of individual patients offers the possibility to examine differences in the sensitivity of developmental processes to specific environmental agents between autistic and normal individuals. Armed with a complete clinical history of the patients, iPS cell studies can compare patient populations showing sensitivity to particular environmental agents. Currently, neural progenitor cells obtained from postmortem tissue with autism and IDIC-15 are available through the National Neural Stem Cell Resource in collaboration with Philip Schwartz (Children's Hospital of Orange County). In summary, more advanced studies using multiple species will be needed to identify the function of genetic variants, isolate gene × environ-environment interactions relevant to autism, and ultimately test new treatments that may be effective for some, if not all, symptoms. A similar symposium, organized by Jackie Crawley from the University of North Carolina in conjunction with the International Meeting for Autism Research in May of 2008, concluded that incorporation of clinicians into the discussion of behavioral phenotypes in animal models is extremely important. Further refinement of behavioral assays relevant to autism will facilitate research into gene–environment interactions, as well as preclinical treatment. It was concluded from this and previous meetings that animal models must adhere to face, construct, and predictive validity, demonstrating that both environmental agents and novel treatment paradigms may strengthen the model and support use in autism research. Observation of animal behavior should be performed in conjunction with molecular assays in order to help identify potential neurobiological mechanisms, so that targets of neurotoxins, chemicals, psychosocial stressors and other and environmental agents are identified for future study.

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Table 1

Sample behaviors measurable in both humans and animals that may represent discrete features of an ASD.

Behavior	Measurement in humans	Measurement in animals
(A) Social reciprocity		
Prepulse inhibition	Levin et al. (2009)	Smith et al. (2007)
Social avoidance	Budimirovic et al. (2006)	Moy et al. (2008)
Social affiliation/anxiety	Hoge et al. (2008)	Crawley et al. (2007)
(B) Communicative intent		
Olfactory function	Bennetto et al. (2007)	Radyushkin et al. (2009)
Atypical communication	Wang et al. (2006)	Scattoni et al. (2008)
(C) Habitual behaviors		
Stereotyped behavior	Lam et al. (2008)	Martin et al., 2008
Increased anxiety response	Corbett et al. (2009)	Benno et al. (2009)
Hyperacusis	Orekhova et al. (2009)	Chen and Toth (2001)
(D) Other associations		
ADHD symptoms	Fine et al. (2008)	Martin et al. (2008)

Table 2

Synopsis of the significant effects of genotype × prenatal-treatment interaction.

Genotype	**RL**		**HZ**		**WT**	
Prenatal treatment	**VEH**	**CPF**	**VEH**	**CPF**	**VEH**	**CPF**
Body weight	↓↓	↑↑	↓	↑	=	=
Ultrasonic call emission (PND 7)	↓↓	↑↑	↓	↑	=	=
Grasping reflex (PND 3)	=	=	=	↑	=	↑↑
Righting latency, shortest (PND 3)	↑	=	↑	=	=	=
Righting latency, longest (PND 7, 11)	=	↑↑	=	=	=	=
Scopolamine-induced hyper-locomotion (adults)	=	↑	=	↑	=	=
Amphetamine-induced hyper-locomotion (adults)	↓↓	↑↑	↓	=	=	↓
Amphetamine-induced behavioral stereotypy (adults)	↑↑	↓↓	↑	=	=	↑

↑ increased or ↓ decreased when compared to WT, within the VEH-pretreatment group ↑ increased or ↓ decreased in the CPF-versus VEH-pretreatment, within each genotype (reprinted with permission from Behavioral Brain Research).

Table 3

Examples of Ca^{2+} regulating and Ca^{2+}-regulated genes linked to autism.

Gene (map)	Function	Mutation (dysfunction)
CAC NA1C (12p1 3.3)	L-type voltage-dependent Ca^{2+} channel (CaV1.2)	G406R-delayed inactivation (Timothy syndrome)
CAC NA1H (16p1 3.3)	T-type voltage-dependent Ca^{2+} channel (CaV3.2)	R212C; R90 2W, W962C, A1874V-altered activation (autism)
SLC2 5A12 (2q24)	Ca^{2+}-dependent mitochondrial aspartate/glutamate carrier	SNPs (autism)
KCNMA1 (10q22.3)	Ca^{2+}-activated K^+ channel ($BK_{Ca^{2+}}$)	Balanced 9q23/10q22 translocation
PTEN (10q23.3)	Ca^{2+}-regulated PI-3-phosphatase; regulates CaV1.2	H93R, D252G, F241S (macrocephaly; autism)
MECP2 (Xq28)	DNA methylation (Ca^{2+}-dependent phosphorylation)	Down regulated/mutations-altered DNA methylation (autism, RETT syndrome)
MET (7q2 1.1)	Tyrosine receptor kinase for hepatocyte growth factor	Polymorphism-down regulation (autism)
CADP S2 (7q31-q32)	Ca^{2+}-dependent activator protein for secretion	Aberrant alternative splicing lacks exon 3 (autism)
NL-1; NL-3 3q26.31; Xq1 3.1	Neuroligin synapse formation/function EF-hands	NL-1 R47 6C (autism) NL-3 R471 C (autism)
Reelin/APOE (7q22)	Extracellular matrix/RTK signaling	Association-genome wide scan heterozygous Reeler Mouse