Title

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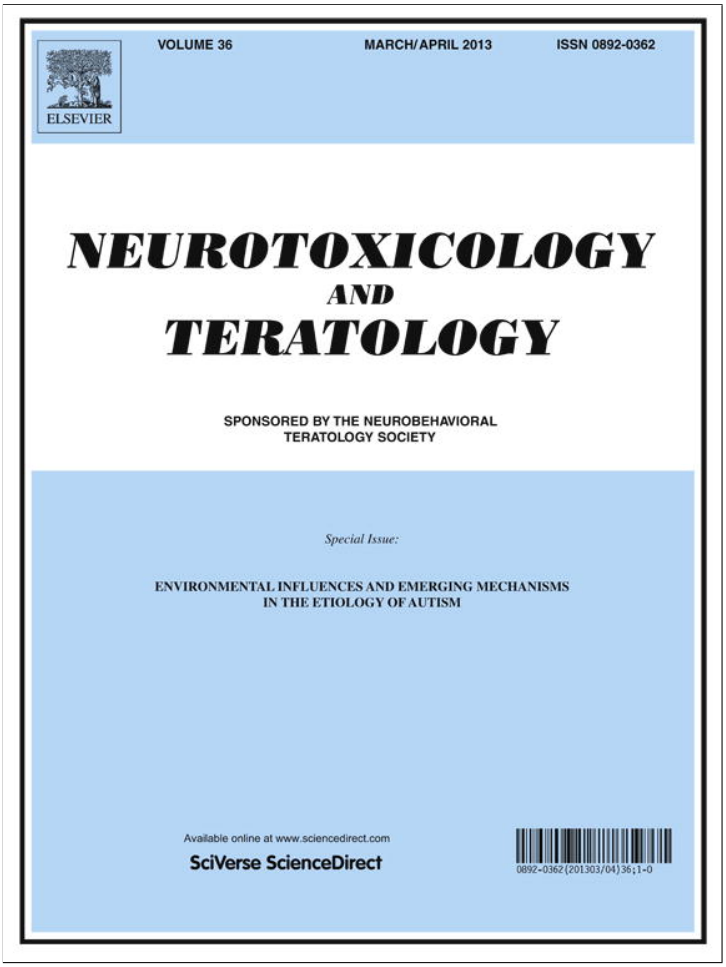
Publication Date

2013-03-01

DOI

10.1016/j.ntt.2012.12.001

Peer reviewed



Neurotoxicology and Teratology 36 (2013) 3–16

Contents lists available at SciVerse ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

Review article

Neuronal connectivity as a convergent target of gene × environment interactions that confer risk for Autism Spectrum Disorders

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ARTICLE INFO

Article history:
Received 20 August 2012
Received in revised form 12 November 2012
Accepted 17 December 2012
Available online 23 December 2012

Keywords:
Autism Spectrum Disorders
Gene–environment interactions
Neuronal connectivity
Organophosphorus pesticides
Polyaromatic hydrocarbons
Polychlorinated biphenyls

ABSTRACT

Evidence implicates environmental factors in the pathogenesis of Autism Spectrum Disorders (ASD). However, the identity of specific environmental chemicals that influence ASD risk, severity or treatment outcome remains elusive. The impact of any given environmental exposure likely varies across a population according to individual genetic substrates, and this increases the difficulty of identifying clear associations between exposure and ASD diagnoses. Heritable genetic vulnerabilities may amplify adverse effects triggered by environmental exposures if genetic and environmental factors converge to dysregulate the same signaling systems at critical times of development. Thus, one strategy for identifying environmental risk factors for ASD is to screen for environmental factors that modulate the same signaling pathways as ASD susceptibility genes. Recent advances in defining the molecular and cellular pathology of ASD point to altered patterns of neuronal connectivity in the developing brain as the neurobiological basis of these disorders. Studies of syndromic ASD and rare highly penetrant mutations or CNVs in ASD suggest that ASD risk genes converge on several major signaling pathways linked to altered neuronal connectivity in the developing brain. This review briefly summarizes the evidence implicating dysfunctional signaling *via* Ca^{2+}-dependent mechanisms, extracellular signal-regulated kinases (ERK)/phosphatidylinositol-3-kinases (PI3K) and neuroligin–neurexin–SHANK as convergent molecular mechanisms in ASD, and then discusses examples of environmental chemicals for which there is emerging evidence of their potential to interfere with normal neuronal connectivity *via* perturbation of these signaling pathways.

Contents

Abbreviations: AChE, acetylcholinesterase; A1254, Aroclor 1254; ASD, Autism Spectrum Disorders; B(α)P, benzo(α)pyrene; BDNF, brain-derived neurotrophic factor; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CAPS2, calcium-dependent activator protein for secretion 2; CNV, copy number variation; CDC, Centers for Disease Control and Prevention; CNTNAP2, contactin-associated protein-2; CREB, cAMP response element binding protein; CRU, Ca^{2+} release unit; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; fMRI, functional magnetic resonance imaging; FMR1, Fragile X mental retardation 1; GABA, γ-aminobutyric acid; HGF, hepatocyte growth factor; iPSC, induced pluripotent stem cells; MET RTK, MET receptor tyrosine kinase; MRI, magnetic resonance imaging; mTOR, mammalian target of rapamycin; NDL, non-dioxin-like; OPs, organophosphorus pesticides; PAHs, polyaromatic hydrocarbons; PBDEs, polybrominated diphenyl ethers; PCBs, polychlorinated biphenyls; PI3K, phosphatidylinositol-3-kinase; PON-1, paraoxonase-1; RTK, receptor tyrosine kinase; RYR, ryanodine receptor; SHANK, SH3 and multiple ankyrin repeat domain proteins; Trk, tyrosine kinase receptor.

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http://dx.doi.org/10.1016/j.ntt.2012.12.001

1. Introduction

Autism Spectrum Disorders (ASD) is a group of neurodevelopmental disorders defined by core deficits in social reciprocity and communication, restrictive interests and repetitive behaviors. The severity of core symptoms and co-morbidities (mental retardation, seizures, gastrointestinal symptoms) as well as the response to treatment varies considerably, and this clinical heterogeneity is a hallmark characteristic of these disorders (Geschwind, 2009; Geschwind and Levitt, 2007; Nazeer and Ghaziuddin, 2012). ASD continues to increase at an alarming rate with the most recent statistics released by the Centers for Disease Control (CDC) indicating an incidence of 1.14%, or one in every 88 neurotypical children (CDC, 2008). Considering the sex-bias of ASD, which has a male to female ratio of approximately 4:1, this translates into an even higher incidence (1 in 54) for boys (Baron-Cohen et al., 2011). Recent findings from a study of more than 6 million ASD patients indicate that the prevalence of ASD seems to be increasing linearly from year to year (Keyes et al., 2012). While increased awareness, improved detection and broadening of diagnostic criteria for ASD contribute to the progressive rise in ASD incidence, several studies indicate that there is indeed a true increase in the frequency of this disorder, with factors other than diagnostic drift likely accounting for more than half of new cases (Grether et al., 2009; Hertz-Picciotto and Delwiche, 2009; King and Bearman, 2009).

While ASD is considered to be one of the most heritable complex neurodevelopmental disorders (El-Fishawy and State, 2010; Geschwind, 2011), genes linked to ASD rarely segregate in a simple Mendelian manner (El-Fishawy and State, 2010). Thus, it has been widely posited that multiple genetic etiologies, including rare, private (*de novo*) single gene mutations that are highly penetrant, interactions between inherited, common functional variants of multiple genes with small to moderate effects on ASD, or copy number variation (CNV), occur independently or in combination to determine ASD risk (Abrahams and Geschwind, 2008; Judson et al., 2011; Levitt and Campbell, 2009; O'Roak and State, 2008; Veenstra-Vanderweele et al., 2004). An alternative but not mutually exclusive hypothesis is that environmental factors interact with genetic susceptibilities to influence ASD risk, clinical phenotype and/or treatment outcome (Herbert, 2010; Pessah and Lein, 2008). Early indications of an environmental contribution to ASD came from observations of a high incidence of autism associated with *in utero* exposure to valproic acid (Rodier et al., 1997) or congenital rubella (Chess et al., 1978). However, consistent findings from twin studies of incomplete monozygotic concordance (Herbert, 2010) together with the observation that even in genetic syndromes highly associated with ASD, a significant percentage of carriers do not express autistic phenotypes (Levitt and Campbell, 2009), suggest that environmental risk factors for ASD are not limited to these well-defined *in utero* exposures. In one of the largest twin studies conducted to date, 192 mono- and dizygotic twin pairs were analyzed to quantify the relative contributions of genetic heritability versus the shared environment. The findings from these analyses suggested that 38% of ASD cases are attributable to genetic causes whereas 58% are linked to the shared *in utero* environment (Hallmayer et al., 2011). While the model used in this study had a number of inherent biases (*e.g.*, it was assumed there were no gene × environment interactions, monozygotic and dizygotic twins were assumed to share the environment to the same extent, and there are questions regarding the validity of the values used for the prevalence for autism and ASD), and these findings have yet to be replicated in an independent study of a different cohort of children, the conclusion from this study that environmental modifiers contribute to ASD risk is certainly consistent with the clinical heterogeneity of ASD. A significant contribution from environmental factors would also provide a plausible explanation for the rapid increase in the incidence of ASD over the past few decades (Hertz-Picciotto and Delwiche, 2009).

How environmental factors might interact with genetic susceptibilities to increase ASD risk remains largely unknown. Answering this question is complicated by the fact that efforts to identify specific environmental risk factors for ASD have produced a number of candidates but few definitive hits (DeSoto, 2009; Herbert, 2010; Kalia, 2008; Landrigan, 2010; Landrigan et al., 2012; Pessah and Lein, 2008; Shelton et al., 2012). If the complexity of heritable factors contributing to autism susceptibility creates a range of sensitivities of the developing brain to the adverse effects of environmental factors (Herbert, 2010; Levitt and Campbell, 2009; Pessah and Lein, 2008), then establishing clear associations between exposure to environmental factors and ASD diagnosis will be challenging. This argues for the critical need to employ "reverse epidemiology" in which experimental models are used to identify relevant gene × environment interactions and this information is then used to inform and focus subsequent epidemiological studies. We propose that one approach for applying this strategy is to identify environmental factors that modulate the same signaling pathways as ASD susceptibility genes to cause adverse neurodevelopmental outcomes of relevance to ASD (Pessah and Lein, 2008). The rationale behind this approach is that genetic susceptibilities may amplify the adverse effects of environmental exposures if both factors (genes and environment) converge at critical times during neurodevelopment to interfere with the same signaling pathway.

The feasibility of this experimental approach is strengthened by recent advances in defining the molecular and cellular pathology of ASD. Genetic, histological, electrophysiological and functional imaging studies of children and adults with ASD all point to altered patterns of neuronal connectivity in the developing brain as the neurobiological substrate underlying these disorders (Bourgeron, 2009; Geschwind and Levitt, 2007; Judson et al., 2011; Zoghbi and Bear, 2012). Importantly, there is increasing evidence from both human genetics and experimental models that many ASD risk genes converge on several major signaling pathways that play key roles in regulating neuronal connectivity in the developing brain (Bourgeron, 2009; Krey and Dolmetsch, 2007; Levitt and Campbell, 2009; Pardo and Eberhart, 2007; Zoghbi and Bear, 2012). This review will present a brief summary of the evidence implicating dysfunctional Ca^{2+}-dependent signaling (Krey and Dolmetsch, 2007; Pessah and Lein, 2008), signaling through extracellular signal-regulated kinases (ERK) and phosphatidylinositol-3-kinases (PI3K) (Bourgeron, 2009; Judson et al., 2011; Levitt and Campbell, 2009), and neuroligin–neurexin–SH3 and multiple ankyrin repeat domain (SHANK) interactions (Bourgeron, 2009; Sudhof, 2008) in ASD followed by examples of environmental chemicals for which there is emerging evidence of their potential to interfere with normal neuronal connectivity *via* perturbation of these signaling pathways.

2. Neuronal connectivity as a convergent target in ASD

While the pathogenesis of ASD has yet to be determined, it is clear that altered neuronal connectivity is involved. Functional magnetic

resonance imaging (fMRI) studies have demonstrated altered patterns of cortical activation in diagnosed children and adults with ASD during the performance of social and cognitive tasks, as well as abnormal intrinsic functional connectivity (anterior–posterior and/or interhemispheric connections) [reviewed in Judson et al., 2011; Rudie et al., 2012]. These functional deficits include reduced long-range functional connectivity in the cortex and enhanced local connectivity in multiple brain regions [reviewed in Amaral et al., 2008; Geschwind and Levitt, 2007; Judson et al., 2011]. Diffusion tensor imaging studies (DTI) demonstrate abnormalities in anterior–posterior and interhemispheric white matter tracts [reviewed in Judson et al., 2011; Rudie et al. 2012]. In some cases of ASD, aberrant functional connectivities are postulated to reflect an increased ratio of excitation to inhibition in sensory, mnemonic, social and emotional systems (Rubenstein and Merzenich, 2003). This hypothesis is derived from clinical studies indicating an imbalance of excitatory to inhibitory neurotransmission in brain regions of relevance to ASD (DeVito et al., 2007; Lewine et al., 1999; Page et al., 2006; Wheless et al., 2002), as well as recent research indicating dysfunctional $GABA_A$ receptors in ASD patients (Mendez et al., in press).

Postmortem studies of brain tissue from ASD subjects provide evidence of perturbed neuronal connectivity at the structural level. Brain tissue from adult ASD subjects exhibits ultrastructural characteristics suggestive of local hyperconnectivity and distal underconnectivity in prefrontal regions involved in attention, emotion and executive function. This involves changes in the proportion of small, medium, large and extra-large diameter axons between the anterior cingulate cortex, the orbitofrontal cortex and the lateral prefrontal cortex in ASD subjects compared to matched controls, and reduced myelin thickness in axons below the orbitofrontal cortex (Zikopoulos and Barbas, 2010). Increased spine density in Golgi-impregnated pyramidal neurons of layer II in the frontal, temporal and parietal lobes and layer V of the temporal lobe in ASD patients suggests skewed cortical synaptic activity towards excitation, although functional evidence of excitation is needed to further support this hypothesis (Hutsler and Zhang, 2010). Abnormal spine morphology is also found in the temporal and visual cortices of subjects with Fragile-X, a syndrome with high incidence of ASD (Irwin et al., 2001). Collectively, histological evidence combined with findings from imaging studies strongly indicate impaired neuronal network connectivity as the basis of behavioral and cognitive abnormalities in ASD. The heterogeneous clinical phenotypes that are characteristic of ASD (Geschwind, 2009; Geschwind and Levitt, 2007; Nazeer and Ghaziuddin, 2012) presumably reflect differences in when, how and which brain circuits are affected (Judson et al., 2011).

A number of neurodevelopmental processes ranging from early events of cell proliferation and differentiation to migration and axonal outgrowth to late events involving maturation of the dendritic arbor, synaptogenesis and myelination are critical to setting up mature patterns of neuronal connectivity in all regions of the brain, including those of relevance to ASD. Perturbations of the spatiotemporal patterns or magnitude of any of these events could theoretically interfere with the formation of meaningful networks and give rise to the deficits in functional connectivity associated with ASD (Belmonte and Bourgeron, 2006; Lein et al., 2005). Insight as to which of these neurodevelopmental processes may be preferentially targeted in ASD comes from genetic studies of ASD and neuropathological evidence from syndromic disorders with a high incidence of ASD diagnosis, including Angelman syndrome, Fragile X syndrome, Rett syndrome, Smith Lemli–Opitz syndrome, Timothy syndrome, neurofibromatosis and tuberous sclerosis. Data from these studies suggest that the late stages of neurodevelopment, *e.g.*, dendritic growth, synaptogenesis and myelination, are probably most vulnerable [reviewed in Levitt and Campbell, 2009]. This is consistent with the fact that ASD is typically diagnosed within the first three years of life, a period characterized by extensive formation and refinement of synaptic connections in the human brain (Huttenlocher and Dabholkar, 1997). Research findings from studies of transgenic animal models expressing ASD-associated genes further support the hypothesis that the changes in synaptic connectivity observed in children at risk for ASD arise from perturbations of dendritic growth, synapse formation and synapse stabilization (Bourgeron, 2009; Zoghbi and Bear, 2012). It is these critical later neurodevelopmental events that will be the focus of this review.

Data emerging from studies of syndromic ASD and rare highly penetrant mutations or CNVs in ASD have identified distinct adhesion proteins needed to maintain and modify synapses in response to experience, as well as intracellular signaling pathways that control dendritic arborization and/or synaptogenesis signaling systems that appear to represent convergent molecular mechanisms in ASD. The former includes neuroligins, neurexins, contactin-associated protein-2 (CNTNAP2) and SHANK proteins (Abrahams and Geschwind, 2008; Bourgeron, 2009; O'Roak and State, 2008; Sudhof, 2008); the latter, Ca^{2+}-dependent signaling (Krey and Dolmetsch, 2007), as well as ERK and PI3K signaling (Levitt and Campbell, 2009).

2.1. Synaptic proteins in ASD

Rare mutations in genes that encode synaptic proteins, specifically neuroligins 3 and 4 (*NLGN3*, *NLGN4*), neurexins 1 and 3 (*NRXN1*, *NRXN3*), CNTNAP2 (*CNTNAP2*) and Shank3 (*SHANK3*), are overrepresented in ASD compared to neurotypical children (Alarcon et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008; Durand et al., 2007; Jamain et al., 2003; Kim et al., 2008; Laumonnier et al., 2004; Szatmari et al., 2007; Yan et al., 2005). Neuroligins are expressed in postsynaptic membranes and function as cell adhesion molecules, bridging the synaptic cleft by binding to neurexins expressed on the opposing presynaptic membrane (Sudhof, 2008). CNTNAP2 is a synaptic protein that is homologous to neurexin, and SHANK3 is a scaffolding protein in the postsynaptic density that functions downstream of neuroligin–neurexin binding (Bourgeron, 2009). Neuroligin–neurexin–SHANK interactions regulate activity-dependent synaptogenesis and synapse maintenance in the developing brain (Craig and Kang, 2007; Scheiffele et al., 2000). There are five known isoforms of neuroligin, each encoded by a separate gene. These isoforms differentially regulate the formation of excitatory and inhibitory synapses *in vitro* (Craig and Kang, 2007; Scheiffele et al., 2000). In particular, neuroligin-2 enhances the formation of GABAergic inhibitory synapses whereas neuroligins 1 and 3 favor induction of glutamatergic excitatory synapses (Song et al., 1999; Varoqueaux et al., 2004). Results from *in vitro* studies demonstrated that neuroligin–neurexin interactions promote synapse formation in hippocampal neurons (Chih et al., 2004; Graf et al., 2004; Scheiffele et al., 2000). Genetic ablation of specific isoforms of neuroligin or neurexin in mice did not cause obvious changes in synapse number *in vivo* but did impair synaptic function, leading to the hypothesis that the predominant *in vivo* function of neuroligin–neurexin interactions is to regulate synapse stabilization and activity-dependent synaptic remodeling (Missler, 2003; Sudhof, 2008; Varoqueaux et al., 2006). The finding that chronic inhibition of NMDA receptors suppresses the synaptogenic activity of neuroligin-1 *in vitro* further supports the idea that neuroligins contribute to activity-dependent modification of synapses (Chubykin et al., 2007). More recently, it has been demonstrated in the *Aplysia* model that neuroligin–neurexin interactions are critically important in learning-related synaptic remodeling *in vivo* (Choi et al., 2011). Collectively, these observations suggest that neuroligin–neurexin interactions function to stabilize nascent synapses and modulate activity-dependent synaptic plasticity.

Although *NLGN* gene mutations contribute to $<1\%$ of ASD diagnoses, the association supports the hypothesis that synaptic abnormalities contribute to ASD (Bourgeron, 2009; Sudhof, 2008). Functional studies of the *NLGN3* R451C and *NLGN4* D936X mutations identified in ASD subjects reveal defective trafficking and synaptogenic properties of

the mutant proteins (Chih et al., 2004; Comoletti et al., 2004; Khosravani et al., 2005). Primary cultures of hippocampal neurons derived from *Nlgn3* R471C (the rat substitution that corresponds to the human R451C missense mutation) exhibit reduced synchrony of spontaneous activity patterns relative to neurons cultured from wildtype rats (Gutierrez et al., 2009). The functional relevance of these phenotypes to ASD is suggested by reports that *Nlgn4* knockout mice display reduced social interactions and ultrasonic vocalizations as adults (Jamain et al., 2008), and *Nlgn*1 overexpressing mice (an alternative paradigm for achieving the imbalance between neuroligin isoforms that presumably results when *Nlgn3* and/or *Nlgn4* are underrepresented) show significant deficits in memory acquisition consistent with a shift of synaptic balance towards increased excitation and impaired long-term potentiation (Dahlhaus et al., 2010). In mice, expression of ASD-linked *Nlgn3* and *Nlg4* mutations is associated with abnormal synaptic function, imbalance between excitatory and inhibitory synapses and severe autism-like phenotypes (Ey et al., 2011; Jamain et al., 2008; Tabuchi et al., 2007), and expression of the *NLGN3* R451C mutation in the *Aplysia* motor neuron blocks long-term facilitation (Choi et al., 2011). Mice null for *Shank1* exhibit increased anxiety-related behavior (Hung et al., 2008; Silverman et al., 2011) and communication impairments (Wohr et al., 2011). Collectively, these studies suggest that altered expression and/or function of these synaptic adhesion proteins likely contributes to the functional connectivity problems associated with ASD and perhaps also to the imbalance of excitatory to inhibitory neurotransmission thought to exist in a significant percentage of ASD subjects, particularly the 30% of ASD subjects with seizures (Rubenstein and Merzenich, 2003). In support of this hypothesis, recent functional imaging studies of human brain have demonstrated that expression of ASD-linked allelic variants of *CNTNAP2* is linked to altered frontal lobar connectivity and white and gray matter reductions in brain areas normally affected in ASD (Dennis et al., 2011; Scott-Van Zeeland et al., 2010; Tan et al., 2010). Thus, a risk allele for autism is associated with significant cerebral morphological variation. Interestingly, this association was observed in individuals that did not exhibit overt symptoms or behavioral abnormalities characteristic of ASD, suggesting that additional factors interact with these genetic susceptibility factors to determine ASD risk.

2.2. ERK and PI3K pathways in ASD

Data from studies of syndromic ASD also identify the ERK and PI3K intracellular signaling pathways as a second set of convergent molecular targets in ASD (Bourgeron, 2009; Levitt and Campbell, 2009). ERK and PI3K signaling link extracellular signals to mTOR activation, and the primary membrane receptors that transduce signals through ERK and PI3K are receptor tyrosine kinases (RTK). ERK (Sweatt, 2004) and PI3K/mTOR (Jaworski et al., 2005; Kumar et al., 2005; Li et al., 2010) signaling pathways mediate transcriptional and translational mechanisms of experience-dependent dendritic remodeling and synapse formation (Li et al., 2010). Disruption of ERK and PI3K signaling either directly or *via* altered function of the upstream RTK occurs in several syndromic disorders with high penetrance of ASD (Levitt and Campbell, 2009). With respect to the former, mutations in *TSC1*/*TSC2*, *NF1* or *PTEN* are associated with syndromic ASD in the context of tuberous sclerosis, neurofibromatosis or macrocephaly (Bourgeron, 2009). The protein products of these genes function as negative effectors of the mTOR-raptor complex (mTORC); thus, mutations in *TSC1*/*TSC2*, *NF1* or *PTEN* activate mTOR signaling. Mice with conditional *Pten* knockout in the cortex and dentate gyrus exhibit reduced social interactions, increased activity in novel environments, impaired sensorimotor gating coincident with macrocephaly and alterations in spine density and morphology (Kwon et al., 2006). Administration of rapamycin, a specific inhibitor of mTORC1, can reverse aberrant neuronal morphologies and behavioral defects observed in transgenic mice expressing *Pten* (Zhou et al., 2009) or *Tsc1*/*Tsc2* (Ehninger et al., 2008) mutations and reverse abnormal synaptic functions in adult mouse models of Fragile X (de Vrij et al., 2008; Dolen et al., 2007) or Rett syndrome (Guy et al., 2007).

An example of an RTK implicated in the genetic etiology of ASD is the MET RTK (Judson et al., 2011; Levitt and Campbell, 2009). Three allelic variants of *MET*, all located in the putative 5′ regulatory region have been associated with increased ASD risk, and at least one variant, the functional rs1858830 'C' variant reduces transcriptional efficiency of MET *in vitro*, consistent with decreased levels of MET protein expression in the temporal neocortex in ASD post-mortem cases [reviewed in Judson et al., 2011]. MET is activated by binding of its endogenous ligand, hepatocyte growth factor (HGF), which in turn activates signaling through PI3K, ERK and p38 (Trusolino et al., 2010). *In vitro* studies demonstrate that MET signaling regulates dendritic outgrowth of neocortical and hippocampal pyramidal neurons and synaptic connectivity between forebrain neurons [reviewed in Judson et al., 2011], but whether MET signaling mediates similar responses *in vivo* has yet to be determined. However, it has recently been determined that *Met* mRNA is expressed in the right cells during the right time in development to influence synapse formation in the developing mouse forebrain, and that kinase-dead, conditional *Met* mutant mice exhibit altered dendritic morphology and abnormal dendritic spine morphology in neocortical pyramidal neurons and enhanced local circuitry in restricted layers of the cortex [reviewed in Judson et al., 2011]. Interestingly the neural circuits impacted in the *Met* mutant mouse are those that are important for processing socially relevant auditory and visual cues in the primate (Judson et al., 2011). Recent structural and functional imaging studies of the human brain reveal that expression of the cMet allele correlates with altered structural and functional connectivity in heterozygotic or homozygotic children and adolescents (Rudie et al., 2012). Interestingly, the MET risk genotype predicted atypical fMRI activation and deactivation patterns to social stimuli in both neurotypic and autistic individuals; however, effects were more pronounced in ASD subjects (Rudie et al., 2012). This is consistent with a multiple hit model for ASD, in which genetic and environmental risk factors interact to determine ASD risk.

2.3. Intracellular Ca^{2+} in ASD

While experimental data demonstrate that both the initial formation of dendrites (Higgins et al., 1997; Lein et al., 1995; Metzger, 2010) and the latter stages of dendritic branching and spine formation can be driven by activity-independent mechanisms (Frotscher et al., 2000; Kossel et al., 1997), a principal determinant of neuronal connectivity in the developing brain is electrophysiological activity. Activity influences the initial architecture of neural systems and is critical for refining neural circuits to form functionally integrated networks (Aamodt and Constantine-Paton, 1999; Cline, 2001; Komuro and Rakic, 1998; Levitt, 2003; Moody and Bosma, 2005). Activity modulates not only structural aspects of neuronal connectivity, such as dendritic branching and spine formation, but also neurochemical aspects of neuronal connectivity, specifically neurotransmitter phenotype (Borodinsky et al., 2004; Spitzer, 2012). The effects of activity on neuronal connectivity are mediated primarily, if not exclusively, by changes in intracellular Ca^{2+} levels (Lohmann and Wong, 2005). A number of candidate risk genes for ASD encode proteins that have a primary role in generating intracellular Ca^{2+} signals or are themselves tightly regulated by local fluctuations in Ca^{2+} concentrations (Krey and Dolmetsch, 2007; Pessah and Lein, 2008). These genes encode Ca^{2+} ion channels, neurotransmitter receptors and Ca^{2+}-regulated signaling proteins such as BDNF, CREB, Wnt2, ERK1/2 and PI3K.

Emerging evidence suggests that defective neuronal connectivity associated with ASD is mediated in part by defects in neuronal Ca^{2+} signaling (Lohmann, 2009). One illustration of this derives from recent studies of a gain-of-function missense mutation in the L-type Ca^{2+} channel CaV1.2 that causes Timothy syndrome, which has a

60% rate of co-morbidity with autism (Splawski et al., 2004), making it one of the most penetrant monogenic forms of ASD. Neurons differentiated from induced pluripotent stem cells (iPSC) derived from Timothy syndrome patients revealed increased Ca^{2+} oscillations at rest and persistent Ca^{2+} signaling upon challenge with a depolarizing stimulus (Pasca et al., 2011). Additionally, neurons expressing the Timothy syndrome mutation exhibited increased neuronal complexity and upregulated expression of genes linked to Ca^{2+}-dependent regulation of CREB, including CaMK, which is consistent with studies demonstrating that CaMK signaling through CREB links activity to increased dendritic growth (Wayman et al., 2006). Collectively, these studies suggest a mechanism underlying the increased local connectivity associated with ASD. A second example is provided by recent work with CGG trinucleotide expansion repeats within the 5′ noncoding portion of the Fragile X mental retardation 1 (*FMR1*) gene. CGG-repeat expansions in the premutation range (55–200 CGG repeats) not only give rise to the neurodegenerative disorder, Fragile X-associated tremor/ataxia syndrome (FXTAS) but also increase the risk of developing ASDs early in life (Chonchaiya et al., 2012; Hagerman et al., 2011). *FMR1* expansions with >200 CGG repeats give rise to Fragile X syndrome (FXS). Collectively, expansion mutations in *FMR1* are the most prevalent single gene disorder contributing ASD risk (Krueger and Bear, 2011; Leehey and Hagerman, 2012). Hippocampal neurons cultured from a *FMR1* premutation knockin mouse model exhibited impaired dendritic growth and complexity (Chen et al., 2010) as well as aberrant electrical spiking patterns and synchronized Ca^{2+} oscillatory behaviors (Cao et al., 2012b). Similar to observations of iPSC-derived neurons expressing the Timothy syndrome mutation, iPSC-derived *FMR1* premutation neurons also exhibited abnormal Ca^{2+} transients of higher amplitude and increased frequency and a sustained Ca^{2+} elevation after glutamate application relative to neurons expressing the normal *FMR1* allele (Liu et al., 2012). Such studies clearly establish these Ca^{2+} signaling molecules as possible convergence points for genetic variants linked to ASD risk.

3. Environmental chemicals as risk factors for ASD

Over the last few years, a range of non-genetic factors have been associated with increased risk for ASD, including pesticides, air pollution, drugs, paternal age and maternal nutritional, medical or metabolic status (Herbert, 2010; Krakowiak et al., 2012; Landrigan et al., 2012; McCanlies et al., 2012; Schmidt et al., 2011, 2012; Shelton et al., 2012; Volk et al., 2011; Zerbo et al., 2012). In this review, we will focus on environmental chemicals as potential risk factors for ASD, using organophosphorus pesticides (OPs), polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) as examples. Human exposures to these environmental chemicals are widespread and, with the possible exception of PCBs (but see Section 3.2.), generally have increased from the 1980s to the 2000s. Although their direct involvement in ASD pathophysiology has yet to be proven, there is experimental evidence that these environmental chemicals modulate signaling systems implicated in the pathogenesis of ASD.

3.1. Organophosphorus pesticides (OPs)

OPs are currently the most commonly utilized pesticides in the world (Zaim and Jambulingam, 2007). In the United States, about 73 million pounds of OP pesticides were used in 2001 (70% of all insecticides; Kiely et al., 2004). There is widespread exposure of children to these compounds (Adgate et al., 2001; Barr et al., 2004; Curl et al., 2003; Davis and Ahmed, 1998; Eskenazi et al., 1999; Landrigan et al., 1999; Lu et al., 2001; Whyatt and Barr, 2001), and OP residues are detected in the blood and/or urine of nearly all children sampled in the United States (Barr et al., 2005). OPs are well documented developmental neurotoxicants. Animal studies show that the developing nervous system is especially sensitive to the neurotoxic effects of OPs (Moser et al., 1998; Moser and Padilla, 1998; Pope et al., 1991). In rats, exposure to OPs during critical stages of brain development can cause persistent behavioral and cognitive deficits in the absence of significant cholinesterase inhibition (Jett et al., 2001; Levin et al., 2001, 2002; Slotkin et al., 2001, 2009). Consistent with these experimental animal studies, a number of epidemiological investigations have reported a positive correlation between chronic exposure to low-level OPs and behavioral and cognitive problems in children (Bouchard et al., 2011; Engel et al., 2007, 2011; Eskenazi et al., 2007; Kofman et al., 2006; Lizardi et al., 2008; Lovasi et al., 2011; Rauh et al., 2006, 2011; Rohlman et al., 2005). While each individual study has limitations, including exposure quantification (limited to no data regarding the type of OP, the timing and duration of exposure) and potential confounding factors (*e.g.*, concomitant exposure to other environmental factors and socioeconomic factors), collectively, results from these studies suggest OP exposure represents a potential threat to the developing human nervous system.

Several of these epidemiological studies have identified a link between OP exposure and outcomes that are relevant to ASD. For example, in a study of a longitudinal birth cohort of primarily Latino farm worker families in California, levels of urinary OP metabolites in mothers and their children were positively associated with increased risk of pervasive developmental disorder at 24 months of age and with attention problems at 5 years of age (Eskenazi et al., 2007; Marks et al., 2010). In a study of low income communities in New York City, prenatal exposure to the OP chlorpyrifos, as measured in umbilical cord plasma, was significantly associated with increased risk of mental delay, attention problems and pervasive developmental disorder in exposed children at 3 years of age, and with deficits in intellectual development at the age of 7 (Rauh et al., 2006, 2011). Additional epidemiological evidence hinting at a link between OP exposures and ASD risk is the reports that ASD susceptibility is influenced by functional polymorphisms in *PON1*, which encodes the major enzyme involved in OP detoxification, paraoxonase (PON-1) (D'Amelio et al., 2005; Gaita et al., 2010; Pasca et al., 2006). However, PON-1 not only hydrolyzes organophosphate esters but also plays an important role in lipid metabolism (Androutsopoulos et al., 2011), which has been suggested to be altered in ASD (Szatmari et al., 2007). Whether *PON1* polymorphisms influence ASD risk *via* effects on OP detoxification, lipid metabolism or both remains an unanswered question. However, the potential for *PON1* polymorphisms to influence OP neurotoxicity is suggested by the Mount Sinai Children's Environmental Health study of a multiethnic population in New York City. In this study, prenatal urinary and blood levels of OP metabolites were negatively associated with cognitive development at 12 and 24 months of age in the Black/Hispanic group, with effects being more pronounced at 12 months in children of mothers who carried the *PON1* Q192R polymorphism associated with a slower rate of OP detoxification (Engel et al., 2011). Interestingly, the association between OP metabolite levels and cognitive development was reversed in the White group. The significance of these differences across ethnic groups is difficult to assess since the *PON1* polymorphisms were not studied in the White group, and the authors did not consider environmental parameters known to affect PON1 status (Androutsopoulos et al., 2011).

A biological mechanism supporting an association between developmental exposures to OPs and ASD has yet to be described. It seems unlikely that OPs cause ASD *per se* but rather OPs interact with ASD susceptibility genes to drive neurodevelopmental systems over the threshold for ASD expression. Inherent imbalances in synaptic connectivity in children at risk for ASDs (see Section 2) are likely to provide the biological substrate for enhanced susceptibility to environmental triggers that interfere with synaptic connectivity. OPs have been shown to interfere with normal patterns of neuronal

connectivity in experimental models. The OP chlorpyrifos decreases axonal but enhances dendritic growth in cultured neurons (Howard et al., 2005; Yang et al., 2008) and disrupts axonal growth in developing zebrafish (Jacobson et al., 2010; Yang et al., 2011). Developmental exposure to chlorpyrifos also alters levels of presynaptic markers of cholinergic neurons, *e.g.*, choline acetyltransferase and hemicholinium-3 binding in rats (Dam et al., 1999; Slotkin et al., 2001), suggesting that OPs interfere with synapse formation in the intact mammalian brain. Consistent with these observations of altered neuronal connectivity in experimental models of OP developmental neurotoxicity, a recent magnetic resonance imaging (MRI) study of children between the ages of 6 and 11 reported a significant association between structural anomalies of cortical surface features and prenatal exposure to the OP chlorpyrifos as quantified in umbilical cord blood (Rauh et al., 2012). The mechanisms by which OPs interfere with neuronal connectivity remain speculative; however, experimental evidence suggests that OPs may modulate the same signaling pathways linked to genetic risk factors for ASD.

One intriguing possibility is that OPs alter normal synaptogenesis by interfering with neuroligin–neurexin interactions. This hypothesis derives from observations that chlorpyrifos and its oxon metabolite inhibit axon outgrowth in primary cultured neurons by interfering with the morphogenic activity of acetylcholinesterase (AChE) independent of effects on the enzyme's hydrolytic activity (Yang et al., 2008). Interestingly, the morphogenic domain of AChE shares striking structural and functional homology with the extracellular domain of neuroligins (Dean and Dresbach, 2006; Graf et al., 2004). Deletion mutation studies show that synaptogenic activities of neuroligin are mediated by specific sequences within its AChE-homologous extracellular domain, which are necessary and sufficient when expressed in HEK293 cells to induce presynaptic differentiation in axons (Scheiffele et al., 2000). Conversely, transfection with neuroligin restores neurite outgrowth in PC12 cells whose expression of AChE is suppressed by antisense treatment (Grifman et al., 1998), confirming functional redundancy. Considered in aggregate, these observations suggest the possibility that OPs interact with the esterase domain of neuroligins to interfere with their synaptogenic activity. If true, then OPs would be predicted to exacerbate ASD-related *NLGN* mutations, which decrease expression of neuroligin at the cell surface (Sudhof, 2008), by further decreasing the availability of functional neuroligin molecules. This hypothesis has yet to be tested experimentally. An alternative mechanism by which OPs may disrupt neuroligin–neurexin interactions is suggested by a recent microarray study in which repeated postnatal exposure to chlorpyrifos-oxon was reported to significantly decrease the expression of *Nlgn2* in the cerebellum of mice (Cole et al., 2011). These results were confirmed by quantitative PCR, and were related to PON1 activity.

OPs have also been shown to alter the expression and/or function of various proteins that influence neuronal connectivity *via* ERK and PI3K signaling, specifically reelin and neurotrophins. Reelin and neurotrophins have both been implicated in genetic ASD etiologies. Reelin, which is encoded by the *RELN* gene, is an extracellular protease that activates PI3K signaling *via* binding to the very low-density lipoprotein (VLDL) receptor and the apolipoprotein E receptor 2 (APOE-R2) (Forster et al., 2010). Reelin promotes maturation of dendrites and dendritic spines and modulates synaptic function; these effects are mediated by both its protease activity and signaling through PI3K (Forster et al., 2010; Quattrocchi et al., 2002). Variants of the *RELN* gene that express ≥12 repeats of a polymorphic GGC repeat found in the 5′-UTR of the *RELN* gene immediately adjacent to the AUG initiator codon blunt *RELN* gene expression by 25–50% both *in vitro* (Persico et al., 2006) and *in vivo* (Lugli et al., 2003). Long GGC repeat variants have been associated with ASD in North American and Slovakian populations (Kelemenova et al., 2010; Persico et al., 2006; Serajee et al., 2006; Skaar et al., 2005; Zhang et al., 2002); however, these findings were not replicated in studies conducted in Italy, France, England, Germany or the Chinese Han population (Bonora et al., 2003; Devlin et al., 2004; He et al., 2011; Krebs et al., 2002; Li et al., 2004). The identification of geographic differences between these epidemiological studies suggests the possibility that an environmental factor interacts with the long *RELN* allelic variants to confer susceptibility to ASD (Pardo and Eberhart, 2007; Persico and Bourgeron, 2006). It has been proposed that OPs may be one such factor (Persico and Bourgeron, 2006). OPs have been shown to decrease *Reln* mRNA expression in the developing rat forebrain (Betancourt et al., 2006), and OPs potently inhibit the proteolytic activity of reelin (Quattrocchi et al., 2002). Considered in light of data indicating greater household use of OPs in North America compared to Europe during the period corresponding to the infancy and early childhood of the study population (Persico and Bourgeron, 2006), these observations suggest that exposure to OPs during critical periods of neurodevelopment pushes individuals carrying the long *RELN* allelic variants below a threshold of reelin expression required for the development of normal neuronal connectivity.

Neurotrophins, which include brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3), are soluble trophic factors that play a critical role in determining both local and long-range neuronal connectivity (Chapleau et al., 2009; da Silva and Wang, 2011; McAllister, 2000). Developmental exposure to OPs has been shown to decrease expression of BDNF and other neurotrophins in the brain of rodents and chicken (Betancourt et al., 2006; Betancourt et al., 2007; Pomeroy-Black et al., 2007; Slotkin et al., 2008). This effect could theoretically contribute to the reduced long-range connectivity associated with ASD and potentially also to the enhanced local connectivity associated with ASD. Neurotrophins elicit complex responses from central neurons and a single neurotrophin can have opposing effects on dendritic growth (McAllister et al., 1997). In slice cultures of the visual cortex, BDNF stimulates dendritic growth of layer 4 neurons whereas NT-3 inhibits their growth; in contrast, BDNF is inhibitory and NT-3 stimulatory for neurons in layer 6 (McAllister et al., 1997). This effect was observed with endogenous or exogenous neurotrophins and required neural activity (McAllister et al., 1996). BDNF modulates dendritic growth and plasticity by activating PI3K/mTOR signaling downstream of binding to the tyrosine kinase (Trk) receptors, which are members of the RTK family of transmembrane receptors (Gong et al., 2006; Jaworski et al., 2005; Kumar et al., 2005; Takei et al., 2004). This suggests the possibility that OPs interact with ASD susceptibility genes at the level of the PI3K/mTOR/PTEN signaling pathway. These signaling molecules mediate BDNF effects on neuronal connectivity and as discussed above (Section 2), they have been implicated in several forms of syndromic ASD (Levitt and Campbell, 2009). Decreased neurotrophin expression caused by developmental OP exposure may also push individuals with inherited deficiencies in neurotrophin expression, such as Rett syndrome patients, closer to the threshold for ASD. Rett syndrome, which has a high penetrance of ASD, is associated with mutations in the transcription factor MeCP2 (Amir et al., 1999). *Bdnf* mRNA expression is regulated by MeCP2 (Chen et al., 2003; Martinowich et al., 2003), and *Bdnf* transcript expression is diminished in mice nullizygous for *Mecp2* (Chang et al., 2006) or expressing Rett-associated *Mecp2* mutations (Larimore et al., 2009). *Bdnf* overexpression prevents dendritic atrophy caused by Rett-associated *Mecp2* mutations in hippocampal neurons (Larimore et al., 2009) and rescues MeCP2 null mice from behavioral and electrophysiological deficits (Chang et al., 2006). *Bdnf* mRNA expression is lower in brain samples from Rett subjects (Abuhatzira et al., 2007); however, protein expression is not changed (Lappalainen et al., 1996; Leblond et al., 2012; Riikonen, 2003; Riikonen and Vanhala, 1999; Vanhala et al., 1998). The relevance of the latter is not clear since it is well established that local translation of *Bdnf* mRNA in dendrites in response to activity is critical for synaptic plasticity (Chapleau et al., 2009).

OPs may also interact with heritable deficiencies in Ca^{2+} signaling to confer increased ASD risk. Calcium-dependent activator protein for secretion 2 (CAPS2) enhances the release of BDNF and NT-3 from vesicles in parallel fibers in the developing cerebellum and mutations in the gene that encodes CAPS2 has been associated with increased ASD susceptibility (Sadakata and Furuichi, 2009). Mice that are nullizygous for *Casp2* exhibit impaired BDNF release and they express autism-like behaviors, including decreased social interaction, hyperactivity in the home cage but decreased exploratory behavior and/or increased anxiety in a novel environment (Sadakata and Furuichi, 2009). While there is considerable debate regarding the validity of animal models of ASD (Halladay et al., 2009), it would be interesting nonetheless to determine whether OP-induced reductions in neurotrophin levels exacerbate these behavioral phenotypes in the *Casp2* null mice. Additional Ca^{2+}-regulated proteins that are modulated by OPs include cAMP response element binding protein (CREB) and Wnt. OPs significantly increase phosphorylation of CREB in primary cultures of hippocampal and cortical neurons with EC_{50} values in the femtomolar to picomolar range (Schuh et al., 2002). CREB is a pivotal signaling molecule in transcriptional mechanisms of activity-dependent growth of dendrites, dendritic spine formation and synaptic plasticity (Wayman et al., 2008). Its relevance to ASD is suggested by the report of aberrant CREB activity in neurons differentiated from iPSC of Timothy syndrome patients (Pasca et al., 2011). Wnt is another Ca^{2+}-dependent signaling molecule implicated in transcriptional mechanisms of activity-dependent dendritic arborization and synaptogenesis (Salinas and Zou, 2008; Wayman et al., 2006) that has been associated with increased ASD risk (De Ferrari and Moon, 2006; Krey and Dolmetsch, 2007; Okerlund and Cheyette, 2011). Toxicogenomic studies of mice exposed gestationally to chlorpyrifos or diazinon identified significant changes in the expression of the Wnt signaling pathway and these changes occurred independent of AChE inhibition (Moreira et al., 2010; Slotkin et al., 2008).

Collectively, these observations suggest multiple mechanisms by which OPs might interact with ASD risk genes to exacerbate defects in neuronal connectivity. Individuals with heritable deficiencies in neuroligin–neurexin–SHANK signaling, reelin or BDNF signaling through the PI3K/mTOR pathway or Ca^{2+}-dependent signaling molecules that link neural activity or experience to changes in neuronal connectivity may be particularly vulnerable to the adverse effects of OPs. In addition to testing these hypotheses, it will also be important to determine whether interactions are unique to a given subset of OPs or can be generalized across all OPs, particularly since much of the data currently available regarding the developmental neurotoxicity has focused on chlorpyrifos with significantly fewer data available on other commonly used OPs.

3.2. Polychlorinated biphenyls (PCBs)

PCBs are a group of organic compounds classified according to their molecular structure as dioxin-like or non-dioxin-like (NDL). Congeners with >1 *ortho*-substituted chlorine are typically NDL, while only twelve PCB congeners with <2 *ortho*-chlorine substituents are dioxin-like in their toxicity (Safe, 1993). Industrial PCB mixtures were used mostly as coolants and lubricants from the 1930s until 1977, when their production was banned in the United States. However, PCBs are still in use in the United States as dielectric fluids in transformers and capacitors. While environmental and human PCB levels dropped significantly between 1970 and 1995, recent studies of temporal trends show no or only a slight decrease in environmental PCB levels since the mid-1990s (Hornbuckle et al., 2006), which is thought to be due to secondary PCB sources. For example, PCBs were heavily used in large metropolitan areas and are now released into the environment from building materials and other sites of PCB use (Jamshidi et al., 2007). Furthermore, PCB contamination is not static. For example, mass flux studies demonstrate that large quantities of PCBs are deposited and volatilized every year from Lake Michigan (Hornbuckle et al., 2006). These studies suggest that there is still considerable risk for human exposures to PCBs, which is corroborated by studies demonstrating the persistence of high residue levels in foods, particularly fish, and in human tissue samples (DeCaprio et al., 2005; Humphrey et al., 2000; Park et al., 2007).

Critical reviews of epidemiological studies have concluded that the weight of evidence indicates a negative association between developmental exposure to environmental PCBs and measures of neuropsychological function in infancy or childhood (Carpenter, 2006; Korrick and Sagiv, 2008; Schantz et al., 2003; Winneke, 2011). Combined *in utero* and lactational PCB exposure correlates with decreased scores on IQ tests, impaired learning and memory, psychomotor difficulties, and attentional deficits. While discrepancies between studies with respect to the spectrum and persistence of adverse neurobehavioral outcomes, the confound of co-exposures and differences in congener profiles that comprise the exposure raise questions about the causative role of PCBs in human developmental neurotoxicity (Winneke, 2011), experimental findings in animal models confirm that developmental PCB exposure causes deficits in learning and memory (Hany et al., 1999; Sable et al., 2006; Schantz et al., 1989; Widholm et al., 2001; Yang et al., 2009) and sensorimotor functions (Nguon et al., 2005; Powers et al., 2006; Roegge et al., 2004). While it is posited that developmental exposure to PCBs contributes to increased ASD risk (Grandjean and Landrigan, 2006; Landrigan, 2010; Landrigan et al., 2012), there are as yet no epidemiological data to support this hypothesis.

Recent studies of the cellular and molecular mechanisms by which PCBs interfere with neurodevelopment reveal that NDL PCBs modulate Ca^{2+}-dependent signaling pathways that are implicated in ASD. Ca^{2+} imaging studies of cultured rat hippocampal neurons (Wayman et al., 2012a) revealed that acute exposure to the NDL congener PCB 95 promotes the same bursting type of Ca^{2+} activity as was reported in neurons expressing gene mutations that confer ASD susceptibility, specifically the gain-of-function missense mutation in the L-type Ca^{2+} channel CaV1.2 that causes Timothy syndrome (Pasca et al., 2011) and the *FMR1* premutation (Cao et al., 2012b; Liu et al., 2012) (see Section 2). Consistent with the established role of intracellular Ca^{2+} signaling in activity-dependent dendritic growth (Lohmann and Wong, 2005) and with the morphogenic analyses of neurons expressing either the Timothy syndrome mutation or *FMR1* premutation, PCB 95 also triggered increased dendritic complexity in cultured rat hippocampal neurons, and these effects were significantly different from untreated controls at picomolar to nanomolar PCB concentrations (Wayman et al., 2012a, 2012b). PCB 95 effects on dendritic arborization were blocked by pharmacological antagonism or siRNA knockdown of the Ca^{2+}/calmodulin kinase-I (CaMKI)–CREB–Wnt signaling pathway (Wayman et al., 2012a), which is the Ca^{2+}-dependent signaling pathway previously shown to link activity to dendritic growth in cultured hippocampal neurons (Wayman et al., 2006). Genes encoding these same Ca^{2+}-dependent signaling molecules are implicated as ASD susceptibility genes (Krey and Dolmetsch, 2007; Pessah and Lein, 2008), and the proteins encoded by these genes are upregulated in iPSC-derived neurons from Timothy syndrome patients (Pasca et al., 2011) (see Section 2). Thus, this Ca^{2+}-dependent signaling pathway represents a potential convergent molecular target for both NDL PCBs and ASD risk genes that interfere with a final common path of activity-dependent dendritic arborization and plasticity.

The human health relevance of this hypothesis is supported by observations that developmental PCB exposure modulates dendritic growth *in vivo*. Gestational and lactational exposure to Aroclor 1254 (A1254) in the maternal diet significantly increased dendritic arborization of pyramidal neurons in the CA1 region of the hippocampus of weanling rats (Wayman et al., 2012b). In a separate study of experience-dependent dendritic growth, gestational and lactational

exposure to Aroclor 1254 promoted dendritic growth in cerebellar Purkinje cells and neocortical pyramidal neurons among untrained animals but attenuated or reversed experience-dependent dendritic growth among Morris water maze-trained littermates (Yang et al., 2009). A1254 is comprised predominantly of NDL PCB congeners (Kostyniak et al., 2005), and consistent with the hypothesis that these congeners are primarily responsible for the effects of A1254 on neuronal connectivity, developmental exposure to PCB 95 in the maternal diet significantly increased dendritic growth of CA1 pyramidal neurons in the hippocampus of untrained weanling rats at the low (0.1–1.0 mg/kg/d in the maternal diet) but not at the highest dose tested (6.0 mg/kg/d in the maternal diet) (Wayman et al., 2012b). Developmental exposures to A1254 and PCB 95 also cause performance deficits in the Morris water maze task in weanling rats (Yang et al., 2009) and alter activity levels and behavior in the radial arm maze in adult rats (Schantz et al., 1997). Perinatal exposure to a mixture of the NDL PCB 47 and the dioxin-like PCB 77 has recently been reported to alter social behaviors in rats (Jolous-Jamshidi et al., 2010). Another example of PCB interference with neuronal connectivity in the developing brain of significant relevance to ASD is the finding that developmental exposure to PCB 95 interferes with the topographic organization of the primary auditory cortex and creates an imbalance between excitation and inhibition in the auditory cortex of weanling rats (Kenet et al., 2007). If similar effects occur in children, it seems plausible that these could contribute to deficits in communication and social interactions that are core to ASD diagnoses and symptomology.

Additional mechanistic studies establish that the effects of NDL PCBs on dendritic arborization and Ca^{2+}-dependent signaling pathways that regulate neuronal connectivity are mediated by ryanodine receptor (RyR)-dependent mechanisms. While several mechanisms have been shown to contribute to PCB effects on intracellular Ca^{2+} (Kodavanti, 2005; Mariussen and Fonnum, 2006), the most sensitive is RyR sensitization (Wong et al., 1997a, 1997b; Wong and Pessah, 1996). RyRs are microsomal Ca^{2+} channels that are broadly expressed throughout the mammalian brain and associate with cytosolic, endoplasmic reticulum (ER)-anchored and ER luminal proteins to form local Ca^{2+} release units (CRUs). These CRUs regulate Ca^{2+} release from the ER and modify gating responses and signal gain of plasma membrane ion channels, including the NMDA receptor, thereby determining the amplitude and spatial and temporal fluctuation of intracellular Ca^{2+} during cell activation (Pessah et al., 2010). Nanomolar concentrations of PCB congeners with multiple *ortho* substituents interact with CRUs to dramatically sensitize their activation by nanomolar Ca^{2+} and attenuate their inhibition by high micromolar to millimolar Ca^{2+} and Mg^{2+} (Wong et al., 1997a; Wong and Pessah, 1996), thus stabilizing the RyR in its full open conformation (Samso et al., 2009). A stringent structure–activity relationship was identified for this effect with the NDL congener PCB 95 being the most potent and efficacious congener identified to date (Pessah et al., 2010). Blocking RyR activity using either pharmacological approach or siRNA knockdown of RyR prevents PCB 95 enhancement of both synchronized Ca^{2+} oscillations (Wayman et al., 2012a) and dendritic growth (Wayman et al., 2012b), and blocks activation of the CaMK–CREB–Wnt2 signaling pathway (Wayman et al., 2012a).

So, are studies with PCB 95 relevant to human exposures and to ASD? The answer is an unqualified yes. There is increasing evidence that NDL PCBs predominate in environmental samples (Hwang et al., 2006; Kostyniak et al., 2005), human blood (DeCaprio et al., 2005; Stewart et al., 1999) and brain samples from weanling rats developmentally exposed to A1254 (Yang et al., 2009). An intriguing example from human studies is the recent finding that PCB 95, the most active PCB congener in dysregulating ASD-relevant Ca^{2+}-dependent signaling pathways (Pessah et al., 2010; Wayman et al., 2012a) is found in significantly higher levels in postmortem brains of children with a syndromic form of autism, but not idiopathic autism, as compared to neurotypical controls (Mitchell et al., 2012). The samples with detectable PCB 95 levels were almost exclusively those with maternal 15q11–q13 duplication (Dup15q) or deletion in Prader–Willi syndrome. When sorted by birth year, Dup15q samples represented five of six samples with detectable PCB 95 levels and a known genetic cause of ASD born after the 1976 PCB ban. Dup15q was the strongest predictor of PCB 95 exposure across age, gender, or year of birth. Dup15q brain samples had lower levels of repetitive DNA methylation measured by LINE-1 pyrosequencing, but methylation levels were confounded by year of birth. These results suggest a novel paradigm by which specific NDL persistent organic pollutants (POPs) may predispose to genetic CNVs of 15q11–q13.

NDL PCB congeners with the highest activity towards RyRs, including PCB 95, collectively represent 40–50% of total PCBs currently found in environmental and biotic samples, and their net effects are likely to be additive (Pessah et al., 2006). However, even low levels of exposure to these PCB congeners might adversely influence neuronal connectivity in the developing brain of genetically susceptible individuals, such as those with heritable deficiencies in Ca^{2+} signaling. In addition to ASD risk genes that encode molecules that influence the CaMK–CREB–Wnt2 signaling pathway that converges on dendritic arborization (see Section 2), mutations in *RYR* genes suggest another genetic susceptibility that might amplify the adverse effects of RyR-active PCBs on neuronal connectivity. *RYR* mutations have been linked to environmentally-triggered disorders in humans including malignant hyperthermia (Gronert et al., 2004), cardiac arrhythmias (Wehrens et al., 2005), and sudden death (Laitinen et al., 2004). Recent studies demonstrate that specific *RYR* mutations confer sex- and gene dose-dependent susceptibility to pharmacological (halogenated anesthetic) and environmental (heat) stressors that trigger malignant hyperthermia and muscle damage in otherwise asymptomatic individuals (Barrientos et al., 2012; Yuen et al., 2012). Importantly, PCB 95 is significantly more potent and efficacious in disrupting cation regulation of mutant R615C-*RYR1* compared to wild type RyR1 (Ta and Pessah, 2007). While a previous single nucleotide polymorphism study concluded that there was no evidence of an association between *RYR3* and autism in a Japanese population (Tochigi et al., 2008), a joint association test of results from a genome wide association study identified RyR2 as an ASD candidate gene by using sex as an additional risk factor (Lu and Cantor, 2012).

Considered in aggregate, these observations provide important new clues about the possible role of RyRs in contributing to heritable and environmentally triggered neurodevelopmental deficits, and identify PCBs, and in particular NDL PCBs with high RyR activity, as candidate environmental risk factors in ASD. It also suggests that other non-coplanar POPs that sensitize RyR, such as PBDEs (Kim et al., 2011) and triclosan (Cherednichenko et al., in press), should be evaluated as potential environmental risk factors for ASD by determining whether they, like NDL PCBs, also interfere with late neurodevelopmental processes that define neuronal connectivity.

3.3. Polyaromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous environmental pollutants generated by the incomplete combustion of organic chemicals. Major sources of human exposure to PAHs, such as benzo(α)pyrene (B(α)P), are motor vehicle exhaust, contaminated food and water, tobacco smoke and wood smoke. Research on the toxicity of B(α)P and other PAHs has focused primarily on their role as cancer risk factors. However, several recent epidemiological studies have turned attention to the potential for these environmental chemicals to influence ASD risk.

A prospective cohort study of nonsmoking African-American and Dominican mothers and children in New York City evaluated prenatal exposure to airborne PAHs monitored during pregnancy by personal air sampling and the measurement of DNA adducts specific to B(α)P. Child behavior was assessed using the Child Behavior Checklist. At age 3, prenatal exposure to PAHs was not associated with psychomotor development index or behavioral problems; however, high prenatal exposure to PAHs (upper quartile) was associated with lower mental development index (Perera et al., 2006). At age 6, high prenatal PAH exposure was positively associated with symptoms of Anxious/Depressed and Attention Problems (Perera et al., 2012). A parallel study of Caucasian mothers and children in Krakow, Poland similarly reported a positive association between prenatal exposure to airborne PAHs (as determined by 48 hour personal air monitoring as well as a maternal blood sample and/or cord blood sample) and adverse effects on cognitive behavior in children at 5 years of age (Edwards et al., 2010). A recent study examining the association between traffic-related air pollution and ASD diagnoses identified a positive association between residential proximity to freeways during the third trimester of pregnancy and autism (Volk et al., 2011). This study did not, however, find an association between ASD diagnoses and living near other major roads, suggesting that other confounders not included in the study may have contributed to the association noted in the study participants living near freeways. However, because specific contaminants were not identified in air samples from the residences of study participants, it is possible that the different outcomes reflect the differences in contaminant profiles or burdens between sampling sites. PAHs are a major constituent of motor vehicle exhaust, thus these studies are not inconsistent with the proposal that PAHs contribute to ASD risk.

Rodent studies of perinatal exposure to B(α)P have reported a variety of effects on neuronal connectivity *in vitro* and *in vivo*, such as: (1) downregulation of cortical and hippocampal ionotropic glutamate receptor subunit expression and altered cortical electrophysiology (Brown et al., 2007); (2) impaired long-term potentiation in the hippocampus and deficits in behavioral learning (Wormley et al., 2004); and (3) impaired NMDA-mediated somatosensory cortex neuronal activity (McCallister et al., 2008). Emerging evidence suggests that B(α)P may interact with the rs1858830 'C' allele of the RTK MET to amplify ASD risk. As discussed in Section 2, the C-*MET* allele has been identified as a candidate autism gene and it is associated with decreased expression of MET and altered neuronal connectivity in cortical regions. Mouse pups exposed to B(α)P *via* oral gavage of dams on embryonic days 14 through 17 resulted in a significant downregulation of both transcriptional and translational products of *Met* in the cortex during the first two weeks after birth, which corresponds to the time of peak synaptogenesis in the developing mouse brain (Sheng et al., 2010). In addition, prenatal B(α)P exposure reduced the binding of the transcription factor SP1 to DNA targets (Hood et al., 2000). Similarly, the C allele of *MET* is associated with reduced SP1 binding to DNA consensus sequences (Marshall et al., 2008). These observations suggest the testable hypothesis that B(α)P converges on the MET signaling pathway to amplify the dysregulated neuronal connectivity associated with expression of the C-*MET* allele. Positive results would suggest MET signaling as a convergent molecular target to develop as a screen for environmental chemicals that interact with genetic susceptibility factors to increase ASD risk.

4. Conclusions

A significant contribution from environmental factors in determining ASD risk is consistent with both the rapid increase in ASD incidence and the clinical heterogeneity that is the hallmark of this neurodevelopmental disorder. However, this phenotypic heterogeneity together with the complex multigenic etiologies of ASD significantly increases the challenge of identifying specific environmental factors that confer increased risk for ASD. We suggest that resources be focused on identifying mechanisms by which environmental factors interact with genetic factors to influence adverse neurodevelopmental outcomes of relevance to ASD in experimental models. Such mechanistic insights can then be used to develop rapid throughput screens for potential environmental risk factors, which in turn will inform and focus epidemiological studies.

While a number of mechanisms have been proposed to explain gene × environment interactions that influence ASD risk (Herbert, 2010), one fundamental way in 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 during development (Pessah and Lein, 2008). Several signaling pathways have emerged from genetic studies as convergent molecular mechanisms or targets in ASD, and data is already accumulating of environmental chemicals that potentially interact with those signaling pathways. These emerging data suggest a biological framework not only for studying mechanisms by which specific environmental and genetic factors interact to influence adverse neurodevelopmental outcomes of relevance to ASD, but also for setting up screening of environmental chemicals to identify potential ASD risk factors. While we have focused on structural components of neuronal connectivity in this review, this biological framework can be readily adapted to studies of other parameters of neuronal connectivity that are regulated by these signaling pathways, *e.g.*, Ca^{2+}-dependent regulation of neurotransmitter specification (Borodinsky et al., 2004; Spitzer, 2012).

The success of this strategy is predicated on choosing appropriate model systems. While a number of transgenic animals (mice, and more recently, rats), expressing ASD-linked genetic mutations have been or are being developed, the costs associated with the use of these models limit their utility for early mechanistic and screening approaches (Lein et al., 2005). Such models do, however, serve an important role in confirming putative gene × environment interactions identified in simpler systems. But for initial mechanistic and screening studies, we propose the use of *in vitro* models (such as primary neuronal cell cultures) or simple systems-based models (such as *Caenorhabditis elegans* or *Danio Rerio*) that recapitulate the late stages of neurodevelopment thought to be disrupted in ASD (see Section 2). Ongoing research demonstrates that such models can be adapted for rapid throughput screening across multiple culture wells simultaneously using, for example, high content imaging systems to analyze dendritic morphology and synaptic density (Harrill et al., 2011), microelectrode arrays to measure network activity as a measure of functional connectivity (Johnstone et al., 2010; Robinette et al., 2011), or the FLIPR® to assess spontaneous calcium oscillations (Cao et al., 2012a).

Another consideration is the role of cross-talk between signaling systems that influence neuronal connectivity. For example, Ca^{2+} plays a significant role in both the neuroligin–neurexin–SHANK and the ERK/PI3K molecular pathways. Neuroligins contain an EF-hand metal binding motif and neuroligin–neurexin binding requires the binding of Ca^{2+} to neurexin (Nguyen and Sudhof, 1997). Additionally, Ca^{2+} regulates the activational status of both ERK and PI3K. Ca^{2+} signaling also regulates the expression of the MET RTK (Dai et al., 2012), and changes in the levels of intracellular Ca^{2+} derived from either external or internal sources influence MET activation *via* tyrosine phosphorylation (Gandino et al., 1990, 1991; Naldini et al., 1991). Furthermore, dendritic growth and branching triggered by binding of HGF to the MET RTK is mediated by Ca^{2+}-dependent mechanisms (Tyndall et al., 2007). Conversely, SHANK regulates the synaptic clustering of CaV1.3 L-type calcium channels, and interfering with the binding of SHANK to these ion channels attenuates CaV1.3 channel activation of CREB (Zhang et al., 2005). It is perhaps not surprising that there is significant crosstalk between these molecular pathways,

and thus risk factors that primarily target one molecular pathway may have indirect effects on the other molecular pathways thereby potentially amplifying or attenuating effects on neuronal connectivity. Thus the net outcome of environmental exposures on neuronal connectivity will reflect the balance between these signaling pathways, which are likely to be differentially impacted depending on the type, duration and timing of exposure.

Clearly, work is urgently needed to better predict which combination of defective genes and environmental exposures pose the greatest autism risk. The fact that chemical exposures are more readily controlled than genetic factors to prevent or mitigate the expression of ASD-related traits coupled with the significant toll that ASD places on children with autism and their families, provides a compelling reason to engage in this endeavor.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgments

Supported by the National Institutes of Health (R01 ES014901, R01 ES017425, P42 ES04699, P01 ES011269), the United States Environmental Protection Agency (R833292 and R829388) and the J.B. Johnson Foundation. M. Stamou received a predoctoral fellowship from the UC Davis Superfund Basic Research Program (NIH grant ES04699); K.M. Streifel, a postdoctoral fellowship from ; and P.E. Goines a Hartwell Foundation Biomedical Research Postdoctoral Fellowship. These funding sources had no involvement in the study design; the collection, analysis and interpretation of data; the writing of the report; or the decision to submit the paper for publication.

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