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Genetic Epidemiology and Insights into Interactive Genetic and Environmental Effects in Autism Spectrum Disorders

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Abstract

Understanding the pathogenesis of Neurodevelopmental Disorders (NDDs) has proven to be challenging. Using Autism Spectrum Disorder (ASD) as a paradigmatic NDD, this paper reviews the existing literature on the etiologic substrates of ASD and explores how genetic epidemiology approaches including gene-environment interactions (GxE) can play roles in identifying factors associated with ASD etiology. New genetic and bioinformatics strategies have yielded important clues to ASD genetic substrates. Next steps for understanding ASD pathogenesis require significant effort to focus on how genes and environment interact with one another in typical development and its perturbations. Along with larger sample sizes, future study designs should include sample ascertainment that is epidemiologic and population-based to capture the entire ASD spectrum with both categorical and dimensional phenotypic characterization, environmental measurement with accuracy, validity and biomarkers, statistical methods to address population stratification, multiple comparisons and GxE of rare variants, animal models to test hypotheses and, new methods to broaden the capacity to search for GxE, including genome-wide and environment-wide association studies, precise estimation of heritability using dense genetic markers and consideration of GxE both as the disease cause and a disease course modifier. While examination of GxE appears to be a daunting task, tremendous recent progress in gene discovery opens new horizons for advancing our understanding the role of GxE in the pathogenesis of, and ultimately identifying the causes, treatments and even prevention for ASD and other NDDs.

Keywords

neurodevelopmental disorders; Autism Spectrum Disorders; genetic epidemiology; genes; environment; interactions

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Pathogenesis of psychiatric disorders is complex but gene discovery has provided insight into biological mechanisms underlying neurodevelopmental disorders (NDDs), including Autism Spectrum Disorders (ASD), Intellectual Disabilities (ID) and Schizophrenia.

Gene discovery has led to only a modest understanding of ASD biological pathways, however, recent work with rare, *de novo* mutations is pointing the field in new directions, including converging actions of ASD-associated mutations, and the rapidly evolving progress in the use of translational strategies (in animals and humans) to understand the effects of replicated ASD-associated mutations (1). Understanding roles of rare variants in ASD has highlighted the multifactorial etiology of NDDs characterized by pleiotropy (diverse phenotypes from identical genetic factors), genetic heterogeneity (different genes causing same phenotypes), and interactions: Epistasis (between genes) and gene-environment interactions (GxE) (2–10). Evidence also suggests that environmental factors lead to diverse phenotypes, depending upon the developmental timing of exposure (11–13). Research to disentangle this complexity requires strategies that specifically incorporate both genetic and environmental factors.

This paper provides: (1) Overview of the role of genetic epidemiology in identifying both genetic and environmental factors and their joint roles in NDD etiology; (2) Evidence regarding genetic and environmental influences on NDDs; and, (3) Research strategies to advance our understanding of NDD etiology. We use ASD as an exemplar of the broader group of NDDs.

Genetic Epidemiology in ASD

Genetic epidemiology utilizes disparate data from bioinformatics, population genetics, epidemiology, and molecular genetics to elucidate roles for genes and their interactions with environment in the occurrence of disease in populations (14). Genetic epidemiology: i) Focuses on systematic sampling to enhance generalizability of research findings; ii) Studies joint effects of genes and environment; and, iii) Incorporates disease biology into conceptual models (15). Twin, family, linkage and association studies are among study designs that allow examination of genetic and/or environmental factors in diseases. With increasing evidence for NDD heterogeneity, genetic epidemiologic studies must attend to ASD phenotypic variability in both sampling and phenotype definition.

(1) Genetics

Twin studies with sample sizes of 11–67 monozygotic (MZ) and 9–210 dizygotic (DZ) twin pairs, yield 47–96% ASD concordance rates for MZ, and 0–36% in DZ twins, for autism and broader ASD phenotypes, suggesting strong heritability associated with ASD (16–21). Sibling relative risk (λ_s) (ratio of ASD prevalence among siblings of ASD individuals to general population) ranges from 1.5 to 19.4 (22–26).

Initial genome-wide linkage studies were underpowered for detecting genes of small effect, leading to inconsistent findings across the samples and resistance to replication. Linkage analyses using larger samples, endophenotypes and/or quantitative traits yielded positive findings in specific chromosomal regions: 2q, 5, 7q, 15q and 16p (27, 28).

Candidate gene association studies are used for ASD because they are more powerful than linkage, at a given locus, allowing detection of genes of weaker effect. Due to limited knowledge about ASD pathophysiology and gene functions, only a small number of genes including SLC6A4, GABR, RELN, NLGN, MET or EN2, have been examined with infrequent and inconsistent replications (27, 29). Meta-analysis of 14 family-based association studies of 5HTTR, using 1,000 reported findings from each study as inconsistent, with main analyses showing no association (30).

In contrast, genome-wide association studies (GWAS) exploit strengths of association studies without guessing the identity of causal genes, *a priori* (hypothesis-free approach); this leads to unbiased, comprehensive searches for susceptibility alleles (31). GWAS yields successes in medicine (e.g., age-related macular degeneration, obesity, hypertension, diabetes) (32–35), but GWAS with samples >2,000 failed to identify replicable common variants for ASD (36–39).

Scarce replications in searches for ASD risk alleles result from: 1) Sample sizes insufficient to detect modest effect sizes; 2) Poor control for population stratification in case-control studies; 3) Overly-permissive approaches in multiple comparison corrections, especially in early candidate gene studies; 4) Varied ASD phenotype definitions; and, 5) Diverse samples primarily selected from non-representative sources (40, 41).

Significant increases in Copy Number Variations (CNVs; submicroscopic variations in chromosomal structure), especially *de novo* CNVs, in simplex ASD families (i.e. only one affected individual) have been identified (42). Several investigators reported structural variations on the short arm of chromosome 16 associated with idiopathic ASD (42–45). This 16p11.2 CNV includes ~600kilobases and ~29 genes, 22 of which are expressed in human fetal brain (46). Studies have demonstrated 16p11.2 deletions in ~0.1–0.7% of ASD and 16p11.2 duplications in ~0.1–0.5%, a rate 10-times greater than base rates for this CNV in the general population (7, 47–49).

The 16p11.2 CNV is associated with other phenotypes: ID, developmental delay, speech problems, schizophrenia, seizures, increased body weight/obesity, and increased head circumferences (4–9, 48–54). Similar phenomena have been also reported for a large number of ASD-associated CNVs, including CNVs on 1q21.2, 3q29, 7q11.23, 7q36.3, 15q11.2, 15q13.3, 16p13.11, 17p12, 17q12, and 22q11.21 (3).

Excess *de novo* single nucleotide variant (SNVs) burden has been observed *only* for loss of function (LoF; i.e. nonsense, canonical splice site and frameshift mutations) (55–60). Multiple *de novo* SNVs at the same locus, compared to the null distribution in controls, has allowed identification of genome-wide significant loci, including LoF mutations in: SCN2A, CHD8, DYRK1A, GRIN2B, KATNAL2, POGZ, CUL3 and TBR1(55–58, 61). Like CNVs, some SNVs initially associated with single disorders are now associated with other disorders (e.g., SCN2A in ASD and epilepsy) (55, 57, 62).

Recent studies confirm ASD-related genetic heterogeneity found in earlier twin, family and linkage studies. ASD-related genes seem to converge on a few pathophysiological pathways related to synaptic function and plasticity, GTPase/Ras signaling, and neurogenesis (45, 63–

67). Phenotype pleiotropy suggests interaction with additional genetic/non-genetic factors, acting at various developmental time points resulting in divergent phenotype manifestations of single genetic variant (68).

(2) Environmental Factors

Twin studies provide strong evidence equally for genetics and environmental factors in ASD risk. High levels of heritability (phenotypic variance due to genetic factors), in the range of ~90%, were reported in early twin studies; a recent twin study found larger environmental influences on ASD risk - 37% heritability and 55% shared environmental liability (20, 69, 70). These findings have been replicated in a large independent population-based Swedish National Registry study of 2,049,973 siblings including DZ and MZ twins, yielding 50% heritability and 50% non-shared environmental influence for ASD (26). Diagnostic disparities in some MZ twin pairs also suggest that environmental factors contribute to both liability for and expression of autism-related traits (71).

Progressively higher ASD prevalence estimates (0.07–2.6%) suggest that most of the increase is attributable to greater public awareness, better case ascertainment, broadening of ASD diagnostic construct, and/or diagnostic substitution (72–74). If increasing ASD prevalence is even partly caused by increasing incidence, environmental factor(s) and/or their interactions with as-yet-unknown genetic vulnerability may represent other ASD risk mechanisms.

Nutrients, smoking, alcohol, medications and pesticides are the most commonly examined exposures during pregnancy, due to their known neurotoxicity and/or specific adverse/protective impacts on developing brains (75). Epigenetics (long-term and/or heritable changes in function of a locus/chromosome without alteration of underlying DNA) may represent one pathway for GxE (76). ASD is associated with Fragile X, Retts, and Angelmans syndromes, each of which involves epigenetic mechanisms (77–79). Associations have been also reported between ASD and parent-of-origin syndromes (e.g., 15q11.3, Turners) (10, 75, 80). Two recent studies report differences in DNA methylation profiles between individuals with and without ASD; one studied 20 postmortem brains of individuals with ASD and 21 controls, finding differentially methylated regions (DMR) in/near genes implicated in cell signaling, synaptic function, plasticity, imprinting and metabolism (80). Analyses of 6 discordant and 44 concordant MZ twins found numerous DMRs associated with ASD in within-twin and between-group analyses. Significant correlations between DNA methylation and quantitative autistic traits were also found (81); these findings should be interpreted with caution due to methodological limitations, including small, non-representative samples, inadequate case-control sample comparability, multiple comparisons, and cross-sectional design that cannot discern causal relationship. The substrate for epigenetic dysregulation is unknown, however, environmental factors are associated with epigenetic changes, including correlations between folate levels and global DNA methylation (75). This provides evidence that environmental factors may contribute to ASD etiology through epigenetic processes (82, 83).

Studies report associations between ASD and prenatal toxic exposure, ranging from thalidomide to maternal, intrapartum rubella (84–88), suggesting that environmental

exposures during critical periods contribute to ASD susceptibility. How this occurs, the timing, and direct effects causing ASD remain unknown.

Two meta-analyses of 24 studies examined associations between ASD and perinatal complications (89, 90), finding modest increases in ASD risk. Odds Ratios (OR) from 1.4 to 1.8 were associated with abnormal fetal presentation, umbilical cord complications, fetal distress, multiple birth, maternal hemorrhage, summer birth, small-for-gestational age, congenital malformation, meconium aspiration, ABO/RH incompatibility and hyperbilirubinemia, with the largest ORs for birth injury (OR=4.9), low birth weight (<1500g) (OR=3.0), and neonatal anemia (OR=7.87). While disaggregating perinatal complications from ASD is challenging because susceptibility might have led to perinatal complications and ASD, they point to two hypotheses:

Hypoxia: Obstetrical complications related to hypoxia pose significant risk in meta-analyses (90). This is supported by a recent twin study indicating that respiratory distress and other markers of hypoxia increased ASD risk (91). Relationships between brain hypoxia and social behavior deficits warrant further attention.

Immunologic: Studies link maternal infections during pregnancy to ASD in offspring (89, 90). While not well-established, this may be a direct effect of the infectious agent and/or the resulting activation of the maternal immune system leading to increased ASD risk (92–95). Approximately 10% of mothers of children with ASD harbor Anti-Brain Antibodies (ABA) that bind to fetal brain (96, 97); ABA assays were conducted only in mothers of case children, but not in control. When these antibodies were administered to gestating mice and monkeys, the offspring exhibited abnormal behaviors, including social deficits (98–101). These studies suggest that maternal antibodies can alter fetal development resulting in ASD-like behaviors.

Immunologic hypotheses are appealing when combined with transcriptomic analyses of postmortem brain of individuals with autism (102). Compared to individuals without ASD, brains of those with ASD demonstrated downregulation of 209 genes, enriched for gene categories related to synaptic function, while 235 genes implicated in immune and inflammatory response were upregulated. The former group was significantly enriched for association signals in GWAS studies while the latter was not. These studies suggest that relevant immunologic changes are likely caused by environmental factors: however, the roles of rare variants resulting in observed findings cannot be ruled out.

Two studies report associations between ASD and folate exposure. Each study reported protective effects of intake at different times: 1st trimester vs. preconception, suggesting that there may be multiple points during fetal development when folate is protective (103, 104).

The roles of advanced maternal and paternal age at pregnancy on their offspring's ASD risk have been consistently reported (105, 106). Suggested mechanisms include increased rates of *de novo* mutation, epigenetic dysfunction, and cumulative exposure to environmental toxins (60, 105, 107).

Studies examining relationships between maternal smoking and/or drinking during pregnancy and ASD risk are inconsistent and inconclusive. No studies examined the impact of paternal exposure on ASD risks (108).

Exposures to antiepileptic medications and antidepressants, before and during pregnancy, suggest increased risk for ASD, however, exposure timing differed in each study, making sound conclusions challenging (108). Studies of pesticide exposure are still too few to draw conclusions.

Inconsistencies in findings and methodological differences in ASD environmental studies make it challenging to reach solid conclusions; inconsistent results are not surprising given significant methodological differences in work-to-date. Phenotype measurement differences occur because: 1) Quality of ASD diagnoses vary greatly; 2) Comorbidity, especially ID, is not considered, making it difficult to disaggregate risk factors for ID/comorbidity from those for ASD; and, 3) Case ascertainment from administrative datasets increases likelihood of ascertainment bias due to factors associated with accessibility/eligibility for the inclusion. Administrative datasets have great diversity because inclusion criteria are administratively but not clinically salient and may change over time. Measurement differences in these studies include: 1) Varying methods to measure perinatal risks; 2) Timing and dose of exposure not consistently measured, making it difficult to align exposure with specific developmental processes and to compare exposures across studies; 3) Validity of exposure data jeopardized by retrospective recall; and 4) Ecological fallacy present when group level exposures are obtained.

(3) GxE

Genes and environment rarely act alone to create NDD/ASD (7). Despite many studies exploring roles of genes or environmental factors in ASD, few examine GxE. Since development is a dynamic process reflecting a constant interplay between genes and environment, these interactions occur constantly. Specific perturbations in this process likely play roles in ASD etiology; ignoring these interactions may obscure independent genetic/environmental effects, leading to false negative and inconsistent findings (109). Both human and animal studies also suggest that GxE plays a role in ASD pathogenesis (10).

When considering GxE, it is important to distinguish: (a) Statistical vs. Biological Interaction; (b) Additive vs. Multiplicative Interaction; and (c) Gene-Environmental Correlation (rGE) vs. GxE.

Statistical interaction of multiple factors is the coefficient of the product term of the risk factors. While convenient for identifying interactions, it ignores biological plausibility of interaction mechanisms and depends on statistical models (110). Statistical interactions can be artifactual by data anomalies, including data distribution problems, making a careful examination of data characteristics essential when statistical interactions are detected (111). Biological interaction refers to multiple biological factors acting together to increase/decrease disease risk or the pathophysiologic substrate of disorders (112). Example is phenylketonuria (PKU), caused by genetic mutation. Having the mutation is not sufficient; it requires exposure to environmental phenylalanine plus the genetic anomaly to create the

disease phenotype. Disruption of that interaction (phenylalanine-restricted diet) is sufficient intervention to treat the condition.

Interactions can be detected as multiplicative or additive. Multiplicative interactions exist when the relative risk (RR) of having multiple factors does not equal the product of the RRs associated with each factor separately (113). Logistic regression implicitly utilizes multiplicative scales (112). Additive interactions exist when the excess risk attributable to multiple factors does not equal the sum of excess risk caused by each factor separately (113). Despite the analytic convenience of multiplicative interactions, use of additive interactions is advocated when examining biological interactions due to its public health implications (114). Because detection of interactions is model-dependent, it is possible to have one significant additive or multiplicative interaction but not the other (111). For example, bladder cancer risks (ORs) in individuals with chr1p13.3 deletion, smoker and the joint effect are 1.70 (95% Confidence Interval: 1.38–2.09), 3.30 (2.71–4.03), and 4.69 (3.86–5.69), respectively; the null risks under additive and multiplicative interaction models are 4.00 and 5.61, respectively, making GxE significant only in the additive model (115).

GxE is genetic difference in vulnerability to particular environmental factors that contribute to a phenotype. rGE is genetic difference that causes differential exposure to particular environment factors: rGE may be heritable, even if consequences of exposure are not (116, 117). For example, individuals with antisocial behavior are more likely to engage in risk-taking behaviors like drinking and driving (118); however, increased risk for automobile accidents associated with drinking and driving is an rGE and not genetic. To avoid mistaking rGE as genetic effects or GxE, investigators must be aware that even small rGE may cause type I errors in GxE studies with case-only or case-control designs (117).

Several research designs are useful for GxE studies. When shared/non-shared environment data are available, twin studies can provide inferences about specific GxE (119). Discordant MZ twin, adoption and half-sibling studies can identify environmental factors and GxE (120). Nested case-control studies offer advantages by minimizing selection and recall biases, however, they are not optimal for rare diseases, and selection bias can occur from low participation rates (119). While case-only designs are efficient for examining GxE, they are based on no rGE and rare disease assumptions; even minor violations of these assumptions causes significant bias (121, 122).

In discordant sibling studies, unaffected siblings had fewer perinatal complications than ASD probands but more than controls, suggesting that individuals with ASD react differently to the same environmental stimuli and have less tolerance to perinatal environment when compared to siblings (10, 123).

Inconsistent findings regarding parental smoking and ASD risks may be explained by GxE. One study comparing stillbirths of smoking and non-smoking mothers found EN2 strongly expressed in arcuate nucleus neurons in non-smokers' fetuses, but not in 11/12 fetuses from smoking mothers, however, small sample size and multiple comparisons hamper the interpretations of the results (124). Intrauterine smoke exposure also damages serotonin projections in cortex and striatum, producing sex-selective changes in 5HT1A and 5HT2

receptor expression, and inducing adenylyl cyclase causing sensitization of heterologous inputs in this signaling pathway (125, 126). EN2 and SLC6A4 were associated with ASD in candidate gene studies, but not in GWAS. GWAS replication failures might partially stem from failure to consider interactions between environmental events (*in utero* nicotine exposure) and genetic vulnerabilities (EN2 or SLC6A4 variants) in increased ASD risk. These GxE hypotheses require further examination.

Animal studies support GxE in ASD pathogenesis. Pletnikov et al. reported differences in brain pathology, behavior, neurochemistry and drug response in rats exposed to Borna Disease virus, a teratogen causing neurodevelopmental damage and behavioral deficits analogous to ASD (127). Other animal models, ranging from *C. elegans* to mice, also demonstrate that genetic defects in synaptic function alter sensitivity to environmental factors, suggesting plausible GxE mechanisms (10).

Using a case-control design, one group reported three GxEs from the same study population. In the study of 429 ASD and 278 typical children, maternal MTHFR 677TT (rs1801133), CBS rs234715 GT+TT and child COMT 472AA (rs4680) genotypes conferred greater ASD risk when mother did not take vitamins periconceptionally (128). However, observed GxEs lost significance after multiple comparison corrections. Also, population stratification was not controlled when investigators reported differences in racial composition between cases and controls. In the second study of 429 children with ASD, 130 with developmental disorders and 278 typically developing, the strongest protective effects of maternal folate intake during first month of pregnancy were reported in mothers and children with the MTHFR 677C>T variant (103). Again, multiple comparisons were not addressed, and residual population stratification, resulting from crude racial categories and retrospective exposure data collection, are sources of Type I error. The third study reported interactions between high pollution and NO₂ and the MET CC genotype (rs1858830) in 251 cases and 156 controls. Air pollution was determined using public air quality data for the area where individuals reported residence at-birth (129). In addition to uncorrected multiple comparisons and population stratification, pollution exposure was measured at a group level leading to concern about ecological fallacy and Type I error. All three studies used a candidate gene approach prone to false positive findings (130). None of these GxE findings have been independently replicated.

(4) Challenges in GxE Research and Future Suggestions

GxE studies are power-intensive. Even testing for a single GxE specified *a priori*, the exponential growth in the number of comparisons requires large samples (131). For example, in an unmatched case-control study with a log-additive inheritance model, sample sizes required to examine a GxE effect size of OR 1.5 and 2.0 with 80% power and 2-tail p-value 0.05 (without multiple comparison corrections) are 31,084 and 9,550, respectively, when prevalence of a disease, environmental risk, and a risk allele are 1, 5 and 5%, respectively, and main effects of environmental and genetic risks are OR 1.2 and 1.2, respectively (132). Researchers attempt to overcome this challenge in two ways: Increasing sample size by establishing large consortia that share data, and Meta-analyses. Several large-scale, population-based genetic epidemiological ASD studies are underway: Norwegian

Autism Birth Cohort Study and Mother and Child Cohort Study, Danish Birth Cohort Study, Finnish Birth Cohort Study, and Swedish Registry-Based Studies, as well as US-based Childhood Autism Risks from Genetics and Environment (CHARGE) Study and Early Autism Risk Longitudinal Investigation (EARLI) Study (92, 104, 133–137). All these studies, except CHARGE, use prospective cohort designs; European studies use population-based representative samples. Biological specimens are collected during pregnancy with repeated phenotype and exposure measurements during follow-up. When combined with attempts to harmonize the exposure and phenotype measurements across studies, power to detect GxE is significantly increased.

There are not enough methodologically-sound GxE ASD studies to conduct meta-analyses. Investigators wishing to conduct valid meta-analyses need ASD GxE studies with ample size, appropriately-ascertained samples, detailed phenotypes, sound environmental measurement, state-of-the art sequencing data and strong statistical analyses. Investigators and editors must publish both positive and negative findings to minimize publication bias (131).

Research identifying how environmental factors impact ASD pathogenesis has often been characterized by relatively low levels of accuracy and reliability in the measurement of environmental exposures, leading to both Type I and II errors in GxE studies. Environmental measures in ASD GxE studies were based on questionnaire or ecological measures rather than necessary measurement at the individual level. In the cited air pollution GxE study, linking group-level exposures of air pollution to individual-level exposure is difficult because it depends on specific information on exposure duration and individual biological factors, including inhalation/absorption of pollutants, activities, ages, and preexisting health conditions (138). There is no information about how maternal air pollutant exposure is correlated to fetal exposure in humans. Solving these problems is critical for identifying biomarkers of individual exposures (parental and child) and understanding specific environmental risk and its influence on ASD pathogenesis. Forthcoming technologies, such as geographic information systems and biological monitoring/sensing, enable more precise measurement of environmental exposure at individual levels (138). Meanwhile, researchers must use established methods to reduce measurement error, including validity and reliability studies for questionnaires and ecological data (139).

Systematically ascertained population-based samples representing the entire spectrum of the ASD phenotype are optimal for GxE studies. For cost and convenience, clinical/administrative samples are used more commonly even though they are more vulnerable to selection bias in terms of case severity, comorbidity, and factors associated with access to services (140, 141). For example, two studies revealed that sex prevalence discrepancy decreased from 4–5:1 to 2–2.5:1 in epidemiologically ascertained children with ASD (26, 73). Similarly, the proportion of ASD children with ID decreases significantly as community ascertainment is more complete (73, 142).

ASD phenotype heterogeneity poses challenges in etiological research, including GxE studies. Dimensional phenotyping using tools already available (e.g., Social Responsiveness

Scale) is less vulnerable to phenotype heterogeneity and can complement traditional diagnostic approaches (143, 144).

Methodological advances can address preexisting challenges in GxE studies. Experimental models will allow GxE testing in animal/cellular systems allowing subsequent population-based GxE studies with more specificity (145). Advances in technology will allow examination of genetic and environmental factors for ASD risks without *a priori* hypotheses about candidate genetic and environmental factors (146). There is already successful gene discovery using agnostic approaches through GWAS (147). Similarly, proof-of-principle environment-wide association studies (EWAS) are being examined (146). When methods are well-established, EWAS+GWAS will provide opportunities for identifying novel GxE mechanisms (148).

Conclusions

New genetic and bioinformatics strategies have yielded important clues to ASD genetic architecture. Recently, a large, epidemiologically-ascertained ASD sample estimated liabilities of 2.6% from rare *de novo* mutation, 3% from rare inherited variants and 49% from common inherited variants (149). Understanding ASD pathogenesis will require more sophistication. Significant effort must focus on how genes and environment interact with one another in typical development and its perturbations, including those that yield ASDs. Larger sample sizes will be necessary but not sufficient to address complex questions. Study designs should include: (1) Sample Ascertainment that is epidemiological and population-based to capture the entire ASD spectrum; (2) Phenotypic Characterization that is both categorical and dimensional; (3) Environmental Measurement with accuracy, validity and biomarkers; (4) Statistical Methods that address challenges such as population stratification, multiple comparisons, and GxE with rare genetic variants (e.g., rare variant burden analyses stratified by environmental exposure status in cases); (5) Animal Models to test hypotheses before and after going into field; and, (6) New Methods, including GWAS and EWAS to broaden the capacity to search for GxE, precise partitioning of heritability with the use of dense genetic marker from GWAS to refine the search for GxE, and consideration of GxE not only as the cause of disease but also as a disease course/outcome modifier (150).

We have the tools to study and understand GxE with recently identified genes; application of methodologically rigorous studies have major potential for discovering new ASD etiologic substrates. While examination of GxE appears to be a daunting task, tremendous recent progress in gene discovery opens new horizons for advancing our understanding the role of GxE in the pathogenesis of, and ultimately identifying the causes, treatments and even prevention for ASD and other NDDs.

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