The Emerging Picture of Autism Spectrum Disorder: Genetics and Pathology

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Annu. Rev. Pathol. Mech. Dis. 2015. 10:111–44

The *Annual Review of Pathology: Mechanisms of Disease* is online at pathol.annualreviews.org

This article's doi: 10.1146/annurev-pathol-012414-040405

Keywords

human genetics, molecular pathology, neuropathology, mouse models, systems biology

Abstract

Autism spectrum disorder (ASD) is defined by impaired social interaction and communication accompanied by stereotyped behaviors and restricted interests. Although ASD is common, its genetic and clinical features are highly heterogeneous. A number of recent breakthroughs have dramatically advanced our understanding of ASD from the standpoint of human genetics and neuropathology. These studies highlight the period of fetal development and the processes of chromatin structure, synaptic function, and neuron-glial signaling. The initial efforts to systematically integrate findings of multiple levels of genomic data and studies of mouse models have yielded new clues regarding ASD pathophysiology. This early work points to an emerging convergence of disease mechanisms in this complex and etiologically heterogeneous disorder.

INTRODUCTION

Autism spectrum disorder (ASD) comprises a group of developmental disabilities characterized by impaired social interaction and communication accompanied by stereotyped behaviors and restricted interests (1). Previous definitions included the presence of language dysfunction, which although frequently observed is no longer required for diagnosis. Once thought to be relatively rare, affecting fewer than 1 in 1,000 children, more recent studies have estimated the prevalence of ASD at 1 in 68 (2). Since autism was first described in 1943 by Kanner (3), who even at the time suggested a genetic etiology, our understanding of ASD continues to grow from genetic, neuropathological, and neuroimaging observations. However, the fundamental molecular pathways involved in ASD are still largely uncharacterized. Consequently, clinical diagnosis on the basis of behavior is considered the gold standard (4), and the few available pharmacologic interventions target symptomatology (e.g., risperidone for the treatment of irritability and aggression) rather than the underlying biology (5).

ASD has been relatively resistant to classification into more homogeneous clinical or pathological subgroups. The text revision to the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) subclassified ASD into several categories (autistic disorder, pervasive developmental disorder/not otherwise specified, and Asperger's syndrome). However, the fifth edition (DSM-5) removed these groupings in favor of a unified autism spectrum disorder in light of low interrater agreement in clinical assessments (4) and unreliable distinguishing features between subcategories in terms of neuropathology or response to treatment (6). Thus far, no unique ASD subtypes with distinct pathological features have been robustly demonstrated.

The extreme heterogeneity of ASD further complicates our understanding of its biology. The very concept of an autism spectrum acknowledges the fact that clinical phenotypes (including core phenotypes, such as social function and mental flexibility, and associated phenotypes, such as language delay and IQ) of ASD patients can vary dramatically, even among disease-concordant monozygotic twins (7). In addition, there is variation in the timing of onset, as about a quarter of children with ASD "regress" after developing normally for as long as 2 years, whereas others miss their developmental milestones all along (8). ASD on a genetic level is arguably even more complicated; ASD has been associated with numerous Mendelian diseases, and genetic evidence suggests that 200–1,000 genes are involved in ASD susceptibility (9).

Therefore, it is not surprising that the systematic study of ASD pathology has been hindered by the disorder's heterogeneity and by relatively small sample sizes. However, dramatic advances over the past decade promise to shift our understanding from a smattering of tantalizing clues to a coherent understanding of the circuits, cells, and pathophysiological processes affected in ASD that belies its apparent heterogeneity. We start by reviewing exciting studies in human genetics that serve as a causal anchor for ASD biology. Because genotype precedes phenotype, evidence of genetic contributions to ASD has supported a molecular element to the disease and identified specific genes that drive its pathogenesis. We then summarize recent findings in other aspects of the molecular pathology (in particular, transcriptomics of postmortem brain) and neuropathology (from the microscopic to the macroscopic scale) that yield insight into higher-order levels of brain dysfunction in ASD. Finally, we touch on work in animal models and systems biology that has begun to integrate each of these levels, and that hints at a bigger picture in spite of extreme genetic and clinical heterogeneity.

HUMAN GENETICS: A FOOTHOLD ONTO BIOLOGY

Although Kanner first assumed that autism was an inborn (i.e., genetic) disturbance, he also noted commonalities in how autistic children were raised and questioned whether their environment

could also contribute (3). Since then, many hypothesized environmental contributions have been thoroughly debunked (for example, Bettelheim's "refrigerator mother" hypothesis or Wakefield's vaccine hypothesis). Environmental factors likely still account for some cases, though few have been clearly defined (10). Notable examples include prenatal exposures such as valproic acid, which is associated with significantly increased rates of ASD when exposure occurs in early fetal development (11). Environmental factors may also interact with genetic factors; for example, patients with phenylketonuria have a high risk of developing ASD and other manifestations of the disease if they consume diets high in phenylalanine (12). A similar interaction has been proposed for a recently discovered metabolic form of ASD caused by mutations in *BCKDK*, which can cause ASD in a recessive manner but has been treated in a mouse model with high consumption of branched-chain amino acids (13).

The effect of genetic factors has been more explicitly quantified, and a large accumulation of evidence points to a strong genetic component of ASD. One line of evidence comes from Mendelian diseases associated with ASD, showing that single genes can greatly increase risk for the disorder. These include mutations in *FMR1* (fragile X syndrome), *MECP2* (Rett syndrome), *TSC1/TSC2* (tuberous sclerosis complex), and *CACNA1C* (Timothy syndrome), as well as chromosomal abnormalities such as maternally inherited 15q11–13 duplications (dup15q syndrome), and many others, which may collectively account for 10–20% of all ASD cases (9, 14). Indeed, new syndromic forms of ASD continue to be identified, such as cortical dysplasia–focal epilepsy syndrome, caused by mutations in *CNTNAP2* (15), and a new syndrome characterized by ASD and specific facial dysmorphism, caused by mutations in *ADNP*, that was described as this review was being prepared (16).

Epidemiological evidence from family and twin studies also supports a strong genetic component in ASD etiology. Strong familial clustering has been observed—approximately 20% of infants with an affected older sibling also develop ASD, and the rate is even higher for boys (17). Further, the same study observed that as the number of ASD probands increases in the family, so does the recurrence risk in subsequent children, reaching almost 50% in a male born into a family with more than one affected proband. Twin studies also support a genetic etiology, revealing high concordance of ASD diagnosis in monozygotic twins relative to dizygotic twins—ranging from 30% to 90% in monozygotic pairs compared with 0% to 30% in dizygotic pairs (18–20). Overall, the estimated heritability of 0.7–0.8 leaves room for environmental factors but defines a strong role for genetic causes. Additionally, the dizygotic twin concordance rate is similar, or only slightly higher, than the recurrence risk in siblings, suggesting a small contribution from shared (maternal) environment.

Although epidemiological studies have convincingly demonstrated a strong genetic component to ASD, pinpointing the responsible genetic variants has been difficult. Two decades of linkage, candidate gene, and common variant association studies have revealed daunting genetic heterogeneity with multiple modes of inheritance (encompassing rare monogenic causes with partial penetrance, common variants of small effect, and inherited and de novo copy number variation that impacts multiple genes) but few clear solid findings, mostly due to small sample sizes and lack of power (14). Here, we focus on work over the past decade that has resulted in definitive identification of some ASD gene candidates, as these can help us understand pathophysiology. Such significant leaps have been made possible by technological advances such as dense array-based genotyping and next-generation sequencing (see the sidebar, Strategies for the Identification of Genetic Variants). Implicated genetic variants can be either inherited or de novo, each with characteristic experimental design and subject cohorts.

STRATEGIES FOR THE IDENTIFICATION OF GENETIC VARIANTS

Single-nucleotide variants. Oligonucleotide arrays can rapidly and economically genotype several million common single-nucleotide variants (SNVs; i.e., polymorphisms) in a large cohort of patients. Rare SNVs are more challenging to pinpoint because of their high numbers and uncertain positions. Some arrays—such as the so-called exome chip—genotype a subset of rare SNVs. However, other SNVs may have never been previously observed. Sequencing approaches provide DNA sequences over large swaths of the genome, which can then be compared to a reference to find variants. To cover the whole human genome or coding region (exome), next-generation sequencing is most practical.

Structural variants. Structural variants (SVs) include insertion or deletion variants that can span many base pairs. SVs larger than several million base pairs can be detected using light microscopy. Submicroscopic copy number variants (CNVs) can be detected by high-density genotyping arrays (by changes in signal over a number of contiguous genomic positions) and comparative genomic hybridization. Next-generation sequencing can also identify CNVs at high resolution, but thus far has not been widely utilized for this purpose in ASD. Smaller insertions or deletions (indels) are difficult to detect and have not been well characterized in ASD; however, with next-generation sequencing, their role may be soon clarified.

Common Inherited Variation

The advent of high-throughput genotyping of single-nucleotide polymorphisms (SNPs) using microarrays opened the era of genome-wide association studies (GWAS). Five GWAS have examined the association between common variants and ASD diagnosis using family-based cohorts (21–24), identifying two genome-wide significant risk loci—intergenic polymorphisms between *CDH10* and *CDH9* at 5p14.1 (21) and within *MACROD2* (but which may correlate with expression of *PLD2*) (25). Suggestive signals have been reported at 5p15.31 (between *SEMA5A* and *TAS2R1*) (23) and 7q35 (within *CNTNAP2*).

Overall, although recent studies estimate that 15–40% of the genetic risk of ASD is tagged by common variants (26–28), only two modest genome-wide significant loci have been found. Furthermore, even these loci have been problematic to reproduce. For example, the *MACROD2* association has failed to replicate in well-powered cohorts (24, 29). One might wonder why ASD GWAS have yielded relatively few loci of small effect considering the high heritability and prevalence of the disease. First, it is critical to recognize that high heritability does not imply a specific genetic model, Mendelian or otherwise, and the genetic contribution of common variants to ASD is likely mediated by a large, heterogeneous group of variants, each conferring a miniscule amount of risk individually (24). Second, compared with studies of similarly prevalent common diseases, the sample sizes in ASD GWAS are relatively small—only up to approximately 1,500 cases, compared with more than 5,000 cases in discovery cohorts and more than 10,000 cases in meta-analyses for type 2 diabetes (30) and schizophrenia (31). A GWAS of schizophrenia identified only two significant risk loci with a sample of 5,001 cases and 6,243 controls; however, 24 loci reached statistical significance in a meta-analysis of 21,246 cases and 38,072 controls, and modeling based on these results predicts almost a thousand loci in a sample of 60,000 cases and 60,000 controls (31). Much larger sample sizes are needed, and we expect that many new loci will be identified as ASD cohorts similarly expand.

Rare Inherited Variation

Although common variants collectively explain much of the genetic risk for ASD, the effect size of individual polymorphisms is small based on the GWAS cited above. This is also supported by

genetic linkage analysis based on SNP genotyping, which has identified a genome-wide significant signal at 20p13 and a suggestive signal at 6p27 (23). These results follow a decade of previous linkage studies that identified loci at 7q31–35 and 17q11–21, as well as a number of other loci that were not replicated, likely due to genetic heterogeneity and small sample sizes (14). However, in those regions that reach genome-wide significance or are most often replicated, common variants accounting for the linkage signal have not been identified (32–34, 68). This, and genetic models in families (35), has fueled the search for rare deleterious variants that may confer greater risk, due to selective pressures that prevent such variants from propagating into a large fraction of the population. The identification of inherited risk variants has traditionally relied on their segregation according to Mendelian patterns through large families or through linkage disequilibrium with common polymorphisms, thereby generating a large effective sample size in the population. In small families or a cohort of unrelated patients and controls, the heterogeneity of ASD makes it difficult to pinpoint rare ASD risk variants with a high degree of confidence, though signals from synaptic genes (36), genes shared with other neuropsychiatric diseases (36, 37), and an excess of loss-of-function variants can be detected (38). However, elegant strategies to find rare inherited variants in smaller families have emerged. For example, multiplex families (those with multiple members with ASD) and families with consanguinity are enriched for causal inherited variation. Bioinformatically, identification of very rare, recessive variants (therefore necessitating two rare events in a single gene in a single individual) can help narrow the search space and thereby provide power.

Several studies have recently exploited these strategies to yield new leads in the search for inherited variation. Morrow et al. (39) used homozygosity mapping in families with consanguinity to find large homozygous deletions that included the *C3ORF58*, *NHE9*, and *PCDH10* genes. Nava et al. (40) searched for inherited variants on the X chromosome by identifying families with two affected male ASD probands consistent with an X-linked pattern of inheritance, thereby identifying recurrent mutations in the *TMLHE* gene. Yu et al. (41) used linkage in families with consanguinity and multiple affected individuals to further uncover rare inherited variants. Because consanguinity implies identity by descent at a number of alleles, and the presence of multiple affected individuals implies an inherited genetic contribution to ASD, this sample highly enriches for causal homozygous variants. Indeed, cosegregating homozygous missense variants in *AMT*, *PEX7*, and *SYNE1* were observed. A screen of 70 candidate genes in 163 additional families revealed cosegregating variants in *NLGN4X*, *MECP2*, *PAH*, and *VPS13B/COH1*. Lim et al. (42) used a similar strategy and searched for rare "complete knockouts" in exome sequencing data. These compound heterozygous and homozygous loss-of-function variants are very rare in the population, and were enriched in ASD patients. Several knockout genes were found to overlap those from de novo variant studies (see below), including *IFIH1*, *ABCC12*, *PKHD1L1*, and *PCDH11X*, as well as recurrent mutations in *SLC22A14*, *LUZP4*, and *DGAT2L6*.

De Novo Variation

One genetic cause of ASD that does not register in heritability estimates is de novo variants, which originate from mutations in the parental germ line or early somatic cells of the developing individual. The large mutational target size, reduced fecundity, and high prevalence of ASD indicate a priori that de novo genetic variants may play an important role (43). The search for de novo causal variants is simplified compared with the search for inherited variants because of their relative rarity. An individual's genome will only have a handful of de novo copy number variants (CNVs) and single-nucleotide variants (SNVs); thus, if a recurrent CNV or SNV is observed within a particular gene, it is likely involved in ASD susceptibility because of the very small likelihood of this event occurring by random chance. Furthermore, cohorts such as the Simons

Simplex Collection (44) can enrich for ASD cases caused by de novo variants. These so-called simplex families have a single affected proband with unaffected parents and siblings, and the cases of ASD in these families are therefore more likely to be caused by de novo, rather than inherited, genetic variation. Many de novo variants that contribute risk to ASD have been identified using this strategy, opening up a new area of research.

The first de novo events convincingly observed in ASD were CNVs. Several large deletions and duplications detected by standard cytogenetics studies were already known by the 1990s to confer increased risk for ASD, such as dup15q (45), 22q11.2 deletion syndrome (46), Xp22.3 deletions (47) (now known to contain the ASD susceptibility genes *NLGN3* and *NLGN4*), and others (48), which can be inherited but often occur de novo. Efforts in narrowing down chromosomal regions associated with ASD implicated a small number of ASD susceptibility genes—for example, *NLGN3*, *NLGN4* (49), and *SHANK3* (50). The widespread use of dense oligonucleotide arrays for genotyping further enabled facile detection of submicroscopic CNVs, and it was reasonable to assume that novel CNVs that confer risk for ASD could be discovered.

Although inherited CNVs have been demonstrated in ASD (51, 52), the rarity of de novo CNVs provides strong proof for their causal involvement. This is evident in studies comparing CNV number in ASD patients versus controls; whereas no difference is found for inherited CNVs (51, 53), a strong signal is found for de novo CNVs. Whereas only approximately 1% of controls carry a de novo CNV (54), their frequency in ASD patients is 2- to 7-fold greater, and possibly higher in simplex than in multiplex families (53–57). De novo CNV size and the number of genes within each CNV are also increased in ASD patients (56, 58). Therefore, genes affected by even nonrecurrent de novo CNVs have a relatively high prior probability of pathogenicity. Recurrently identified CNVs, such as those at 7q11.23 (the Williams syndrome region), 15q11–13 (the dup15q region), 16p11.2, and 16p13.2 (51–53, 55, 57, 59), are then highly unlikely to occur by chance and are considered causal.

Recent technological advances have reduced the cost of acquiring sequencing data at single-base resolution in large portions of the human genome, or the coding fraction of the genome (exome), thereby enabling the study of de novo SNVs. Whole-genome sequencing studies have estimated the germ-line mutation rate of SNVs on the order of 1×10^{-8} per nucleotide per generation (60–63); an even smaller number fall within the coding regions of the genome, and therefore de novo SNVs can be examined systematically in an unbiased search for ASD risk genes. Interestingly, these studies show that de novo variants occur preferentially on the paternal chromosome and increase as a function of paternal age; this fact explains the observation that children born to older fathers may have a higher risk for ASD and implies a large contribution of de novo variants as a class to ASD risk. Furthermore, the mutation rate of genomic regions can vary by more than two orders of magnitude, and regions near ASD risk loci seem to be hypervariable (63). This evidence points to the involvement of de novo SNVs in ASD. Unlike CNVs, which contain many genes, SNVs can point directly to a single base and therefore provide a more powerful implication of a particular gene's involvement in ASD.

In this regard, four groups used exome sequencing studies of nonoverlapping trios, primarily from the Simons Simplex Collection, to reveal a number of new ASD susceptibility genes (58, 64–66). In the combined cohort, recurrent de novo SNVs were found in *DYRK1A*, *POGZ*, *CHD8*, *NTNG1*, *GRIN2B*, *KATNAL2*, and *SCN2A* among the sequenced subjects. A follow-up targeted gene-resequencing study of 44 genes that were hit at least once in initial sequencing studies used molecular inversion probe technology in a larger cohort of 2,446 ASD probands to identify recurrent de novo mutations in six genes: *CHD8*, *DYRK1A*, *GRIN2B*, *TBR1*, *PTEN*, and *TBL1XR1* (67). Subsequent exome and whole-genome sequencing studies have identified recurrent de novo SNVs in *GPR98* and *KIRREL3* in ASD (63).

Common Themes Emerging

A large number of new genes have been implicated by inherited and de novo variants in recent human genetics studies, but the amount of work to be done remains humbling. First, some of these genes have not been studied in vitro, and their functions remain to be clarified. Second, the genetic architecture of ASD has been proven complicated, with contributions from highly penetrant one-hit and two-hit variants, common variants with small effect, de novo variants, inherited variants, and combinations of these, but the large majority of cases of ASD will still have no identifiable genetic cause (9, 27). Third, study designs still lack the power to detect many kinds of potentially causal variants, particularly those with a low effect size, or complicated inheritance patterns modified by factors such as genetic and environmental interactions, parent-of-origin effects, and especially gender (68). Despite these limitations, each newly implicated ASD-causing gene provides a clue to the molecular mechanisms at play, and these clues have begun to outline functional groups such as neuronal activity, transcriptional regulation, neuronal cell adhesion and synaptic function, and excitation/inhibition balance (**Figure 1**) (9). Although the pathogenicity of each of the identified variants is not known with complete certainty, in aggregate they represent a gene list that is highly enriched for causal genetic variation in ASD and in which we can begin to look for common function.

POSTMORTEM BRAIN TRANSCRIPTOMICS: BRIDGING HUMAN GENETICS AND NEUROPATHOLOGY

Another way to study ASD pathophysiology is to focus on its molecular pathology. As such, several studies have looked at gene expression in postmortem ASD brain using transcriptome-wide tools such as microarrays and RNA sequencing to identify molecular commonalities. Remarkably, similarities were found not only within syndromic forms of ASD, but also in idiopathic cases. Because there is convergence at the level of the transcriptome, gene expression can connect genetic data and neuropathology. One can identify the nature of gene expression differences among ASD susceptibility genes and use transcriptomic data to provide insight into larger neurological variation such as cell type or synaptic densities. In this section, we discuss gene expression in postmortem ASD brain.

Early studies of brain gene expression in ASD focused on single genes (69, 70) or groups of genes (71), identifying small gene expression differences in genes encoding proteins such as Reelin and those involved in neurotransmission. Small sample sizes, limited number of targeted genes, and the fact that gene expression changes could result from cell composition of the assayed tissues have limited the conclusions that could be drawn from such experiments. The first relatively large study of idiopathic ASD transcriptomics profiled gene expression in three regions—superior temporal gyrus, prefrontal cortex, and cerebellar vermis—from 19 ASD patients and 17 neurotypical individuals (72). This study found coexpressed modules of genes that were correlated with presence or absence of ASD, and two of these (one upregulated and one downregulated in ASD) were additionally uncorrelated with technical variables such as postmortem interval and RNA integrity number or with biological covariates such as age, sex, comorbidity of seizures, family history of psychiatric disorders, and medication. This study also reported attenuated differences in gene expression between frontal and temporal cortex in ASD, which could be related to the finding that frontal cortical regions are more structurally similar in ASD (73), suggesting distortion of cortical patterning. The downregulated module identified by Voineagu et al. (72) was heavily enriched for synaptic genes and genes found in parvalbumin-positive interneurons. Importantly, the genes in this module were significantly more likely to have alleles modulating ASD risk in a large ASD

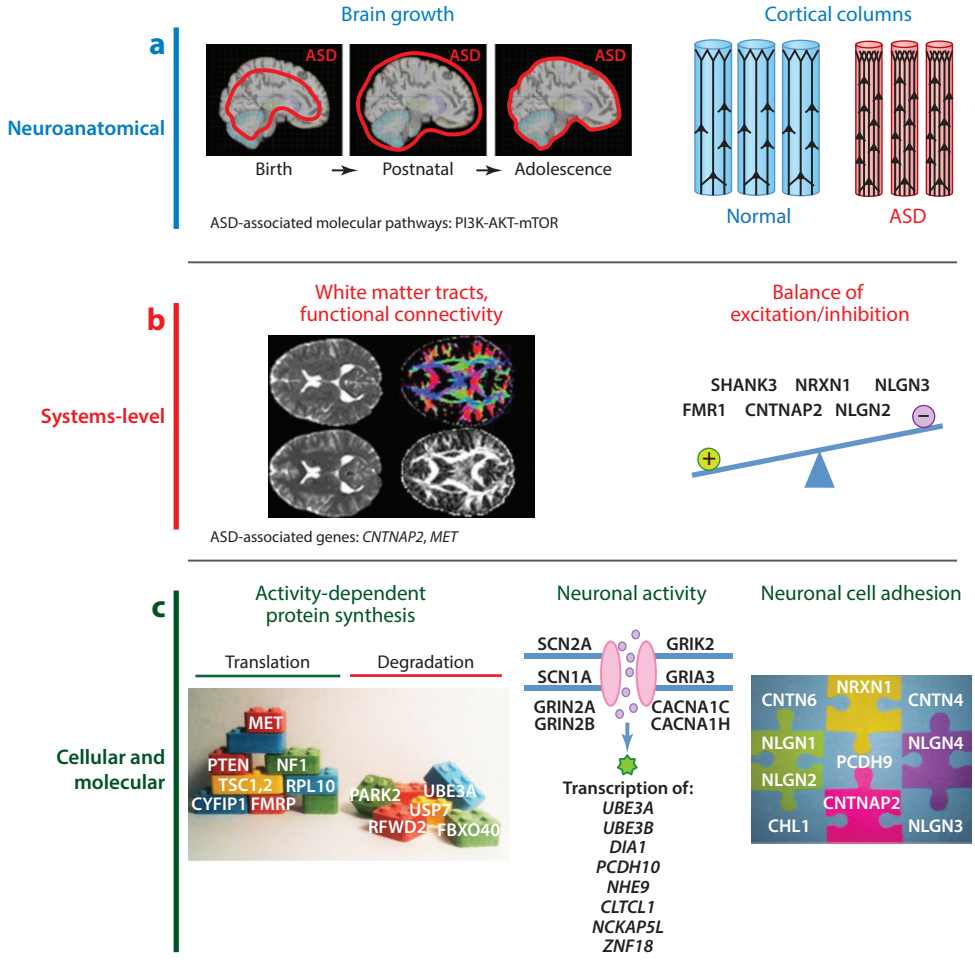

Figure 1

Biological convergence in autism spectrum disorder (ASD). (*a*) Studies of the neuropathology of ASD have consistently identified abnormal brain growth trajectories (*left*) and disordered cortical organization (*right*). (*b*) Systems-level studies of the brain point to higher-level disturbances in brain connectivity on a cognitive level, and to alterations in excitatory/inhibitory neurotransmission on a cellular level, as important features of ASD. (*c*) Although a diverse set of ASD susceptibility genes have been identified from human genetics studies, many of them can be grouped into common molecular pathways. So far, activity-dependent protein synthesis, neuronal activity, and neuronal cell adhesion seem to be particularly central to ASD etiology. Figure adapted from Reference 9 with permission from BioMed Central.

GWAS data set, suggesting a causal role for this transcriptional program. Two of these genes encoded key GABA-producing glutamate decarboxylases, GAD65 and GAD67, consistent with preceding studies in cerebral and cerebellar cortices (74–76).

In contrast, genes in the upregulated ASD-associated module showed no such genetic enrichment, suggesting these changes in gene expression were environmental or secondary to any genetic drivers. These genes were overwhelmingly glia- and immune-related and included core marker genes of microglia and astrocytes, consistent with upregulation of these cell types in ASD brain. This finding is corroborated by an earlier, smaller microarray study reporting increased

expression of several glial genes (74) and a study reporting GFAP elevation (77). These gene expression differences could reflect glia-related pathological findings in ASD. A similar pattern of gene expression changes was also seen at a higher average level in eight out of eight brains from people with dup15q syndrome (T.G. Belgard & D.H. Geschwind, unpublished results), ultimately suggesting a genetic, rather than environmental, cause. Importantly, these glial and immune changes are not correlated with epilepsy, which is a known cause of reactive astrocytosis.

A second study of gene expression in postmortem ASD prefrontal cortex focused on changes over time in order to better understand differences in trajectories. In a microarray study of gene expression, Chow et al. (78) analyzed samples from 15 autistic individuals and 18 neurotypical individuals between 2 and 56 years old and reported a striking age-dependence effect. Specifically, they found dysregulation in pathways affecting cortical patterning, cell number, and differentiation in the young set and dysregulation of signaling and repair pathways in the adult set. These cell cycle genes were enriched for ASD-specific CNVs and were also associated with ASD in GWAS data, complementing a study reporting 67% more neurons in the prefrontal cortex of male children with ASD in comparison with male neurotypical children (79). However, the finding is based on fewer than 10 ASD cases per age group. Furthermore, given that the authors did not rely on strict false discovery rate cutoffs to correct for multiple comparisons in this study (78), the specific differentially expressed genes need to be independently confirmed.

Studies have also looked at other features of the transcriptome. For example, there is evidence that the neuron-specific splicing factor RBFOX1 is differentially expressed in ASD, where it triggers changes in splicing and, more generally, regulates the splicing of many key neurodevelopmental genes (72, 80). In the future it may also be informative to look at RNA editing, as a recent study found extreme (both abnormally high and low) rates of A-to-I RNA editing in ten synaptic genes in postmortem cerebellum in 3 of 11 autistic individuals (81).

NEUROPATHOLOGY: MICROSCOPIC AND MACROSCOPIC MANIFESTATIONS

Even after the widespread acceptance of a biological (versus a psychogenic) view of ASD, its neuropathological features were poorly understood. However, modern stereological techniques have allowed detailed characterization of the microscopic alterations involved in ASD. Although study of postmortem brain tissues is limited by small sample sizes, retrospective phenotyping, comorbidities, and other technical issues, several neuropathological features have been frequently observed. The most consistent and apparent alterations localize in the limbic system and cerebellum; however, more subtle examinations and preparations have identified abnormalities in cerebral neocortex and other brain structures (**Supplemental Table 1**; follow the **Supplemental Material link** in the online version of this article or at **http://www.annualreviews.org**). These findings provide clues to the ultimate source of cognitive deficits in ASD.

Supplemental Material

Limbic System

Some of the earliest pathological studies showed abnormalities in limbic structures, finding neurons in the hippocampus and amygdala to be smaller and more densely packed compared with those in control subjects (82, 83). Indeed, similar pathological findings have been reported for other limbic structures, including entorhinal cortex, mammillary body, anterior cingulate cortex, and medial septal nucleus (83–85). Despite concerns that the concomitant seizures in these patients would likely affect amygdala and medial temporal lobe structures and therefore confound the analysis, pathological alterations of these structures have been repeatedly demonstrated

Table 1 Examples of disruptions of cortical connectivity and organization in ASD

Modality		Findings	Patient characteristics	Reference
Neuropathology	Immunocytochemistry, cytokine profiling	Astrogliosis in subcortical white matter of MFG, ACG, and CB; microglial activation in cortical regions and predominantly CB; increased levels of proinflammatory cytokines in MFG, ACG, CB, and CSF	Postmortem brain from 15 ASD (5–44 years) and 12 TD (5–46 years); CSF from 6 ASD (3–10 years) and 10 TD (12–45 years)	96
	Light microscopy with stereological analysis	Decreased minicolumnar width; increased density and number of minicolumns	Postmortem brain from 6 ASD (4–24 years) and 6 TD (4–25 years)	221
	Light microscopy with stereological analysis	Decreased layer 3 neuron density, decreased layer 3–5 neuron number, and decreased layer 4–5 neuron volume in FFG but not BA17	Postmortem brain from 7 ASD (mean 12.1 years) and 10 TD (mean 30.1 years)	107
	RNA in situ hybridization	"Patches" of aberrant cellular organization in PFC	Postmortem brain from 11 ASD (2–15 years) and 11 TD (4–15 years)	114
Structural imaging	Structural MRI	Altered growth trajectory of both white and gray matter	60 male ASD (2–16 years) and 52 TD (2–16 years)	129
	Structural MRI	Anatomic shifting of superior frontal sulci, right Sylvian fissure, STS, left IFS, and intraparietal and collateral sulci	22 ASD (mean 10.7 years) and 20 TD (mean 11.3 years)	238
	DTI	Increased FA in genu and body of CC, SLF, and cingulum	22 ASD (mean 3.2 years) and 32 TD (mean 3.4 years)	239
	DTI	Decreased FA in CC, anterior and posterior limbs of IC, ILF, SLF, cingulum, anterior thalamic radiation, and corticospinal tract	26 ASD (mean 12.8 years) and 24 TD (mean 13.0 years)	240
	fMRI, DTI	fMRI hyperactivation and reduced deactivation in an emotional faces paradigm; decreased DMN functional connectivity; decreased FA in white matter tracts—splenium of CC, SLF, ILF, and cingulum; effects observed at intermediate levels in *MET* risk allele carriers and at higher levels in ASD patients	66 ASD and 78 TD children across three genotypes	241
	Structural MRI	Abnormal cortical geometry and decreased mean separation distances on left frontal and temporal cortical surfaces	34 male ASD (mean 26 years) and 34 male TD (mean 28 years)	242

(*Continued*)

Table 1 (*Continued*)

Modality		Findings	Patient characteristics	Reference
Functional imaging	EEG	Increased theta-range (local) coherence, particularly in left frontal and temporal cortex; decreased alpha-range (long-range) coherence	18 male ASD (mean 22.66 years) and 18 male TD (mean 24.93 years)	243
	fMRI	Abnormal FFG activation and smaller, less organized functional connectivity networks in an *n*-back working memory task	11 male ASD (mean 24.5 years) and 11 TD (mean 28.7 years)	244
	fMRI	More widespread frontal connectivity in *CNTNAP2* risk allele carriers in a reward feedback processing task	16 ASD (mean 12.4 years) and 16 TD (mean 12.3 years)	159
	fMRI	Broadly decreased iFC in corticocortical connections; decreased iFC in subcortical regions	360 male ASD (7–58 years) and 403 male TD (6.4–48 years)	245
	MEG	Decreased coherence between fusiform face area and other cortical regions; decreased phase-amplitude coupling (local connectivity) during a face viewing task	17 male ASD (14–20 years) and 20 TD (age matched)	246

Abbreviations: ACG, anterior cingulate gyrus; ASD, autism spectrum disorder; BA, Brodmann area; CB, cerebellum; CC, corpus callosum; CSF, cerebrospinal fluid; DMN, default mode network; DTI, diffusion tensor imaging; EEG, electroencephalography; FA, fractional anisotropy; FFG, fusiform gyrus; fMRI, functional magnetic resonance imaging; iFC, intrinsic functional connectivity; IC, internal capsule; IFS, inferior frontal sulcus; ILF, inferior longitudinal fasciculus; MEG, magnetoencephalography; MFG, middle frontal gyrus; MRI, magnetic resonance imaging; PFC, prefrontal cortex; SLF, superior longitudinal fasciculus; STS, superior temporal sulcus; TD, typically developing.

(84, 86–89). The hippocampus also shows distorted cytoarchitecture and heterotopia (90) and, on a neuronal level, reduced dendritic arborization (88). The amygdala and other limbic structures subserve social processing, emotional processing, and other functions that are deficient in ASD, and the pathology observed here may provide a basis for some of these symptoms (91, 92).

Cerebellum and Brainstem

Aside from identifying pathological changes in limbic structures, early studies have commented on abnormalities in the cerebellum (83). Many subsequent investigations have also identified decreases in the size and number of Purkinje cells in the cerebellum in a subpopulation of patients, most prominently in the neocerebellar hemispheres, and these decreases are among the most robust neuropathological changes of ASD (86, 93, 94). The loss of Purkinje cells may be a reactive response due to postnatal immunological processes; although some authors have emphasized the absence of gliosis (95), many recent studies have observed its presence along with widespread neuroinflammatory processes in other brain regions (77, 86, 96). The presence of oxidative stress markers (97), apoptosis-related proteins (98), and normal numbers of associated GABAergic interneurons (99) further strengthens the evidence for a reactive mechanism, but the prevalence of cerebellar dysplasia, such as flocculonodular dysplasia, is compatible with a developmental disturbance (90). Synaptically related to the cerebellum, the inferior olivary nucleus also may exhibit pathological changes (86). This may be a result of retrograde degeneration stemming from the

connection between inferior olivary nucleus and cerebellar Purkinje cells via climbing fibers and the cerebellar nuclei.

The classical role of the cerebellum is its involvement in motor control and learning, and there is some suggestion of weak motor coordination in ASD patients (100). However, higher functions of the cerebellum and its connections to cerebral cortex are becoming increasingly recognized, and it is now believed that the cerebellum may play a role in social and emotional processing, language, and cognition (101, 102). Deficits in these areas may explain how the abnormal pathological findings contribute to expression of symptoms.

Cerebral Cortex

Postmortem studies on the cortical neuropathology of ASD highlight the disorder's heterogeneity, with few abnormalities consistently identified among studies. Several studies have reported no obvious differences in the organization of cerebral cortex between ASD patients and controls (103–105). Multifocal cortical pathology, such as altered cytoarchitecture in the anterior cingulate cortex (84, 85), posterior cingulate cortex (106), fusiform gyrus (107, 108), and prefrontal cortex (79, 109), is sometimes reported. This aberrant organization of the cortex may result in decreased interareal differences between prefrontal regions (73) and frontal and temporal cortex, consistent with a developmental patterning defect (72). These regions are involved in social cognition and executive functions, which are closely related to the characteristic deficits of ASD.

More generally, signs of subtle cortical dysgenesis, such as gray matter heterotopia and irregular lamination, have been observed in a subset of ASD cases (86). On a quantitative level, minicolumn organization is perturbed in a number of cortical regions, with increased density and decreased size of minicolumns in ASD (110, 111). These differences were particularly pronounced in the inferior frontal gyrus pars opercularis, a part of Broca's area (112). Dendritic spine densities in layer 2 of frontal and parietal cortex and in both layers 2 and 5 in temporal cortex were found to be greater in ASD subjects versus controls (113). Abnormalities in cortical organization have also been recently observed as "patches" of aberrant cellular organization as identified by RNA in situ hybridization of cortical layer– and cell type–specific markers (114). These patches were found throughout the layers of prefrontal and temporal cortex, but not occipital cortex, and affected a high percentage of ASD patients in a small cohort (10 of 11 ASD cases, compared with 1 of 11 controls).

Furthermore, emerging evidence implicates increased microglia infiltration and activation in cerebral cortex, consistent with the transcriptomic analysis of postmortem brain (72). Increased microglial density and activation have been observed in the prefrontal cortex (96, 115) and frontoinsular and visual cortex (116) of ASD patients. Similar findings have also been observed in vivo in young men with ASD by using positron emission tomography (117). These findings may also underpin long-range underconnectivity in ASD (118). Reactive astrocytosis in cortical regions has also been reported (77). The nature of these cortical alterations and their widespread distribution may indicate profound abnormalities in corticocortical connections and normal brain functioning, which have been further demonstrated using structural and functional brain imaging (**Table 1**).

Macroscopic Pathology

Kanner's original 1943 study identified that 5 of the 11 children had enlarged heads (3), a finding that has since been consistently associated with ASD. In general, an estimated 15–20% of children with ASD will also have macrocephaly, although estimates vary widely depending on age and genetic background (119–123). Aside from increased head circumference, some studies have shown

that ASD subjects have heavier brains than controls (86, 95, 124). In young children, the brain largely molds the shape of the skull; therefore, the macrocephaly associated with ASD can be explained by accelerated postnatal brain growth. However, these volumetric changes seem to slow or even disappear by mid-childhood; whereas some adult ASD cohorts demonstrate increased brain volume (125–128), in others no difference is visible (129, 130).

The observation of macrocephaly has been further refined through the use of medical imaging technologies. In parallel with the finding of macrocephaly, magnetic resonance imaging (MRI) studies have shown increased total brain volume in autistic children (121, 129, 131). The excellent spatial resolution of MRI also allows the distinction of white versus gray matter and parcellation of anatomical brain regions. Some reports have suggested that the increase in volume may not be uniform across the brain; in young children, growth may be more pronounced in white matter than in gray matter (129, 131). The brain enlargement may also preferentially affect particular brain regions, such as frontal and temporal lobes, whereas parietal and occipital lobes remain more normal in size (132). At even finer resolutions, structural MRI techniques have identified specific enlargement of dorsolateral prefrontal and medial frontal cortex (132), basal ganglia (133, 134), amygdala (135), and hippocampus (134, 135). MRI studies have also found brain regions that are smaller in ASD patients compared with controls, including lobules VI–VII of the cerebellar vermis (133, 136), corpus callosum (133, 137), and basal ganglia (138, 139). However, some of these findings have not been consistently reproduced and must be interpreted with caution; a detailed treatment of the vast literature on neuroimaging findings in ASD is beyond the scope of this review.

In summary, neuropathological studies in ASD, although in their early stages, have yielded a number of consistent findings that may be informative about the biological basis of the disorder (**Supplemental Table 1**; **Figure 1**). Given the suggestion that ASD is a disorder that is caused by many genes and exhibits a wide range of clinical phenotypes, the existence of characteristic neuropathological findings among cases at the molecular, microscopic, and macroscopic levels is remarkable in itself. Furthermore, a number of syndromic forms of ASD include some of the characteristic neuropathological changes in the limbic system and cerebellum, as we have reviewed for idiopathic ASD (15, 140–142), further lending credence to the idea of common neuropathology. However, most studies have primarily focused on areas that are thought to be related to the core deficits of ASD, which precludes an unbiased interpretation of the findings. Therefore, the causal relationship of the pathological abnormalities remains to be determined.

Supplemental Material

ANIMAL MODELS: FROM GENES TO MECHANISM AND TREATMENT

Human genetics and pathology studies have revealed changes in autistic patients at multiple levels, from molecular to microscopic to macroscopic. However, the mechanisms by which disruptions in gene function lead to brain pathology and, ultimately, to cognitive deficits are still unclear. Animal models of neuropsychiatric disorders are thus an essential component of research and allow us to study gene function at a level not possible in humans, so as to demonstrate causal mechanisms. Furthermore, reversal of behavioral symptoms in animal models helps to evaluate potential pharmacological treatments (143, 144).

Establishing an animal model of a psychiatric disorder invites consideration of validity at three different levels: construct validity (derived from an underlying cause of the disease), face validity (reflects key aspects of the human symptoms), and predictive validity (responds to treatments that are effective in the human disease). Though they are separated from humans by 60 million years of evolution, mice have emerged as the predominant model owing to practical considerations and their genetic tractability. Remarkably, mice can demonstrate analogs of behavioral deficits

ASSESSMENT OF SOCIAL FUNCTIONING IN MICE

Though their behaviors differ drastically from those of humans, mice are highly social animals that nest in groups, communicate with scent marks and vocalizations, and engage in other social interactions. Therefore, social deficits in mouse models analogous to those in human ASD can provide face validity. Tests of social approach, such as the three-chamber test (which measures a mouse's preference to engage with another mouse rather than an object), and more complex assessment of reciprocal social interaction provide quantification of mouse sociability (145).

Most ASD mouse models with construct validity have demonstrated deficits in social behavior as measured by such tests (**Table 2**). Some have not yet been tested for social deficits, such as mice with disruptions in *Chd7*, *Grin2b*, and *Met*. Others have shown apparently normal social approach but exhibit other social abnormalities. This is the case for the *Itgb3* (254) and *Sema5A* (255) mouse models, which have normal sociability as measured by the three-chamber test but show a deficit in social novelty preference. However, it is unclear how this phenotype relates to ASD, highlighting the need to define what aspects of sociability in a mouse are relevant to humans and to standardize tests among laboratories.

associated with ASD (including social interaction, vocal communication, repetitive behavior, and restricted interests) (145). However, only a small subset of current mouse models have been proven to have construct, face, and predictive validity. In addition, only models with mutations in *Cntnap2* (146), *Shank2* (147), *Shank3* (148), and *Ube3A* (149) have been shown to display abnormalities in all three core domains defining ASD, suggesting that these genes play a major role in circuits involved in social, communication, and repetitive behaviors.

Below, we discuss the main pathological findings in the currently proposed mouse models of genes that have been linked to ASD in humans and that have been behaviorally characterized by at least deficits in social interaction, the core deficit in the disorder (**Table 2**) (see the sidebar, Assessment of Social Functioning in Mice). Several non-genetic-based mouse models of ASD have also been proposed, including mouse strains that naturally display ASD-related behaviors, such as the inbred strains BTBR T^{+} *tf*/J, BALB/cByJ, A/J, and 129S1/SvImJ (150), and mice with environmental exposures such as in utero exposure to valproic acid (151) and viral infections (152); these models fall outside the scope of this review. For a comprehensive list of the available mouse models related to ASD, please see the SFARI gene database (**https://gene.sfari.org**). Experimental findings in mouse models currently point toward four main biological mechanisms affected in ASD: synapse development and function, growth regulation, serotonergic neurotransmission, oxytocin/vasopressin signaling, and neuron-glia signaling.

Synapse Development and Function: Abnormal Excitatory/Inhibitory Neurotransmission

Formation of synapses involves the temporal and spatial coordination of different biological processes such as axonal growth and pathfinding, assembly of protein complexes, pruning, and maturation (153). Critical for synapse formation and maintenance are cell-adhesion molecules (CAMs), many of which have been found mutated in ASD, suggesting that the development and/or maintenance of synaptic contacts is a key factor in ASD pathophysiology (154). Among these are the neurexin family of presynaptic CAMs; their postsynaptic partners, the neuroligins; the SHANK family of postsynaptic scaffolding proteins, which have a key role in anchoring CAMs and other molecules to the actin cytoskeleton; and contactin and contactin-associated proteins, which are CAMs essential for different axonal and dendritic molecular organizations.

Table 2 Pathology associated with genetic mouse models of ASD with reported social deficits

Biological process	Gene	Pathology	Reference(s)
Synaptic signaling	*CADPS2*$^{-/-}$	Decreased number of PVALB cells in cortex and CALB cells in cerebellum Impairment in short-term plasticity in parallel fiber–PC synapses Decreased number of dendritic protrusions and spines on DG	172
	CACNA1C$^{+/-}$ (Timothy syndrome)	Not reported	247
	CNTNAP2$^{-/-}$ (cortical dysplasia–focal epilepsy syndrome)	Neuronal migration deficits Decreased number of interneurons Decreased neuronal synchrony	146
	EN2$^{-/-}$	Abnormal cerebellum patterning, reduced number of PCs Decreased number of interneurons	174 175
	EXT1$^{-/-}$ pyr cells	Decreased frequency and amplitude of mEPSCs in BLA Reduced AMPAR	248
	FMR1$^{-/y}$ (fragile X syndrome)	Enhanced LTD, impaired LTP Increased spine density, abnormal spine morphology Altered GABAergic transmission Loss of PVALB cortical interneurons	166 167 171
	MECP2 dup	Increased dendritic arborization and spine turnover	249
	NLGN3 (R451C)	Enhanced GABAergic transmission in cortex Enhanced excitatory transmission in HPF	156 157
	NLGN4$^{-/-}$	Decreased brain size	155
	NRXN1a	Decreased frequency of mEPSCs in HCF	250
	SCN1A$^{-/-}$	Decreased frequency of IPSCs in CA1 and PFC Increased frequency of EPSCs in CA1 and PFC	251
	SHANK2$^{-/-}$ exon 7	Increased NMDA/AMPA ratio in CA1 Enhanced LTP Reduced mEPSC frequency in CA1 Decreased spine density in CA1	147
	SHANK2$^{-/-}$ exon 6–7	Decreased NMDA/AMPA ratio in CA1 Impaired LTP	162
	SHANK3$^{-/-}$ (Phelan–McDermid syndrome)	Decreased frequency and amplitude of mEPSCs of MSNs Increased dendritic complexity, reduced spine density in MSNs	148
	SHANK3$^{+/-}$	Impaired LTP Decreased amplitude and increased frequency of mEPSCs in CA1	160
	SHANK3$^{-/-}$ exon 4–9	Normal mEPSCs and mIPSCs Impaired LTP Altered dendritic spine morphology	161
	UBE3A triplication (dup15q syndrome)	Decreased mEPSC amplitude and frequency Decreased spontaneous EPSC amplitude and frequency	149

(*Continued*)

Table 2 (*Continued*)

Biological process	Gene	Pathology	Reference(s)
mTOR pathway	*CDKL5*$^{-/-}$	Decreased oscillatory strength in the delta, theta, and alpha frequency bands Decreased phosphorylation of mTOR	186
	PTEN$^{-/-}$ cortex, hippocampus (Cowden syndrome)	Macrocephaly, increased soma size Increased spine density in cortex and DG	177
	TSC1$^{-/-}$ PC	Decreased PC excitability Decreased PC number; bigger soma and increased apoptosis Increased spine density in PCs	182
	TSC2$^{+/-}$ (tuberous sclerosis)	Impaired LTD Abnormal LTP	184, 252
	TSC2$^{-/-}$ PC	Decreased PC number	183
Serotonin	*MAOA*$^{-/-}$	Decreased corpus callosum thickness Increased dendritic branches in orbitofrontal cortex	195
	SLC6A4$^{-/-}$	Decreased serotonin neuron number in dorsal raphe nucleus Decreased firing rate in dorsal raphe nucleus	193
	SLC6A4 (SertAla56)	Decreased firing rate in dorsal raphe nucleus Increased serotonin clearance rate in hippocampus	194
OXT/AVP	*AVPR1A*$^{-/-}$	AVP signaling	205
	CD38$^{-/-}$	Increased number of OXT vesicles in neurohypophysis Decreased OXT levels in plasma and CSF	201
	OXT$^{-/-}$	OXT signaling	253
	OXTR$^{-/-}$	OXT signaling	200

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AVP, arginine vasopressin; BLA, basolateral amygdala; CSF, cerebrospinal fluid; DG, dentate gyrus; GABA, γ-aminobutyric acid; HPF, hippocampal formation; mEPSCs, miniature excitatory postsynaptic currents; mIPSCs, miniature inhibitory postsynaptic currents; LTD, long-term depression; LTP, long-term potentiation; MSN, medium spiny neuron; NMDA, *N*-methyl-D-aspartate; OXT, oxytocin; PC, Purkinje cell; PVALB, parvalbumin.

Mutations in any of these proteins could lead to abnormal circuit wiring and altered information processing. In fact, several genetic mouse models of CAMs have been generated that show ASD-like features (**Table 2**).

Among the neurexins and neuroligins, mutations in *NRXN1*, *NLGN3*, and *NLGN4* have been linked to ASD in humans. However, knockout of these genes in mice shows less convincing evidence for their role in regulating the core social behaviors in ASD. Of these, only knockouts of *Nlgn4* show deficits in sociability (155). Interestingly, *Nlgn3*-knockin mice carrying a point mutation found in humans with ASD (R451C) display deficits in social interaction, a phenotype not found in *Nlgn3* knockouts, suggesting a gain-of-function or dominant-negative effect for this mutation. The R451C-knockin mice also demonstrated a specific increase in inhibitory GABAergic synaptic transmission in somatosensory cortex, without effects on excitatory synapses (156). Interestingly, this same mutation caused a rather different phenotype when recording was performed from hippocampal area CA1, with increased AMPA receptor–mediated excitatory synaptic transmission and enhanced long-term potentiation (LTP). These electrophysiological changes were accompanied by increased dendritic complexity and abnormal spine morphology in this brain area (157). These results seem to indicate that the same mutation could have different effects depending on the brain region that is being studied. How these different findings lead to the specific ASD-associated behavioral deficits remains to be elucidated.

Animal models of the SHANK family of scaffolding proteins also highlight the variability of the pathophysiology associated with a single gene. Deletion of the *SHANK3* gene, such as in 22q13 deletion/Phelan–McDermid syndrome, is highly associated with ASD (158). *Shank3*-knockout mice show the triad of core ASD symptoms (148). In addition, they have a striatocortical synaptic dysfunction, a neural circuit strongly implicated in ASD in humans (159), with decreased miniature excitatory postsynaptic currents (mEPSCs) and a correlated reduction in spine density of medium spiny neurons (148). Similarly, mice heterozygous for *Shank3* show altered glutamatergic transmission with reduced LTP and amplitude of mEPSCs (160). However, mice lacking exons 4–9 in *Shank3* (encoding the ankyrin domain, which interacts with cytoskeletal proteins in the postsynaptic density) show reduced LTP, but normal mEPSCs (161). These mice also displayed subtle morphological alterations in dendritic spines, which were longer and thinner than in wild-type mice but occurred at normal density.

Mouse models of different *Shank2* mutations exhibit similar phenotypic variability. *Shank2*-knockout mice that carry a deletion in exon 7 have been reported to show abnormal excitatory neurotransmission with reduced mEPSCs, reduced spine density in hippocampal CA1, and increased NMDA/AMPA ratio and LTP (147). On the contrary, when the deletion is in exons 6–7, *Shank2*-knockout mice show reduced NMDA/AMPA ratio and LTP (162). Although the cause of these apparently paradoxical results remains to be elucidated, these studies highlight the role of *Shank2* in NMDA signaling. One issue that needs to be formally addressed is that different laboratories use different protocols for measuring in vivo or slice electrophysiology, so analysis of these animals using a uniform physiological framework would be helpful. Nevertheless, despite the variability observed, a common theme among the described animal models is a reduction in excitatory neurotransmission.

A third group of neural CAMs, comprising contactin and contactin-associated proteins, has also been studied in mouse models of ASD. The protein encoded by *CNTNAP2* (contactin associated protein-like 2, or CASPR2), is a member of the neurexin family. CASPR2 binds to contactin and plays an important role in axonal molecular organization (163). *Cntnap2*-knockout mice recapitulate the key features observed in patients with cortical dysplasia–focal epilepsy syndrome, caused by rare homozygous mutations in this gene (15, 146). Neuropathologically, *Cntnap2*-mutant mice show defects in the migration of cortical projection neurons and a reduction in the number of GABAergic interneurons. Synchronous electrical activity in layer 2/3 neurons in the somatosensory cortex is dramatically reduced, presumably due to an abnormal wiring of neuronal circuits (146). These data highlight the role of this protein in circuit assembly, in addition to its known function in clustering potassium channels in myelinated axons (163).

Despite some phenotypic variability across these mouse models, even for disruptions of the same gene, the most common electrophysiological and neuroanatomical findings are altered glutamatergic synaptic transmission, loss of inhibitory GABA interneurons, and impairments in synaptic plasticity, attributable to dysfunction of NMDA and AMPA receptors. Interestingly, similar findings have been reported in mouse models of genes not directly implicated in neuronal signaling, such as the ubiquitin ligase gene (*UBE3A*) in the dup15q syndrome region. Increasing the dosage of *Ube3A* in mice recapitulated the three core ASD traits, accompanied by decreased glutamatergic synaptic transmission (149). The fact that synaptogenesis might represent a common etiological pathway suggests that ASD might be treatable with drugs that specifically target glutamatergic transmission. In this regard, selective mGluR5 (metabotropic glutamate receptor type 5) receptor antagonists and modulators constitute a promising pharmacotherapy in ASD. *Fmr1*-knockout mice, which mimic fragile X syndrome, show hyperactivity of mGluR5 signaling (164), leading to excessive protein synthesis at the synapse and increased trafficking of AMPA receptors (165). Accordingly, they show enhanced mGluR-dependent long-term depression (LTD) and deficits in

cortical LTP (166). In addition, *Fmr1*-knockout mice show increased density in dendritic spines and altered spine morphology (167). Decreasing mGluR5 activity in this mouse model rescued protein synthesis, dendritic spine alterations, and multiple behavioral phenotypes (168).

Reductions in the expression of $GABA_A$ receptor subunits (169, 170) and in parvalbumin cortical interneurons have also been reported in *Fmr1*-null mice (171). Reduced parvalbumin interneuron density has been observed in other mouse models of ASD; CNTNAP2 deficiency in mice leads to reduced expression of GAD1 and parvalbumin inhibitory interneurons (146), and absence of CADPS2 (Ca^{2+}-dependent activator protein for secretion 2) results in reduced numbers of cortical and hippocampal parvalbumin interneurons as well as cerebellar Purkinje cells (172). Similarly, mice lacking the transcription factor Engrailed-2 (*En2*), which has been associated with ASD in humans in a candidate gene study (173), display abnormal cerebellar development and reductions in the number of Purkinje cells (174), with associated deficits in social and motor tasks. Recently, Sgado et al. (175) found a reduced number of interneurons, including the parvalbumin subtype, in cortex and hippocampus in these mice.

Growth Regulation and Protein Synthesis: The mTOR Pathway

The signaling pathway containing mTOR (mechanistic target of rapamycin) is critical for cell growth, proliferation, and survival. mTOR is a component of at least two functionally distinct protein complexes: mTORC1 and mTORC2. mTORC1 lies downstream from proteins encoded by risk genes implicated in ASD, including PTEN (phosphatase and tensin homolog) and TSC1/TSC2 (which are mutated in tuberous sclerosis complex, a neurocutaneous disorder associated with ASD). TSC1 and TSC2 form a heterodimer that negatively regulates mTOR signaling and thereby controls protein translation. Protein synthesis within synaptic spines is necessary for neuronal plasticity and is required for proper cognitive function. PTEN, an upstream regulator of TSC1/TSC2-mTOR is a lipid phosphatase that antagonizes signaling through the PI3K pathway and affects cellular proliferation, differentiation, and migration (176). Although PTEN mutations are found in a small subset of children diagnosed with ASD and accompanying macrocephaly, they account for enough cases to warrant specific genetic testing in these patients. In mice, broad ablation of *Pten* in the brain causes dramatic anatomical disruption and premature death. Therefore, recent studies have focused on deleting *Pten* in limited cell populations or restricted brain regions using conditional knockout technology. Conditional deletion of *Pten* in mature neurons in the cerebral cortex and hippocampus results in dendritic and axonal overgrowth, increased synapse number, neuronal hypertrophy, and macrocephaly (177). Interestingly, rapamycin, a specific inhibitor of mTOR, can reverse many of the behavioral abnormalities seen in PTEN-deficient mice (178). In vivo knockdown of *Pten* in hippocampal granule cells results in an increased excitatory synaptic function (179). Similar results are found when knocking down the gene in basolateral amygdala (180). However, contrary to previous studies, the latter study found overall decreased spine density in this brain region, but increased number and function of mature spines. These findings raise the possibility that PTEN knockdown may increase synaptic activity by inducing morphological and functional maturation of spines. Thus, attenuating PTEN function in neurons has profound effects on neuronal morphology and circuitry.

Mouse models deficient in TSC1 or TSC2, which lie downstream of PTEN in regulating mTORC1 activity, show some aspects of ASD behavior, and studies in TSC patients demonstrate that cerebellar pathology correlates with increased ASD severity (181). Remarkably, selective knockout of either *Tsc1* or *Tsc2* in cerebellar Purkinje cells leads to Purkinje cell degeneration. Interestingly, treatment of these mice with rapamycin prevented the pathological and behavioral deficits (182, 183). Recent studies also indicate that downregulation of mGluR5 signaling can

contribute to synaptic and behavioral deficits in TSC, providing an intersection between the FMR1 and TSC pathways and highlighting the role of aberrant protein synthesis in ASD pathology. Auerbach et al. (184) found that *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mutations result in opposite hippocampal LTD dysfunction in mice, with increased LTD in the former and decreased LTD in the latter. In addition, synaptic and cognitive defects in these mutants are corrected by treatments that modulate mGluR5 in opposite directions, and deficits disappear when the mice are bred to carry both mutations, raising the possibility of using mGluR5 agonists to revert ASD symptoms in TSC.

Another ASD candidate gene that has been shown to affect the mTOR pathway is the X-linked *CDKL5* (cyclin-dependent kinase-like 5). Mutations in this gene in humans have been associated with ASD and other neurodevelopmental disorders, including atypical Rett syndrome and early infantile epileptic encephalopathy (185). Mice lacking *Cdkl5* show deficits in social interaction and disruption of the mTOR signaling pathway, among others. The authors found reduced phosphorylation of the S2448 residue in mTOR, which is necessary for mTORC1 assembly and is thus potentially correlated with reduced mTORC1 activity (186). This study raises the possibility of targeting the mTOR pathway as a potential treatment of patients with CDKL5-related disorders. Lastly, recent work from our lab implicates mTOR signaling in dup15q syndrome, which is among the most common recurrent forms of ASD (256). This, and other genetic evidence (39), suggests that one convergent pathway may involve regulation of neuronal protein synthesis.

Serotonin Signaling

Alterations in the serotonergic system were among the earliest evidence of abnormal neurotransmission in ASD. Elevated levels of serotonin in blood have been reported for up to 45% of ASD subjects (187, 188). Serotonin signaling mediates many neurodevelopmental processes including neurogenesis, cell migration, cell survival, synaptogenesis, and plasticity (189), raising the possibility that defects in this system could lead to abnormalities in circuits important for ASD. Genetic variants affecting the serotonin system, such as in *SERT/SLC6A4* (the serotonin transporter gene) and in *MAOA* (the monoamine oxidase A gene, involved in the degradation of serotonin), have been tentatively linked to ASD in humans (190–192). Alongside deficits in social behavior, mutant mice lacking or harboring mutations in these genes show abnormal serotonin neurotransmission (193, 194) and neuroanatomical abnormalities such as reduced corpus callosum thickness and increased dendritic complexity (195), supporting the role of serotonin in brain development. Serotonin reuptake inhibitors might reduce symptoms of ASD in certain patients as well as potentially affect brain development.

Oxytocin and Vasopressin Signaling

The neuropeptides OXT (oxytocin) and AVP (arginine vasopressin) are evolutionarily conserved regulators of social behavior. Genetic variation in the genes that encode the neuropeptides, their receptors, or enzymes involved in their secretion have been proposed to contribute to autistic features in humans (196–198). Although the evidence for these variants being involved in ASD is currently weak, genetic mouse models affecting the OXT and AVP systems, which were developed to study the basic neurobiology of social cognition, exhibit ASD-like behaviors. Three different knockout models affecting OXT signaling have been developed: knockouts of the peptide itself (199), its receptor (200), and the enzyme CD38, which regulates calcium-dependent secretion of OXT (201). Impairments in social cognition have been identified in all three models of altered OXT signaling. In addition, these deficits can be rescued in all three models by administration of

OXT (202, 203). The efficiency of OXT administration in the OXT receptor knockout suggests that an alternative pathway, possibly through the AVP receptor signaling pathway, might be implicated in this model. In fact, administration of AVP in the OXT receptor knockout also rescues the social deficits, and coadministration of a V1aR (AVP receptor 1a) antagonist along with OXT prevents the rescue (204). Because AVP-V1aR knockouts also show deficits in social behavior (205), it is important to study the individual neurobiology of these systems in order to develop the right pharmacotherapies for ASD and to discover the potential alteration of both neuropeptide systems in the disorder (206).

Neuron-Glia Signaling

Glial cells were originally thought to be connective tissue that glues and holds neuronal circuits together. Today, we know that glial cells have important roles in genesis, development, and functional remodeling of the highly complex neural cellular networks (207). Because abnormal formation of neuronal networks and abnormal neurotransmission are central to the pathophysiology of ASD, it has recently been considered that the disruption of neuron-glia interactions could be a primary event in the neurobiology of ASD. Reactive astrocytosis and gliosis have also been observed in neuropathological studies of the cerebellum and neocortex of ASD patients.

The impact of astrocyte activation and reactive gliosis on the pathogenesis of neurological disorders is not yet fully understood, but these processes are generally accepted to have many protective effects. However, if not resolved in time, reactive gliosis can exert inhibitory effects on several aspects of neuroplasticity and central nervous system regeneration and thus might become a target for future therapeutic interventions (208). Changes in microglial morphology and gene expression have been associated with neurodevelopmental disorders, including ASD (209). However, it remains unknown whether these changes are a primary cause or a secondary consequence of neuronal deficits. Remarkably, there are no published studies of microglial or astroglial pathology across the validated ASD mouse models based on human genetic findings, although reactive astrocytosis likely due to seizures has been observed (146). Zhan et al. (210) have recently shown in mice that deficits in neuron-microglia signaling result in impaired social behavior as well as increased repetitive behaviors. Mice lacking the chemokine receptor gene *Cx3cr1*, which plays a developmental role in microglia migration, exhibit a transient reduction of microglia during the early postnatal period and a consequent deficit in synaptic pruning associated with weak synaptic transmission and decreased functional brain connectivity in addition to behavioral deficits. These findings build upon evidence from human transcriptomic and histological studies for the possibility that microglial pathology could contribute to ASD (72) and, therefore, that modulators of microglial function may represent a novel therapeutic strategy.

SYSTEMS BIOLOGY: DEVELOPING A COHERENT MODEL OF AUTISM SPECTRUM DISORDER PATHOPHYSIOLOGY

Despite the advances in our understanding of disease mechanisms afforded by elegant studies in mouse models of ASD, the genetic heterogeneity of the human disorder still dwarfs the number of animal models. Will we have to understand ASD gene by gene in hundreds of models, or might there be some convergent biology that will limit the search space in the quest for mechanistic insight? Such questions necessarily invoke pathway analysis, in its various manifestations, to answer the question of whether there is molecular pathway convergence in ASD (211). Analyses that integrate multiple levels of these data in the paradigm of systems biology will allow us to get a better

sense of this whole picture, and of whether or where ASD biology converges on pathophysiological mechanisms.

The wisdom of a systems biology approach becomes apparent in attempts to find commonalities among the genes implicated in ASD (212, 213). It has been repeatedly demonstrated that ASD genes group together in terms of functional annotations (such as Gene Ontology and KEGG pathways) and interactions (such as protein-protein interactions and coexpression). As mentioned above, Voineagu et al. (72) identified enrichment of ASD-associated genes in a module of coexpressed genes related to synaptic function in the brains of autistic individuals. Ben-David & Shifman (213) used a similar approach, termed WGCNA (weighted gene coxpression network analysis), to identify enrichment of ASD rare variant– and GWAS-implicated susceptibility genes in gene expression profiles from different brain regions. Two modules were highlighted, one related to neurogenesis and brain development and another related to sensory cortex and clathrin-dependent endocytosis. Gilman et al. (214) developed another method, termed NETBAG (network-based analysis of genetic associations) and based on a gene network derived from functional descriptors, gene interactions, and coevolutionary patterns, that identified significant grouping of genes falling within CNVs identified in ASD patients in one study; the cluster was also closely related to synaptic function. Exome sequencing studies have demonstrated grouping of genes using protein-protein interaction networks (65, 66), which are enriched for involvement in β-catenin and p53 signaling, chromatin remodeling, ubiquitination, and neuronal function. Each of these methods also demonstrates that the relationships among ASD susceptibility genes in a given gene network is greater than expected by chance; these methods therefore simultaneously validate the existence of a biological relationship among identified genes (implying their involvement in the disorder) and provide hints toward their function.

A complementary approach involves asking whether ASD genes converge on a specific developmental time window or cellular or molecular process. Two groups provide a road map for this area by integrating gene expression data representing normal human fetal development to clarify the developmental timing and cellular specificity of the molecular pathways disrupted in ASD (215, 216). Willsey et al. (215) mapped the spatiotemporal expression pattern of nine well-validated ASD susceptibility genes to overlap with the gene expression patterns of layer 5–6 glutamatergic neurons at mid-fetal development. Using a different approach, Parikshak et al. (216) found that genes implicated by rare de novo variants, syndromic forms of ASD, and common variation were concentrated in fetal brain gene coexpression modules that corresponded spatially to superficial cortical glutamatergic neurons (layer 2–4) and temporally to the 10th to 20th week of gestation (**Figure 2**). These modules were also enriched in genes that interact with FMRP (the protein encoded by *FMR1*), suggesting convergence at common pathways of synaptic plasticity and activity-dependent gene regulation that are mediated by this protein (217) and agreement with previous findings (64). This finding was corroborated by a demonstration that genes disrupted by CNVs in autistic probands are enriched for FMRP interactors, particularly those that are specifically expressed in fetal brain (217). An increased burden of mutations disrupting FMRP interactors in ASD probands was also identified, suggesting a multihit etiology in at least a subset of patients.

Taken together, these recent studies identify an important role for fetal glutamatergic neuron development in ASD based on the causal genes identified thus far (215, 216). Parikshak et al. (216) were also able to distinguish ASD from intellectual disability (ID) on the basis of the pattern of fetal brain coexpression, indicating more circuit specificity in ASD than in ID. Coupled with the neuropathological findings reviewed herein, there is growing evidence for convergence of function for ASD-causing genes. Moreover, the recent gene expression studies implicate large modules of coexpressed or interacting genes with similar spatiotemporal expression patterns that are highly

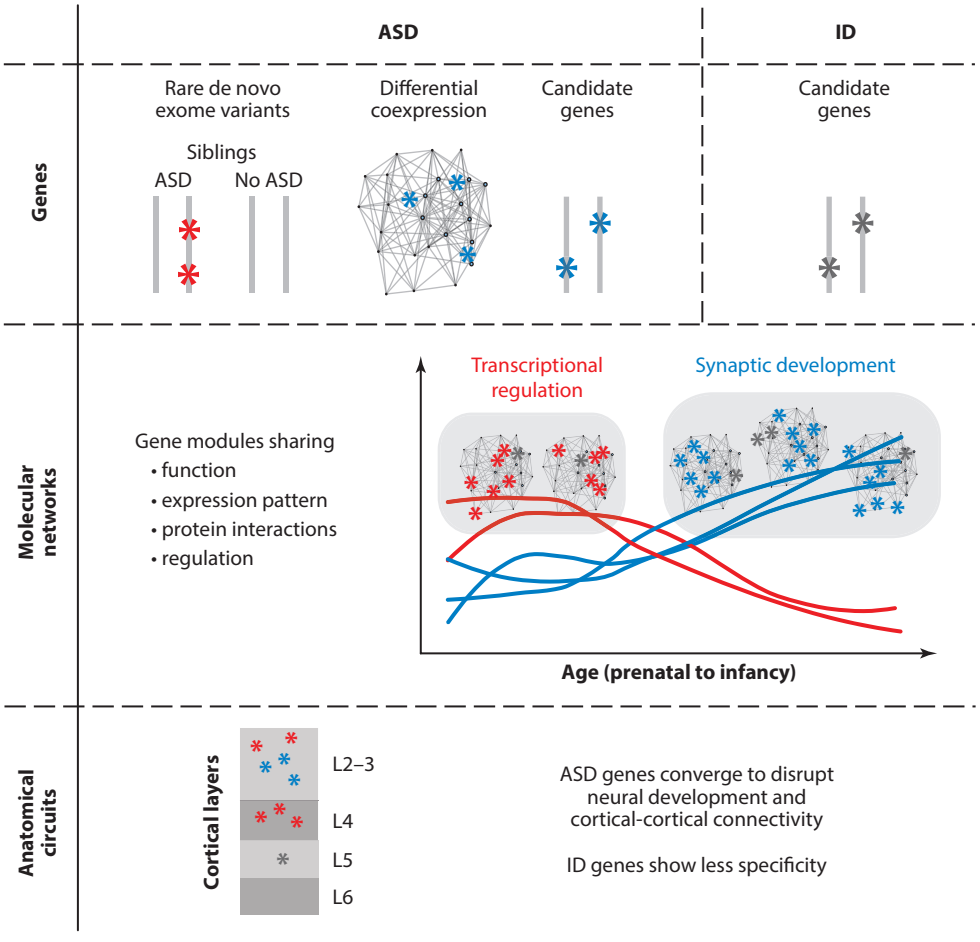

Figure 2

A systems biology approach integrating human genetics and transcriptomics to implicate molecular pathways involved in autism spectrum disorder (ASD). Putative ASD susceptibility genes (*top left*) are enriched in two coexpression modules of genes that share regulatory and functional characteristics in the developing human brain (*middle*). ASD susceptibility genes are also enriched in genes that are preferentially expressed in superficial neocortical layers, in contrast with intellectual disability (ID) genes (*top right*), for which such enrichment is not observed (*bottom*). These results identify a molecular signature of ASD related to transcriptional regulation and synaptic development, and localized temporally to prenatal development and spatially to layer 2–4 glutamatergic projection neurons. Figure adapted from Reference 216, with permission from Elsevier.

enriched for ASD genes. Therefore, these networks may also be helpful for prioritizing genetic variation found within ASD and identifying new genetic risk factors. With the explosion of new findings on the molecular, microscopic, and macroscopic levels, the framework of systems biology is just beginning to piece together the mechanisms at play in ASD. Coupled with future studies in human neurons derived from patient induced pluripotent stem cells or primary tissue, systematic integration of multiple levels of anatomical, physiological, and genomic data in a systems biology framework promises to rigorously test the notion of convergence in ASD (218). However, to what extent these systems faithfully model in vivo human development remains an open question.

CONCLUSION: FINDING CONVERGENCE AMONG HETEROGENEITY

The highly variable genetics and phenotypes observed in ASD patients would at face value weigh against the possibility of convergence in biology, yet recent studies using different methods have organized ASD etiologies into common themes. We have reviewed a growing body of evidence from human genetics, neuropathology, animal models, and systems biology that identify a striking degree of convergence at intermediate biological levels (**Figure 1**). Studies from these disciplines generally corroborate a "many genes, common pathways" hypothesis (211) emphasizing a primary deficit early in neural development and in the development of synaptic function resulting in abnormal cortical development in superficial cortical layers (**Figure 2**) (216). Continuing comparisons to other neuropsychiatric diseases, including schizophrenia and ID, will also be important to identify features specific to ASD.

One unifying concept consistent with the genetic and neuropathological abnormalities found in ASD patients broadly fits within the narrative of a "developmental disconnection syndrome" (219), in which long-range neural connections between normally cooperative brain regions fail to fully form, with a corresponding abundance of abnormal short-range connections (220). Cortical abnormalities, such as patches or migration defects, increased synaptic spine density, and increased minicolumn density, may reflect this abnormal organization (90, 114, 221). As a result, the integration of information in the frontal lobe (incidentally, one of the focal points of cerebral pathology) would become disorganized. This theory has been further investigated using functional imaging modalities, which have demonstrated deranged functional connectivity along frontoparietal (137, 222–224), frontotemporal (225), frontostriatal (226, 227), and interhemispheric (228) circuits (**Table 1**). The cerebellum, another region harboring pathological changes in ASD, may also contribute to symptomatology via connections with the frontal lobe (229). In essence, in the formulation of developmental disconnection, the cerebral cortex becomes disorganized, and long-range corticocortical connections are diminished, especially those affecting frontal and anterior temporal lobe function (219).

At a molecular level, activity-dependent protein synthesis and synapse development seem to be important, as implicated by a high prevalence of ASD among patients with fragile X syndrome, Timothy syndrome, Rett syndrome, and Angelman syndrome, all caused by mutations in genes important for synaptic activity–dependent gene regulation, as well as highly penetrant ASD-causing mutations in synaptic adhesion and scaffolding genes such as neuroligins and neurexins (230). Many genes with newly discovered roles in ASD susceptibility also function within this pathway (231). Neuropathological findings in postmortem human brain (232), mouse models of fragile X syndrome with autism-like behavioral and neuropathological phenotypes (167), and enrichment of FMRP interactors in ASD-related developmental brain gene expression modules (216) further corroborate this hypothesis.

Importantly, these common pathways represent a potential therapeutic target for ASD. Therapies targeted toward these specific points of convergence have already been attempted; for example, the mGluR5 modulators, which are thought to normalize aberrant synaptic protein synthesis, have already blocked ASD-like phenotypes in the FMR1 and TSC mouse models. Similar findings have been reported in the BTBR (233) and valproic acid (234) mouse models, which are not based on monogenic human disorders, speaking to the potential generalizability of mechanism in the face of diverse genetic and environmental etiologies. It is exciting to think about the possibility of other pharmaceutical agents targeted toward common pathways of convergence, such as synaptic gene regulation (235), excitation-inhibition balance (236), and other pathways identified in an unbiased manner using genetic screens (237). As more pieces of the puzzle are put into place, we are optimistic that the emerging picture of disease pathophysiology will

begin to translate into effective diagnostics and treatments to improve the welfare of ASD patients.

DISCLOSURE STATEMENT

D.H.G. serves on the Scientific Advisory Board of SynapDx Corp. The authors are not aware of any other affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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