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Autism Spectrum Disorders: From Genes to Neurobiology

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Abstract

Advances in genome-wide technology, coupled with the availability of large cohorts, are finally yielding a steady stream of autism spectrum disorder (ASD) genes carrying mutations of large effect. These findings represent important molecular clues, but at the same time present notable challenges to traditional strategies for moving from genes to neurobiology. A remarkable degree of genetic heterogeneity, the biological pleiotropy of ASD genes, and the tremendous complexity of the human brain are prompting the development of new approaches to translating genetic discoveries into therapeutic targets. Recent developments in systems biology approaches that 'contextualize' these genetic findings along spatial, temporal, and cellular axes of human brain development are beginning to bridge the gap between high-throughput gene discovery and testable pathophysiological hypotheses.

Introduction

It is a very exciting time for the translational neuroscience of autism spectrum disorders (ASD). Advances in genomic technology, coupled with the availability of large study cohorts, are leading to a rapidly expanding pool of reliable ASD genes and presenting the field with new opportunities and challenges in conceptualizing the path forward from genetic data to an actionable understanding of pathophysiology. Conventional approaches to translating gene discoveries into therapeutic targets have developed largely in the context of Mendelian genetics, typically moving directly from gene identification to single gene manipulations in model systems. However, the extraordinary degree of etiological heterogeneity underlying common forms of ASD, the biological pleiotropy of the implicated genes, the difficulty in distinguishing primary from secondary effects in developmental syndromes, the finding that a myriad of divergent outcomes may be associated with a given ASD mutation, and the likely role that polygenic background plays in this diversity, all suggest that systems biological approaches that can integrate diverse genetic data and capture key dimensions of brain development and function will become an increasingly

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important complement to the existing armamentarium for moving from genes to neurobiology.

Gene Discovery

ASDs are highly heritable, with a substantial contribution from common genetic variation [1,2]. However, while inherited polymorphisms are likely to account for the majority of heritability, genome wide association studies (GWAS) have yet to lead to replicable risk loci [3-6]. This is almost surely a consequence of small effects and an attendant lack of statistical power in current study cohorts. Conversely, while estimated to account for a smaller proportion of ASD population risk [1], the search for large effect, rare mutation in the coding portion of the genome has proven to be a highly productive avenue of investigation.

The first rare variant discoveries in ASD involved genes contributing to monogenic syndromes characterized both by intellectual disability and an increased risk for social impairment. For example, the cloning of the genes *FMR1* [7-9], *PTEN* [10-12], and *TSC1* [13] and *TSC2* [14] are now appreciated to represent the earliest successes in ASD gene discovery. Subsequently, the first replicated findings in 'idiopathic' or 'nonsyndromic' ASD emerged in the early part of this century. Rare point mutations in the genes *Neuroligin 3X* and *4X* (*NLGN3X and NLGN4X*) [15,16] were identified by targeted sequencing of segments of the X chromosome, based on the observation of rare chromosomal abnormalities in affected females. Importantly, the *NLGN4X* finding was quickly replicated in a linkage study of a large multiply-affected pedigree [16]. The neuroligins encode neuronal adhesion molecules at the post-synaptic density (PSD), leading to an early interest in synaptic pathology in ASD [17-19].

In fact, the biological relevance of the first genes identified in ASD for the understanding of common, genetically complex forms of the syndrome has become increasingly apparent over time. However, the path forward toward a systematic approach to reliable gene discovery did not emerge until rare *de novo* mutations could be readily detected at high throughput, high resolution, and at a genome-wide scale. Using microarray technology, Sebat et al [20] in 2007 showed that *de novo* variations in submicroscopic chromosomal structure carried substantial risk for ASD in simplex families (those with only a single affected child). This finding has now been widely replicated [21-26]. Moreover, this discovery was followed quickly by a series of observations that *de novo* CNVs cluster in specific segments of the genome in affected individuals [27,28], and that this could be leveraged to pinpoint risk regions. Dozens of ASD-associated CNVs have now been confirmed, including 16p11.2, 15q11.2-13.1, 15q13.2-q13.3, 7q11.23, NRXN1, 1q21.1, 22q11.2, 16p13.11, Xp22.1 (*PTCHD1-PTCHD1AS*), and *AGBL4* [21-30]. Current estimates suggest that several hundred ASD-related regions will eventually be identified [24,25].

While CNV analyses represented a critical step in clarifying the genomic architecture of ASD, they have proven challenging for translational neuroscience, in large part due to the multigenic nature of many of the relevant risk regions. However, more recent exome sequencing studies have now confirmed that *de novo* coding single nucleotide variants (SNVs) and *de novo* insertion deletions (indels) similarly contribute substantial risk for ASD

[31-36]. Moreover, a subset of these *de novo* mutations—specifically premature stop codons, canonical splice-site mutations, and frameshift indels—has been found to carry the largest effects. Similar to CNVs, the search for multiple *de novo* SNVs and indels at the same locus in unrelated individuals has proven to be a powerful approach to gene discovery [33,37]. Currently, eleven genes have been found, in recent analyses of *de novo* LGD/LoF mutations, to be strongly associated with ASD [31-36,38-40] (Table 1). Overall, current estimates suggest that up to 1,000 risk genes will carry *de novo* coding mutations [33,35,41]. Importantly, the path to their identification is now readily apparent [37], dependent only on available cohorts and the resources necessary to sequence and analyze them.

From Genes to Neurobiology

Single Gene Syndromes and ASD models

In depth studies of model systems that recapitulate monogenic ASD syndromes have yielded critical insights into neurobiological mechanisms. The details of these efforts have been well reviewed elsewhere (e.g. [17,19,42-47]) and will not be considered here in depth. It is useful, however, to briefly consider some of the key conceptual advances that have attended studies of, for instance, Fragile X and Tuberous Sclerosis complex, among the earliest single gene syndromes found to be associated with an increased risk for autism.

In 1991 the relationship between the fragile X mental retardation 1 (*FMR1*) gene, and fragile X syndrome (FXS) [7-9] was discovered, followed subsequently by the finding that *FMR1* encodes an RNA-binding protein (Fragile X Mental Retardation Protein; FMRP) [48]. Over the last several decades, a host of studies have confirmed that individuals with FXS demonstrate markedly elevated rates of ASD (e.g. [49-52]). Studies in model systems have shown that a key function of FMRP is regulation of protein translation at the synapse, acting in opposition to metabotropic glutamate receptors (mGluRs). These insights led to the mGluR theory of FXS, postulating that the CNS pathology results from an inability to regulate protein synthesis downstream of mGluR activation (reviewed in [44,46]). A series of seminal subsequent findings have revealed that both genetic and pharmacological inhibition of mGluR5 rescues key aspects of the phenotype in FXS models, even in adulthood (reviewed in [44,46]). These observations have led to several clinical trials of mGluR5 antagonists and negative allosteric modulators, both with regard to FXS as well as idiopathic ASD (http://www.clinicaltrials.gov) [44,46].

Similarly, it has long been observed that individuals with Tuberous Sclerosis complex (TSC) show elevated rates of ASD (e.g. [53-57]). Subsequent to the cloning of causal mutations in the genes *TSC1* [13] and *TSC2* [14], it was determined that the encoded protein products function as negative regulators in the Akt/mTOR pathway, also involved in protein synthesis. Notably, an increased incidence of ASD has also been found in patients carrying rare mutations in the genes *PTEN* [58-61] and *NF-1* [54,62], which are likewise negative regulators of the Akt/mTor signaling pathway. Mouse models of these mutations recapitulate many of the phenotypes observed in affected individuals, some of which are reversed or ameliorated by treatment with inhibitors of the mTor pathway, such as rapamycin or lovastatin (reviewed in [44]). Accordingly, clinical trials with these drugs are similarly underway (http://www.clinicaltrials.gov).

These discoveries, along with multiple additional examples of phenotypic rescue in adult models of monogenic intellectual disability (ID) / ASD syndromes (reviewed in [17,19,43,45,47]), have led to a marked shift in thinking about the opportunities for treating neurodevelopmental disorders. Certainly a decade ago, Fragile X, TSC, and other ID/ASD syndromes were considered largely static entities offering little hope for substantial reversal after birth. These are now widely thought of as reflecting a combination of developmental as well as ongoing functional deficits, opening the door to somatic treatments across the lifespan. Not surprisingly, such discoveries have had a profound influence on thinking about the possibility that common forms of ASD may reflect similar mechanisms and opportunities.

Idiopathic ASD, Genetic Complexity and Neurobiology

The extraordinary degree of locus heterogeneity so far identified in idiopathic ASD presents clear challenges. However, in addition, the combination of biological pleiotropy of risk genes, the (incompletely characterized) cellular diversity of human brain, the difficulty in distinguishing primary from secondary effects in developmental syndromes, and the dynamic nature of brain development, all underscore the challenge of pinpointing key pathological mechanisms in ASD. The issue of characterizing the relevant dysfunction in animal models of ASD mutations has become particularly pressing in light of findings demonstrating that an identical variant carrying risk for ASD may also carry substantial risk for phenotypes with strikingly distinct symptom profiles and natural histories, ranging from schizophrenia, to specific language impairment [63]. This overall complexity is forcing a reevaluation of the strategies that will be necessary to tackle the next phases of translational neuroscience of ASD.

Not surprisingly, in the face of an over-abundance of rare, large effect ASD-related mutations, there have been multiple attempts to identify unifying characteristics among risk genes. Initially this has involved querying sets of genes against pre-existing databases of biological information to determine if particular characteristics, processes, or relationships are overrepresented. For example, in ASD, recent results of gene ontology and protein-protein interaction analyses have focused attention on chromatin biology, neuronal development, Wnt/beta-catenin signaling, FMRP binding, and synaptic functioning as important in pathogenesis (e.g. [26,32,35,64,65]).

These findings, while clearly important, provide limited information with regard to when and where to look in model systems for specific ASD related mechanisms. Moreover, based on the hypotheses that developmental processes are important in ASD pathophysiology, it would follow that being able to specify spatial and temporal variables while modeling specific mutations could help constrain the search for relevant phenotypes.

Until recently, such ‘contextualized’ analyses have not been possible, due to the fact that most available biological databases draw information from a wide variety of sources, but without allowing for the parsing of relevant variables such as cell or tissue type, brain region or developmental stage. Consequently, the results of many current systems biological analyses have tended to define a general and static topology of ASD pathology (Figure 1A). However, recent foundational efforts are rapidly leading to the creation of multidimensional

'omics' datasets, reflecting the entire course of brain development. For example, the BrainSpan developmental transcriptome project summarizes gene expression from early fetal to late adult stages across multiple distinct anatomical regions in 57 typically-developing human brains [66,67]. Similar datasets are now becoming available in primate[1] and mouse[2]. These provide the substrate to begin to capture developmental dimensionality in systems biological analyses of ASD-related genes.

Indeed, several groups have utilized the BrainSpan data set to evaluate genetic findings and these studies have already begun to point to convergent neurobiology. For example, Ben-David et al [65] found that a large proportion of the set of genes identified by whole-exome sequencing [32-35] are present in a single co-expression module that is most highly expressed before birth. Several analyses have now extended and refined these findings, implicating specific brain regions, developmental epochs, and cell types in pathology (Figure 1B-E). For instance, by sub-setting the BrainSpan expression array data into smaller spatiotemporal windows, constructing gene co-expression networks around only the strongest set of ASD genes, and then assessing each for enrichment of additional ASD risk genes, our group found that deep layer cortical projection neurons in midfetal prefrontal cortex are one important nexus for a subset of ASD risk genes [40]. In a similar vein, Parikshak et al [68] used BrainSpan RNA-seq data, a broad range of input genes, and weighted gene co-expression network analysis, and found evidence for the importance of developmental epochs encompassing both early fetal and late fetal to early infancy, as well as projection neurons. In this case, their findings pointed to both deep and superficial cortical layers (III-IV), however the latter were found to be most relevant for ASD pathology.

Xu et al subsequently developed an approach, known as specific expression analysis (SEA) [69], for associating candidate gene lists with a particular context based on expression profiles across multiple contexts (e.g. cell type, brain region, time period). They applied SEA to interrogate the genes identified in exome studies [32-35] and, similar to the findings noted above, found enrichment in the developing midfetal cortex, as well as midfetal striatum. Finally, Uddin and colleagues [70] utilized exon level expression data from BrainSpan set to assess the relationship between exon expression and relevance to ASD. The authors observed a strong relationship between brain-expressed exons under purifying selection and ASD risk—an effect strongest in prenatal development, and in particular the orbital frontal cortex.

These studies collectively begin to give a sense of new opportunities in addressing the path from rare variant discovery in idiopathic ASD. The findings with regard to cell-type, regional, and developmental variables should begin to narrow the search for mechanisms and treatment targets in ASD. One can envision either focused investigations of single genes in particular cell types, developmental stages and brain regions, or, alternatively, using this

[1]Website: ©2014 Allen Institute for Brain Science. NIH Blueprint Non-Human Primate (NHP) Atlas [Internet]. Available from:http://www.blueprintnhpatlas.org/

[2]Website: ©2013 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas [Internet]. Available from: http://developingmouse.brain-map.org.

spatiotemporal information as the basis for the simultaneous examination of multiple mutations in (apparently) functionally distinct genes.

Finally, while the genes so far discovered have all pointed to some degree to fetal cortical development, they also have shown some divergence (Figure 1B-E). Given the heterogeneity of ASD, this is not surprising as there will likely be multiple regions and time periods involved, some of which have yet to be identified. Finally, while current studies have found that risk mutations appear to converge in terms of their function in early brain development, this does not necessarily argue against the notion, supported by studies of monogenic syndromes, that the phenotypic manifestations may be a consequence both of developmental as well as ongoing functional deficits.

Future Directions

There are, of course, multiple areas of investigation that will be essential to support continued progress. First, ongoing gene discovery is essential. As noted above, many of the promising recent findings in translational neuroscience have relied on identifying either functional or spatiotemporal convergence of multiple risk loci. It follows that the more rare, large effect ASD mutations that are confirmed, the more inputs there will be for future studies. Similarly, it is clear that a substantial portion of ASD risk is carried in common polymorphisms. While these variants will undoubtedly confer smaller biological effects, clarifying this source of risk will be critical, including for determining the role of polygenic factors in dictating widely divergent outcomes from identical high effect mutations. Finally, as gene discovery in ASD continues to advance, the likelihood that population level sequencing will lead to the discovery of protective alleles increases, as does the potential that these will provide another path to therapeutics development.

An additional area of tremendous need is the development of additional ‘omics’ datasets that capture key aspects of brain development across species, including, but not limited to, isoform-level gene expression, ChIP-seq, methyl-seq, and proteomics data. These resources must ultimately include cell-type specificity to allow for the type of high resolution analyses that are likely to be essential for the next steps in clarifying when and where ASD pathology may be most productively studied.

Finally models systems will clearly be a critical resource. The addition to this armamentarium of induced pluripotent stem cells, brain organoids, genome editing tools, and methods to capture data from increasingly large groups of neurons will play a key role in allowing the field to move forward. While there are currently well-known limitations to any given system, the increasing ability to model multiple mutations simultaneously, across the range of systems, from fly to human cells holds real promise for the successful navigation of the difficult path from genes to neurobiology.

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Highlights

- High throughput genomic methods are rapidly increasing the pool of ASD genes
- Heterogeneity, pleiotropy, and brain complexity complicate translation to biology
- Monogenic syndromes have provided the first insights into the neuropathology of ASD
- Leveraging multidimensional data from the brain offers a new complementary approach
- Recent results localize a subset of ASD risk to specific spatiotemporal windows

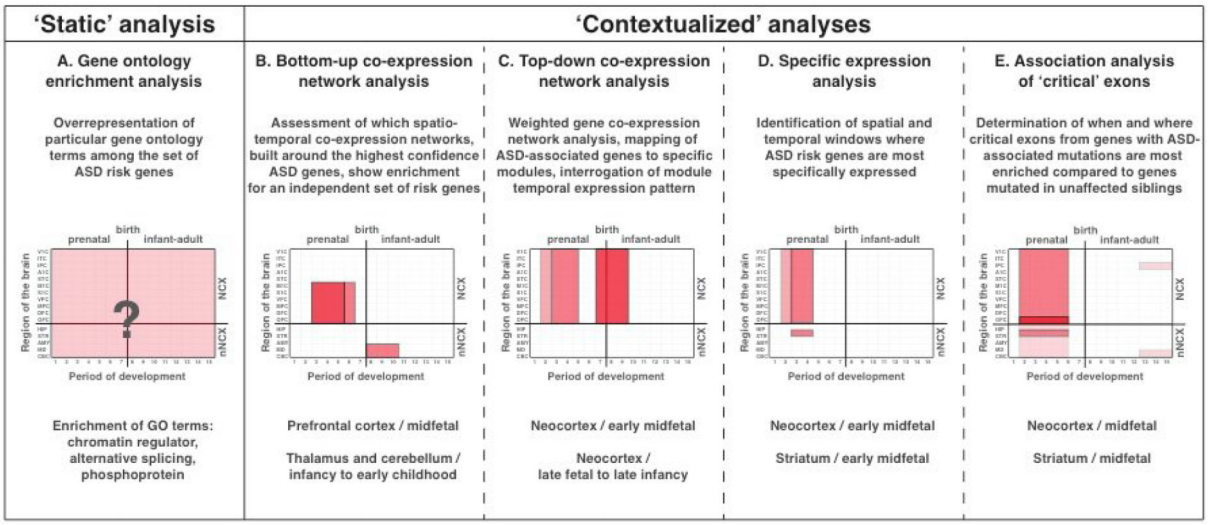

Figure 1. Systems biology approaches in ASD

Two main types of systems biology analysis, which we refer to as 'static' versus 'contextualized', have been recently applied to ASD genes. **Panel A**: A set of 131 ASD risk genes [40] evaluated for enrichment of gene ontology terms using DAVID [71,72] identifies 'chromatin regulator', 'alternative splicing', and 'phosphoprotein' as enriched terms. The grids illustrate temporal (X-axis; developmental periods as defined in [66]) and spatial (Y-axis; anatomical brain regions) variables, and the intensity of red indicates the strength of evidence in each panel. The '?' in panel A reflects the absence of data on these dimensions from static enrichment analyses. **Panels B-E**: illustrate several recent approaches to contextualized analyses: **Panel B**: BrainSpan exon array expression dataset was used to create co-expression networks based on a small number of high confidence ASD genes [40]. Each of the spatially and temporally defined networks was then evaluated for the presence of additional ASD associated genes. Two spatiotemporal windows were found to be significantly enriched for ASD genes: midfetal prefrontal cortex and thalamus/cerebellar cortex during neonatal to early childhood. The enriched networks were then assessed for additional properties with regard to cortical layer and cell type [66]. **Panel C**: Weighted gene co-expression network analysis was conducted with BrainSpan RNA-seq expression data from the neocortex, and resultant modules were assessed for enrichment of ASD-associated genes [68]. Based on the temporal expression pattern and biological properties of the genes within enriched modules, biological processes likely key during early fetal development and late fetal to early infancy were identified. Laminar and cellular specificity were also assessed. **Panel D**: A specificity index was calculated by brain region and developmental epoch for expression of ASD risk genes identified from whole-exome sequencing [31-35]. The authors determined that expression of the set of ASD genes is particularly specific to early midfetal neocortex as well as the early midfetal striatum [69]. **Panel E**: A third recent analysis utilized exon transcriptome-mutation contingency indices [70]. After summarizing exon level mutation rates based on population controls, the authors identified critical exons (defined as having high expression but low mutational burden) and

observed that enrichment of critical exons in genes mutated in ASD cases versus unaffected sibling controls [33,35] is strongest in prenatal neocortex and striatum.

Table 1

ASD risk genes discovered through recurrent *de novo* loss of function (dnLoF) mutations identified by whole-exome and whole-genome sequencing.

Gene name	Gene symbol	Recurrent dnLoF	False discovery rate[a]	References
Chromodomain helicase DNA binding protein 8	*CHD8*	3	0.0002	[32,39]
Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	*DYRK1A*	3	0.0002	[32,35,39]
Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	*GRIN2B*	3	0.0002	[31,32,39]
Sodium channel, voltage-gated, type II, alpha subunit	*SCN2A*	3	0.0002	[33,34]
Ankyrin 2, neuronal	*ANK2*	2	0.02	[35,40]
Cullin 3	*CUL3*	2	0.02	[32,38]
Katanin p60 subunit A-like 2	*KATNAL2*	2	0.02	[32,33]
Lysine (K)-Specific Methyltransferase 2E	*KMT2E (MLL5)*	2	0.02	[35,36]
Pogo transposable element with ZNF domain	*POGZ*	2	0.02	[34,35]
Regulating Synaptic Membrane Exocytosis 1	*RIMS1*	2	0.02	[35,36]
T-box, brain, 1	*TBR1*	2	0.02	[32,39]

[a]False discovery rate estimated as in [40].