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Unlocking the Treasure Trove: From Genes to Schizophrenia Biology

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Significant progress is being made in defining the genetic etiology of schizophrenia. As the list of implicated genes grows, parallel developments in gene editing technology provide new methods to investigate gene function in model systems. The confluence of these two research fields—gene discovery and functional biology—may offer novel insights into schizophrenia etiology. We review recent advances in these fields, consider the likely obstacles to progress, and consider strategies as to how these can be overcome.

Key words: schizophrenia/genomics/CRISPR/sequencing/model systems

Introduction

Two factors will be critically important in understanding the molecular basis of schizophrenia: identifying genetic etiology and having technologies to investigate gene function in model systems. Significant progress is being made on both fronts. The last 5 years has seen advances in our appreciation of the genetic architecture involved, with large numbers of confirmed risk variants.[1–3] Illustrative of progress in the methodologies available to functional biologists are developments in precision genome engineering, identified by the journal *Science* as research breakthroughs of 2012 and 2013.[4] The most recent of these is based on a naturally observed phenomenon in prokaryotic organisms: the facility of the type II prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR)/Cas adaptive immune system for site-specific DNA cleavage. Technology based on CRISPR promises rapid, robust engineering to allow investigation of single genetic variants, but also assays of multiple genetic risk variants in a model system.[5,6] The confluence of gene discovery and functional biology offers the potential for novel insights into schizophrenia etiology. In this article, we consider how this can be achieved and focus on strategies to deal with 3 of the main obstacles: the selection of target risk variants, what model systems will be required, and how to model genetic complexity.

Genetic Targets: Where to Begin?

Having had precious few targets before this decade, deciding which genetic variants to prioritize for functional studies is now a real challenge. It is likely that common single nucleotide polymorphisms explain ~25% of the variance in schizophrenia genetic liability[7] and >100 independent risk variants have been identified, with confidence, by genome-wide association studies (GWAS) and meta-analytical heft.[2,51] Echoing the experience of other complex disorders, only a small minority of common risk variants are nonsynonymous, exonic polymorphisms with obvious functional effects. Of the remainder, ~40% can possibly be mapped, with some degree of confidence, to single genes.[51] It is presumed that many of these variants have regulatory functions, but how this is achieved is, in most cases, poorly understood although this is changing as the integration of genome sequence and functional data improves (eg, see Lappalainen et al[8] and Maurano et al[9]). As entry points for functional biology, common variants make a small contribution to risk, and it has been argued that their impact on phenotype will be difficult to model and more difficult to interpret. A counter argument is that the known contribution of this class of variation to risk is hard to ignore, and there are examples where such variants identify important therapeutic mechanisms for common diseases (see Plenge et al[10] for review).

An alternative strategy, articulated elsewhere, is to simplify the problem by restricting investigation to mutations that are highly penetrant with a potentially clearer relationship to disease risk.[11] This is a pragmatic argument for managing limited resources, particularly weighed against the low throughput, but increasingly sophisticated tools available to probe neural circuits in vivo.[12] A target of about a dozen such models was proposed by

the "Genetic and Neural Complexity in Psychiatry 2011 Working Group,"[11] and this, conveniently, approximates the current number of rare, highly penetrant schizophrenia mutations. All of these are structural risk variants (copy number variants) involving deletions or duplications of large genomic regions (>100 kb). In most cases, they represent recurrent de novo events involving many genes: a challenge for downstream biological research. However, there are examples where inherited events or the accumulation of different copy number variations (CNVs) at loci implicate specific genes (*NRXN1*, *VIPR2*, *TOP3B*, *PAK7*).[13–17] In our view, a much greater portfolio of mutations will be required to identify convergence across genetic models, and this will be important in estimating the number of potential disease mechanisms involved. From the structural variants, we have learned that almost all of these mutations are moderately penetrant for schizophrenia (odds ratios in the range of 2–30) but are highly pathogenic because they are known to be associated with other phenotypes including developmental delay, autism, and more subtle cognitive impairment.[18,19] As such, the mutations are likely to model aberrant developmental processes where schizophrenia is only one of a number of possible phenotypic outcomes. It is far from clear whether the same finding will apply for other classes of rare mutations (eg, sequence variants). However, we suspect that more complex systems will be required to model combinations of risk factors (eg, genetic, environmental) because this may be important in determining and discriminating between phenotypic outcomes.

The Promise and Challenge of Sequence Data

Because discovery shifts to genome sequencing, the list of risk mutations is likely to expand. Two recent studies illustrate the promise of exome sequencing but also the challenges.[20,21] An order of magnitude larger than previously reported analyses, the study by Purcell et al,[20] identified enrichment of rare disruptive mutations across a number of different gene sets in schizophrenia patients (detailed below). Across these sets, they identified 990 mutations in cases ($n = 2536$), but far from being confined to this group, they also identified 877 mutations in the same gene sets in the control group ($n = 2543$). The study was underpowered to detect which (pathogenic) mutations were contributing to this enrichment. Apart from the (ongoing) brute-force approach of increasing sample size,[22] which was successful for common variant analysis, other methodological approaches may be helpful in identifying the best candidates for functional follow-up.

Lessons can be learned from other neurodevelopmental disorders (NDDs) that have yielded evidence for new mutations recurring at specific genes.[23–26] Both recent schizophrenia exome article, while unable to identify statistical association with individual genes, highlight loss-of-function mutations in confirmed autism spectrum disorder (ASD) genes (*SYNGAP1*, *SYN2A*, *POGZ*, *DLG2*, *SHANK1*) that seem to be in keeping with the observed overlap in structural variation studies.[20,21] Investigators of other NDDs have also found evidence for convergence of mutations on functional annotations (eg, pathways).[27] The 2 schizophrenia article report analyses of pathways implicated by extant schizophrenia and NDD data. The group who published the Fromer paper[21] previously reported that schizophrenia risk CNVs were enriched for *N*-methyl-D-aspartate receptor/activity-regulated, cytoskeleton-associated (ARC) protein complex genes.[28] In their exome analysis, they found significant enrichment for nonsynonymous mutations in this gene set ($P = .0008$). Looking to the NDD literature, they also identified involvement of a network of brain-expressed genes repressed by the fragile X mental retardation protein (FMRP) gene ($P = .009$). Purcell et al[21] confirmed enrichment of likely functional mutations in NMDAR, ARC, and FMRP. They found mutations in genes related to calcium channels, which had previously been implicated by GWAS analyses of schizophrenia and other psychiatric disorders.[2,29] Further support is required to confirm perturbations of these networks in disease pathogenesis. Taking the same FMRP gene set, we analyzed 16 additional exome-based cohorts of NDD or healthy controls, of varying but generally small sample sizes.[23–25,30–34] We found enrichment of disruptive de novo mutations among FMRP-related genes in 5/10 NDD cohorts but in none of the 6 control data sets.

Convergence on particular networks or with known NDD risk genes increases the prior probability for involvement of a mutation in disease but does not particularly help with the identification of poorly characterized or novel mechanisms. Identifying distinctive features of schizophrenia, rather than commonalities with other NDDs, may be vital for developing specific interventions. There is certainly a role for alternative approaches that are agnostic to disease pathogenesis. These include probabilistic strategies, based on other characteristics of genes, such as their rates of, and tolerance to, mutations. Although we know that some genes tolerate protein-disrupting mutations without obvious phenotypic consequences, others are very intolerant of the impact of mutation and may be subject to moderate degrees of selection.[35] Recent analyses have used estimates of gene mutation rates derived from analysis of species divergence or analyses of the tolerance of genes to new or rare variants based on the distribution of population mutation frequencies. For example, O'Roak et al[25] demonstrated that the likelihood of observing *N* or more specifically classed mutations compared with the expected mutation rates for these classes can help to prioritize recurrently mutated genes. This work highlighted de novo loss-of-function mutations in *CHD8* as one of the most frequent mutational events in ASD. Investigators of epileptic encephalopathy have demonstrated that within the genome, genes known to be associated with

that disorder are among the most intolerant to mutation.[36] Examining the distribution of de novo mutations (DNMs) within the 25th centile for intolerance (n = 4264 genes), the authors demonstrated a significant shift from the null distribution, indicating a subset of genes among the intolerant gene list that confer substantial epileptic encephalopathy risk. In a similar vein, taking a relatively small cohort of 57 exome-sequenced schizophrenia trios, we observed a higher than expected proportion of de novo nonsense mutations in genes with significantly higher probability of haploinsufficiency. These findings suggest that gene-based scores of intolerance to mutation, such as the Residual Variation Intolerance Score (http://chgv.org/GenicIntolerance/), may be useful in interpreting sequence data for disease studies.

The emergence and progression of schizophrenia must also be seen in a dynamic context. A recent ASD study of *FMRP* genes argued for different etiologies depending on whether genes were specifically expressed during early development (where a single hit may be particularly penetrant) or were more generally expressed in adolescence or adulthood (where multiple pathway disruptions may be important).[37] With available exome data, it is increasingly possible to examine similar hypotheses in schizophrenia. Because transcriptome databases of the human brain become more sophisticated, it is possible to examine how potential candidate genes cluster and are coexpressed spatially and temporally during neurodevelopment.[31,38–40] Identifying a modest enrichment of DNMs in schizophrenia cases compared with controls, Gulsuner et al[40] reported that the DNMs in cases were significantly more likely to cluster in protein-protein interaction networks. The DNMs in cases were more likely to be coexpressed in fetal frontal cortex. The overlapping network of interacting and coexpressed genes had known functions including neuronal migration, synaptic transmission, signaling, transcriptional regulation, and transport. By combining these approaches, we can begin to learn when, where, and how aberrant network function happens and model how this contributes to disease pathogenesis.

What Model Systems Do We Need?

The number of known genetic risk factors and evident genetic complexity speak to a need for high-throughput mutation screening to understand disease pathogenesis. So where, and how, do we begin to unlock this treasure trove? Limited by difficulty, cost, and time involved in assessing the impact of mutations in model systems, progress has been slow. There are considerable challenges: to model human mutations in other systems, to model the complexity of multiple mutations, and to functionally evaluate epistatic or environmental interactions. Importantly, the rate at which hypotheses can be tested will determine the number of mutations that can be investigated, and this may be critical for enumerating the main disease mechanisms. As a first pass, characterizing likely gene disruptive mutations, haploinsufficient or intolerant genes, or developmentally regulated genes may simplify the "screen space." This can be followed by a more comprehensive investigation of mutations clearly damaging to protein function or critical to function of a molecular network in mouse, or other, models.

To date, the molecular mechanisms of disease mutations have been explored mostly in vertebrates such as mice, rats, and more recently zebra fish, but also in invertebrates such as *Drosophila melanogaster*. The relative advantages and disadvantages of these systems have been reviewed extensively elsewhere.[41,42] However, the difficulty of modeling mutations in these organisms, as well as the complexity of the nervous system in some, has limited screening potential and biological interpretation. *Caenorhabditis elegans* has a number of key features that makes it an attractive model organism for high-throughput screening of the neuronal effects of (at least a subset of) human disease mutations: the fate of all 302 neurons have been completely mapped, it can be genetically manipulated to quantify and visually study aspects of development and behaviors, and it is inexpensive to maintain. The organism has been extensively studied for other neurological conditions including Alzheimer's disease and epilepsy, as well as processes such as memory formation. For a complete review, see Bessa et al.[43] To date, most studies have involved endogenous gene knockout or establishing transgenic strains expressing the human homolog. The delivery of RNA interference (RNAi) targeting thousands of human-worm orthologs is also possible. However, while most neurons are refractory to RNAi, alterations to post embryonic dendritic development of the bilateral polymodal nociceptive for mechanosensation and thermosensation neurons are feasible, which maybe very suitable for rapidly identifying genes regulating neuronal development, arborization, synaptic formation, and synaptic positioning.[44]

The time-consuming reprogramming of fibroblasts to induced pluripotent stem cells (iPSCs) and neuronal differentiation using transcriptional factors is a barrier to high-throughput screening of mutations in patient material.[45] Methodological progress in establishing pluripotency of cells could make large-scale characterization of patient-derived neurons and patient-specific mutations more feasible. The nuclear reprogramming of somatic cells in response to external triggers presents the unique opportunity to generate patient stem and dedifferentiated cells by a simple physical stimulus, eliminating the need for genetic manipulation of nonpatient cell lines to study specific mutations. Such an approach has recently been demonstrated and is termed stimulus-triggered acquisition of pluripotency (STAP), based on the exposure of cells to physical stress in the form of a low pH solution.[46,47] STAP presents a number of significant advantages over iPSC conversion of somatic cells. Reprogramming is possible for many cell types: brain, skin, lung, liver, and T-cells. If

progress can be made, the higher conversion pluripotency rate by STAP may substantially reduce the time necessary to generate patient-derived neurons compared with iPSCs and enable high-throughput characterization and mutation screening of existing patient samples.

Facing the Challenge of Complexity

Combining recent developments in CRISPR gene-targeting technologies with the promise of high-throughput screens in *C. elegans* and STAP-derived patient neurons enables modeling of specific mutations and also provides the ability to directly address several challenging and long-standing questions about the complexity of genetic disease. Importantly, the synergy of CRISPR's multiplex gene-targeting ability[48,49] and high-throughput screens is a broad platform to model and dissect the genetic contribution of multiple mutations. We believe that this work needs to be grounded in statistical evidence rather than biological plausibility. Rare, highly penetrant risk mutations will be important starting points. However, a more ambitious program to sequentially target a spectrum of multiple risk variants, taking a small number of variants per assay, may be important to define the molecular etiology critical to a across a pathway, eg, the *NMDAR*/*ARC* gene complex or components of the *FMRP* network. Substituting sequences of multiple risk variants (common and/or rare) in patient-derived neurons with wild-type or protective alleles may be a useful strategy to partition the main effects of mutations from epistatic interactions while preserving the "isogenic" background. A complementary strategy would be to introduce combinations of risk variants into neurons derived from unaffected relatives, especially discordant twins, which would also serve to validate main or epistatic effects of mutations across pathways.

Over the next 5 years, we see the possibility for a tremendous expansion in our knowledge of genetic disease pathogenesis. Achieving this may require the pace of gene discovery to be matched with a rapid growth in high-throughput functional studies. While the most obvious starting point may be with single variants, perhaps of higher penetrance, a more nuanced understanding of the effects on biological systems may come from investigations of multiple mutations through in vivo or in vitro systems. Also important will be our ability to disambiguate models with more general effects on neurodevelopment (eg, structural variants), from those that may be more disease specific. This should inform better, hypothesis-driven models for the study of behavior in intact biological systems and ultimately reap benefits for affected individuals and their families.[50]

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