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Enlisting hESCs to Interrogate Genetic Variants Associated with Neuropsychiatric Disorders

Steven E. Hyman[1,2,*]
[1]Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA
[2]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA
*Correspondence: seh@harvard.edu
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Connecting rare genetic variants to neuropsychiatric disease mechanisms remains a significant challenge. In this issue of *Cell Stem Cell*, Pak et al. (2015) combine gene targeting and stem cell technologies to identify a significant cellular effect of rare penetrant NRXN1 mutations in human neurons, which was found to cause a defect in neurotransmitter release.

The genetic analysis of schizophrenia, autism spectrum disorders (ASDs), and other neuropsychiatric disorders is yielding a potential trove of molecular clues to understand pathogenesis and identify possible therapeutic targets. However, significant scientific hurdles stand between emerging genetic findings and their biological exploitation. Major challenges are posed by the polygenic architecture of neuropsychiatric disorders (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Purcell et al., 2014); by the incomplete penetrance of most disease-associated alleles; and by their pleiotropic effects, whether single nucleotide variants (SNVs) or copy number variants (CNVs) (Lee et al., 2013, Stefansson et al., 2014). In this issue of *Cell Stem Cell*, Pak et al. (2015) describe the use of human stem cell-derived excitatory neurons to study two heterozygous NRXN1 mutations associated with ASDs and schizophrenia, one that truncates and one that deletes the gene.

Damaging NRXN1 mutations result most often from CNVs in the human genome. As is typical for CNVs associated with neuropsychiatric disorders (Stefansson et al., 2014), those causing NRXN1 mutations result in multiple disease phenotypes (Enggaard Hoeffding et al., 2014). NRXN1 encodes a presynaptic cell adhesion molecule, neurexin-1, which interacts with postsynaptic partners including neuroligins, which have been independently associated with ASDs (Südhof, 2008). The precise boundaries and other features of CNVs at any chromosomal locus differ within populations, making specific alleles very rare. Given, in addition, the incomplete penetrance of CNVs including those affecting NRXN1—and thus the importance of genetic background—biological analysis of their effects is markedly benefitted by use of isogenic cells. Thus Pak et al. introduced two different heterozygous conditional NRXN1 mutations into isogenic H1 human embryonic stem cell lines using a recombinant adeno-associated viral (AAV) vector. The two resulting embryonic stem cell lines were induced to become excitatory cortical neurons by forced expression of the transcription factor Ngn2. During neuronal differentiation, cells were treated with appropriate recombinases to yield two different sets of matching control and NRXN1 mutant neurons. The mutant lines, one a knockout and the other a truncation, proved haploinsufficient in neurexin-1 protein, mimicking the situation of patients with heterozygous loss-of-function mutations.

Neuronal differentiation, morphology, and nonsynaptic physiology of the NRXN1 mutant neurons did not differ from that of controls; however, both of the NRXN1 mutant lines demonstrated significant alterations in synaptic physiology. Pak et al. observed a decrease in the frequency of miniature excitatory postsynaptic currents (mEPSCs) without a change in their amplitude. mEPSCs result from spontaneous release of single neurotransmitter-containing vesicles from presynaptic terminals. Evoked EPSCs, in contrast, result from simultaneous release of many neurotransmitter-containing vesicles, typically produced by an action potential (neuronal firing) or in vitro by a suprathreshold electrical or chemical stimulus. The decrease in mEPSC frequency suggested that the NRXN1 mutant neurons might have fewer synapses, fewer vesicles per synapse, or alternatively, a decrease in the probability of vesicular release. There was no morphological evidence of alterations in the number or structure of synapses, suggesting that reduced neuroexin-1 protein levels specifically affected synaptic release mechanisms.

When the authors stimulated the induced neurons, they observed that both of the NRXN1 mutations caused a decrement in EPSC amplitude at the beginning of a 10 Hz spike train. After the initially low amplitude, with continued stimulation, the size of the evoked EPSC returned to control levels; nonetheless, the effects on neurotransmitter release were biologically significant. Pak et al. also found a 70%–80% increase in protein, but not mRNA, levels of CASK, a cytoplasmic scaffolding protein that interacts with neurexin-1, suggesting that neurexin-1 interactions destabilize CASK. Interestingly, CASK mutations have also been associated with intellectual disability and ASDs (Asadollahi et al., 2014).

An important strength of this analysis is the use of an appropriate cell type, a particularly challenging matter for human brain disorders. This is because (1) the brain has myriad cell types; (2) circumstances under which living cells can be obtained from the human brain are vanishingly rare; and (3) the human cerebral cortex has undergone recent evolutionary change, limiting the utility of animal models as a source of fully relevant cells. Indeed the highly significant abnormality in stimulus-dependent neurotransmitter release that Pak et al. found in

their induced human neurons was not observed in primary cultures of cortical neurons from heterozygous or homozygous NRXN1α knockout mice (see their Supplemental Information). Such species differences have important implications not only for pathophysiologic studies but also for drug discovery. Insofar as the correction of cellular defects, such as those reported here, become a basis for development of neuropsychiatric therapeutics, it will be critical to identify and employ the "right" cells for use in assays—not only with respect to species, but also with respect to specific neuronal or glial cell type. Without recent advances in stem cell biology that have paralleled progress in genomics, the ability to progress from neuropsychiatric-disorder-associated gene lists to informative biology would be very much limited (McCarroll and Hyman 2013).

The use of isogenic cell lines with appropriate control conditions makes the identification of a robust synaptic defect convincing. However, given the importance of genetic background in penetrance and expressivity of ASD- and schizophrenia-associated CNVs, future analyses would benefit from comparisons that added additional isogenic lines. Indeed when robust phenotypic differences can be found, as with these NRXN1 mutations, it would also be worth making comparisons with neurons derived from patients with similar mutations. While the overall genetic differences between lines might prove insuperably confounding, it is also possible that comparisons could inform the generalizability of a particular human cellular model as an assay for drug discovery. In one speculative scenario that takes an analogy from oncology (recognizing differences from the penetrant somatic mutations that characterize cancer), it might be possible to target treatments for neuropsychiatric disorders to the correction of pathologic cellular phenotypes even when the causative mutations produce diverse cognitive and behavioral phenotypes in patients.

Another caveat is important. Biological study of most of the coding variants that have been identified by whole-exome sequencing, for example in schizophrenia (Purcell et al., 2014), will be more difficult than studying inactivating mutations of NRXN1. The penetrance of most such variants is quite modest, and their rarity means that their disease association will remain cloudy. Use of isogenic cellular models to look for their biological effects would likely need to be engineered with "sensitized" genetic backgrounds likely gleaned from patients. One initial strategy might be to look for shared biological effects using allelic series of rare coding variants in genes already known to be disease associated by GWAS. Even against "risky" genetic backgrounds, the biological effects of most rare coding variants may be far less robust than those described here for NRXN1. However these technologies are ultimately deployed, Pak et al. (2015) have taken an important step in the analysis of rare variations associated with neuropsychiatric disease.

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