Understanding Rare Variation

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Rare Variants

Shared Paths for Therapeutic Development and Neurobiological Investigation

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Abstract

The revolution in human genetics has led to the identification of hundreds of rare genetic variants that underlie neuropsychiatric disorders. This technological leap presents both an opportunity and a dilemma for developing new therapies. Monogenic diseases are simpler to study and can be used to develop a road map for progressing from a genetic cause to both an understanding of neurobiology and a disease-modifying therapy. This trajectory involves the development of cellular and animal models, the understanding of the natural history of a disease, and the identification of biomarkers and clinical endpoints. However, the large number of mutations and the rarity of the diseases requires criteria for prioritization and strategies for connecting these diseases to more common causes of neuropsychiatric disorders. The goal of this chapter is to provide a road map to help prioritize investments that will improve our understanding of rare neuropsychiatric diseases, connect these diseases to common disorders, and help to catalyze the development of new therapies.

Introduction

Therapeutic development relies either on serendipity or on understanding the pathophysiology of a disease sufficiently to design a rational intervention. For most of history, the development of therapies for diseases of the central nervous system has been based on serendipity combined with astute clinical observation. However, over the past decade, advances in genetics and neurobiology combined with new therapeutic modalities have led to some successes based on rational drug design, including:

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- Nucleic acid therapies: Nusinersen (Spinraza™), Onasemnogene abeparvovec (Zolgensma™), and Risdiplam (Evrysdi™) in spinal muscular atrophy (Vignette 5.1) (Finkel et al. 2017; Gidaro and Servais 2019; Keinath et al. 2021; Kim et al. 2019; Messina and Sframeli 2020),
- Selective immune-modulating antibodies: Ocrelizumab in multiple sclerosis (Juanatey et al. 2018), and
- A GABA-receptor modulating neurosteroid: Brexanolone in postpartum depression (Faden and Citrome 2020; Leader et al. 2019).

Our goal is to leverage the tremendous progress in understanding the genetics of neuropsychiatric disorders to extend these successes to other rare diseases and, ultimately, to common psychiatric disorders such as depression.

Rare variants with large effect sizes at well-defined genomic loci represent a highly tractable starting point for both investigating neurobiology and developing therapeutics. These quests are highly synergistic. Many of the characteristics that make a specific rare variant suitable for neurobiological inquiry also make it suitable for therapeutic discovery. Furthermore, many of the investigative steps are also shared, including understanding genotype-phenotype relationships and natural history, delineating the direction of effect, assessing whether mechanisms are preserved across species, and identifying endpoints and biomarkers. However, we also face substantial challenges, including a limited understanding of how the brain functions or develops, highly pleiotropic effects as well as incomplete penetrance from the rare variants, the inaccessibility of the human brain, and the absence of clearly defined equivalents of

Vignette 5.1 Spinal muscular atrophy (Bonanno et al. 2022).

Symptoms	• Early-onset, lower motor neuron atrophy. • Resulting paralysis leads to respiratory failure. • Severe forms are usually fatal by two years of age.
Genetics	• Recessive loss-of-function variants in the *SMN1* gene (often deletions of exon 7) lead to loss of the SMN protein, which leads to progressive lower motor neuron degeneration. • The neighboring *SMN2* gene encodes an identical protein but with substantially reduced efficiency due to a cryptic splice site in exon 7. • Copy number of *SMN2* varies substantially across the population and modifies the severity of spinal muscular atrophy symptoms (type 0–IV).
Incidence	• 10 in 100,000 in Europeans. • Carrier frequency of 1 in 35 in Europeans but penetrance modified by *SMN2* copy number. • Lower incidence in other ancestral groups.
Endpoints	• Mortality, respiratory support, motor milestones
Therapies	• AAV gene replacement (Zolgensma™) • Splice modifying ASOs to increase *SMN2* translation efficiency (Spinraza™) • *SMN2* splice modifying small molecule (Evrysdi™).

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neuropsychiatric phenotypes in experimental model systems. To overcome these challenges, we need large-scale concerted efforts to better define the impact of rare variants across multiple dimensions of neurobiology in humans and multiple experimental model systems.

This chapter summarizes our discussions at the Forum, which were guided by the following key questions:

- With the goal of improving therapeutics, what are the key dimensions to prioritizing genes and loci for neurobiological investigation?
- What are strategies for testing for convergence/divergence in mechanisms between genetic loci? How do we determine meaningful convergence?
- Which models should be used to interrogate genetic loci and how should they be leveraged? How do we validate their predictions in humans?
- What are key advances required in natural history, biomarkers, and clinical endpoints to optimize the probability of success in clinical trials?
- How do we establish infrastructure and incentives to generate rigorous reproducible findings?

As each topic is considered in turn, we propose an optimal study design for achieving testable hypotheses regarding the neurobiology of rare, highly penetrant variants, across multiple models, and levels of analysis.

Prioritizing Genes and Loci for Neurobiological Investigation

What are the key dimensions to prioritizing genes and loci for neurobiological investigation?

To improve therapeutics, we need to explore the multiple dimensions necessary to prioritize genes and loci for neurobiological investigation. Prioritization strategies vary based on the characteristics of the genes and loci. Recognizing that all neuropsychiatric disorders are polygenic, but that the genomic architecture varies between them (see Robinson et al., this volume), we considered three levels of complexity: single gene disorders, structural variants with multiple risk genes, and common variants.

Single Gene Disorders

To date, most single gene disorders are in childhood-onset phenotypes, including neurodevelopmental delay and autism spectrum disorder (ASD). Recent, large-scale cohorts have demonstrated that single gene disorders also play a role in schizophrenia and bipolar disorder, albeit in a very small number of individuals (see Robinson et al., this volume). As a consequence of natural selection, the effect sizes of rare variants associated with neuropsychiatric

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disorders on neurobehavioral phenotypes are estimated to be high, with odds ratios over 10, and several variants with odds ratios in excess of 50 (Fu et al. 2022; Marshall et al. 2008; Sanders et al. 2017; Satterstrom et al. 2020; Singh et al. 2022). These effect sizes are substantially larger than the odds ratios below 1.05 observed for the majority of common variants associated with neuropsychiatric disorders (Robinson et al., this volume), but smaller than those observed in Mendelian disorders (e.g., ≥ 500), in keeping with a substantial contribution from genomic background, environmental factors, and stochastic effects, even in individuals with a rare variant. These large effect sizes provide two major opportunities:

1. Human cellular and animal model experimental systems with the genetic variant are likely to provide an opportunity to acquire knowledge about the pathophysiology of the disorder.
2. Rescuing the genetic variant, or restoring the relevant gene's function, within the right neurodevelopmental window is likely to improve symptoms.

Modeling a genetic variant whose impact is sufficient to cause the phenotype in a suitable genetic background simplifies subsequent investigation. In theory, each disorder has only one therapeutic target and needs only one experimental model system. This, however, makes the assumption that a gene is the appropriate level of resolution, which may not always be the case. For example, numerous genes have multiple transcription start sites and variable splicing isoforms, and these can impact protein function (Araki et al. 2020; Chau et al. 2021; Dai et al. 2019; Liang et al. 2021). Alternatively, if a gene is a transcription factor, it may impact multiple other genes, and in this case, rescuing downstream targets may be more effective. Similarly, functional and phenotypic consequences can vary between missense variants in the same gene (Sanders et al. 2018). Attention to genotype-phenotype relationships in human populations is key to ensuring that research insights represent the observed disorder.

Gene Prioritization

In considering criteria for rare variant prioritization, we drew inspiration from the successes and obstacles encountered in developing therapies for rare neurodevelopmental disorders, reviewed by Sahin (this volume), and the implications of these on understanding neurobiology. We identified numerous factors (detailed below) to consider in gene prioritization and were struck by how similar the weighting of these factors was between the two goals of therapeutic development and understanding neurobiology (Figure 5.1).

Phenotypic Association. In an era in which there are hundreds of genes associated with neurodevelopmental phenotypes at genome-wide significance, crossing this threshold is a requirement for a gene to be prioritized (unless

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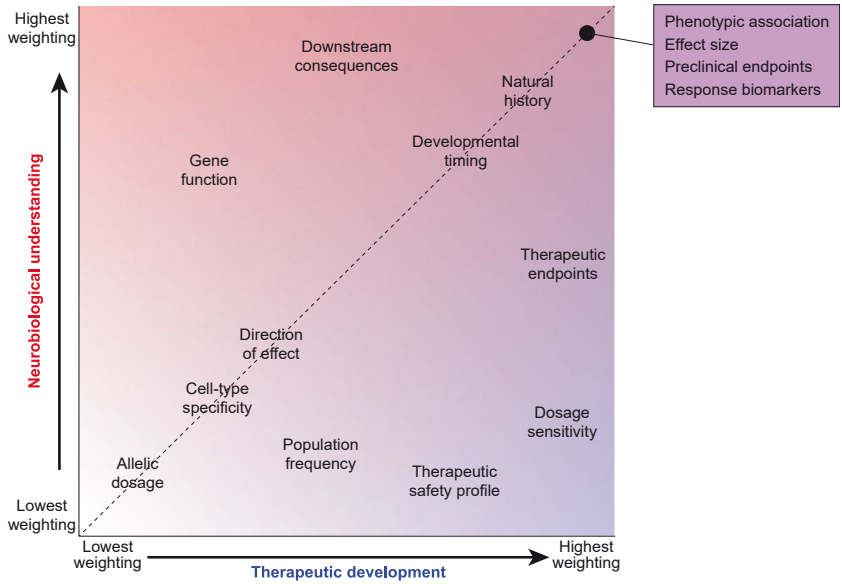

Figure 5.1 Gene prioritization in single gene disorders. The relative weighting for 15 factors in determining gene priority is shown qualitatively for therapeutic development (X-axis) and neurobiological understanding (Y-axis).

hundreds of genes are being assayed, and a more liberal threshold is appropriate). Statistical techniques exist for integrating multiple modalities of genetic data (e.g., *de novo* and inherited variation; Fu et al. 2022; He et al. 2013), but exceptions requiring human curation remain (e.g., triplet repeats in Fragile X syndrome undetected by exome sequencing; see Vignette 5.2). Further ranking by evidence of association is driven largely by population frequency and effect size, which we will consider separately.

Population Frequency. High population frequency simplifies natural history studies, genotype-phenotype analysis, clinical trial design, and commercialization of therapies and offers an opportunity to help more individuals. While a higher population frequency is useful for understanding neurobiology, it is a more important consideration for developing therapeutics. From a therapeutic perspective, the *prevalence of diagnosed individuals* matters more than the incidence; for example, there are larger cohorts of individuals with tuberous sclerosis (*TSC1*, *TSC2*) due to the longstanding clinical awareness of these disorders than there are with *CHD8* mutations, despite the predicted incidence of *CHD8* mutations being higher than *TSC1/2* due to the larger gene length. However, even N-of-one studies can be informative if there is clear natural history and a large effect size of the therapy (Gupta et al. 2020), so this criterion may carry less weight than others in specific cases.

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Vignette 5.2 Fragile X syndrome (Bear et al. 2004; Berry-Kravis et al. 2016).

Symptoms	• Developmental delay, intellectual disability, ASD, ADHD, motor coordination impairment. • Distinctive physical signs may be present.
Genetics	• Expansion of CGG repeats (typically ≤44, premutation 55–200, FXS >200) in the 5′UTR of the *FMR1* gene on chromosome X leads to methylation of the promoter, silencing transcription and reducing levels of the FMRP-encoded protein. • Females have milder symptoms due to the second chromosome X. FMRP regulates translation of multiple genes, including group 1 metabotropic glutamate receptors (mGluR1/mGluR5). • In preclinical models of FXS, inhibition of mGluR5 corrects multiple phenotypes.
Incidence	• 20 in 100,000 males; 10 in 100,000 females.
Endpoints	• Behavioral measures: aberrant behavior checklist (ABC), Clinical Global Impression–Improvement (CGI-I) scale, repetitive behavior scale.
Therapies	• mGluR5 antagonists (e.g., mavoglurant/AFQ-056) did not modify the ABC endpoint in phase 2b clinical trials of adults (18–45 years) or adolescents (12–17 years). • Preclinical trials are also focusing on reactivation or replacement of the *FMR1* gene.

Effect size. With larger effect size of the variant, we would expect greater benefit from therapy and more dramatic downstream consequences for understanding neurobiology. Accurate estimates of effect size are often challenging due to the absence of sufficient numbers of unaffected carriers with phenotype data. For haploinsufficient genes (i.e., when the loss of one allele leads to clinically relevant functional impairments, often leading to monoallelic or dominant inheritance patterns), the "loss-of-function observed/expected upper bound fraction" (LOEUF) score—an estimate of constraint based on the extent to which loss-of-function mutations in genes are under purifying selection in large population cohorts (Karczewski et al. 2020)—may help quantify this.

Natural history. Detailed natural history studies are critical to defining prognosis, phenotypic endpoints, therapeutic requirements of the affected individuals, genotype-phenotype correlations, and potential biomarkers (Gupta et al. 2020; Zerres and Rudnik-Schöneborn 1995). The availability of these data is highly beneficial and often essential for therapeutic development since the data helps select appropriate age ranges and therapeutic endpoints specific to the disorder. They are equally important for neurobiology, where understanding genotype-phenotype relationships and relating data from experimental model systems to human phenotypes are key.

Dosage sensitivity. Haploinsufficiency (i.e., sensitivity to low gene expression) underlies the majority of neurodevelopmental disorders (Fu et al. 2022).

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In a subset, overexpression can also lead to symptoms; for example, insufficient MeCP2 protein leads to Rett syndrome but duplications of the *MECP2* gene also cause neurodevelopmental delay. Similarly, both deletions and duplications at the 16p11.2 locus are associated with neurodevelopmental sequelae. In contrast, moderate overexpression (in contrast to gain-of-function) of *SCN2A* does not appear to have functional consequences (Tamura et al. 2022). For neurobiological inquiries, this can be controlled (or may even aid experimental design) so it plays little role in prioritization. For therapeutic development, genes with bidirectional dosage sensitivity (e.g., the "Goldilocks effect") might be expected to have a narrow therapeutic window, and techniques to exert fine control over expression are in their infancy. For now, genes with unidirectional dosage sensitivity should be prioritized over bidirectional dosage sensitivity for therapeutic development.

Allelic dosage. Since effect size and dosage sensitivity are considered separately, we felt that both biallelic (autosomal recessive) and monoallelic (dominant or X-linked) disorders were appropriate starting points for both therapeutic development and understanding neurobiology, giving this factor a lower weight.

Functional understanding. To date, progress in therapeutics and neurobiology has followed a detailed understanding of gene/protein function, direction of effect, cell-type specificity, and downstream consequences. These factors are essential steps in leveraging the gene to understand neurobiology, while only "sufficient" detail is required to aid therapeutic development; for example, the mechanisms by which survival motor neuron (SMN) protein deficiency leads to lower motor neuron death are still not clear. Consequently, the following factors were weighted slightly higher in prioritizing genes for understanding neurobiology:

- *Gene/protein function*: Genes with specific downstream functions (e.g., ion channels) or clearly annotated functions in well-known pathways (e.g., *CUL3*) may be more tractable for understanding neurobiology and defining the direction of effect than others (e.g., transcription factors).
- *Direction of effect*: The effects of rare variants can be loss-of-function, gain-of-function, dominant negative, or increased-function, either alone or in combination (Sanders et al. 2018). Once these effects are defined (which relies on defining and assaying gene function), they can be factored into experimental design or therapeutic development. Gain-of-function effects may require allele-specific or DNA/RNA-editing therapies. In theory, DNA and RNA editing could succeed without prior knowledge of the direction of effect.
- *Downstream consequences*: Defining the impact of the rare variant on brain function and development is critical to understanding neurobiology. For therapeutic design, it can help identify preclinical and

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therapeutic endpoints. Of note, if a therapy is directed at downstream consequences rather than the underlying genetic variant (e.g., mGluR5 in Fragile X), then the evidence base underlying these consequences should be a major consideration in gene prioritization.

- *Cell-type specificity*: Like direction of effect, it is important to characterize the cell types in which the gene/protein is expressed, and this needs to be factored into experimental and therapeutic design.
- *Developmental timing*: Like cell-type specificity, it is critical to define the developmental timing; however, this is often more challenging and harder to control experimentally. Disorders with substantial prenatal pathology may be especially challenging to treat (Herzeg et al. 2022; Zylka 2020); however, without validated preclinical endpoints, only successful therapies in humans would truly resolve the developmental critical window.

Preclinical endpoints. A robust preclinical endpoint relevant to the human disorder greatly benefits both neurobiological understanding and therapeutic development. The efficacy of such endpoints is not always immediately obvious. For example, SMN levels and tail necrosis in the mouse model of spinal muscular atrophy successfully predicted the benefits of antisense oligonucleotide (ASO) therapy in humans with spinal muscular atrophy (Finkel et al. 2017; Hua et al. 2010), while it is less clear that behavioral assays in rodents with particular genetic mutations (e.g., Fragile X syndrome) reflect intellectual disability or ASD in humans (Berry-Kravis et al. 2016).

Therapeutic safety profile. The field of nucleic acid therapies in the human central nervous system is in its infancy (Kuzmin et al. 2021) and may be associated with substantial risk, which must be balanced against potential benefit. In contrast, repurposing existing drugs with known safety profiles carries substantially lower risk. These considerations are critical for therapeutic development but matter less for understanding neurobiology.

Therapeutic endpoints. Clinical trials must predefine a single primary endpoint by which they are assessed; thus, the choice of endpoint is critical. Quantitative and "clean" endpoints, such as seizure frequency, are likely to be better powered than qualitative and "noisy" endpoints, such as behavioral symptoms. At the same time, given the urgent need to develop therapies for neurobehavioral manifestations of these conditions, the development of improved quantitative behavioral endpoints is a high priority. Precedence that the endpoint can be modified at a specific developmental stage is also an advantage. Like the safety profile, this is a critical consideration for therapeutic development and may also impact neurobiological inquiry, by demonstrating the relevance of findings to the human disorder.

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Response biomarkers. The availability of objective evidence that a therapy is engaging with the target and having the desired effect (such as a response biomarker) enables critical insight into dosing, duration of therapy, and patient stratification. At best, a response biomarker can act as a surrogate endpoint (e.g., HbA1c in diabetes) and be translated between experimental model systems and humans to act as a preclinical endpoint. Effective biomarkers for spinal muscular atrophy are emerging (Pino et al. 2021). For other neurodevelopmental and neuropsychiatric disorders, we are unaware of any robust biomarkers (Parellada et al. 2023; Sahin et al. 2018).

Application to Spinal Muscular Atrophy, Fragile X Syndrome, and Angelman Syndrome

Comparing these prioritization categories for (a) spinal muscular atrophy (Vignette 5.1), where there are widely adopted therapies, (b) Fragile X syndrome (Vignette 5.2), where phase 2 clinical trials failed to meet the endpoint, and (c) Angelman syndrome (Vignette 5.3), with promising interim data in phase 1 and 2 trials, many similarities can be identified (Table 5.1). In Fragile X syndrome, the relatively wide age ranges in developmental timing of disease onset, the lack of certainty that mGluR underlies symptoms in humans (downstream neurobiology), and the challenging nature of identifying robust

Vignette 5.3 Angelman syndrome (Judson et al. 2021; Noor et al. 2015; Wolter et al. 2020; Ultragenyx Pharmaceutical Inc. n.d.).

Symptoms	• Neurodevelopmental delay, often with ataxia, seizures, and microcephaly.
Genetics	• Loss-of-function of the maternal copy of *UBE3A*, which is imprinted in neurons. • Can be caused by *de novo* deletions, *de novo* small disruptive mutations in *UBE3A*, or uni-parental disomy. • *UBE3A* encodes an E3 ubiquitin ligase, which impacts the levels of multiple proteins. Overexpression of *UBE3A* has also been implicated in neurodevelopmental disorders.
Incidence	• 6 in 100,000 across ancestry groups. Deletion is the most common mechanism (70%).
Endpoints	• Developmental milestones, Clinical Global Impression–Improvement (CGI-I) scale.
Therapies	• Antisense oligonucleotides directed at the *UBE3A* antisense transcript (*UBE3A-AS*) that mediates imprinting can reactivate the paternal copy of *UBE3A.* • Several such therapies are under development, including GTX-102 that shows promise in a phase 1/2 open label clinical trial (NCT04259281). • CRISPR and gene replacement approaches are in preclinical development.

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Table 5.1 Comparison of therapeutic development in spinal muscular atrophy, Fragile X syndrome, and Angelman syndrome.

	Spinal Muscular Atrophy	Fragile X Syndrome	Angelman Syndrome
Phenotypic association	Robust	Robust	Robust
Population frequency	10 in 100,000	20 in 100,000	6 in 100,000
Effect size	Very high	Very high	Very high
Dosage sensitivity	Unidirectional	Unidirectional	Bidirectional
Allelic dosage	Biallelic (recessive)	X-linked	Monoallelic (dominant), but imprinted
Functional understanding	Low SMN protein and lower motor neuron death but the mechanism is unclear	Low FMRP affects translation of multiple proteins; preclinical evidence for mGluR5 being key	Low *UBE3A* in neurons impacts the levels of numerous proteins; unclear how this leads to symptoms
Preclinical endpoints	Mortality, SMN levels and tail necrosis in rodents	Behavioral measures in rodents	Motor performance, seizure susceptibility, and behavioral measures in rodents
Therapeutic action	Genetic rescue of causal gene or paralogue (*SMN1/2*)	Antagonist of downstream protein (mGluR5)	Genetic reactivation of imprinted causal gene (*UBE3A*)
Therapeutic safety profile	High risk: first ASO in human central nervous system	Low risk: repurposed drug	High risk: ASO in human central nervous system
Therapeutic age	After birth	12–45 years	4–17 years
Therapeutic endpoints	Mortality, respiratory support	Behavioral measures	Clinical impression
Response biomarkers	None	None	None
Natural history	Well-characterized	Well-characterized	Well-characterized

preclinical and therapeutic endpoints in neurodevelopmental delay probably contributed to failure (Berry-Kravis et al. 2016; Grabb and Potter 2022). These lessons helped refine subsequent studies, including for Angelman syndrome, which used younger ages, alternative endpoints, and gene-directed therapy. However, it is easy to draw such conclusions in retrospect. We can imagine a version of events where we are drawing inspiration from Fragile X syndrome and critiquing the early developmental onset and the high risk of ASOs in both spinal muscular atrophy and Angelman syndrome. Selecting the most tractable disorders for therapeutic development now helps pave the way for other disorders in the future.

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Structural Variants

Structural variants (SVs) include copy number variants (CNVs, e.g., deletions and duplications), inversions, translocations, and aneuploidies, either alone or in combination. Due to hypermutability, some SVs have population frequencies that are substantially higher than expected (e.g., non-allelic homologous recombination leading to 16p11.2 CNVs or meiotic nondisjunction leading to Trisomy 21). In addition to the factors already listed, we considered the impact of known "causal genes" when prioritizing SVs:

Causal genes. In some SVs, most of the phenotypic risk is mediated by a single gene (e.g., *NRXN1* in 2p16.3 deletions, *SHANK3* in 22q13 deletions, *UBE3A* in 15q11-13 maternal deletions). In these SVs, exome-sequencing data of cohorts with neurodevelopmental delay independently identify the single gene but not others within the SV (Fu et al. 2022; Sanders et al. 2015; Satterstrom et al. 2020). For these SVs, our prioritization schema for single gene disorders, above, applies.

In contrast, other SVs, such as CNVs at 16p11.2 and 22q11.2, appear to have a more complex risk architecture. Exome-sequencing data do not suggest a single gene is mediating risk or may implicate multiple genes (Sanders et al. 2015; Satterstrom et al. 2020). Systematic knockouts of each gene in experimental model systems have claimed to identify individual genes; for example, *KCTD13* at the 16p11.2 locus (Golzio et al. 2012) or *FZD9* at the 7q11.23 Williams syndrome locus (Chailangkarn et al. 2018). However, these results are not easily reconciled with the absence of gene association in human exome-sequencing data (Fu et al. 2022; Sanders et al. 2015; Satterstrom et al. 2020) or other similar analyses (Escamilla et al. 2017; Qiu et al. 2019). A polygenic or oligogenic model is a parsimonious explanation. However, it is possible that the genes within these SVs have combinatorial effects (i.e., interactions; Corominas et al. 2014; Lin et al. 2015) that individual gene knockouts miss or that risk is mediated by noncoding regions or surrounding loci. We considered how rare variant prioritization should be weighted by a polygenic SV locus.

Therapeutic development. Without a single gene target, nucleic acid therapies are challenging to apply, therefore we felt that SVs currently should not be prioritized for therapeutic development. We saw three possibilities for future therapeutics in polygenic SVs:

1. identifying and targeting the gene mediating the most risk,
2. targeting multiple genes, and
3. identifying downstream targets that might be amenable to repurposed drugs or small molecule screens.

Neurobiological understanding. Several SVs mediate high effect sizes with high population frequencies (e.g., 16p11.2, 22q11.2), providing unparalleled

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opportunities to study genotype-phenotype relationships and natural history in rarer variant disorders in human populations (Cable et al. 2021; Jacquemont et al. 2022). However, with current techniques it is challenging to induce, rescue, or manipulate large mutations, complicating many lines of inquiry. Similarly, questions of cell-type specificity, gene function, and development timing are complicated by the numerous genes involved; combinatorial effects may also be present.

In summary, due to the complexities of multiple genes within SV loci, we think that SVs without a clear single gene locus should be down-weighted for both therapeutic development and neurobiological understanding. Across SVs with multiple genes, the factors and weights we described for single gene disorders (Figure 5.1) can be used to prioritize them, though we note that the inclusion of more than one causal gene complicates several of these.

Common Variants

The majority of trait liability in neuropsychiatric disorders is thought to arise from numerous common variants, each mediating small effects (Corvin and Sullivan 2016; Gaugler et al. 2014; Owen et al. 2009; Sullivan 2005). The utility of these variants *en masse* to investigate neurobiology is considered by Won et al. (this volume). Below, we focus on which individual common variant loci, if at all, should be prioritized for therapeutic development or understanding neurobiology (cf. Won et al., this volume).

Fine mapping. As described above, the absence of a single gene target complicates experimentation. With sufficient genomic or functional genomic data, many common variants can be fine-mapped to individual genes (Sekar et al. 2016) with a specific mechanism (e.g., decreased expression), direction of effect, effect size, or causal variant (see Ronald et al. and Won et al., this volume). In prioritizing individual common variant loci, we would want to include a factor relating to whether the locus has been resolved to a single gene or variant in this manner and carries a high weight (Trubetskoy et al. 2022).

Overlapping loci. We observe substantial overlap in the genes from rare variant analyses of ASD and schizophrenia (Fu et al. 2022; Singh et al. 2022) and loci from common variant analyses of schizophrenia (Trubetskoy et al. 2022). Where such overlap exists for a given disorder between individual genes or loci arising from both common and rare variant studies, this adds to the prioritization of both the gene and locus.

Therapeutic development. There was consensus in our group that individual common variants should not be prioritized for therapeutic development due to the small effect sizes. We acknowledge, however, that a therapy with a large impact on a gene identified by a common variant with a small effect size might

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be beneficial, especially if there was overlapping rare variation. Further, in the presence of a rare variant, polygenic risk scores might be beneficial to consider for patient stratification (Davies et al. 2020).

Neurobiological understanding. The small effect sizes of individual common variants also pose a challenge for exploring neurobiology, compounded by the lack of conservation between species of noncoding regions, where most common variants are found. Investigating the gene targeted by a common variant provides a simpler starting point and may lead to larger effect sizes (Sekar et al. 2016). This strategy would add the additional requirement of demonstrating that the observed effects were relevant to the human disorder. As such, we felt that individual common variants should not be prioritized for investigating neurobiology, but this should be reevaluated as more data becomes available.

Gene Prioritization

While we qualitatively defined weights for the prioritization factors (Figure 5.1), we did not apply these to rank single gene disorders. We note that this ranking would vary based on requirements (e.g., a specific phenotype) and therefore be somewhat subjective. As such, the ranking would best be performed by a group of domain experts guided by these factors rather than by applying an algorithm based on the qualitative weights. We also note that these factors are dynamic. For example, the discovery of a response biomarker or changes in the perceived safety profile of a therapeutic modality could change the ranking of a gene radically.

Convergent Neurobiology

What are the strategies for testing for convergence/divergence in mechanisms between genetic loci? How do we determine meaningful convergence?

Therapy aimed at a specific gene offers hope for the minority of patients with a single gene disorder. For the majority of patients that lack such a clear target, therapies will need to focus on the neurobiological pathways that causally mediate phenotype. Single gene disorders can be used to illuminate these neurobiological pathways, leveraging the large effect sizes to identify causal pathways from genotype to phenotype (Figure 5.2a). In neuropsychiatric disorders, this process is complicated by pleiotropy (Figure 5.2b)—many functional consequences for each genetic variant—and the genetic heterogeneity (Figure 5.2c)—many genetic loci associated with each neuropsychiatric disorder.

In the absence of a clear endpoint (e.g., face-valid behavior such as seizures or pain response) or biomarker (e.g., cholesterol), the identification of convergent consequences across multiple genetic variants offers a mechanism to distinguish the biological processes by which genetic information influences

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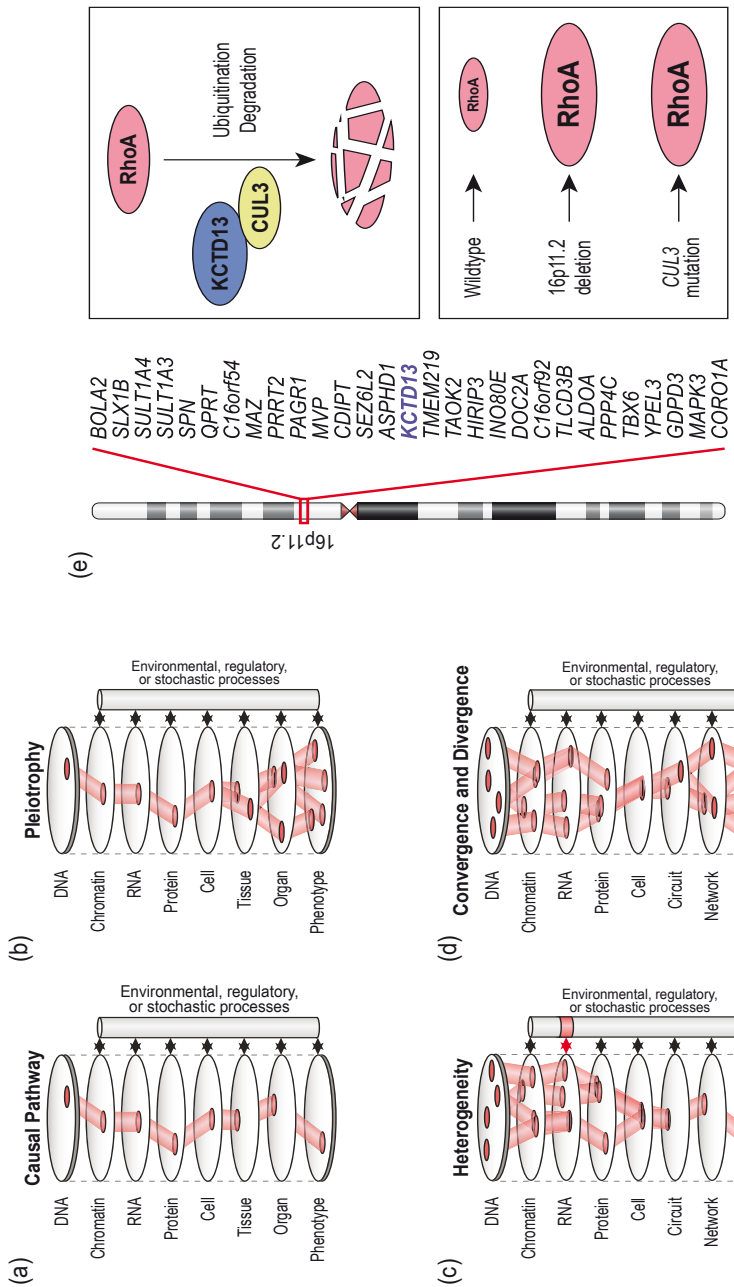

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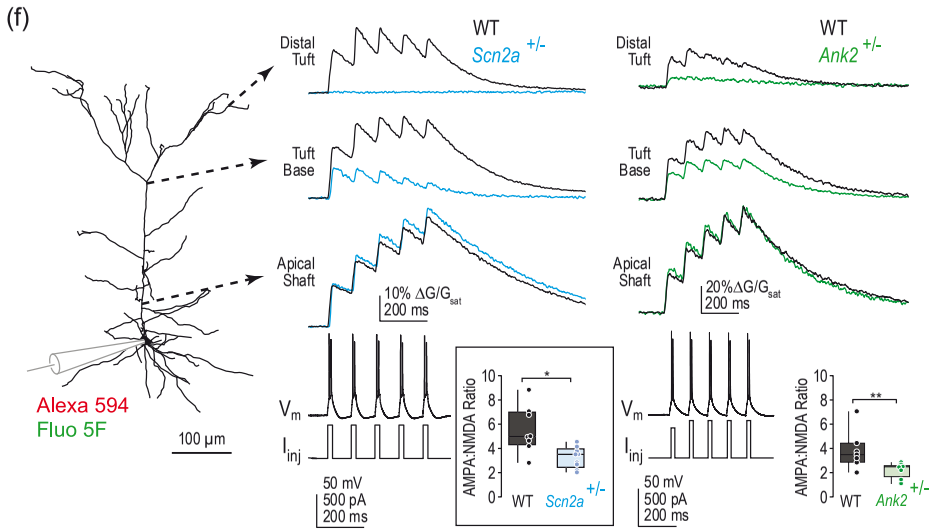

Figure 5.2 Causal pathways and convergence across causal factors in neuropsychiatric disorders. (a) We hypothesize that the mechanisms underlying genotype-phenotype associations act via different levels of biology that are discoverable. (b) Due to the complexity of the brain, each causal factor is likely to be pleiotropic (i.e., it can contribute to numerous phenotypes and multiple intervening biological processes). (c) Similarly, neuropsychiatric phenotypes are heterogeneous with multiple causal factors (both genetic and environmental). (d) We expect that a large number of causal factors might converge on a smaller number of intervening biological processes such that convergence might help guide the discovery of the causal pathway; see (a). However, divergence might be observable at other levels of analysis. (e) The gene *KCTD13* in the ASD-associated 16p11.2 microdeletion/duplication region has been implicated as a contributor to phenotype. The KCTD13 protein interacts with CUL3, encoded by the *CUL3* ASD-associated gene, and both proteins regulate the degradation of RhoA. This is an example of experimental "micro-convergence" providing a shared mechanism between two ASD-associated loci. (f) A second example of micro-convergence: heterozygous knockouts of both *Scn2a* and *Ank2* in mice lead to reduced backpropagation, with action potentials unable to reach the distal end of the dendritic tree. In both knockouts, the AMPA:NMDA ratio is reduced, suggesting immature synapses and a deficit in synaptic plasticity.

phenotype (Figure 5.2). This concept rests on the assumption that shared phenotype reflects some degree of shared neurobiology and that a large number of genetic loci act via a smaller number of biological processes, necessitating similarities in downstream processes between some genetic variants. Such convergence may be detectable at one or more levels of analysis (e.g., RNA, protein, subcellular location, pathway, cell type, circuit, brain region, developmental stage) but may not be readily detectable at others (Figure 5.2d). A biological process (e.g., excitatory neuron function) that truly mediates risk arising from combined effects across multiple loci might be expected to show convergence across experimental models of these loci. However, a closely related biological process (e.g., serotonergic neuron function) might also show a degree of convergence due to this similarity. Convergence is thus relative: we are searching for the processes with the greatest degree of convergence at each level of analysis, rather than any degree of convergence. Furthermore, convergence may be dynamic; for example, observable only at a specific stage in development or under specific conditions. This suggests the need for systematic analysis across multiple levels of analysis (e.g., cell types) to detect the biological process with the strongest convergent evidence based on a measure of effect size (e.g., correlation or enrichment) or variance (e.g., degree of phenotypic variance explained).

Multiple Autism Spectrum Disorder Genes

To date, 72 genes have been associated with ASD at genome-wide significance (Fu et al. 2022; Satterstrom et al. 2020); the majority act via haploinsufficiency (i.e., pathogenic for ASD when one of the two alleles does not produce a functional protein). While analysis of each gene independently can provide insight into the downstream neurobiological consequences, pleiotropy (Figure 5.2c) makes it hard to distinguish which of these consequences are relevant to neuropsychiatric symptoms. Again, the absence of a clear endpoint or biomarker prevents definitive experiments.

An alternative approach is to assess convergence across multiple ASD genes, and there is some evidence that this concept can provide insights. Many of the genes associated with ASD are enriched for *de novo* protein-truncating variants in genes that have been shown to be intolerant of such variants in the general population; this suggests convergence in a loss-of-function effect in haploinsufficient genes (Fu et al. 2022; Satterstrom et al. 2020). At the level of protein function, we observe the majority of genes associated with ASD-encoding proteins with a role in gene regulation (e.g., transcription factors, chromatin modifiers) or neuronal communication (e.g., synaptic proteins, ion channels; De Rubeis et al. 2014; Sanders et al. 2015; Satterstrom et al. 2020). Integrating these results with gene expression data from the developing human cortex shows that ASD-associated genes are enriched during mid-fetal gestation (Chang et al. 2015; Parikshak et al. 2013; Willsey et al. 2013), though

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the majority of genes continue to be highly expressed in the postnatal cortex. Single-cell transcriptomic data from the human brain shows strong convergence across ASD-associated genes in cortical excitatory and inhibitory neurons in the developing brain (Fu et al. 2022; Satterstrom et al. 2020).

These data provide some initial insights into key questions of function, cell type, and developmental period—questions critical to consider for the development of therapeutics (Dominguez et al. 2016; Hua et al. 2007; Liang et al. 2017; Muntoni and Wood 2011; Qi et al. 2013; Rinaldi and Wood 2018). The predominant heterozygous loss-of-function effects suggest that overexpression of the wildtype allele (e.g., CRIPSRa or knockdown of a repressor of the ASD-associated gene using antisense oligonucleotides) could be beneficial in many single gene disorders associated with ASD and neurodevelopmental delay. Animal models already show promise for several causes of neurodevelopmental delay (Colasante et al. 2020; Luoni et al. 2020; Schmid et al. 2021; Valassina et al. 2022); however, therapies for haploinsufficient genes may fail without sufficient knowledge of the disease-relevant biology (Hill and Meisler 2021).

While the assessment of convergent patterns has provided some insights, we must be mindful of the limitations of this approach. For example, cell-type enrichment strongly implicates cortical neurons. The developing brain is enriched for neurons compared with the mature brain: Is the prenatal enrichment of ASD-associated genes due to the high proportion of neurons, or is the neuron enrichment a reflection of the prenatal onset, or does ASD act through neurons in the prenatal cortex? Such questions are now being addressed with newer single-cell RNA-sequencing methods (e.g., Velmeshev et al. 2019). Transcription-based enrichment studies act on the assumption that high gene expression correlates with "cause," but there is no guarantee that this is true. If we were to study neurons in a different brain region (e.g., postnatal striatum), would we observe a higher degree of convergence that might change our conclusions? At present, we do not have a clear experimental or mathematical framework to address these issues.

Micro-Convergence across Autism Spectrum Disorder Loci

16p11.2 and CUL3. The concept of convergence can also be applied to experimental data from small numbers of loci associated with neuropsychiatric disorders (Figure 5.2e). Deletions and duplications at the 16p11.2 locus are associated with multiple neuropsychiatric disorders (see Vignette 5.4). A recent study using patient-derived induced pluripotent stem cells and brain organoids identified dysregulation of the neuronal cytoskeleton, neuronal migration, and RhoA signaling as potential biological processes underlying the 16p11.2-associated brain phenotypes (Urresti et al. 2021). Interestingly, one of the 16p11.2-encoded proteins, KCTD13, is a direct interacting partner of Cullin 3 (CUL3) ubiquitin ligase encoded by the *CUL3* gene (Lin et al. 2015), which has been associated with neurodevelopmental delay at genome-wide

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Vignette 5.4 16p11.2 deletion and duplication syndromes (D'Angelo et al. 2016; Martin-Brevet et al. 2018; McCarthy et al. 2009; Weiss et al. 2008).

Symptoms	• Both deletions and duplications are associated primarily with developmental delay, intellectual disability, and ASD. • Deletion is associated with increased head circumference and body mass index. • Duplication is associated with reduced head circumference, reduced BMI, and schizophrenia risk.
Genetics	• Loss (deletion) or gain (duplication) of ~600 kb in the short (p) arm at the 11.2 position on chromosome 16. • Surrounding segmental duplications create a locus susceptible to non-allelic homologous recombination, leading to the high population frequency. • Unlikely that any single gene underlies all the symptoms (e.g., exome sequencing has not identified a risk locus). • Risk mediated by two or more genes remains possible.
Incidence	• 60 in 100,000, split equally between deletion (30 in 100,000) and duplication (30 in 100,000).
Endpoints	• Language, social communication, motor milestones.
Therapies	• Behavioral focused (symptom-based) interventions (at present).

significance (Fu et al. 2022). Of note, the haploinsufficient *Cul3* mouse model also has defects in neuronal cytoskeleton and RhoA signaling, similar to those observed in 16p11.2 organoids (Amar et al. 2021). Furthermore, the resulting complex between KCTD13 and CUL3 regulates RhoA levels by ubiquitination and degradation: RhoA is known to be involved in neuronal migration, axon growth, dendrite formation, and cytoskeleton remodeling during brain development (Govek et al. 2011). RhoA activation has also been observed as a consequence of *CACNA1C* disruption (encoding the $Ca_V1.2$ calcium channel) in Timothy syndrome (Krey et al. 2013), which is associated with ASD. Some of the 16p11.2-related neuron migration phenotypes observed in organoids were rescued using a small molecule inhibitor of RhoA activity Rhosin (Urresti et al. 2021); this approach is currently being tested in the *Cul3* mouse model. This "micro-convergence" provides a complementary experimental approach to complement the larger-scale convergent patterns observed across many genes.

SCN2A and ANK2. A second example of micro-convergence has recently been described between two other ASD-associated genes. Heterozygous loss-of-function mutations in *SCN2A* (encoding the voltage-gated sodium channel $Na_V1.2$) are a major cause of ASD (see Vignette 5.5). Characterization of the *Scn2a* heterozygous knockout mouse ($Scn2a^{+/-}$) has demonstrated a persistent deficit in action potential backpropagation by which action potentials reach the dendrites of the cell that generated the action potential, in contrast to forward propagation down the axon to other neurons (Figure 5.2f) (Spratt et al.

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Vignette 5.5 *SCN2A* mutations (Li et al. 2021c; Sanders et al. 2018; Tamura et al. 2022).

Symptoms	• Seizures, ranging from severe epileptic encephalopathy to late-onset seizures. • Developmental delay, ASD, movement disorders, ataxia.
Genetics	• *De novo* mutations in the *SCN2A* gene that encodes the $Na_V1.2$ voltage-gated sodium channel. • Protein-truncating variants and loss-of-function missense variants lead to developmental delay, ASD, ±late-onset (≥ 3 years) seizures. • Gain-of-function missense variants lead to early-onset (≤ 6 months) epileptic encephalopathy. • Mixed gain/loss-of-function missense variants lead to seizures with an onset of 6 months to 3 years and developmental delay ± movement disorders. • Inherited mild gain-of-function missense variants lead to benign infantile seizures.
Incidence	• 9 in 100,000 across populations • 7.5 in 100,000 with loss-of-function • 1.5 in 100,000 with gain-of-function or gain or loss-of-function
Endpoints	• Mortality (epileptic encephalopathy), seizure frequency, developmental milestones
Therapies	• Sodium channel blocking antiepileptics for early onset seizures. • Non-sodium channel blocking antiepileptics for late-onset seizures. • PRAX-222 ASO nonselectively decreases *SCN2A* expression for gain-of-function therapy and is beginning clinical trials. • CRISPRa therapy upregulates *SCN*2A expression for loss-of-function therapy and is in preclinical development.

2019). This reduction in backpropagation leads to a reduced AMPA:NMDA ratio, suggesting immature synapses and a deficit in plasticity. In the *Scn2a*$^{+/-}$ mice, this synaptic phenotype can be rescued by a single *in vivo* CRISPRa injection via the tail vein (Tamura et al. 2022). The gene *ANK2* encodes the protein ankyrin-B, which has recently been shown to be essential for scaffolding $Na_V1.2$ to the dendritic membrane. Like *SCN2A*, *ANK2* is associated with ASD and neurodevelopmental delay at genome-wide significance (Fu et al. 2022). Analysis of the *Ank2* heterozygous knockout mouse (*Ank2*$^{+/-}$) reveals the same deficit in backpropagation and synaptic plasticity (Figure 5.2f) (Nelson et al. 2022).

Neurexins and neuroligins. Mutations in neurexins (*NRXN1*, *NRXN3*) and neuroligins (*NLGN2*, *NLGN3*, *NLGN4*) are associated with ASD and neurodevelopmental disorders. *NRXN1* is also associated with schizophrenia (Pak et al. 2015; Tromp et al. 2021). All these genes encode proteins that form transsynaptic signaling complexes anchored on the pre- and postsynaptic membrane that shape the properties of synapses (Eichmüller et al. 2022; Südhof 2008, 2017b).

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Divergence across Levels of Analysis

Fragile X syndrome provides a counterexample, in which functional "divergence" emerges across levels of neural organization despite "convergence" at the molecular level (see Figure 5.2d, Vignette 5.3, and Sahin, this volume). Our current understanding of the pathogenesis of Fragile X syndrome and the role of group I metabotropic glutamate receptors (including mGluR5) is based primarily on studies in the hippocampus and neocortex in rodents (Bear et al. 2004). In these regions, Fragile X syndrome leads to increased signaling through mGluR5, which leads to elevated synaptic plasticity as evidenced by an enhancement of long-term depression (LTD) (Fitzjohn et al. 2001; Hou et al. 2006; Huber et al. 2002; Nakamoto et al. 2007; Palmer et al. 1997). As predicted, downregulating mGluR signaling in mice corrects multiple abnormalities induced in Fragile X syndrome in these regions, including the LTD phenotype, impaired inhibitory avoidance extinction, audiogenic seizures, and enhanced cortical spine density (Dölen et al. 2007). However, the opposite effect occurs in the lateral amygdala, where mGluR5 mediates reduced synaptic plasticity via long-term potentiation (LTP) (Rodrigues et al. 2002; Suvrathan et al. 2010). Accordingly, an mGluR antagonist failed to reverse deficient amygdalar LTP in *Fmr1*$^{-/y}$ mice (Suvrathan et al. 2010).

In a more recent study using a rat model of Fragile X syndrome, activation of mGluRs reversed LTP impairment in the lateral amygdala as well as its behavioral correlate: impaired recall of conditioned fear (Fernandes et al. 2021). Interestingly, this study also revealed the presence of presynaptic mGluR5 at the same thalamic inputs that mediate LTP in the lateral amygdala. In contrast, much of the earlier work on synaptic deficits associated with Fragile X syndrome and their reversal in the hippocampus focused primarily on postsynaptic mechanisms. In other words, while mGluR-dependent synaptic signaling mechanisms in Fragile X syndrome pathophysiology are a point of convergence in the hippocampus and amygdala, the pharmacological correction of synaptic defects serves as an example of divergence: mGluR-inactivation is effective in the hippocampus, whereas mGluR-activation has been shown to *reverse* synaptic and behavioral deficits in the amygdala of Fragile X syndrome rats. Further, while we observe molecular convergence across brain regions at the level of mGluR, we observe subsequent divergence at the level of synaptic plasticity and behavioral outcomes (Figure 5.2d). Notably, this also highlights the importance of modifying the prevailing mGluR-based framework for therapeutic strategies to include circuit-specific differences in Fragile X syndrome pathophysiology.

Summary

The identification of convergent functional consequences across multiple genetic loci associated with a disorder increases the likelihood that the specific

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functional consequence is on the causal pathway between genotype and phenotype (Figure 5.2). However, convergence is relative and, if demonstrated, a greater degree of convergence in a related process might dramatically change interpretation. Identifying convergent effects requires systematic analyses across genes at multiple levels of analysis and across development. There is considerable scope to improve the analytical framework of convergent analyses to facilitate better comparisons and aid causal inference.

Models to Interrogate Genetic Loci

Brain development is extremely sensitive and complex. This process is crucial to future brain function, and investigating it is key to understanding how neurodevelopmental disorders alter brain function. Given the uniqueness of the human brain across species, the best tissue for enquiries to illuminate these developmental processes is from humans. There is a critical need for more molecular, structural, functional, and clinical information on typical and atypical human brain function across development as the primary source of information on these processes and as a comparator to validate experimental model systems. However, we cannot experimentally manipulate human subjects or gain access to living tissue with sufficient resolution. As such, we advocate for a systematic analysis of human-induced pluripotent stem cells (hiPSCs) as well as rodent and nonhuman primate (e.g., marmoset) models of single gene disorders in tandem with human studies. While there are concerns about the predictive efficacy of rodent studies for therapies of neuropsychiatric disorders in humans (see Vignette 5.2), there is limited data on how nonhuman primate models will fare. Here, we consider how data from experiment model systems can be used to make and validate predictions of human phenotypes (see also Brennand and Kushner, this volume).

Rodent Models

Since the publication of the first knockout mouse in 1987 (Thomas and Capecchi 1987), mice have been the mainstay for modeling most rare genetic disorders. More than 99% of mouse genes have human homologs; thus, genetically engineered mouse models have construct validity (or etiologic validity), which refers to how closely the molecular underpinnings of a disease in an animal model mirror those in humans. Whether those models have face validity as well, such that the phenotype in an animal model has significant overlap with the phenotype in humans, is still open to investigation. There is substantial skepticism about the behavioral traits observed in neuropsychiatric disorders. Finally, predictive validity (predicting successful therapy in humans) of these models does not yet exist for the most part because there are no FDA-approved treatments for most neuropsychiatric symptoms associated with rare genetic

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disorders; there is also an absence of validated preclinical endpoints and biomarkers. Nonetheless, mouse models can be extremely helpful in providing insights at the level of cellular function. They may be less useful, however, at the level of circuits or behavior. Additionally, genetic background effects play a substantial role in many phenotypes, consequently demonstrating consistent findings across mouse strains is critical.

Rats have been used extensively for pharmacological modeling but less so in the genetic modeling of brain disorders. Extension of CRISPR editing and advances in embryonic stem cell methods now allow for the efficient generation of rat models of single gene disorders. Rat models have the advantage that they display more complex behaviors and communications than mice (Ellenbroek and Youn 2016). Comparison of mouse and rat models of genetic diseases is starting to reveal that while cellular functions may be conserved, behavioral manifestations may differ across species in response to genetic manipulations (Till et al. 2015). Whether rat models will have improved face and/or predictive validity compared to mice in terms of developing new treatments for rare genetic disorders is not yet known.

Prairie voles have also been proposed as a suitable model for neuropsychiatric disorders because of the long-term social attachments formed between mating partners (Berendzen et al. 2023). Again, face and/or predictive validity remains to be demonstrated.

When modeling disorders with rodents, several steps can be taken to increase rigor and reproducibility (Gulinello et al. 2019). The issue of strain-specificity can be overcome by using multiple background strains, outbred mouse models, and multiple genetic models for the same disease. Issues related to statistical power can be addressed by preregistering behavioral assays and metrics (similar to clinical trials), increasing sample sizes, and replicating significant positive findings independently before moving to clinical trials (Howe et al. 2018). Finally, translational biomarkers shared by humans and rodents are needed to assess the therapeutic efficacy of an intervention (Modi and Sahin 2017).

Human-Induced Pluripotent Stem Cell Models

To investigate the molecular mechanisms of neurodevelopmental disorders, hiPSC-based models are now widely used. Given the limited opportunity for studying fetal brain tissue from individuals with neurodevelopmental disorders, hiPSC technology provides a unique opportunity to study developmental processes in a human context. Somatic cells derived from individuals are reprogrammed and differentiated into two-dimensional neuronal cultures, three-dimensional models (organoids), or more complex models, such as assembloids (Kelley and Pașca 2022; Pașca et al. 2022). Alternatively, specific genetic variants can be engineered into existing hiPSCs lines. It has been demonstrated that organoids are particularly well-suited for studies of prenatal

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neurodevelopmental disorder studies because their transcriptional profiles, cellular composition, and even electrophysiological properties largely capture those of the human early- to mid-fetal brain, despite certain noted limitations (Amiri et al. 2018; Bhaduri et al. 2020; Camp et al. 2015; Luo et al. 2016; Trujillo et al. 2019; Velasco et al. 2019). Many studies to date have used 3D cortical organoids to model neurodevelopmental disorders, such as lissencephaly (Bershteyn et al. 2017), idiopathic ASD (Mariani et al. 2015), microcephaly (Lancaster et al. 2013), Timothy syndrome (Birey et al. 2017), and 16p11.2-associated ASD (Urresti et al. 2021). These models may enable high- and medium-throughput drug screening for preclinical endpoints that emerge early in neurodevelopment. In addition, recent technological developments allow transplantation of organoids and assembloids into animal brains to create grafted "chimera" models that could provide further insights into diseases within more physiological conditions, including later stages of neurodevelopment, vascularization, and integration of more diverse cell types (Daviaud et al. 2018; Mansour et al. 2018).

Despite their many advantages, hiPSCs have some substantial limitations. It is hard to differentiate the cells beyond the equivalent of mid-fetal development, limiting insights into postnatal development, let alone the adolescent, adult, or elderly brain. For organoid models, there is substantial variability in cellular composition between organoids from the same hiPSC line, adding heterogeneity to assays. The use of hiPSC-derived models is further limited by patient availability or the heterogeneous genetic backgrounds of the patients, which can complicate the investigation of genetic variants of small effect (e.g., common variants from genome-wide association studies), and the prohibitive cost of producing such models on a large scale (e.g., hundreds to thousands of patients). Some of these limitations (e.g., accessibility to relevant patient populations) could be overcome, for example, by generating isogenic models by CRISPR engineering the desired mutation(s) into control iPSC lines (Ben Jehuda et al. 2018) or correcting the mutation from patient lines to independently assess the role of genetic background. Further avenues to pursue include lowering the cost of hiPSC reprogramming and organoid production and adapting the technology to a high-throughput low-volume format. Overall, despite many interesting insights into neurodevelopmental disorders achieved with hiPSC-derived models, the current state of the field still suffers from small sample sizes and variability in protocols that are used to generate these models, which makes cross-comparison difficult (Anderson et al. 2021). The application of appropriate statistical models that account for technical and biological replicates from different iPSC clones, and mixed models that treat the individual as a random-effect variable should be used in the analyses (Hoffman et al. 2019). As with rodent models, predictive validity remains to be demonstrated, though there are examples of treatments that have gone directly from hiPSC models to clinical trials without testing in an animal model (Wainger et al. 2014). These potential pitfalls notwithstanding, hiPSC-derived models clearly

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serve as an essential asset for future studies and therapeutic interventions for neurodevelopmental disorders.

Nonhuman Primate Models

Currently, most animal work is conducted in rodents, primarily because there is an extensive range of genetic tools with which to investigate these models and infrastructure to support these efforts. However, the human brain is quite distinct from that of rodents (Marshall and Mason 2019). To fill the gap, it is crucial to have a model animal that is closer to humans, and nonhuman primates represent the closest species. The common marmoset (*Callithrix jacchus*), a small New World monkey, has recently emerged as a new animal model for studies of neurological and neuropsychiatric disorders, basic and behavioral neuroscience, neuroimaging, stem cell research, and drug toxicology (Hikishima et al. 2011; Iwanami et al. 2005; Kishi et al. 2014; Leuner et al. 2007; Mansfield 2003; Mashiko et al. 2012; Poswillo et al. 1972; Sasaki et al. 2009; Tomioka et al. 2010; Yamazaki et al. 2011). Similar to humans, but unlike rodents, wild marmosets live in stable extended families. Group members support infant care by the breeding mother with strong parental and familial relationships. These and other human-like characteristics of marmosets are likely to be advantageous for cognitive behavioral research.

In addition, the recent generation of transgenic marmosets will enable researchers to investigate the molecular genetic basis of higher cognition and complex brain disorders that contain endophenotypes related to human-like conditions (Kishi et al. 2014; Okano et al. 2012; Sasaki et al. 2009). Moreover, comparisons revealed substantial similarities in cell types and gene expression patterns within prefrontal and visual cortices between marmosets, humans, and other species (Kita et al. 2021; Onishi et al. 2022; Ma et al. 2022). The above results suggest that the marmoset is likely to be a good animal model for human developmental brain disorders, and there is a reasonable expectation of face and predictive validity not achieved in other experimental model systems of neuropsychiatric disorders.

Using nonhuman primates for all experimental goals, however, raises ethical and economic issues. It is important to consider what is the best model animal for a given experiment. For example, if a rodent or cellular model recapitulates the human condition, then it is hard to justify a nonhuman primate model. Thus, failure to identify such a feature in other model systems may be a prerequisite for beginning to generate a nonhuman primate model. Currently, there are also practical considerations, as there are few marmoset colonies available for experimentation, limiting the number of conditions that can be modeled.

Nonhuman primates are not a panacea, since humans have capacities not found in marmosets or other nonhuman primates, and their brains differ in important ways. Key to understanding the extent to which marmosets can model human conditions is developing large-scale data sets that can be directly

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compared between species in typical and atypical brains. Such data sets would include whole-genome sequencing, epigenetic (e.g., ATAC-sequencing method), transcriptomic (e.g., single nuclei RNA sequencing and *in situ* hybridization), high-resolution structural and functional imaging, and deep phenotyping. Most of these assays will need to include the developmental axis. As an initial step in this direction, the marmoset ISH database provides an invaluable reference tool that helps translate knowledge from rodents to primates and advance primate molecular neurobiology research.

Summary

It remains unclear whether neuropsychiatric disorders are human-specific (necessitating hiPSC models), brain region-specific (necessitating animal models), or circuit- and behavior-specific (potentially necessitating nonhuman primate models). Until a clear consensus emerges driven by robust predictive validity in neuropsychiatric disorders, there is a role for all experimental models. We identified three advances that are required:

1. Reduced reliance on single strains of inbred animals.
2. Increased use of large mammalian models, especially the marmoset nonhuman primate model including single gene disorder models.
3. Systematic data generation in parallel across multiple models at multiple levels of analysis to provide clear data to judge which processes are conserved across models, which are not, and whether there are biomarkers or endpoints in common between models.

Requirements for Clinical Trials

What key advances are required, in terms of natural history, biomarkers, and clinical endpoints, to optimize the probability of success in clinical trials?

Natural History

Natural history studies define the natural course of a disease, providing critical insights into the needs of the patient population, potential therapeutic endpoints, and potential biomarkers. Obtaining this data requires the collection of standardized prospective longitudinal information, ideally across multiple developmental points in time. Since individuals affected with rare genetic disorders are likely to be geographically distributed, it is essential to have a platform to perform standardized assessments of such individuals across the distributed sites. Such a multisite platform requires tight standardization of neuropsychological assessments and biomarkers (e.g., EEG and MRI). Standardization of protocols is often achieved by using human controls or artificial "human

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phantoms," who travel from site to site at regular intervals (Prohl et al. 2019; Saby et al. 2021). Although such efforts are time- and labor-consuming, they are essential to obtain meaningful and reproducible data from multisite observational or interventional studies.

Biomarkers

Reliable, quantifiable, and translatable biomarkers of disease are needed to transform the investigation, diagnosis, and management of neuropsychiatric disorders. Multiple types of biomarkers have been defined by the BEST (Biomarkers, EndpointS, and other Tools) Resource from the FDA–NIH Biomarker Working Group (see Table 5.2). All of these are valuable (Califf 2018); however, response biomarkers that could assay target engagement of an intervention are especially important from the perspective of drug discovery (Parellada et al. 2023). Having reliable biomarkers and related mechanistic biological understanding is key for establishing target engagement and making "go/no-go" decisions, even in the absence of a clinical effect. With this framework, every failure informs the next study; a single success has the potential to transform the field due to the knowledge gained regarding therapeutic development.

The discovery of biomarkers that translate between species, including humans, would transform the utility of animal models. The utility of rodent behavior varies by phenotype, but measures of pain or seizures have predictive validity as preclinical endpoints in humans (Howe et al. 2018). Nonetheless, behavioral assays are complicated, and response biomarkers that correlate with these endpoints (e.g., neuronal excitability, EEG, or autonomic activation) could lead to more efficient comprehensive therapeutic screens. Other potential response biomarkers include myelination (as measured by MRI) or motor response (as measured by wearable technologies). Since demyelination is a hallmark of several neurological conditions (e.g., multiple sclerosis), validation of *in vivo* biomarkers of myelin is a particularly active area of investigation. A recent systematic review of published quantitative validation studies found that magnetization transfer and relaxometry-based measures showed the strongest correlations with myelin content (Mancini et al. 2020). Currently, however, there is no MRI-based measure of myelin that is true to histology, and more reproducibility studies are needed in the field. Likewise, noninvasive, wearable sensor technologies hold great potential for quantifying and continuously tracking real-world motor function, both in relation to disease progression and treatment response (Ganesalingam and Bowser 2010; Tyler et al. 2020). Greater collaboration across multiple disciplines (clinicians, bioengineers, data scientists, and software developers) from both academia and industry is needed, however, to fulfill their promise.

Developing response biomarkers that could provide reliable and valid measures of cognitive and behavioral phenotypes is of paramount importance for

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Table 5.2 Definitions of biomarker types, adapted from the FDA–NIH Biomarker Working Group (2016).

Susceptibility/risk	Biomarker that indicates one's potential for developing a disease/condition of interest.
Diagnostic	Biomarker used to detect or confirm the presence of a disease/condition or to identify individuals with a disease subtype.
Monitoring	Biomarker that is measured repeatedly to assess the status of a disease/condition or for evidence of exposure to (or effect of) an intervention.
Prognostic	Biomarker used to indicate the likelihood of a clinical event, disease recurrence, or progression in individuals with disease/condition of interest.
Predictive	Biomarker used to identify individual(s) more likely to experience a particular effect from exposure to an intervention.
Response	Biomarker used to show that a biological response (potentially beneficial or harmful) has occurred from exposure or intervention, with the following subcategories: • Pharmacodynamic: reflects the biological activity of an intervention without necessarily indicating efficacy or a particular mechanism of action; could be used to establish proof of concept, dose selection, or to measure treatment response. • Surrogate endpoint: can be used as an endpoint in clinical trials as a substitute for a direct measure of patient response; is expected to predict the clinical benefit or harm.
Safety	Biomarker measure before/after exposure to an intervention to indicate possible toxicity as an adverse effect.

neuropsychiatric and developmental disorders. To date, no response biomarkers have been validated in ASD (Parellada et al. 2022). The majority of studies are underpowered and few studies aim to systematically screen multiple biomarkers with robust statistical thresholds and replication. As challenging as it is to find such biomarkers, their importance merits the large-scale collaborative efforts that will be required to find them.

Importance of Developmental Timing

Interventions for phenylketonuria and amblyopia have identified critical periods for cognitive and visual development, respectively. Whether there are critical periods for the treatment of behavioral symptoms in neurodevelopmental or psychiatric disorders remains unknown; similarly, the extent to which cognitive impairments can be rescued later in development has not been determined. There are specific time periods in which the brain undergoes dramatic shifts in neural organization; specific circuits are likely differentially affected based on developmental timing. This suggests that focusing on time periods involving dramatic shifts in synaptic organization, as well as the early postnatal

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period (in rodents as well as nonhuman primate models), is essential for gaining insight into how specific mutations affect brain development. However, neurodevelopmental periods of the most rapid change (gliogenesis/synaptic organization) may not necessarily correspond directly to the optimal window for treatment (Guy et al. 2007; Koene et al. 2021; Milazzo et al. 2021; Rotaru et al. 2018; Silva-Santos et al. 2015; Tsai et al. 2018; Ure et al. 2016).

There may be points in development where correcting gene function no longer benefits the patient. Thus, pinpointing critical periods for therapeutic intervention for specific outcomes is essential: What is the recoverable fraction of the target outcome at a given time in neurodevelopment? Even for traits like binocular plasticity, there is a gradient of response (Wang et al. 2010). Some circuits are dynamic and continuously remodeled, such as adult mouse hippocampus (Attardo et al. 2015).

The best answers to these questions may come directly from attempting to treat these disorders in humans, as has been done in spinal muscular atrophy. Therefore, the ability of genetic therapies to pinpoint the extent to which neuropsychiatric symptoms can be modified across developmental stages and degrees of severity may inform the potential of future therapies aimed at idiopathic cases lacking a simple single gene target.

The Rocky Road to Treatment Success

There are many potential pitfalls on the path to therapeutic success (see Sahin, this volume). The cumulative impact of all these pitfalls leads to a low probability of success, even when the probability of making a correct decision at each stage is relatively high (Table 5.3). For example, if there were nine major decisions to be made and each had an 80% probability of being correct, the overall chance of success is only 13%.

In the face of these challenges, it is clear that we need to attempt more "shots on goal" to maximize chances of successful treatment; we also need to optimize decision making at each stage. More attempts should be made in the context of repurposed (i.e., safe) drugs, where prior experience may help increase the probabilities of success at several stages. Genetic therapies have the advantage of near certainty on the choice of "target," marginally increasing the overall chance of success as well as the confidence that even an unsuccessful trial has lessons to refine future trials. Accordingly, the sharing of data for negative outcomes is essential. A successful trial of even a single neurodevelopmental disorder would provide critical insights that could increase the probability of success at multiple stages of future trials. There is also an urgent need to improve efficiency in clinical trial design to reduce resource needs. A closer relationship with industry would be mutually beneficial, creating a precompetitive space for characterizing disorders in model systems and humans, and helping train the next generation of researchers for clinical translation.

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Table 5.3 Hypothetical probability of success at each stage of therapeutic development and overall.

Decision	Probability of Correct Decision				
Target	95%	90%	80%	70%	60%
Therapy	95%	90%	80%	70%	60%
Dose	95%	90%	80%	70%	60%
Duration	95%	90%	80%	70%	60%
Population	95%	90%	80%	70%	60%
Age range	95%	90%	80%	70%	60%
Sample size	95%	90%	80%	70%	60%
Endpoints	95%	90%	80%	70%	60%
Side effects	95%	90%	80%	70%	60%
Total:	63%	39%	13%	4%	1%

Implementation

How do we establish infrastructure and incentives to generate rigorous reproducible findings?

Better alignment of incentive structures between academia and industry is key (see Figure 5.3). There is a precompetitive need for understanding biology, and establishing training programs with a translational focus (e.g., T32 training grants to gain experience in clinical trial implementation) will benefit both. This approach should meet the synergistic goals of research findings with higher clinical impact, more successful therapies for industry, and better treatments for people who need them. In addition, as a global effort, implementation is key to the success of the field, as it is critical to increase

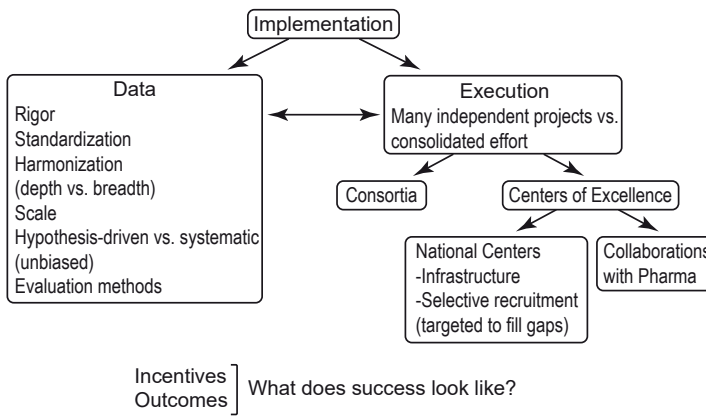

Figure 5.3 A model for implementation. Incentive structures between industry and academia must be better aligned to optimize chances of successful therapeutics.

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the representativeness and diversity of the workforce, as well as to ensure widespread access throughout the world to innovative therapies. Training programs that better integrate trainees and mentors from industry and academia are needed to establish greater synergy. For example, industry could provide funding for internships in academic centers with a translational focus.

Rigor and Reproducibility

Presently, across science, the novelty of experimental proposals and methodologies may be prioritized ahead of investigative rigor. In practice, this leads to a paucity of studies that are able to confirm or refine new findings, even in cases when this may be most warranted. In concept, this may continue to support the phenomenon that "new" is assumed to be equal to "true." Yet the penalties for potential errors that could arise under this mantra are nontrivial, and they could be especially costly for large-scale studies or consortia charged with creating resources to be used broadly across diverse scientific disciplines. Fortunately, there are standout examples where truth by replication and revision is appreciated (e.g., the Human Genome Project). Still, there remains a strong need for journals and funding bodies to incentivize programs for multiple labs to create, check, and replicate crucial data iteratively and collaboratively.

In a similar example from translational studies, a recent comprehensive review of potential biomarkers for evaluating ASD diagnosis and treatment found that no current candidate biomolecule has sufficient evidence to inform clinical decisions in trials related to symptom reduction (Parellada et al. 2022). This review highlighted a major problem in the field; namely that, of the nearly 1,000 candidate biomarkers evaluated, 80% were unique to a single publication. This points to the urgent need for greater standardization and collaboration to solve these challenges for achieving treatment success.

Large-scale examples of collaborations prioritizing experimental rigor and reliability that may provide a model for smaller-scaled studies include the brain structure and gene atlases created by the Allen Brain Institute (Hawrylycz et al. 2012; Li et al. 2018), and the PsychENCODE Consortium (2015) among others. These collaborations are notable for their operating plan which coordinated, organized, and enabled identical usage of the best technology possible in concert with the best labs possible. In these cases, data generation and validation occur through multiple streams, in parallel and recursively. This model also requires that data, code, and other resources are made public rapidly and from a single, easily available point of access. Since stringent quality control and data availability are intrinsic to the mission of these projects, their track record of creating products widely valued and credited with advancing the field may be a direct consequence.

Another collaborative framework that provides a model for other studies of practical and translatable scientific rigor is the Rare Diseases Clinical Research Network—an NIH-supported consortium of 20 research groups that are

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specified to include scientists, patients/community members, and clinicians, each focused on a group of rare disorders. Since such groups and the diseases of interest are extremely unique and heterogeneous by definition, care is taken by the network to assure that methods are validated in all cases and unified whenever possible. Furthermore, when singular findings are encountered that may hold great scientific interest but cannot meet statistical significance owing to rare diagnoses and current sample availability, oversight by the network assures that expanded studies have a higher likelihood of definitive results.

Studies within the network of nonhuman primate research centers may also exemplify policies, ideals, and incentive structures that could promote scientific reproducibility and increase overall productivity. In these settings, research ethics and relative resource limitations predetermine that experiments are planned and carefully coordinated within groups so that each part of the model system (the animal, its exposures, and/or behavior) is used optimally. This process is organized both within and between national primate research centers to assure that such models and methods are standardized and that integration with preclinical and clinical studies is consistent. Thoughtful translation of these principles to other experimental platforms can assure that rigor and reproducibility, as well as the speed, simplicity, and lower costs inherent in other models, all can be maximized.

An Experimental Model for Investigating Rare Variants

Across the five questions that we addressed in our discussions, we identified multiple opportunities to refine the analysis of rare variants and inform the study of neurodevelopmental and psychiatric disorders. Here, we combine these insights to propose an optimal experimental model to advance the field (Figure 5.4).

As we considered the prioritization of genes, we appreciated that the dual goals of neurobiological insight and therapeutic discovery share many synergies and lead to similar rankings of genes for investigation. In our discussion of the search for convergent biology, we observed utility from both large-scale systematic analysis of many genes as well as "micro-convergence" from in-depth systematic analyses of small numbers of genes. Regarding the utility and validation of models and recognizing our ignorance about how neurodevelopmental and psychiatric symptoms emerge, we saw value in both human cellular and animal models. We felt there was an urgent need to embrace nonhuman primate models, such as marmosets, in the hope that they might yield the necessary preclinical endpoints that are essential to demonstrate causal relationships and effective therapies. As we considered how to optimize the probability of success in clinical trials, we described the need to perform natural history studies, search for biomarkers, and define therapeutically actionable windows of development. Finally, to improve the rigor and reliability of experiments,

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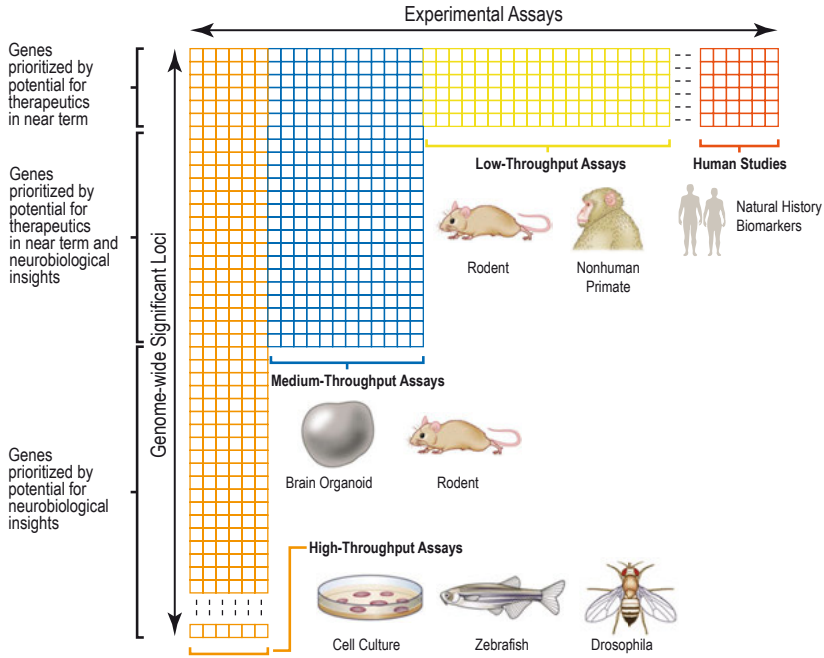

Figure 5.4 A proposed model for collaborative investigations of rare variants.

we stress the contributions that large-scale collaborative endeavors can contribute to help achieve this.

We envision our proposed framework as a large-scale collaborative endeavor, one that aims to generate systematic data across multiple levels of analysis. We start by prioritizing all genome-wide significant loci from rare variant discovery efforts according to the schema described earlier (see section, Prioritizing Genes and Loci for Neurobiological Investigation). We aim to define three groups:

- Group 1: a small number of single gene disorders with the greatest short-term therapeutic potential,
- Group 2: a medium number of genes prioritized for therapeutic and neurobiological potential, and
- Group 3: a large number of genes prioritized for neurobiological potential (Figure 5.4).

Next, we identify experimental assays to help understand these single gene disorders and split these into three groups:

- High-throughput assays that can be applied to groups 1, 2, and 3 for large-scale convergent analyses,

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- Medium-throughput assays to be applied to groups 1 and 2 for in-depth convergent analyses,
- Low-throughput assays and preclinical translational assays to be applied to group 1 for therapeutic development, biomarker discovery, assessment of predictive validity of model systems, and examination of micro-convergence across multiple levels of analysis.

It is important to note that this experimental design is modular. For example, if a technological advance enabled a medium-throughput assay to be deployed at higher throughput, or if an assay showed such clear potential in group 1 or 2 that it should be deployed in a larger group, it would be clear which genes remained to be assayed. Furthermore, the resulting data would still be systematic, allowing convergent analyses across multiple genes. Similarly, if a preclinical therapy was developed for a gene in group 2, there would be a clear roadmap of how to perform the additional group 1 assays for clinical translation. The experimental design also lends itself to generating comparable data across model systems and humans, allowing the model systems to be validated.

For low-throughput assays in group 1, human and nonhuman primate studies are essential. These activities would include natural history studies (standardized across multiple genes to allow comparisons) and assays across multiple brain regions and developmental stages in cellular and animal studies with a particular emphasis on nonhuman primates.

To utilize these data to assess convergent patterns, it is essential that we do not rely on shared controls. If all assays are compared to the same set of pooled controls, it is inevitable that similar patterns will be seen across the genes assayed, leading to false convergent signals. Therefore, substantial resources will need to be devoted to data generation in wildtype models and healthy human control subjects. This, however, will create a data set that provides critical insights into the typical distribution of values in the assay. It also offers a chance to create reference data across brain regions and developmental stages in typically developing animals and cells that can be compared directly with equivalent data from typically developing humans. Thus, the extensive control data are critical to both validating experimental model systems and systematically quantifying convergence patterns across the entire experimental framework.

Conclusions and Major Outcomes

At present, variants with large effect sizes represent the most tangible starting point for both investigating neurobiology and developing therapeutics. The dual quests for treating single gene disorders and understanding neurobiology are highly synergistic, as exemplified by the urgent need to identify reliable endpoints, biomarkers, natural history, and cross-species similarities. The development of a successful therapeutic for a single gene disorder (the

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best-case scenario) would galvanize the field to further understand neurobiology and enhance therapeutic development since it demonstrates that such a quest can yield tangible results and likely provide important lessons and caveats. A successful therapeutic could also serve as a tool for neurobiological inquiry, enabling interrogation of critical period windows and helping to exclude neurobiological processes that are not targeted by the therapy. Characterizing normal development in animal models and humans (e.g., via transcriptomics and neuroimaging) is vital to interpreting how rare variants impact development. We are now poised at a critical juncture, as a multitude of therapies for neurodevelopmental and neuropsychiatric disorders are currently under development. To realize these goals, we urgently need systematic and standardized data, analyzed in rigorous ways. This requires careful experimental design, well-powered analyses, researchers focused on rigor, and an emphasis on replication. We propose an optimal experimental design that matches gene prioritization to assay throughout to generate a data set that can be used to identify convergent patterns of neurobiology, assess the validity of model systems at different levels of analysis, and accelerate therapeutic development.

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