Turning Strains into Strengths for Understanding Psychiatric Disorders

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Abstract

There is a paucity in the development of new mechanistic insights and therapeutic approaches for treating psychiatric disease. One of the major challenges is reflected in the growing consensus that risk for these diseases is not determined by a single gene, but rather is polygenic, arising from the action and interaction of multiple genes. Canonically, experimental models in mice have been designed to ascertain the relative contribution of a single gene to a disease by systematic manipulation (e.g. mutation or deletion) of a known candidate gene. Because these studies have been largely carried out using inbred isogenic mouse strains, in which there is no (or very little) genetic diversity among subjects, it is difficult to identify unique allelic variants, gene modifiers, and epigenetic factors that strongly affect the nature and severity of these diseases. Here we review various methods that take advantage of existing genetic diversity or that increase genetic variance in mouse models to (1) strengthen conclusions of single gene function; (2) model diversity among human populations; and (3) dissect complex phenotypes that arise from the actions of multiple genes.

Keywords
strain differences; neuropsychiatric; isogenic; congenic; selective breeding; outbred; quantitative trait locus

1. Introduction

Psychiatric disorders account for approximately 13% of all disease in the world¹,². They are also a leading cause of death and disability³, and incur staggering economic costs, with global estimates for psychiatric illnesses (in 2010) reaching 2.5 trillion U.S. dollars⁴.

To better understand psychiatric conditions, a standard research method is to genetically alter the function or expression of a candidate gene in model organisms, such as transgenic mice. Testing whether the resulting phenotype in these mice mimics the symptomology of a
human condition is used to determine if a gene is ‘causative’ of disease. Results from single-gene studies like these have led to key insights into several fundamental mechanisms underlying basic brain function\(^5\) and the identification of genetic insults\(^6\) that correlate with the expression of traits consistent with psychiatric disorders.

However, experiments targeting single genes in animal models also have notable limitations. For example, psychiatric diseases are characterized by complex and multifaceted symptoms involving changes in cognition, motivation, and affect, which likely arise from the interaction of multiple different genes (e.g.\(^7\)). Thus, while a single-gene approach may establish the relative contribution of a single gene to a phenotype in question, it may not fully describe an entire disease state. In addition, many psychiatric disorders (e.g. autism or schizophrenia) are spectrum disorders, in which individuals diagnosed with the same disorder exhibit distinct attributes and impairments. While the different presentations may ultimately be linked to a common underlying mechanism, the final expression is likely modified by a combination of multiple genetic, epigenetic, or environmental factors. Importantly, because of interactions between different allelic variants and/or modifying genes, the genetic background of the mouse model being used, while often overlooked, can profoundly impact the effect of the gene under investigation. There are many examples where similar experiments conducted in transgenic mice harboring the same mutation on different genetic backgrounds have produced discordant results, such as differences in the severity or types of traits expressed (e.g.\(^8\)–\(^13\)). Finally, even when the effects of a mutation are solely related to the function of the gene (i.e. not influenced by genetic background), the resulting phenotype(s) produced in a homogenous population of inbred mice may be incongruent with the effects of the same mutation in humans, where genetics and environmental effects vary widely from person to person\(^14\)–\(^18\).

Rodent models have been used very successfully to address these challenges. The approaches we present here have been deployed in both rats and mice, each with their own advantages and disadvantages\(^19\). Due to space constraints, we primarily focus this review on mice, but also highlight one example using rats. We will start by presenting a broad overview of inbred isogenic mouse strains, and identify some of the strengths and weaknesses of their use in neuroscience research. We then provide descriptions of alternative strategies and their advantages, including leveraging multiple isogenic strains and generating hybrid or outbred mouse lines. Throughout the review, we highlight exemplar studies which have used these strategies to uncover neurobiological mechanisms underlying the expression of complex phenotypes.

2. **Using isogenic strains to establish single-gene function**

Isogenic strains are defined as a group of mice resulting from 20 or more generations of full sibling mating (i.e. inbreeding) originating from a single breeding pair. All members of an isogenic strain have less than 2% genetic variance, making each mouse a (near) clone of any other mouse in that strain. The first isogenic mouse strain, known as DBA (diluted brown, or non-agouti) was established by Clarence C. Little in 1909; today over 450 isogenic strains are available\(^20\),\(^21\). Initially, the use of isogenic mouse strains was largely promulgated by investigators studying tumor immunology and transplantation biology. These strains were
key to the discovery that genetic matching between donor and recipient determines the success of tumor and nonneoplastic tissue transplantation\textsuperscript{17}. Since then, the use of isogenic mouse strains has become widespread for the study of single-gene function. Theoretically, because every mouse within an isogenic strain is genetically identical, changes in a phenotype can be attributed directly to an engineered genetic alteration (insertion, deletion, or mutation). While this approach has vast utility and has resulted in a number of important mechanistic insights, the use of isogenic strains to elucidate single-gene function is not always appropriate or sufficient.

One obvious drawback to this approach is that it is generally only useful when the phenotype of interest is determined by the function of a single gene (termed “monogenic”). However, many psychiatric and neurological diseases exhibit complex and multi-faceted symptomology that likely arise from the action and interaction of multiple genes (termed “polygenic”). In support of this assertion, results from genome-wide association studies (GWAS) and genomic structural variation analyses have established that psychiatric disorders are best characterized as a collection of genetic alterations, including both rare and common variants (see\textsuperscript{22}). As a result, the single-gene approach may not fully describe the entire disease state.

Another issue arises from the fact that single isogenic strains cannot recapitulate the modifying effects that different genetic backgrounds have on the gene or genes responsible for a particular disease state. For example, about half the cases of Dravet syndrome (a severe form of early-onset epilepsy) are caused by mutations in a single gene, \textit{SCN1A}, that encodes a voltage-gated sodium channel, resulting in haploinsufficiency\textsuperscript{23}. However, in mouse models different phenotypes were observed depending on the background strain used; deleting a single copy of the \textit{Scn1a} gene (\textit{Scn1a}\textsuperscript{+/−}) on the 129S6/SvEvTac background resulted in no overt phenotype but the same mutation introduced on the C57BL/6J background resulted in spontaneous seizures and premature lethality, similar to the phenotype of human patients with Dravet syndrome\textsuperscript{24}.

While discordant results (as illustrated by the \textit{Scn1a} example above) may initially seem to confound interpretation of a single gene’s function, these strain-dependent phenotypic variations can be leveraged to reveal important biological mechanisms. For example, these variations may signal the presence of other genes that are important for modifying phenotype expression\textsuperscript{24,25}. While it is possible to make the same mutation in two independent isogenic lines, practical considerations - including the availability of appropriate embryonic stem (ES) cell lines and transgene insertion effects - make this approach impractical. Instead, it is more feasible to generate congenic mice to identify other genes contributing to phenotype expression. We review this approach in the next section.

3. Using congenic strains to reveal gene-modifier effects

A congenic strain is generated through a breeding strategy that introduces genetic material – for example, an engineered transgene – from one isogenic line (donor) into a different isogenic line (recipient). Donor mice (with the transgene) are crossed with wildtype recipient mice to generate F1 hybrid offspring, which will be heterozygous at all loci (having...
one allele from the donor strain and one allele from the recipient strain); thus each F1 mouse will have one copy of the transgene. Subsequent and successive crossing of generations of offspring (with the transgene) with wildtype mice from the recipient line (called backcrossing) will produce mice that have progressively more genetic material from the recipient line (more than 98% after 5 backcrossed generations), while maintaining the transgene of interest from the donor line (Figure 1 A).

Congenic strains are often used to determine whether an observed phenotype is solely dependent upon the gene of interest, or arises due to the interactions between the gene of interest and other genes in an isogenic background. These interactions are collectively referred to as ‘gene modifier effects’, and are manifested in several ways, including changes in the frequency that a phenotype is expressed among individuals, changes in the severity of a phenotype, loss of a phenotype, or even appearance of an entirely new phenotype. These changes are formally explained as changes in the penetrance, expressivity, dominance, and pleiotropy of a trait and are reviewed in Figure 2.

In fact, congenic mice were used to identify gene modifiers of Dravet syndrome (described above); the Scn1a gene deletion was originally carried out in the 129S6/SvEvTac strain and subsequently transferred to C57BL/6J by backcrossing. In addition to finding strain differences in lethality and seizures among Scn1a+/− mice, researchers also found differences in the physiological function of single neurons in the hippocampus. Altogether, these findings suggested that the C57BL/6J strain carried gene modifiers that altered disease outcomes. Subsequent gene-mapping studies and expression profiling of the 129S6/SvEvTac and C57BL/6J strains identified Gabra2, a gene involved in inhibitory neurotransmission, as a putative regulator of Dravet syndrome penetrance and expressivity.

Isogenic and congenic strains are ideal models for ‘reverse genetics’, or studies where changes in phenotype expression are assessed after the disruption of a gene’s function. However, their use is limited when gene candidates underlying a behavior or disease are unknown. In the following sections we discuss approaches for using distinct pre-existing phenotypes among mouse strains to reveal the underlying neurophysiology and genes that account for trait expression.

4. Using phenotype variation among distinct isogenic strains to reveal underlying neurobiological mechanisms

4a. Targeted neuroanatomical and neurophysiological characterization guided by pre-existing strain-dependent phenotypes

It is clear that distinct isogenic mouse strains exhibit remarkably different behavioral phenotypes. For example, individual strains differ in their spatial learning and memory capabilities, risk-taking, aggression, and stress-responsivity, many of which mimic disease characteristics (see Table 1). Strain-dependent differences in neurophysiology and neuroanatomy have also been identified. Linking these disparate behaviors to neurophysiological mechanisms is now possible with the advent of techniques (e.g. optogenetics; in vivo calcium imaging) that enable precise monitoring and/or...
perturbation of neural circuit function *in vivo*. Physiological differences between strains can then be used to target gene expression profiling to specific brain regions or cell types (Figure 1 B).

An example of an area which has benefited from leveraging innate differences among strains is the investigation and potential treatment of maladaptive fear and anxiety-related disorders. Distinct isogenic mouse strains display different susceptibility to maladaptive forms of fear, such as persistent fear, which remains elevated even in the absence of the threat, or generalized fear, in which related but non-threatful stimuli still produce a fear response. In humans, the development and expression of fear- or anxiety-related disorders is highly individualized, and is strongly influenced by many interacting factors including environmental variables (i.e. early-life experience or trauma type), and biological variables (i.e. sex, genetic makeup, or epigenetic mechanisms). Not surprisingly, estimates of the heritability of anxiety-related disorders varies widely. Thus, isogenic strains with distinct fear learning phenotypes not only serve as models of differential susceptibility (or resilience) to fear- and anxiety related disorders, but can also be used to elucidate how different factors interact to influence disease expression. To investigate maladaptive fear in the laboratory, researchers commonly use the Pavlovian fear learning paradigm. During fear conditioning (FC), rodents increasingly exhibit a conditioned fear response to a stimulus (like a tone) when it consistently predicts an aversive event (ie mild electric shock); after this association is learned, the stimulus (now called the conditioned stimulus [CS]) can elicit fear by itself. However, if the CS is repeatedly presented in the absence of the aversive event, rodents learn to gradually diminish their fear responses to CS presentations, a behavior known as fear extinction (FE).

Researchers have leveraged distinct FE learning phenotypes among strains to identify factors that predispose maladaptive fear behavior. For example, some strains like the DBA/2J, are able to learn FE rapidly, but others, such as the 129S1, exhibit profound deficits in FE, such as persistent fear similar to that observed in anxiety-related disorders. Work in this area has linked the predisposition to maladaptive fear in 129S1 mice to changes in regulation of the hypothalamic-pituitary-adrenal axis (HPA, a central regulator of the body’s response to stress) along with functional and neuroanatomical changes in cortico-amygdala circuitry. These results are aligned with work in rodents and humans, which suggests that stress responsivity mediated by release of cortisol under control of the HPA axis and glucocorticoid receptor (GR) signaling may represent a node of dysregulation or vulnerability. In fact, recent work comparing FE recall between strains discovered a novel gene—*Ppid* (Peptidylprolyl Isomerase D)—that can improve FE learning in a GR-dependent manner. Interestingly, *Ppid* belongs to the same family as FKBP5 of tetratricopeptide repeat proteins that influence stress signaling via GRs and are commonly reported as biomarkers for individuals who experience trauma or are diagnosed with post-traumatic stress disorder (PTSD).

The heterogeneity of symptoms reported by individuals, high level of comorbidities, and variable heritability has made identifying new therapeutic approaches difficult. Further, there is a high non-response or relapse rate to current pharmacological and behavioral treatments. Strain differences can be exploited to evaluate new pharmacological
behavioral\textsuperscript{61}, and genetic/epigenetic approaches\textsuperscript{28,59,60,62} to ameliorate maladaptive fear in mice. For example, we have developed a novel FE protocol, termed Novelty-Facilitated Extinction (NFE), that enhances FE learning. We found that exposure to a fear CS in daily novel environments ameliorates FE deficits in the 129S1 strain\textsuperscript{61}. Another form of NFE training, in which a novel auditory stimulus (instead of a novel context) is paired with a fear CS, is also more effective than standard FE in diminishing conditioned fear in rats\textsuperscript{63,64}. Importantly, NFE has been shown to be effective in human populations. Healthy (control) participants exhibited a diminished conditioned stress response (galvanic skin conductance) to a fear CS paired with a novel stimulus, while exposure therapy carried out in multiple context was more effective therapeutically in phobic patients\textsuperscript{64–67}.

Taken together, these examples provide a powerful illustration of how: (1) phenotype-driven characterization in rodents may engender greater translatability compared to studies focused on a candidate-gene; and (2) how phenotype diversity among strains can be leveraged to elucidate links between behavior, neurophysiology and genetics. These approaches are complementary to “big-data” projects that seek identification of biomarkers and environmental factors that make populations susceptible or resilient to psychiatric disorders (such as GWAS \textsuperscript{68} and other large consortium studies \textsuperscript{69}). While “big-data” projects identify important factors, “focal-data” projects using model organisms can reveal how individual differences (i.e. in genes, sex, environment) alter neurophysiological mechanisms which lead to susceptibility or resiliency (for example, by visualizing neural circuit activity during FE learning\textsuperscript{70}). In addition, “focal-data” projects can inform which combination/sequence of treatments may be most effective at altering specific physiology and phenotypes (see\textsuperscript{59,60,71}). However, it is important to note that one limitation of this approach is that it requires at least two mouse strains that natively exhibit significantly distinct phenotypes. In the absence of preexisting phenotypically divergent strains, investigators can often employ selective breeding to generate the desired phenotypic divergence; this is approach is described in the following section.

4b. Use of selective breeding to reveal underlying mechanisms associated with trait expression

Using selective breeding strategies to isolate behavioral phenotypes is another powerful method for studying the neurobiological substrates of complex traits. In this paradigm, offspring in each successive generation that display the most extreme measures of the behavior of interest are selected for inbreeding\textsuperscript{72}. Over many generations, this strategy produces two distinct lines that exhibit vastly different performance for a specific behavior, such as high/low treadmill/wheel running\textsuperscript{73–76}, high/low levels of exploration in novel environments\textsuperscript{77}, high/low alcohol sensitivity\textsuperscript{78,79}, high/low aggression\textsuperscript{80}, and high/low anxiety\textsuperscript{81}, among others (Figure 1 B).

One such example is the selectively-bred high responder (bHR) and low responder (bLR) rats that were differentiated based on their levels of exploration in novel environments. Interestingly, bHR rats also exhibit impulsive, aggressive, and reward-seeking behavior, while bLR rats exhibit anxiety- and depressive-like behaviors, suggesting that these traits may be genetically related to high or low propensity to explore novel environments\textsuperscript{77,82–84}.
In addition, it was discovered that bHR rats attend more to a stimulus predictive of a reward rather than the location of the reward delivery (termed “sign-tracking”), but that bLR rats attend more to the location of reward delivery (termed “goal-tracking”). In 2011, Flagel and colleagues cleverly utilized the bHR/bLR rats to explore the role of the neurotransmitter dopamine in stimulus reward learning. This study demonstrated that the bHR rats (sign trackers) had increased dopamine release in the nucleus accumbens in response to presentations of the predictive stimulus, whereas bLR did not show preferential dopamine signaling. These results suggest that a stimulus that is predictive of a reward gains greater incentive value for bHR (but not bLR) rats. Interestingly, sign and goal tracking behavior has been characterized in human populations in which sign-tracker individuals are more influenced by stimuli associated with reward and exhibit greater impulsivity.

In summary, native trait differences between isogenic mouse strains and/or selective breeding to generate differential phenotypes can be used to model maladaptive or pathological conditions resembling disease features in humans. These methods are powerful because they can facilitate the identification of genes and neurobiological mechanisms underlying complex phenotypes which are likely polygenic and thus impractical to study using single-gene models or reverse genetics. Further, this approach also facilitates the discovery of genetically related traits and the ability to define physiological properties associated with the studied behavior. However, it is important to note that these approaches are still limited in the amount of genetic diversity accounted for in an experiment, because the comparisons are made between few (typically two) isogenic rodent lines. In the next section we review outbred strains, wherein genetic diversity is maximized, and how the resulting genetic variance can be used to assess the effectiveness of therapeutics and robustness of candidate disease mechanisms.

5. Using the genetic diversity of outbred strains to establish disease mechanisms and potential therapeutics

Outbred strains, which avoid sibling-to-sibling mating, maximize phenotypic variation because each individual animal is genetically unique. The advantage of using outbred rodents is apparent when considered in the context of human clinical trials where genetic background heterogeneity is inherent. Obviously, testing therapeutic efficacy in only one subject (or even many clones of the same subject, which is analogous to isogenic replicates) would not effectively represent the range of potential outcomes and/or drug interactions that may arise in different individuals (with unique genetic backgrounds). Thus, in studies developing or testing potential therapeutic interventions, utilizing outbred rodents as test subjects can maximize the content validity of experimental results.

One strategy to generate an outbred strain is to use many (often 4–8, or more) inbred isogenic parental strains, mated in every pairwise combinations, to produce a variety of F1 hybrid mice; then a complex, rotational breeding scheme, which avoids inbreeding, is followed for 40 or more generations to result in genetically unique mosaic mice (Figure 1 C). In theory, outbred strains can be generated by individual laboratories; however, the cost and organizational effort associated with the number of breeders and unique mating crosses...
required to generate these lines makes this impractical for most investigators. A more feasible option is to obtain outbred rodents from commercial facilities, and then maintain the line by continuous non-sibling mating of a large number (20 or more) of mice. In either case, it is important to remember that all individuals in every outbred line are completely unique and cannot be reproduced at any future time; therefore, it is impossible to fully replicate any study using outbred lines. To maximize both genetic diversity and reproducibility, a number of groups have used specific breeding strategies (such as the four-way cross) to generate mice (or rats) that are genetically heterogeneous at the level of the individual animal while maintaining a fixed gene distribution across the population; these populations can be reproduced by starting with the same parental lines and following the same breeding scheme (Figure 1 C).

An example of the power of this type of approach is provided by the Interventions Testing Program (ITP), developed by the National Institutes on Aging, which aims to identify compounds that can extend lifespan and reduce multiple forms of late-life disease. Testing a candidate compound in a single isogenic strain could lead to spurious strain-specific conclusions, but testing in multiple isogenic strains would be prohibitive in terms of time, effort, and cost. Instead, the ITP employs a four-way cross breeding strategy. In this strategy, the F1 hybrid offspring from C57BL/6J × BALB/cJ matings are bred with the F1 hybrid offspring from C3H/HeJ × DBA/2J matings. This F1 × F1 breeding scheme produces the experimental strain (called UMHET3) in which all mice have 25% of their genetic material from each original strain, but with unique combination profiles. Thus, using UMHET3 mice to evaluate the efficacy of a proposed intervention lessens the chance of missing a truly effective agent because it failed to work in one single isogenic strain (false negative) and likewise reduces the possibility of identifying a compound that is only efficacious in a single strain and does not generalize to other strains or organisms (false positive).

One of the first compounds evaluated by the ITP (resveratrol) illustrates how the outbred strain strategy has lessened the emphasis on treatments that may only be beneficial in specific conditions or isogenic mouse strains. Resveratrol modulates levels of sirtuin proteins, which are thought to mediate anti-aging effects via several mechanisms, including maintaining DNA integrity and reducing oxidative stress (for review, see). Assessing the effect of resveratrol on lifespan in model organisms has produced conflicting conclusions. Some studies have reported beneficial effects on lifespan in nematodes and flies, but these results were not replicated in other studies. In C57BL/6Nia mice fed a high fat diet, resveratrol was reported to extend median lifespan, but failed to affect the lifespan of these mice fed normal (control) chow. The lack of an effect in the control mice was attributed to the late timepoint of treatment initiation (12 months of age). Studies using the UMHET3 mice also failed to find an extension of median or maximum lifespan (in either males or females at three different test sites), regardless of whether resveratrol treatment started at 12 months of age, or even earlier at 4 months of age. These results suggest that resveratrol-mediated upregulation of sirtuin function may help to ameliorate deleterious effects in obese mice, but diminishes the enthusiasm for further testing and development of resveratrol as a general anti-aging therapeutic.
On the other hand, treatments resulting in anti-aging effects in isogenic strains that also extend lifespan in UMHET3 mice greatly bolsters their significance because the efficacy of those interventions is more likely to be generalizable to other populations and organisms, including humans\textsuperscript{97}. To date, seven interventions have been successful in ITP studies, including rapamycin\textsuperscript{98,99} and acarbose\textsuperscript{100,101} (to see all compounds evaluated in the ITP, see https://www.nia.nih.gov/research/dab/interventions-testing-program-itp). The increased interest in these efficacious interventions is evidenced by ensuing studies (both within the ITP and from other researchers) that are evaluating different treatment regimens\textsuperscript{24,102}, investigating the diverse cellular pathways engaged\textsuperscript{103,104} and assessing myriad ancillary age-related phenotypes\textsuperscript{105,106}.

In summary, outbred strains maximize genetic diversity among test subjects and are ideally suited for making robust and generalizable conclusions about the effects of experimental perturbations or effectiveness of treatments. The strong experimental support provided by studies utilizing outbred strains is thus more likely to yield interventions that successfully translate into a therapeutic setting. The advances demonstrated by the ITP in the field of aging research suggest that other areas may also benefit from a similarly-designed program. For example, other government or private foundations could deploy this type of approach to study interventions that modulate diverse physiological phenotypes, such as valence, cognitive, social, and arousal systems\textsuperscript{107}. However, while outbred strains provide a powerful model system that more accurately reflects genetic diversity in human populations, they also have lower genetic tractability, making them less useful for identifying genetic contributions to neurological or psychiatric diseases. A breeding scheme that results in high genetic diversity and high genetic tractability is the recombinant inbred paradigm, which we review in the next section.

6. Using recombinant inbred strains for genome-phenotype association studies

Recombinant inbred (RI) mice are generated by crossing two distinct isogenic strains to produce F1 hybrids; then many individual pairs of F1 hybrids are mated, and each of these pairs become the founders of distinct RI lines. Each line is inbred for 20+ generations to produce distinct isogenic lines that are all each unique genetic mosaics of the parental strains (Figure 1 D). High genetic diversity is achieved because each RI line has a unique genetic mix, and high genetic tractability is achieved because the full sequence of each of the original parental lines is known (meaning each of the RI lines can also be nearly fully sequenced using identified markers through the genome). Therefore, an RI strain can be used to identify genetic sequence(s) that correlate with the expression of a quantitative phenotype (e.g. more or less resiliency to stress). The genetic variants that modulate phenotype expression are termed quantitative trait locus (QTL) (see Figure 1 D). The probability of identifying a QTL depends on the strength of the genetic contribution, which is reflected by the phenotypic variance among the RI lines. The resolution (and statistical power) of the QTL analysis depends on the amount of genetic diversity, which increases with the number of RI lines used\textsuperscript{108}. Further, the inheritance rate of the phenotype, the robustness of the phenotype and the pleiotropy among individual RI lines indicates the
extent to which the trait is mono vs polygenic. RI lines have been previously used to identify genes involved in addiction vulnerability\textsuperscript{109}, persistent maladaptive fear\textsuperscript{28}, hyperserotonemia (a biomarker of autism spectrum disorder\textsuperscript{110}), and resiliency to cognitive decline resulting from familial Alzheimer’s mutations\textsuperscript{111}, among others.

A commonly used RI strain is comprised of the BXD lines (from Jackson Laboratories), which are derived from multiple unique crosses of C57BL/6J and DBA/2J isogenic strains\textsuperscript{112,113}. Because the BXDs comprise more than 120 unique lines, they offer greater genetic diversity that better model genetic variance among human populations\textsuperscript{114,115}. They also possess high genetic tractability because they have been profiled for single-nucleotide polymorphisms (SNPs) at over \textasciitilde 470,000 locations (see \url{http://www.genenetwork.org}), making high-resolution gene mapping possible without full-genome sequencing\textsuperscript{113,116}.

In an intriguing study, BXD lines were used to identify specific gene sets underlying susceptibility to age-related cognitive deficits. Neuner and colleagues\textsuperscript{117} measured variance in cognitive aging by testing a cohort of middle-aged (15 months old) mice from 21 BXD lines on a hippocampal-dependent, contextual fear memory task. Utilizing the broad range of cognitive performance among these mouse lines, the investigators were able to demonstrate that genetic alterations in a small region (2.8 Mb) of chromosome 4 are highly correlated with differences in cognitive performance. Importantly, this region did not associate with cognitive performance in young-adult mice\textsuperscript{118}. Out of 10 gene candidates, the researchers first focused on \textit{Hp1bp3} (Heterochromatin Protein 1 Binding Protein 3) because they found an age-dependent correlation between hippocampal expression of \textit{Hp1bp3} and cognitive performance. The role of \textit{Hp1bp3} in cognition was further validated using (1) \textit{Hp1bp3} knock-out mice, which exhibited deficits in contextual fear memory\textsuperscript{117} and (2) by viral-mediated knock-down of \textit{Hp1bp3}, which resulted in behavioral and transcriptional changes consistent with advanced aging\textsuperscript{119}. Finally, the investigators discovered \textit{Hp1bp3} expression was correlated with cognitive performance among elderly adults when they examined human tissue samples collected post-mortem\textsuperscript{117}.

In summary, RI strains coupled with QTL analysis can be used to systematically exploit genetic complexity to identify underlying molecular mechanisms that determine complex trait expression. Importantly, studies using RI rodents can fill gaps where human research is impractical or even impossible. For example, the ability to identify disease “resiliency genes” is limited because asymptomatic individuals rarely enter the clinic for treatment and more typically serve as the control subjects for GWAS\textsuperscript{111}.

7. Combining approaches

The strategies presented above leverage both the genetic tractability of isogenic strains and the genetic (and trait) diversity between strains or in hybrid/obtbred mice. Importantly, while we present these techniques as separate approaches, their use for understanding complex phenotypes is by no means mutually exclusive. Below we provide an example where insights into the relationship between the neurotransmitter serotonin (5-HT) and autism spectrum disorder (ASD) were revealed through a series of studies that incorporated
phenotype comparisons between multiple isogenic strains, the use of RI lines, QTL analysis, and generation of congenic strains.

ASD is a male-predominant psychiatric condition characterized by repetitive behavior and deficits in social interaction and communication. An alteration in serotonin signaling is viewed as one of the primary candidates underlying disease expression; in fact, hyperserotonemia—or elevated 5-HT levels in the blood—is used as an effective biomarker for predicting ASD incidence\textsuperscript{120–122}. Human linkage studies also implicate the 17q chromosomal region, which contains the \textit{SLC6A4} gene encoding the serotonin transporter (SERT), in ASD\textsuperscript{123–125}.

To define the links between SERT gene variation and neurophysiological differences, multiple mouse strains harboring polymorphisms in the \textit{Slc6a4} were compared\textsuperscript{126}. These studies found that mouse strains segregated into two distinct haplotypes. The GK haplotype (Gly39/Lys152) exhibited reduced effectiveness for 5-HT uptake as compared to the ER haplotype (Glu39/Arg152)\textsuperscript{126,127}. To further distinguish the specific effects(s) of the haplotypes on complex traits from the effects of genetic background in these strains, researchers turned to a RI strategy. By correlating anatomical, biochemical and behavioral measures across many BXD RI lines (generated by crossing C57BL/6J mice that have the GK haplotype with DBA/2J mice that have the ER haplotype), specific phenotypes were identified that were correlated with either the GK or ER variation in SERT\textsuperscript{126}. The GK haplotype was found to be associated with altered expression of the dopamine transporter (\textit{Slc6a3}) in the caudate putamen as well as altered expression of the D2 (\textit{Drd2}) dopamine receptor in the ventral midbrain. The GK mice also exhibited reduced immobility in a tail-suspension test and lower ethanol consumption\textsuperscript{126}. Further, a QTL analysis was performed to test if hyperserotonemia (an ASD biomarker) was associated with SERT function in the BXD line. Interestingly, the same locus identified in humans, \textit{SLC6A4} (encoding SERT), was identified as a locus for whole-blood 5-HT levels in mice. Taken together, these results indicate that mice represent a faithful model in which to test the links between SERT polymorphisms and complex behavior\textsuperscript{128}.

One well established SERT polymorphism is the Ala56 variant, which in humans is most commonly associated with rigid-compulsive behavior and sensory aversion. Although SERT Ala56 was found to have strong transmission bias (2:1 affected to unaffected children), many individuals harboring the variant were devoid of any ASD symptoms, suggesting that epistatic factors can modify symptom expression\textsuperscript{123}. Thus, to distinguish the specific contribution of the Ala56 variant from gene modifier effects, researchers evaluated this mutation in congenic mouse lines. Knock-in mice for SERT Ala56 were originally generated in the 129S4/S6 background strain, and exhibited hyperserotonemia, altered social behavior, decreased ultrasonic vocalizations, and repetitive climbing/hanging behavior in the home cage\textsuperscript{129}. Subsequently, the Ala56 129S4/S6 knock-in mice were backcrossed to generate a C57BL/6 congenic strain. Across both lines, the tendency for Ala56 mice to withdraw from social encounters remained consistent; however, other traits such as ultrasonic vocalization, hyperserotonemia, 5-HT receptor hypersensitivity, and repetitive behavior were differentially altered\textsuperscript{130}. Taken together, these data suggest that alteration of SERT function associated...
with Ala56 primarily affects social behavior, while other traits are likely a result of more complex polygenic interactions\textsuperscript{130}.

8. Summary and Conclusions

There is an increasing consensus that factors altering risk, resiliency, and the expression of many psychiatric and neurological disorders is polygenic. For psychiatric disease, this consensus is supported by numerous GWAS (see\textsuperscript{131}) and is unsurprising considering that many psychiatric disorders, like schizophrenia, are characterized by complex alterations in both cognitive and affective behaviors. However, polygenicity is also an important factor to consider even for many neurological, ‘single-gene’ or ‘Mendelian disorders’ (such as Huntington’s Disease), where the severity of disease and pleiotropy can be altered by gene modifiers (see\textsuperscript{132}).

While animal models cannot fully recapitulate human disease, using the techniques outlined in this review makes them well suited to identify the relationship between specific gene variants and discrete traits (i.e. stress resilience, maladaptive avoidance, cognition). Since 2005, over 1,800 GWAS have been carried out to identify associations between genes and disease or trait expression\textsuperscript{133}. However, while GWAS can confidently link SNPs to a disease, they do not identify which genes relate to which specific traits within the disease. For example, thousands of SNPs have been linked to schizophrenia\textsuperscript{134}; each of these identified gene variants may be altering multiple molecular mechanisms, modulating multiple neural circuits and contributing to multiple behavioral traits.

The present challenge is to establish a context for how collections of SNPs coalesce into mechanisms and may explain discrete alteration in neural function and behavior. The approaches presented in this review, which focus on systematically leveraging genetic and trait heterogeneity in rodents, may be well suited to decipher the complex relationships between identified gene variants and phenotypes. These approaches are synergistic with genetic studies in humans and help unravel the complex relationships between genes, environments and behavior.

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Figure 1: Experimental strategies for leveraging genetic tractability and diversity to understand complex phenotypes.

While these strategies can be used to study complex behavior, the physical trait of a curly tail has been used for this illustration. (A) **Congenic Strains** are used to test for the presence of gene modifiers on a phenotype of interest by determining if the phenotype is maintained in both the donor and recipient strain. (B) **Phenotype variation among strains.** Targeted characterization by strain differences (top) can be used to link physiological differences among strains to differential phenotypes. When differences between stains are not natively present, selective breeding (bottom) can be employed to generate quantitative divergence for a trait. (C) **Outbred strains** represent an important technique for testing hypotheses that rely on genetic heterogeneity, such as validating the generalizability of experimental treatments. Distinct outbred breeding strategies yield differences in the amount of genetic diversity and reproducibility. For example, a fully outbred scheme has higher diversity, but lower reproducibility, while a four-way cross has lower diversity, but higher reproducibility. Note
that the more unique parental (P1) strains used, the more genetic diversity is generated (D) **Recombinant inbred strains** have the highest amount of genetic diversity while preserving genetic tractability. Typically, they are used for *quantitative trait locus analysis* (QTL) which is a technique that matches variation in gene expression to variation in a quantitative trait or phenotype.
**Figure 2: Effects of modifier genes.**

Modifier genes are those that affect the level of expression of other genes. The existence of gene modifier effects is evidenced by changes in the dominance, expressivity, penetrance and pleiotropy of a phenotype when a transgene is expressed in distinct background strains. In this example, homozygous expression of the hypothetical “a” mutation in Gene X causes a curly tail phenotype in mouse Strain 1. The “a” mutation is assessed among 4 additional strains (in columns) for wildtype (mutation null), heterozygous and mutation “a” homozygous populations (in rows). Some gene modifiers will change the **dominance** (cyan box) of a trait, a measure of the allele dosage needed to cause the curly tail phenotype. For example, in Strains 1–4, a single allele containing the “a” mutation is not sufficient to result in the curly tail phenotype, but in Strain 5 it is. Changes in **expressivity** (green box), or quantitative differences in the trait, can also be evident: while mice in Strain 2 exhibit a curly tail, there are fewer curls per length. **Penetrance** (blue box) refers to the proportion of mice that carry the allele (e.g. mutation “a”) that also display the curly tail phenotype; in Strain 3, only 2 out of 3 mutant homozygous mice exhibit a curly tail. **Pleiotropy** (red box) or the number of phenotypes generated by an allele is also indicative of gene modifiers. In Strain 4, mutant homozygous mice exhibit the curly tail phenotype, but in addition have a change in coat color that is caused by interactions between gene modifier(s) and mutation “a”.
### Table 1.

Example references for strain-dependent effects and features

| Strain Dependent Phenotypes: | Citations |
|-----------------------------|-----------|
| learning and memory         | Colom-Lapetina et al., 2017; Graybeal et al., 2014; Manahan-Vaughan and Schwegler, 2011; Neuner et al., 2016; Turner et al., 2017; Whitehouse et al., 2017 |
| aggression                   | Kessler et al., 1977; Takahashi et al., 2015 |
| fear and anxiety-like behavior | Gunduz-Cinar et al., 2018; Keum et al., 2016 |
| compulsive behavior          | Mitra et al., 2017 |
| locomotor activity           | Crawley et al., 1997; Podhorna and Brown, 2002 |
| parental behavior            | Carola et al., 2006; Chourbaji et al., 2011 |
| vision                       | Mattapallil et al., 2012; Mehalow et al., 2003 |
| hearing                      | Turner et al., 2005; Zheng et al., 1999 |
| responses to pharmaceuticals and substances of abuse | Crabbe et al., 2016; Dockstader and van der Kooy, 2001; Holtz et al., 2015; Mulligan et al., 2008; Surget et al., 2016 |

non-exhaustive list