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Animal Models in Psychiatric Disease: A Circuit-Search Approach

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Abstract

Our collective desire to understand how the "normal" vs. "diseased" brain works drives our ongoing need for nonhuman animal research. Our current understanding of circuits within the brain, and the techniques required to investigate neural activity, stem from animal work. These techniques often require invasive methods, which necessitate animal models. Unfortunately, this means that our investigative approaches are subject to the same limitations that animal models had before these new techniques were developed. In this article I briefly overview these limitations, then outline a relatively new strategy that enables us to establish a causal relationship between a specific neurocircuit abnormality and disease. This approach utilizes novel techniques designed to selectively target mutations to specific brain circuits in the mouse. Such a strategy allows the researcher to "home in" on how a gene affects a single brain circuit. This is powerful because it avoids an often-cited problem that plagues traditional animal models: non-targeted mutations disrupt a myriad of circuits. Rather than mutating all brain cells, targeting a gene known to be highly penetrant for human disease to an individual, relatively conserved, circuit element helps us determine whether that circuit is involved in generating an abnormal behavioral phenotype. This will provide invaluable clues about where and how psychiatric disease originates in humans. Finally, I briefly discuss how computational neuroscience-based techniques and noninvasive, low-risk neuromodulation techniques could be employed to test hypotheses generated by these animal models in humans, leading to both greater understanding of neurocircuits underlying psychiatric disease and possibly new treatments.

Keywords

Animal model; Circuits; Psychiatric disease; Autism; Treatment; Electrophysiology; Mutant; Genetics; Targeting; Biomarker identification; Computational

Introduction

As long as animal models of disease have existed they have been controversial. The arguments are nuanced, but broad themes stand out. One theme involves the fact that animal models do not have the forebrain elaboration found in humans, rendering them incapable of

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fully mirroring the behavioral manifestations of human neuropsychiatric disease. The resulting argument is that behavioral readouts of interventions or explorations of underlying neurocircuit changes must be interpreted with great care. Another theme involves the ability to identify a causal circuit: when a mutation exists in every cell of the animal, as is the case in many animal models, how can one identify *where* in the brain the mutation's effects are most damaging? The overriding questions raised by studies of the traditional animal models are thus:

1. Given the differences between the brains of animals and humans, how can we possibly relate behavioral abnormalities in mutants to human psychiatric disease?
2. Even if we assume that the animal model and human with the disorder both have similar circuit abnormalities, how can we possibly find the relevant circuit(s)?

Here, I will briefly discuss some of the justifications and limitations of our continued reliance on animals as a way to gain insight into human psychopathology. I will then examine how researchers are using recently developed techniques to strengthen our ability to connect animal findings to human diseases. Finally, I discuss an experiment design template that, if widely employed, has the potential to systematically apply a combination of these approaches to identify treatment targets, and human protocols to test treatments of these targets, for any psychiatric disease with identifiable genetic underpinnings. Throughout the reader will find examples that illustrate points made in the text.

Limitations of Human Research

Current investigations of neuropsychiatric disease in humans are significantly limited by available techniques. fMRI data have yielded glimpses of putative physiologic differences between psychiatric patients and controls[1–3], but the spatiotemporal resolution of fMRI is low. Even the best post-processing techniques cannot avoid the fact that a single MRI voxel represents the activity and structure of many circuits. Volumetric MRI studies are similarly limited, as are newer techniques based on diffusion tensor imaging (DTI). Functional connectivity analysis and resting state studies are also necessarily limited spatially, and although connectivity between structures can be analyzed, specific pathways within the myriad of neurons that project via white matter structures cannot be satisfyingly isolated. Studies utilizing EEG and MEG, both low-spatial resolution methods, have better temporal resolution but ultimately yield similarly nonspecific findings in that they measure the summed activity of many neurons, which are part of many circuits. In comparison with our capabilities in animals, our ability to meaningfully interrogate human tissue remains in relative infancy. We are far from the levels of resolution we will need to meaningfully understand how the brain works, let alone what brain activity is dysfunctional in pathological states; thus, animal research is currently needed to understand disease at the level of the circuit.

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Limitations of Animal Models

The recent development and implementation of powerful new experimental techniques including DREADDs[4], CLARITY[5], optogenetics[6,7], and many others has finally opened the door to circuit-level investigations of psychiatric disease in the animal model. These methods, respectively, enable the researcher to chemically modify neuronal activity, visualize long-range projections in intact brains, and control neuronal activity with light. With these tools, we have powerful new ways to probe specific circuit elements, and to view pathways in a fully intact mouse (or, increasingly, rat) brain, generating highly specific data. Unfortunately, these investigative methods still do not solve some fundamental issues with the animal models they are used in. When optogenetics, DREADDs, CLARITY, and the myriad of other novel anatomical and physiological techniques are used to study traditional animal models of disease, in which a genes of interest are knocked out or mutated everywhere in an animal, we run the risk of generating hard-won data that are difficult or impossible to interpret meaningfully.

The chief concern here is that in a traditional knockout animal, all cells in a circuit are modified by mutations, resulting in a great many physiological changes that may or may not be related to a behavioral phenotype. See Example 1, below. A mutation in every cell of the brain yields a complex set of changes that are nearly impossible for the neurobiologist to implicate as causal for phenotypic changes in an animal model. For this reason, even the best studies to date on networks, circuits, synapses, and receptors using anatomy, traditional electrophysiology, calcium imaging, voltage sensitive dye imaging, CLARITY, DREADDs, and optogenetics on traditional animal models of neuropsychiatric disease are subject to caution in their interpretation. These data will ultimately prove to be of significant value, as being able to link a mutation in a specific neuroanatomical pathway to pathology will provide a much-needed frame in which to think about these already accumulated findings.

One way to address the aforementioned problem would be to study an animal in which individual pathways are selectively mutated, whether by point mutation, more extensive alteration, knockout or change in expression, and the resultant neurophysiologic and anatomic changes studied. Such an approach is possible, and is gaining traction (see Example boxes).

Another shortcoming of animal models is that a disease affects humans and other species differently. In fact, >20% of the genes that are "essential" (necessary for an organism to achieve reproductive fitness) in humans can be knocked out in the mouse without such a drastic effect[8]. For example, an autosomal dominant mutation of transforming growth factor-β interacting factor (TGIF1) in the human causes holoprosencephaly (failure of the forebrain of the embryo to develop into two hemispheres), which leads to severely perturbed brain development and often very early death. Strikingly, the same mutation in mice does not appear to affect growth, behavior, or fertility[8]. Such a drastic difference in phenotype for the same gene - one that is essential to survival in one organism but has almost no effect in the other - suggests that when we interpret animal data we need to proceed with extreme caution.

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The Potential of Animal Models

While the degree of cortical elaboration in humans is much greater than in the mouse and other animal models, some basic circuit elements of the mouse brain can still serve as useful models of homologous human circuits. The high degree to which some structures' neurocircuitry is conserved across species[9,10] suggests that fundamental changes to a circuit may also be largely, and meaningfully, conserved across species for a given mutation that affects neuronal development. Thus, while humans may have many more circuit elements, the basic neuron subtypes and connections among them in several brain areas are largely similar to those in mice. Any measured changes that result from mutations in such highly conserved circuits in the mouse are likely to be similar to the anatomical and physiological effects that said mutations would have in human cells (and small networks of cells). Drawing from this principle of evolutionary conservation, animal research has proven to be of incalculable value in understanding basic neurophysiology and developing new pharmaceuticals.

In their 2010 review, Nestler and Hyman[11] provide an illuminating discussion. They suggest that the way one views the utility of an animal model is of significant importance, and that developing discrete goals - e.g., neurobiological hypotheses about the disruptions mutations cause in specific pathways and subsequently investigating them - would be superior to a less targeted approach. They also point out that while the phenotypes of animal models are difficult to connect to disease in humans, studying neurocircuitry that is critical to development of a behavioral phenotype (in a mouse model) may nonetheless prove to be fruitful. In the next section I will outline a strategy that attempts to achieve what Nestler and Hyman proposed.

A series of recently developed techniques have increasingly enabled targeting genes of interest to specific brain structures at different times of development[12,13]. These strategies utilize a variety of techniques, ranging from Cre-recombinase-based methodologies to Clustered Regularly Interspaced Short Palindromic Repeat – CRISPR-associated protein (CRISPR-Cas) based strategies, that are enabling us to target genetic mutations to specific cells with increasing fidelity and specificity[14–17]. Beyond mere deletions, CRISPR-cas techniques enable cell-type specific genetic changes including protein epitope tags, point mutations, and alteration of promotion/enhancer activities[16,17]. In one such paradigm, mice are genetically modified to include regions of DNA that can be "snipped" out when tamoxifen is present. Animals are injected with tamoxifen at a certain phase of embryonic development to induce a mutation in cells after a certain point in development. Thus, if the researcher times it right, injecting tamoxifen can essentially "turn on" the mutation at a time that corresponds to the period of development of interest for individual pathways[18–21]. Researchers use this to temporally limit the mutation's effect on cells (to time periods after tamoxifen injection), but the strategy can now be further enhanced by also targeting specific cell types.

While a few animal models and circuits have been analyzed using these techniques, many animal models of disease, and targets within those models, remain unexplored. Systematically building a library of these changes has the potential to yield individual

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critical circuits that must be disrupted to generate disease. Taking this one step further, one might choose to knock out genes during the critical developmental periods for not just one but for two or more circuits in an attempt to discern whether multiple hits versus single hits of one or more circuits are necessary to produce a behavioral phenotype. Such an approach may also help elucidate whether different circuit changes (via distinct mutations or pathways) can yield behaviorally similar phenotypes. This approach has a greater potential to implicate specific neuroanatomical pathways in disease than trying to sift through a brain that has been "carpet-bombed" by a global mutation, and thus this approach could serve as a critical basis for understanding neuropsychiatric diseases and their variants. Once abnormalities are identified, it should become possible to select drugs or neuromodulatory treatments that target these circuits. Determining which circuits are critical to the development of pathology, and how they are changed, may also lead to entirely different approaches to treatment.

One treatment approach might involve using computational modeling to simulate the effects that the mutation has on a critical mouse circuit, putting the effects into a model of human circuitry, and then applying virtual neuromodulatory algorithms in an attempt to "treat" the effects that the mutation has. Such an approach is undoubtedly a moonshot but might also be a way to more systematically approach protocols for transcranical current stimulation (TMS), focused ultrasound, deep brain stimulation (DBS), and other neuromodulatory techniques. Data collected in selective mutant mice also yield insight into what these circuits *normally* do, by determining what happens when normal function is disrupted.

Operationalization of Mutation Strategies

While a full description of available methods to target genetic modifications to individual neurons or circuits is beyond the scope of this paper, I will briefly discuss the capabilities of a few select strategies here.

Homologous Recombination in embryonic stem cells

To produce what many researchers now might refer to as "classic" mouse models of human disease researchers use homologous recombination[22]. In this method researchers first isolate and culture embryonic stem cells, which colonize all of a host organism's tissues, and introduce mutations by "targeting" a new genetic sequence. This targeting is done by using known, cloned, sequences of a gene, modifying a portion of the gene, then introducing it to the embryonic stem cells with a vector. This strategy can be used to create an organism with a genetic knockout in any cloned gene. Refinements of this strategy have led to the ability to introduce not just knockouts, but also modifications of genes, point-mutations for example, that yield more subtle changes in the organism[23].

Inducible Cre

One issue with a genetic knockout is that it can be lethal if mutated during embryogenesis. Since genes are expressed to various extents in different tissues across development knocking out a gene in all tissues before development begins may lead to a critical system failure. If, however, one were to knock a gene out during a phase of development that occurs

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after the gene is expressed where and when it needs to be for an organism to survive, one might begin to home in on that gene's function with greater and greater degrees of specificity as the organism develops. Another situation that could be mitigated by this strategy would be to induce a mutation at various timepoints in development to track any homeostatic mechanisms that might be compensating for induced mutations.

Temporal control over a genetic mutation is enabled by an inducible Cre strategy. In this paradigm, the gene of interest is first flanked (or "floxed") with sites that can be recognized by recombinase (*loxP* sites). These sites are introduced by homologous recombination (see previous section). One can then use an inducible promoter to express a Cre recombinase transgene. This inducible promoter can vary, and agents used to engage them can have their own drawbacks, but the end result is the introduction of a mutation that is only activated when a researcher injects a chemical[24]. This strategy is useful to control when a mutation occurs during development, and to a certain extent where (ie, if a gene is known to be expressed in certain tissues only at certain points in development, the mutation could be timed to target individual circuits). This strategy can, however, be combined with a cell-specific targeting technique to achieve even greater circuit specificity.

Targeted mutation examples (Cre)

While temporally inducible Cre technologies outlined in the previous section can partially target a gene to an area of the brain using timing of a mutation, other conditional Cre-based strategies can target mutations to specific cell types. In this paradigm Cre is expressed in only certain cells in one mouse, which is mated to a mouse that carries a target gene flanked by two *loxP* sites[25]. This leads to a mutation in the offspring in only cells expressing Cre. Other cells, without Cre gene expression, will be wild-type. Cell-type specific targeting is achieved by putting Cre gene transcription under the control of a promoter of a cell-type specific protein[26]. This strategy is becoming more powerful as we accumulate evidence of various cell types and knowledge of protein expression patterns that make them unique.

Examples of the many identified populations of neurons expressing specific proteins include inhibitory interneurons expressing somatostatin, parvalbumin, and 5HT3aR cells[27], as well as thalamocortical relay cells, which express Gbx2[21]. While agreement on subpopulations of cells and their respective molecular markers in the central nervous system has been elusive in some cases, broad classes of cells have been conclusively defined by virtue of their anatomical and physiological properties and more systematic efforts to develop a cell-type taxonomy have been proposed[28]. A concerted effort to identify and classify neuronal populations will open the door for the Constitutive Cre-based approaches described above to determine what effects genetic modifications have on various neuronal populations. Advances in Cre manipulations are also now enabling efficient cell-type specific targeting of proteins that enable cellular control (optogenetic proteins) and calcium, voltage or glutamate indicators that enable researchers to more readily analyze cellular physiology and perturb cellular function in specific circuit elements[12].

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Rabies Virus-based vectors

While the aforementioned Cre-based techniques enable the researcher to target subtypes of neurons, and provides some control over when during development a mutation occurs, neither technique is able to geographically restrict mutations. Fortunately, restriction to certain cell-types in a specific brain area is now possible with rabies virus-based vectors[29]. In this paradigm, rabies-based viral vectors are injected into a target area. Since rabies is RNA-based it does not lead to mutagenesis in the host, but rather expression of the protein of interest in a cell. While this method enables the researcher to perform multiple tasks, including insert optogenetic capable receptors and fluorescent proteins into local cells, the primary focus here is its ability to carry Cre into local cells. This allows the researcher to genetically engineer a cell-type specific Cre-dependent mutation, and then activate that mutation for cells of that type *only where the rabies virus is injected*. This enables cell-type specific targeting of a mutation to only a specific area of the brain, with spatial resolution down to the level of an individual neuron.

CRISPR-Cas Strategies

While genome editing tools have been around for some time, the emergence of CRISPR-Cas has enabled precise, efficient editing of the genome[14]. As suggested above, CRISPR-Cas takes our capabilities well beyond knocking out a gene, allowing us to manipulate any segment of DNA we choose to. This tool not only allows cell-type specific gene editing, but also enables the study of multigenic disorders. For instance, a large number of mutations with relatively low penetrance for neuropsychiatric diseases like autism spectrum disorder and schizophrenia have been discovered, and this strategy would allow the researcher to generate multiple hit animals.

Briefly, CRISPR is a technique that utilizes a portion of bacterial DNA that is responsible for destroying DNA-based viruses, protecting bacteria from infections[30]. These pieces of DNA contain palindromic repeats with interspersed segments that correspond to viral DNA of previous infections. Essentially, this allows bacteria to recognize a virus, cut open its DNA, and make a change in the DNA where it was cut, killing the virus. This system has been adopted by researchers to recognize any DNA sequence, enabling specific targeting and insertion of new DNA[14,31]. One could envisage an experiment in which CRISPR is used to introduce sequentially greater ‘hits’ in the form of mutations associated with a given disease in a series of mouse litters. Phenotypes could then be monitored for the emergence of symptoms, and these mutations could then be targeted to individual cell populations using Cre-based strategies outlined above.

Proposed Research Template

I propose an approach to researching psychiatric disease in which a gene with high penetrance for human disease is knocked out in multiple brain regions in mice (with targets informed by current knowledge of neurocircuitry and findings in the human literature), and the mice are assessed for behavioral abnormalities. This targeting can be achieved by using any appropriate combination of the aforementioned techniques (and other newly developed techniques) that would achieve the desired circuit specificity. The strategy will vary

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significantly depending on the circuit being targeted, but as the library of cell-type specific markers grows so too will our ability to target individual cell types. After identifying electrophysiological abnormalities, computational models of the neurocircuits are used to develop "rescue" strategies. If compensating for circuit abnormalities (i.e., inhibiting an hyperexcitable circuit or exciting a hypoactive circuit) rescues a "normal" behavioral phenotype, a causal relationship could be determined.

The identified electrophysiological abnormalities that lead to behavioral abnormalities in animals are then fed into computational models that extrapolate to human circuit structures, computationally modeling how the circuit disruption would most likely "look" in a human brain area. Finally, these models are used to develop clinical trials to both test the hypothesis that a critical circuit is responsible for pathology as well as determine treatment efficacy.

Discussion/Conclusion

In this article, I have briefly reviewed key limitations to our collective ability to discern neural activity in the human brain and the constraints of animal models. I then reviewed a sampling of powerful techniques that can be used in animals that maximize our chances of finding abnormalities that can be used to better understand human disease. Throughout, I described an example of the application of tools aimed at isolating the changes that mutations induce in individual projections by selectively knocking out genes during critical periods of development in targeted neurocircuits. If broadly applied, such an approach in the nonhuman animal has the potential to determine whether individual pathways might be causal elements of human pathology. Looking ahead, such data may provide clues about where one might look in humans as techniques to do so are developed, and also have the potential to provide a shortcut to new treatments and even prevention of human disease.

A limitation of this approach is that it requires that the animal exhibit some sort of abnormal behavioral phenotype. If mutations in suspected circuits did not yield behavioral phenotypes in animal models, targets in the mutated area could be systematically expanded, structure-by-structure, until phenotypes emerged. Alternatively, if a pathway were compellingly linked to pathology in humans, and highly conserved in the animal, one could target the circuit and investigate the circuit under the assumption that something similar might be happening in humans.

This "circuit-search" approach might be useful in guiding low-risk neuromodulatory therapy trials, interpreting DTI-based tractography studies in human neuropsychiatric disease, and demonstrating fine-scale anatomical and physiological changes that might account for detected abnormalities. Furthermore, systematically altering genes and their expressionin individual brain structures and studying the resultant electrophysiological changes has the potential to yield individualized treatments, new ways of targeting current treatment modalities (TMS, DBS, etc.), and novel treatments of human neuropsychiatric disease.

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Example 1:Animal models in which the tuberous sclerosis gene TSC1 is knocked out everywhere in the mouse brain have yielded behavioral phenotypes and abnormalities in the way that neurons in the brain communicate with one another. While it is interesting and useful to identify the abnormalities in brain communication, it is impossible to determine which abnormalities are the ones that generate the behavioral abnormalities that we would like to treat as a result of our research. Fortunately, genetic and molecular advances have given researchers a solution to this problem, as will be discussed below.

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Example 2:Normand, et al. 2013 were able use an inducible Cre-Lox strategy to target a mutation to thalamic relay cells. By targeting CreER (which turns on Cre expression when tamoxifen is present) to cells that express Gbx2 (mostly in thalamus), Normand et al. (2013) were able to achieve both spatial and temporal control of excision of the TSC1 gene. This approach essentially targets Cre-Lox, which enables one to carry out deletions or insertions of genes, to specific cells, and “turns on” the mutation at a specific point in development by coupling Cre expression to the presence of tamoxifen, which is injected by the researcher. This has the potential to provide data about when disease-relevant structural and functional changes occur for a given mutation. This mutation causes mice to engage in repetitive grooming and seize -- behaviors linked to the mutation’s effects on this particular pathway at this stage of development. This approach allows for, as best we can with current technology, the relative isolation of changes that a mutation induces at the level of an individual circuit during a certain stage of embryonic development. These circuits can then be analyzed using the powerful aforementioned techniques, with knowledge that *something* about the way this particular pathway differs from its corollary in control animals is yielding a behavioral phenotype. This targeted approach makes the “haystack” dramatically smaller as we search for the needles.