Finding Genes for Bipolar Disorder in the Functional Genomics Era: From Convergent Functional Genomics to Phenomics and Back

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ABSTRACT

Psychiatric genetics, while promising to unravel the mechanisms of psychiatric disorders, has proven to be a challenging field. Psychiatric disorders, like other common genetic traits, are complex and heterogeneous. Psychiatric genetics has also suffered from a lack of quantifiable, biology-based phenotypes. However, the field is currently at an opportune moment. The work of various investigators is on the verge of paying rich dividends. Efforts at positional cloning are being greatly accelerated by the fruits of the Human Genome Project. New tools of functional genomics, such as expression profiling and proteomics, are being applied to animal models. These two methods can complement each other in an approach we have termed convergent functional genomics. Lastly, improvements in the measurement of biologically distinct endophenotypes—or phenomics—will lead to a better understanding of the mapping of genes to phenotypes in both animal and human systems.

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INTRODUCTION

The importance of the genetic contribution to the etiology of bipolar disorder and schizophrenia (SZ), the major psychoses, is evident from numerous genetic epidemiological studies.[1] However, the mode of transmission is likely complex and non-Mendelian. Over the past decade, numerous linkage and association studies have been conducted to identify the susceptibility genes. These studies, that have been reviewed in detail elsewhere,[2-5] have suffered from inconsistent replication. However, as shown in Table 1, a series of genomic loci have emerged as being repeatedly implicated in illness. One of the surprising results is that many of these genomic loci overlap between studies of bipolar disorder and SZ. These recent results have fueled the century-old debate regarding the relationship between these syndromes that began with clinical observation in Kraeplin's time.

Though it is clear that the genetic transmission of bipolar disorder is non-Mendelian, the identification of linked genomic regions shows that some genes are of a large-enough effect size that they can be detected with available samples. Nevertheless, the identified linkage peaks tend to be quite extended, spanning tens of megabases and hundreds of genes. In fact, such a result has been predicted as a theoretical result of the non-Mendelian properties of complex disorders.[6] This presents a formidable problem when attempting to identify the exact gene within the linkage peak.

TOOLS FOR POSITIONAL CLONING

Fortunately, the Human Genome Project has provided powerful tools for these problems. The draft sequence of the human genome was published in February 2001.[7,8] These 3 billion base pairs were found to contain far fewer genes than originally suspected—approximately 35,000 instead of 100,000. However, of these 35,000 genes, the function was known for only about 7,000. Though the process of identifying all the genes is far from complete, it is now possible to know most genes within any genomic region. Hence, the positional candidates within a linkage peak can now be looked up instead of painstakingly cloned. Moreover, an extensive catalog of all known sequence variation is being constructed. Despite these advances, mapping genes for complex traits remains difficult. Any clue from the known biology of the disorder is an invaluable advantage.

FUNCTIONAL GENOMICS

Help is being provided by the next frontier of the Human Genome Project: functional genomics. This term encompasses a broad range of technologies and scientific strategies whose goal is to elucidate the functional role of all genes. The most developed and widely used method is expression profiling. In this technique, DNA microarray technology is employed to simultaneously assess

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expression changes in thousands of genes (and in the not-to-distant future, likely of whole genomes) on a single chip. Two general methods are in common use. In one, oligonucleotides are synthesized on the chip in a massively parallel fashion. An alternative approach involves the use of a robot to spot full-length complementary DNA strands on a glass slide. mRNA from each tissue under study is fluorescently labeled and applied to the chip. The chip is then read using a laser-scanning fluorescence detector. Each approach has its respective advantages and disadvantages, but provide a dramatic new approach to gene expression. These methods have given new impetus to comprehensive, discovery-based, non–hypothesis-driven studies of gene expression in specific brain regions of either animal models or human postmortem tissue.

More broadly, functional genomics also include methods to measure changes in protein in a similar comprehensive fashion termed proteomics. These methods are not yet as well developed but typically include a separation of proteins using 2-dimensional gel electrophoresis and identification of individual spots using mass spectroscopy. Because proteins are inherently more difficult to separate, measure, and more numerous than mRNAs, proteomics is inherently more complicated. However, proteomics will be essential for a comprehensive view of genome function. Massively parallel "protein chips" are also now being developed.

APPLICATION OF FUNCTIONAL GENOMICS TO PSYCHIATRY

Functional genomic methods, such as expression profiling, can be applied to a variety of different tissues derived from illness models to identify biologically relevant candidates for positional cloning studies.

GENE EXPRESSION STUDIES IN ANIMAL MODELS

Animal models of psychiatric disorders can be broadly classified into two groups: pharmacological animal models and genetically altered animal models. Based on analogies with human states, multiple behavioral stress models, including sleep deprivation, have also been proposed. Expression profiling or other functional genomic methods can be applied to any of these models to identify candidate disease genes and to better understand signaling pathways relevant to disease.

Genetically Altered Animal Models

Spontaneous or engineered genetic mutations in rodents such as rats and mice have provided a convenient terrain for behavioral, biochemical, and gene expression studies. Although the ability to engineer genetic changes in rats is at present limited, engineered mice are widely available and have become a fundamental experimental tool.

Mutations in mice have traditionally been targeted to specific genes, such as deleting a gene (knock-out) or inserting an altered copy (knock-in). Tissue-specific and inducible mutants are a newer development in this methodology and may eliminate some of the experimental confounds of constitutive genetic alterations.[9] More recently, an opposite approach has come to the forefront[10]: random germline mutagenesis with the chemical mutagen EMU, followed by identifying interesting mutants based on their phenotype, and working backwards to identify the gene involved.

In terms of applications to the study of bipolar disorder and related disorders, the targeted mutant approach was stymied by the fact that until recently there were few high-probability candidate genes for bipolar disorder. Based on neuropharmacological data in humans and the aminergic neurotransmitter hypothesis of mood disorders, various mutants in aminergic neurotransmitter receptors or transporters have been generated and studied. The dopamine transporter knock-out mouse,[11] which has a persistent extracellular hyperdopaminergic tone, is a particularly interesting model for hyperactivity states, including mania and positive-symptoms psychosis.[12]

PHARMACOLOGICAL ANIMAL MODELS

A series of pharmacological agents, like ouabain[13] and 6-hydroxydopamine,[14] have been used to mimic mania in rodents. By far, the most used and best studied pharmacological agent is amphetamine. Single-dose amphetamine treatment in humans reproduces the core symptoms of mania: increased energy, decreased need for sleep, and psychomotor hyperactivity.[15,16] Chronic use, especially in a binge fashion, also leads to delusions and hallucinations that mimic psychotic mania or the positive symptoms of SZ.[17] However, this model, like any animal model, has limitations. Amphetamine psychosis is more commonly paranoid in nature, whereas that of mania is more typically grandiose. A more prominent limitation is that the primary symptoms in humans involve thought, affect, and speech, and therefore cannot be observed in rats. Rather, the primary similarity is that of increased locomotor activity. These inherent limitations of animal models call for examination of the tissue of relevance in humans as a complementary approach.

continued on page 223

TABLE 1. OVERLAP IN GENETIC LOCI REPORTEDLY LINKED TO BIPOLAR DISORDER AND SCHIZOPHRENIA

Bipolar	Schizophrenia	Bipolar and Schizophrenia
21q21	1q21–22	10p, 10q
4p16	6q	13q14.1–q32
12q24	6p24–22	18p11.2
18q22	8p22–21	22q11–13
Xq26		

Niculescu AB 3rd, Kelsoe JR. *CNS Spectrums.* Vol 7, No 3. 2002.

continued from page 216

GENE EXPRESSION STUDIES IN POSTMORTEM HUMAN TISSUE

Human postmortem brain tissue has been used for protein immunohistochemistry and mRNA determinations by in situ hybridization.[18,19] More recently, microarray technology has been used for expression profiling in such tissues, most notably in SZ[20-23] and alcoholism.[24,25] Though these studies are just beginning, some intriguing findings have emerged. These include the presence of decreased expression of presynaptic rather than postsynaptic genes in SZ and identifications of changes in white matter.

The potential caveats of using human postmortem tissue are numerous: possible uncertainty of the psychiatric diagnosis while the patient was living, incomplete information about other medical or substance abuse problems, medications, and so forth. Tissue degradation and age-related changes, while important factors, can usually be addressed by carefully matched controls. Perhaps more challenging is the degree of genetic heterogeneity likely present in these illnesses and the number of brains that will be necessary to detect the correspondingly small changes in gene expression. Nevertheless, there is no substitute for direct studies of the human organ involved in the disease process, difficult as they may be.

GENE EXPRESSION STUDIES IN HUMAN LYMPHOCYTES

Human lymphocytes are an easily available source of cells from premortem human subjects. Following immortalization in the laboratory with Epstein-Barr syndrome, they constitute a replenishable source of material for DNA and RNA, as well as for biochemical and cell biological assays.

The potential caveats of using lymphoblastoid cell lines to observe RNA level and cell biological assays are numerous. In culture, these are immortalized cell lines that may not necessarily reflect what happens inside the body in general and the brain in particular. Immortalized lymphoblastoid cell lines have the advantage of being easy to grow and limitless in supply. However, other living peripheral tissues are suitable for such studies, including primary lymphocyte cultures, skin fibroblasts, and olfactory neuroblasts. Despite the limitations, such cells provide convenient access to living tissue from affected individuals; though in most cases, gene expression will not resemble that in the brain. In a few instances signaling pathways are similar enough to provide a limited test.

For example, we have recently reported preliminary evidence of reduced levels of G-protein receptor kinase 3 (*GRK3*) in lymphoblastoid cell lines from bipolar disorder patients.[26] As will be described in more detail later, *GRK3* is a protein involved in receptor desensitization that is a candidate gene supported by multiple lines of evidence.

Other possible examples include transient receptor potential channel 7 and altered inositol monophosphate 2, two genes involved in calcium homeostasis. They map near known-linkage loci for bipolar disorder (chromosome [chr] 21q22.3 and chr 18p11.2, respectively) and have been reported to have decreased levels in lymphoblastoid cell lines from bipolar disorder I patients, but not in bipolar disorder II patients or normal controls.[27,28]

Despite the substantial caveats, such tissues may provide a limited model of illness that could be useful for functional genomic studies. Changes seen in such studies could potentially also be used as peripheral cellular endophenotypes of the disorder. That is, different patterns of gene expression might point to different pathophysiological mechanisms that would be useful in distinguishing more genetically homogeneous subforms of illness.

CONVERGENT FUNCTIONAL GENOMICS

Genetic-linkage studies and expression profiling studies each have their strengths and weaknesses. As previously mentioned, linkage studies have as a strength no required a priori mechanistic hypothesis. Hence, truly novel genes and mechanisms may be discovered. Furthermore, genes that transmit the susceptibility within families are clearly of etiologic significance. Yet, the limitations have been that positional cloning of complex traits has proven to be quite difficult, and the linked genomic regions broad and frequently including hundreds of genes. On the other hand, expression profiling is a comprehensive genomic method, but one with a different set of strengths and weaknesses. Its strength: specific genes are studied from the beginning. Its weakness: among the many gene changes detected, it may be difficult to determine which are of primary relevance to the disease mechanism and which are secondary phenomena. Furthermore, if an animal model is employed, an a priori hypothesis regarding disease mechanism of action is required. If postmortem brain or other tissues are employed, the various limitations described previously apply.

We have argued that the relative strengths and weaknesses of these two approaches are complementary, and have described an approach termed convergent functional genomics that intersects data from gene expression profiling with linkage peaks identified in human family studies.[26,29] In this approach, the human chromosomal location of genes identified in expression profiling studies of an animal model are compared with regions in the genome implicated in linkage studies of bipolar disorder or SZ. Genes whose expression is altered in the animal model that also map to linkage peaks are higher probability candidate genes. The power of the approach is to narrow the field of hundreds of possible candidates within a linked region to a smaller and more tractable number of greater biological relevance (Figure 1). This smaller number of candidates can then be examined for potential pathogenic mutations and tested for association to illness in affected individuals.

In our previous application of this approach, we employed single-dose methamphetamine administration in rats as an animal model of mania.[26] The animals were sacrificed 24 hours later, and the prefrontal cortex and amydala brain regions were dissected. RNA was prepared and

expression profiling conducted using an oligonucleotide-based method. The experiment was twice conducted de novo, and only genes whose expression was increased at least two-fold in each of the two experiments were considered reproducibly changed. Out of 8,000 genes examined, the gene with the largest increase in expression was *GRK3*. On the positional cloning side, we had previously completed a genome scan of a set of 20 families with bipolar disorder from the general North American population.[30] The genome-wide maximum evidence for linkage was on chr 22, with a significant lod score of 3.8. As is typical for complex disorders, this linkage peak was quite broad and spanned over 10 megabases. It also had the appearance of two distinct linkage peaks, though this could not be definitively concluded from the available data. The striking convergence of these data with the expression profiling data was that the *GRK3* gene lay extremely close to one of the two potential linkage peaks identified on chr 22q. Along with the gene's known role in receptor desensitization, these results provided strong support for prioritizing it as a positional candidate.

OTHER CONVERGENCES

The power of convergent data concept can be readily expanded to other possible comparisons as illustrated in Figure 2. A comparison between different animal models could lead to the identification of gene pathways that are part of a final common pathway to illness. Comparison of genes altered in an animal model with findings from human postmortem brain could help identify genes relevant to human pathophysiology. Lastly, the same comparison we employed to human linkage peaks could be applied to expression profiling results from postmortem brain. For Mirnics and colleagues,[21] this later approach has been successful in identifying the regulator of G-protein signaling 4 gene. This G–protein-related signaling molecule was found to have altered expression in postmortem brain from SZ subjects and to map to a region on chr 1 implicated in linkage studies.

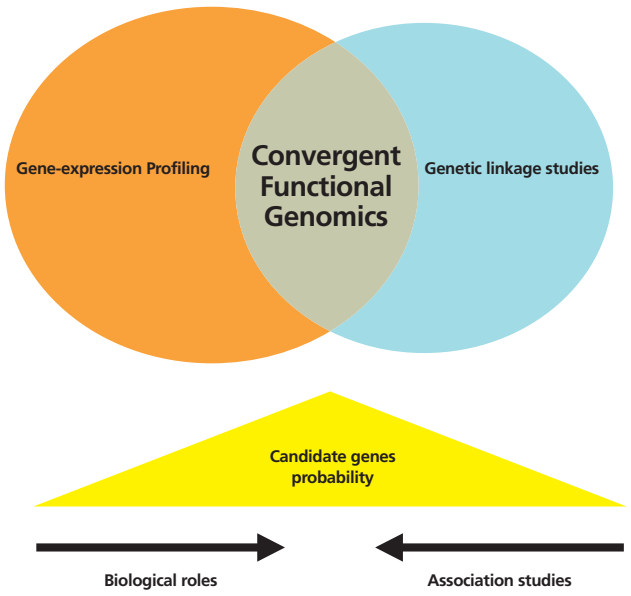

FIGURE 1. Convergent functional genomics: positional cloning and expression profiling have complementary strengths that can be combined to more effectively identify candidate susceptibility genes.

Niculescu AB 3rd, Kelsoe JR. *CNS Spectrums.* Vol 7, No 3. 2002.

CLASSIFYING CANDIDATE GENES: A SIMPLE PARADIGM BORROWED FROM CANCER BIOLOGY

Expression profiling studies of animal models or human tissues present the formidable problem of making sense of the changes observed in numerous genes of unknown function. The aforementioned convergent functional genomics approach is used in an attempt to identify those gene changes that are relevant to the disease process. However, how does one begin to understand the functional role in the pathogenesis of a set of genes of unknown function? As a first approach, we propose to borrow a concept from cancer biology, that of oncogenes and tumor-suppressor genes. By analogy, we term psychogenes as genes whose increased function promotes neural processes leading to mania and psychosis. Similarly, psychosis-suppressor genes describe genes whose increased function inhibits neural processes leading to mania and psychosis.

Though clearly such a dichotomy is simplistic, it may serve as a starting point in classifying genes that are induced or repressed in expression profiling studies. As such, it may have heuristic value for the formation of hypotheses regarding genes of unknown function. Table 2 illustrates this approach by summarizing a selection of genes whose expression is altered in animal models or human tissue and tentatively placing them in one of the two categories. As the function of these genes is at least partly known, there is information to debate their proper

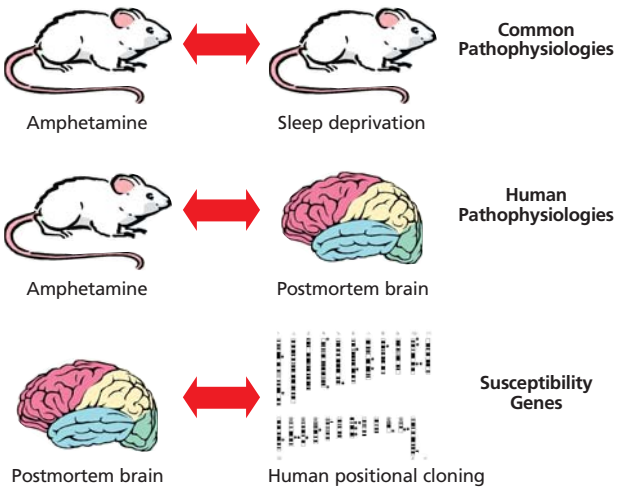

FIGURE 2. Comparisons of expression profiles employing both animal models and human postmortem brain may enable access to different sets of genes involved in pathophysiology.

Niculescu AB 3rd, Kelsoe JR. *CNS Spectrums.* Vol 7, No 3. 2002.

classification or perhaps the inadequacy of such a simple system for any gene. However, for genes of unknown function, such a simple system and corresponding simple hypothesis may facilitate the design of interventional experiments to learn about their role in pathogenesis. Such a simplistic classification must have many limitations and qualifications. For example, different mutations within the same gene may have opposite effects on both disease and gene expression. Similarly, different combinations of alleles may also have very different impact on pathophysiological processes.

GUILT BY ASSOCIATION: COACTING GENE GROUPS

Another clue to discerning the function of unknown genes identified in expression profiling studies may be to examine gene clusters that change together. It seems plausible that genes that change together act together, as part of coacting gene groups. In the absence of any other information about a gene's function, it is reasonable to hypothesize that genes whose expression changes together may be involved in related processes or pathways. This may serve as another starting point for the formation of testable hypotheses regarding the gene's function. This concept may be extended to multiple states, such as coordinated change at different time points, or coordinated change in response to different treatments. For example, genes turned on by an animal model of mania, such as amphetamine, and turned off by a treatment for mania, such as valproate or lithium, are more likely to operate together as part of a pathway involved in disease. Though this rule is rather simplistic and may or may not be generally applicable, it is testable. As a discovery engine, it may help identify possible relationships between genes not previously thought to act synergistically.

TABLE 2. EXAMPLES OF CANDIDATE GENES IN A PROPOSED SIMPLE CLASSIFICATION BASED ON THEIR POSSIBLE ROLE IN MANIA AND PSYCHOSIS

Psychogenes	Psychosis Suppressor Genes
DBP[26]	*GRK3*[26]
FDFT1[26]	*SULT1A1*[26]
MALS-1[26]	*COMT*[35]
IGF-1[26]	PKCγ[36]
GSK3β[37]	*DAT*[11]
PACAP[38]	*RGS4*[21]
IMPA2[28]	*Cdk5*[39]
	TRPC7[27]
	c-fos[40]

DBP=D-box binding protein; GRK3=G-protein receptor kinase 3; FDFT1=farnesyl-diphosphate farnesyltransferase; SULT1A1=sulfotransferase 1A1; MALS-1=vertebrate LIN7 homolog 1; COMT=catechol-*O*-methyltransferase; IGF-1=insulin-like growth factor 1; PKCγ=protein kinase Cγ; GSK3β=G-protein synthetase kinase 3β; DAT=dopamine transporter; PACAP=pituitary adenylate cyclase-activating polypeptide; RGS4=regulator of G-protein signaling 4; IMPA2=altered inositol monophosphate 2; Cdk5=cyclin-dependent kinase 5; TRPC7=transient receptor potential channel 7; c-fos=transcription factor.

Niculescu AB 3rd, Kelsoe JR. *CNS Spectrums.* Vol 7, No 3. 2002.

Such associations may come full circle and providing hypotheses for testing in human populations. Increasingly, it seems likely that in complex human disorders, multiple genes may interact in such a fashion that their combined effect is greater than the sum of their independent effects. This type of superadditive gene interaction is termed epistasis. Epistasis makes sense in terms of the likely underlying biology and represents a nonlinear response to multiple hits in a system of interacting proteins. Much of the difficulty in detecting genes by linkage or association may stem from examining one gene at a time. That is, one gene may have only a small effect on risk, but multiple genes considered together may convey a substantially greater risk. Genes whose expression changes together in expression profiling studies may also interact together at the protein level. In turn, they may be more likely to interact at the genetic level and display epistasis. One of the problems with testing epistatic hypotheses in human genetic studies is the large number of tests required by the many possible combinations of genes. The identification of coacting gene groups from expression profiling studies may suggest a limited number of gene combinations that interact biologically and that deserve prioritized attention in terms of testing for epistasis.

COMING FULL CIRCLE: PHENOTYPES, ENDOPHENOTYPES, AND PHENOMICS

Genomics has been defined as the science of the structure and function of our genes. Similarly, one could define phenomics as the science of the structure and function of phenotypes. Although much progress has been made in genomics, ultimately establishing unambiguous links between genomic data and the corresponding phenotype is necessary to identify which genes are involved in what disease.

All this is as true for psychiatric disorders as it is for metabolic disorders like diabetes or cardiovascular disorders like hypertension. The bad news is that we do not yet have simple quantitative measures in psychiatry like blood glucose levels in diabetes and blood pressure measurements in hypertension. The good news is that there is an appreciation that the same general methodologies are applicable across disorders and organ systems.

The frontline of psychiatric research is thus crucially dependent on having a better description and quantitation of psychiatric phenotypes.[31] Currently, most work in psychiatric genetics employs diagnoses based on impairment as phenotypes. Though it is these conditions that are the ultimate goal, our current diagnostic system does not likely reflect in a detailed way the underlying biological processes of disease. Rather. these diagnostic entities encompass multiple different pathophysiologies, each of which may have a different genetic basis. These different underlying pathophysiologies have been termed endophenotypes or intermediate phenotypes. A more effective approach to positional cloning may be to study such endophenotypes instead of the diagnosis itself to reduce the degree of genetic heterogeneity. This approach has

already been used with some success in SZ[32,33] and is inherently more challenging in bipolar disorder because of the constant variation in state and difficulty in distinguishing state from trait. However, lithium responsiveness is one such possible endophenotype that has recently been employed in a linkage study of bipolar disorder.[34] This endophenotype may be highly useful in distinguishing genetically distinct subforms of illness and providing a method of stratifying families for analysis. Such biologically relevant endophenotypes also may be much more readily examined in animal models and relevant genes identified by expression profiling. Genes whose expression is changed by administration of lithium may be more likely to be involved in the phenotype of lithium-responsive bipolar disorder. Furthermore, using a convergent functional genomics approach, if such genes map to regions identified in human linkage studies using lithium responsiveness as an endophenotype, they become especially attractive candidates.

CONCLUSION

Psychiatric disorders, like other complex human disorders, have proven to be challenging problems for positional cloning. Though powerful tools from the Human Genome Project promise to greatly accelerate the mapping of complex traits, clues from biology must also be employed to facilitate success. Expression profiling and the other tools of functional genomics offer invaluable information regarding the possible pathophysiology when applied to animal models or human tissues. Used in conjunction, these approaches may not only aid in the identification of disease genes but also in understanding the underlying disease mechanisms. CNS

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