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Genetic Neuropathology of Schizophrenia: New Approaches to an Old Question, and New Uses for Postmortem Human Brains

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Abstract

Human postmortem brain studies are critical for elucidating the pathophysiology and etiology of schizophrenia and other major mental illnesses. The traditional approach compares patients and controls, but is potentially confounded by a number of artifacts including medication, substance misuse and other secondary effects of illness. Now, genetic advances make possible a novel approach that focuses on how allelic variation in risk-associated genes impacts on expression and function of transcripts and proteins. These questions can be addressed in normal brain, overcoming to some extent the confounding effects of studying brains from subjects with schizophrenia; equally, extension of the studies to include cases also has advantages. Conceptually, the approach may be seen as the neuropathologic counterpart of genetic neuroimaging, representing a potentially powerful intermediate phenotype. For several schizophrenia susceptibility genes, the data show that risk-associated polymorphisms do affect gene expression or the function of the encoded protein; in some instances expression of downstream or interacting partners of the gene are also altered. A further striking finding is that the implicated transcripts often appear to be enriched in, or specific to, human brain. Some also show enhanced expression in fetal brain. These considerations give a unique importance to postmortem human brain tissue in elucidating the genetic mechanisms underlying schizophrenia, and probably other neurodevelopmental disorders too. Studies of this kind can provide clues as to the biologic mechanisms of genetic association, especially when carried out in conjunction with experimental studies. Moreover, the data, interpreted judiciously, can strengthen the plausibility of the association itself.

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With the exception of neurodegenerative diseases, over a century of postmortem human brain studies have not elucidated clearly the neuropathology of the major psychiatric disorders, nor have they yet to contribute significantly to their etiologic or pathophysiologic understanding, diagnosis, or treatment. To be certain, there has been no shortage of findings, but results have been inconsistent, and their interpretation complicated by artifacts and confounds (e.g. medication, smoking, comorbidity, substance misuse, perimortem events) and compounded by often inadequately sized, described, or matched samples (1,2). As a consequence, it has been difficult to determine the significance of the substantial number of apparent differences between patients and controls in the postmortem human brain literature of psychiatric disorders, particularly for schizophrenia which has received the most attention (3,4).

Recently, advances in genetics have provided new tools to investigate schizophrenia and other psychiatric disorders and are leading, perhaps unexpectedly, to a renaissance of postmortem brain studies and the adoption of innovative study designs. Although failures to replicate genetic association studies raises the question of the legitimacy of a number of candidate genes, postmortem human brain tissue is being used to provide clues to possible mechanisms by which putative schizophrenia susceptibility genes and polymorphisms operate (5). Since most genetic variants associated with schizophrenia to date are non-coding single nucleotide polymorphisms (SNPs), a default explanation for a disease association is that the SNP affects some facet of gene expression, for example by modifying transcriptional activity or alternative splicing. Molecular analyses testing these notions are therefore an essential complement to the statistical demonstration of genetic association. As outlined below, these studies need to include human brain tissue as a central component, because the critical events may not occur in other tissues or species, and *in vitro* or *in silico* analysis alone is insufficient.

The functional correlates of schizophrenia risk alleles have been extensively investigated using neuroimaging, cognitive, and neurophysiologic indices as intermediate phenotypes (6), the notion being that they are ‘closer to the gene’ and therefore may be more easily demonstrated and interpreted than are genetic effects on diagnostic phenotypes. Since risk alleles are possessed by many healthy subjects, and assuming that their biologic correlates are diagnostically agnostic, the approach allows normal subjects to be studied rather than patients, mitigating the difficulties and confounds associated with the latter. These same principles and attractions apply to the genetic neuropathologic studies to be described here, only instead of studying whether a risk allele impacts on MRI signal, cognitive performance, evoked response, etc, the parameters measured are mRNA, protein expression, and function (e.g. enzyme activity). Indeed, mRNA is the ultimate intermediate phenotype, in terms of proximity to the gene, and so the proposed advantages of this strategy (e.g. in terms of sample size required) are maximized.

This review will cover how postmortem brain studies contribute to the understanding of the genetic and molecular basis of schizophrenia. After a general introduction, we focus on how risk alleles in susceptibility genes may increase risk for disease, by using mRNA expression and protein levels as intermediate phenotypes. This approach is providing unique and sometimes dramatic insights into underlying genetic and molecular mechanisms. We illustrate these issues with examples mainly from our own studies of schizophrenia, noting that similar strategies are being adopted by others, and that the principles apply broadly to other disorders too. In addition, we discuss the further potential, and the limitations, of 'genetic neuropathology' in this field.

Genetic contributions to gene expression alterations in schizophrenia

There are many reports of differences in the abundance of mRNAs or proteins between subjects with schizophrenia and controls. Generally, the molecules selected for study in postmortem brains was based on prior reasons for advocating their involvement in schizophrenia pathophysiology. Examples include catechol-O-methyl transferase (COMT), glutamic acid decarboxylase 67 (GAD67/GAD1), and dopamine D2 receptor (DRD2). Recently, microarray studies have identified many other, hitherto unsuspected, differentially expressed genes, including regulator of G protein signalling 4 (RGS4), 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), and oligodendrocyte-lineage transcription factor 2 (OLIG2). For some of these genes, evidence has subsequently emerged that they may be genetically associated with schizophrenia and, as outlined here, genetic neuropathologic studies have investigated whether their expression is related to allelic variation.

COMT is the enzyme primarily responsible for dopamine regulation in the cerebral cortex and as such is of interest with regard to schizophrenia as well as other dopamine-related functions and disorders (7). It has long been known that peripheral COMT activity is trimodally distributed in the population, a finding due primarily to a common, codominant coding polymorphism, Val^{158}Met (8). Val^{158}-COMT has a higher activity than Met^{158}-COMT, with heterozygotes being intermediate. The genotype effect on COMT activity (about a 30% difference between homozygotes) is seen in human postmortem brain (9), providing biologic plausibility to the multitude of studies, showing that this SNP impacts upon a range of cognitive, neuroimaging and other phenotypes (7,10, 11). Genetic neuropathologic studies have also contributed in other ways to the understanding of how COMT and its allelic variants exert these effects. First, Akil *et al*. (12) showed that COMT genotype also affected tyrosine hydroxylase expression in the midbrain, providing a mechanistic link between cortical metabolism of dopamine and this key index of presynaptic dopamine function, complemented subsequently by findings in a functional neuroimaging study (13). The Akil *et al.* report was one of the first to show that allelic variation in one gene can influence expression or function of other genes, a principle now becoming widely appreciated (see below). Second, Tunbridge and colleagues showed that human brain expresses novel COMT protein (14) and mRNA (15) variants, with the isoforms being related to Val^{158}Met genotype and being regionally and developmentally regulated, thereby introducing other themes to which we return later. Third, like COMT activity (9), COMT immunoreactivity in brain is related to COMT Val^{158}Met SNP (16), but COMT mRNA is not (17), supporting findings that the genotype affects peripheral enzyme activity via

differential protein stability and not mRNA expression (8). COMT expression is unaltered in schizophrenia (17, 18), notwithstanding some differences in particular cell populations (18). Together, the postmortem human brain data provide findings, both positive and negative, which have advanced the understanding of the roles which COMT may play in the normal brain and in schizophrenia.

A well replicated postmortem brain finding in schizophrenia is reduced expression of GAD67, the main transcript of the GAD1 gene which codes for the enzyme that converts glutamate to GABA; this has been observed in at least ten studies using tissues from six independent brain collections in multiple brain regions (see [4,19] for review). The reductions have been viewed as primary evidence for dysfunction of GABA interneurons, although there is also some recent evidence that SNPs in the GAD1 promoter region are genetically associated with the disorder (20, 21). Linking these findings, the SNPs are also associated with decreased GAD67 mRNA in the prefrontal cortex (PFC) and hippocampus (20). Thus, the GAD67 postmortem findings in schizophrenia are complemented by results of the genetic studies. It should be noted, however, that the differences in frequency of the risk-associated alleles between patients and controls are insufficient to explain fully the diagnostic difference in expression. The latter must involve an additional mechanism, a consideration that applies to other findings to be described later.

The central role of the DRD2 in antipsychotic drug action, and its candidacy for involvement in schizophrenia pathophysiology, has led to a number of postmortem brain studies. These demonstrated increased DRD2 in striatum and other brain regions, as shown by meta-analysis (22), with some studies also finding elevated DRD2 mRNA (23, 24). Neuroimaging studies of drug-naïve patients have mostly also found increases in DRD2 (25, 26) suggesting that the postmortem brain results are not just the consequences of prior drug treatment, which has always been a concern. Other studies have identified genetic association of the DRD2 gene with schizophrenia (27, 28), with the risk alleles also associated with cognition and activity in striatum and PFC (29,30). Thus, the question arose as to whether the risk alleles might contribute to the DRD2 elevation seen in schizophrenia. In fact, the polymorphisms are associated with decreased DRD2 expression, particularly of the presynpatic isoform (29, 30), whereas the opposite effect might have been predicted to explain the increases in the DRD2 seen in postmortem brain and in vivo neuroimaging. In short, increased DRD2 in schizophrenia do not appear related to DRD2 risk alleles for the disorder. Regardless, the genetic association studies of DRD2 have renewed interest in dopamine receptors.

In an early microarray study of schizophrenia, RGS4 mRNA was reduced in PFC (31), a finding leading to demonstration of genetic association between RGS4 and the disorder (32). Neither observation has been uniformly replicated (33, 34), but RGS4 has remained a gene of interest. The effect of RGS4 SNPs upon RGS4 mRNA expression has been examined (33) and although no such relationship was observed, RGS4 expression was decreased in subjects carrying the COMT Val^{158} allele, linking RGS4 with this well-studied candidate gene mentioned earlier. As a final illustration, other microarray studies have identified two oligodendrocyte transcripts, CNP and OLIG2, decreased in dorsolateral PFC in schizophrenia (35–37, but see 38), and with some subsequent evidence for genetic

association with disease risk (39, 40). Moreover, risk SNPs in CNP and OLIG2 predicted reduced expression of the genes (38). As such, they may contribute to the down-regulation of the transcripts seen in schizophrenia – although as with GAD67, genotype can be at most only a partial explanation for the reduced expression.

Postmortem brains and the molecular basis for genetic associations in schizophrenia

The above examples show that SNPs can be, in some instances, associated with the expression of a gene that is differentially expressed in schizophrenia. A related, novel study design has been to use postmortem brains to interrogate the consequences of allelic variation in putative susceptibility genes, especially where the associated SNPs are non-coding and of unknown function. In this case, the studies are testing the hypothesis that the functionality of the associated SNP (or one in linkage disequilibrium with it) is mediated via an effect on gene regulation or expression. Below we describe a number of studies which illustrate this approach. The results show that many schizophrenia-associated allelic variants modulate indices of gene expression or function, and that these are often subtle and complex, involving novel isoforms which are developmentally enriched and Human brain specific. This approach is limited by the correlative nature of the studies which do not prove causation.

Schizophrenia risk alleles and novel isoforms

Metabotropic glutamate receptor 3 (GRM3) was associated with schizophrenia (41, 42). The implicated SNPs were intronic, had no known function, and the gene was not thought to be alternatively spliced, even though it is now recognized that most genes produce alternative transcripts in human brain (43,44). Thus, Sartorius et al searched for, and found, splice variants of GRM3 mRNA in human brain, including one which lacked exon 4 downstream from the implicated SNP (45). The abundance of this isoform relative to full-length GRM3 was then shown to be affected by two of the risk-associated intronic SNPs (46), supporting the hypothesis that this might be a mechanism underlying the clinical association. Whether this molecular event ultimately influences GRM3 function or signaling is unknown, but the study does illustrate the value of detailed transcript characterization, and the ability to test a genetically-informed hypothesis using postmortem brain tissue.

Neuregulin 1 (NRG1) has been repeatedly associated genetically with schizophrenia, yet its large size, multiple isoforms, and the fact that the associated SNPs are virtually all non-coding, has hindered biologic explanations for the association (47, 48). Law et al (49) noted that most of the associations were in a 5′ region just upstream of an exon which encoded a specific isoform, type IV NRG1 and, using postmortem hippocampus, showed that the risk genotype was indeed associated with differential expression of this isoform, but not type I, II or III NRG1 isoforms. Interestingly, in contrast to these other isoforms, type IV is brain specific and is developmentally enriched (50). The findings highlighted type IV as an NRG1 isoform worthy of study, and provided evidence that the associated SNPs may be functional. Subsequent work, mentioned later, has provided support for these notions and can be traced directly back to the postmortem study. The same strategy was employed with another

schizophrenia candidate gene, the potassium channel gene KCNH2 (51). To explain a genetic association with schizophrenia observed, again with intronic SNPs, the authors used 5′ RACE techniques and transcript cloning in human brain to identify a novel KCNH2 isoform with unique physiologic properties, which was increased in PFC in carriers of the risk allele, and in schizophrenia patients compared to controls (51). Moreover, the novel isoform was brain and primate specific, and preferentially expressed in fetal human brain. Once again, the elucidation of how allelic variation increased risk for schizophrenia required post- mortem human brain.

For the strong schizophrenia candidate gene, DISC1, an initial study did not find that DISC1 risk alleles correlated with expression of any of its known transcripts in either PFC or hippocampus (52), but – like GRM3 and KCNH2 - further study using RACE and transcript cloning then identified multiple novel DISC1 truncated transcripts, which were associated with allelic variants, and which appeared to be brain and primate specific, and preferentially expressed fetally (53). DISC1 also provides examples of additional ways that genotype can affect expression or function. Firstly, DISC1 SNPs modulate expression of known DISC1 binding partners (52), suggesting that genotype effects of DISC1 may extend to interacting genes biologically (54), and thus amplifying the molecular consequences of an individual allelic variant. Similar ‘transgenic’ influences have been seen for NRG1 SNPs with alpha7 nicotinic receptor mRNA (55) and GRM3 SNPs with glial glutamate transporter mRNA (41), in addition to the COMT-RGS4 interaction noted earlier (33). Secondly, DISC1 illustrates that effects of risk SNPs observable in postmortem studies are not limited to gene expression, but can also impact on morphology (56). Pericentriolar material 1 (PCM1) is a centrosomal protein and binding partner of DISC1 (57) and possibly a schizophrenia gene in its own right (58); coding SNPs in DISC1 affect the extent of centrosomal PCM1 immunoreactivity in human brain, revealing a genetic contribution to the DISC1-PCM1 interaction (56). Further postmortem studies of this kind are needed, to show that allelic variants not only affect transcript abundance or splicing, but also result in meaningful downstream effects on gene function.

As a final example, postmortem brains have also been integral to identification of a probable mechanistic basis for the association of polymorphisms in intron 1 of neuregulin 3 (NRG3), another member of the neuregulin gene family and the specific ERBB4 ligand, with risk for, and delusional severity in, schizophrenia (59, 60). Law and colleagues used over 400 human brains to reveal high confidence associations between risk genetic variation in NRG3 and transcript levels of novel, developmentally regulated NRG3 isoforms (60). The significance of these associations (ranging from $p=10^{-12}$ to $p=10^{-15}$) was impressive, if unprecedented, for postmortem brain studies, likely reflecting not only the exceptional sample size, but also the size of the genetic effects.

All the above illustrations arose from work on genes identified as candidate genes or from linkage, but the same approach can be taken to follow up on GWAS-identified SNPs. Notably, a non-coding SNP in ZNF804A has emerged as highly genome-wide significant in a GWAS meta-analysis (61). Initial postmortem brain data suggest that this allele may influence ZNF804A mRNA expression (61, 62), but further work is needed to clarify this statistically robust but biologically obscure association.

The interpretation and future of genetic neuropathologic studies of schizophrenia

A striking aspect of genetic neuropathology in this field has been the productive interplay between postmortem and experimental work, with each informing the other. For example, the novel KCNH2 isoform found in human brain and related to schizophrenia risk (51) was transfected into cells and shown to encode a potassium channel with significantly distinct functional properties affecting firing and depolarization, and was also shown to impact on hippocampal volume and cortical activity (51). Similarly, the finding of the SNP which affected NRG1 type IV expression in human brain (49) was followed by a study which confirmed the effect in transcriptional assays (50, leading to several studies that demonstrate its functionality in a range of domains from cell migration (63) to imaging and cognitive phenotypes (e.g. 64). Complementing these examples where a postmortem finding drove experimental investigations, the reciprocal situation pertained to the study showing a DISC1 SNP effect on PCM1 localization in human brain (58), which was stimulated directly by a finding in cell culture (65).

For balance, we also note that postmortem studies have not always found the hypothesized relationship between risk SNPs and gene expression or function. For example, D-amino-acid oxidase (DAO) has been associated genetically with schizophrenia, and its expression and enzyme activity are increased in the disorder (66). However, no effect of the risk SNPs upon either parameter was observed (67). Similarly, the most significant schizophrenia risk-associated SNP in UMHK1/KIS had no effect on its expression (68). The DAO and UMHK1 findings highlight the issue of how negative results of this kind should be interpreted. Clearly, they might be false negatives, in that more detailed analyses may reveal novel transcripts, or effects of the SNPs earlier in development, etc, as illustrated by some of the examples given earlier. It is also likely that some risk associated SNPs are in linkage disequilbrium and thus proxies for causative SNPs which affect gene function other than expression, e.g. translation or posttranslational modifications. On the other hand, a true negative result may call into question the likelihood that the genetic association is meaningful, in the absence of demonstrable evidence for a molecular correlate of the SNPs concerned – in the same way that we would argue that compelling positive results enhance the statistical evidence that a SNP is associated with schizophrenia because they provide biologic plausibility.

We have emphasized that one attraction of this form of genetic neuropathology is that only normal subjects are required, selected for the presence or absence of a risk polymorphism or haplotype, mitigating the problems inherent when comparing cases with controls, as noted earlier. It should be acknowledged that a SNP-expression association in normal controls only suggests, but does not prove, that this is the mechanism in the disease state. Fortunately, many studies of this kind do also include subjects with schizophrenia (e.g. 46, 49, 51, 56) which may be necessary to determine the disease state mechanism. This has advantages since it allows effects of diagnosis, as well as of genotype to be investigated, and it also increases sample size and thence power. It also raises the possibility of finding a genotype-by-diagnosis interaction; that is, an effect of genotype upon gene expression (or whatever parameter is being measured) which differs between cases and controls (e.g. 69). Such a result may be of great interest, but its interpretation is not straightforward. On the

one hand, it may indicate a novel epistatic or gene-environment interaction that is present in the schizophrenia group more than in the controls (or vice versa), and one worthy of further study. On the other hand, if a genetic effect on gene expression is seen *only* in patients, it may cast doubt on the robustness of the result, since one might expect a 'true' genetic effect on gene expression to be seen, at least to some extent, in normal brain as well. An effect only in patients may reflect a genetic effect not on risk *per se*, but on something that is an illness related epiphenomena, e.g medication effects, smoking effects, etc. A gene x nicotine interaction in fetal and/or postnatal brain is a feasible postmortem study that should allow some of these hypotheses to be tested. Either way, if comparison is to be made between patients with and without risk alleles, the two subgroups should be suitably matched for the potential confounds mentioned earlier. Moreover, the association of genetic variation with expression is a case control association with all caveats thereto, including the need to be aware of potential stratification artifacts related to race, sex and family history.

The existing data show clearly the promise of further genetic neuropathologic studies of schizophrenia. More detailed investigations are needed, prioritizing the most promising risk genes and alleles (in terms of their statistical evidence and their biologic plausibility). Most studies to date have only examined a single brain area, and have not taken regional or cellular heterogeneity of genetic influences into account, even though the evidence for this is now accumulating (e.g. 70). Similarly, temporal variation should be taken into account, and will require a greater availability of fetal and postnatal brains. The work can also expand by parallel assessment of epigenetic processes and other mechanisms that may mediate between genotype and the expression phenotype, and by application of novel technologies (e.g. RNA sequencing). And, although we have focused mainly on analysis of candidate genes, effects of allelic variation on gene expression in postmortem human brain also lend themselves well to the agnostic approaches of microarray studies, with several studies already demonstrating relationships between genome-wide SNP analyses and expression profiling (44,71,72). The existence of these databases makes it possible to interrogate the association of any risk allele with gene expression in postmortem human brain. We assume that many hundreds if not thousands of significant, and in some cases large effect size, associations will prove to exist between SNPs and gene expression; establishing this empirically will be of major value for basic neuroscience as well as for schizophrenia. Finally, although postmortem brain studies are, in our view, essential for identifying the molecular consequences of genetic variation, including the molecular pathogenesis of schizophrenia, they need to be better integrated with other approaches. First, they need to be linked with the findings of neuroimaging and other correlates of allelic variation. For example, in the case of the coding DISC1 polymorphisms mentioned: what connects their effects on transcript abundance (53), centrosomal PCM1 (56), cortical morphology (73), and hippocampal activity (74)? Second, the complementary use of model systems (ranging from in peripheral cells to in vitro assays to humanized transgenics) is essential to test critically hypotheses that emerge from the postmortem studies. Again, using DISC1 as an example, there are many compelling experimental studies showing pleiotropic effects of normal or mutant DISC1 on cellular function, cortical development and plasticity, yet it remains difficult to know their direct relevance to the actual abnormalities of DISC1 expression or function that have been identified in schizophrenia.

Conclusions

Ole Hornykiewicz's finding of decreased dopamine in the postmortem striatum of patients with Parkinson's disease ushered in the modern era of neuropathology (75). The conventional case-control group study design has continued to serve our field quite well for the subsequent 50 years, but unfortunately, the fundamental importance of the discovery (75), and its immediate therapeutic implications, has not been repeated for schizophrenia nor any other 'functional' psychiatric disorder. Whilst searching for the molecular, neurochemical, and morphological, correlates of schizophrenia remains important, postmortem brains coupled with cell culture and animal models can now also be used to investigate the genetic mechanisms underlying the disorder. As the data summarized in this review make clear, they are already proving remarkably valuable in this respect. The fact that the transcripts associated with genetic risk variants appear often to be novel, and tissue and species specific, means that human brain becomes an integral, irreplaceable resource in the undertaking – and brain banking of psychiatric patients and controls must be reinvigorated, prioritized, and supported financially (76). We suggest that further implementation and development of genetic neuropathology will advance our understanding of the etiology and pathogenesis of schizophrenia and other major mental illnesses. It is also likely to lead to identification of novel potential therapeutic targets, which in turn may be realized in the advent of more effective treatments. If and when these outcomes occur, postmortem brain studies of schizophrenia can be said to have made the same fundamental impact as they have in Parkinson's disease and other neurodegenerative disorders.

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