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Three autism candidate genes: a synthesis of human genetic analysis with other disciplines

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Abstract

Autism is a particularly complex disorder when considered from virtually any methodological framework, including the perspective of human genetics. We first present a review of the genetic analysis principles relevant for discussing autism genetics research. From this body of work we highlight results from three candidate genes, *REELIN* (*RELN*), *SEROTONIN TRANSPORTER* (*5HTT*), and *ENGRAILED 2* (*EN2*) and discuss the relevant neuroscience, molecular genetics, and statistical results that suggest involvement of these genes in autism susceptibility. As will be shown, the statistical results from genetic analysis, when considered alone, are in apparent conflict across research groups. We use these three candidate genes to illustrate different problems in synthesizing results from non-overlapping research groups examining the same problem.

However, when basic genetic principles and results from other scientific disciplines are incorporated into a unified theoretical framework, at least some of the difficulties with interpreting results can be understood and potentially overcome as more data becomes available to the field of autism research. Integrating results from several scientific frameworks provides new hypotheses and alternative data collection strategies for future work.

Keywords: Autism; Genetics; Complex disease; Linkage Analysis; Linkage disequilibrium

1. Introduction

This review details how a candidate gene approach can be used to identify genes that contribute to autism spectrum disorder (ASD) susceptibility. However, due to the intrinsic complexity of ASD inheritance, the evidence required to demonstrate that a gene is an ASD susceptibility locus is difficult to attain. To illustrate these points, this review focuses on three genes, *REELIN* (*RELN*), *SEROTONIN TRANSPORTER* (*5HTT*), and *ENGRAILED 2* (*EN2*) that have displayed significant association to ASD. The human genetic analysis for these three genes is apparently conflicting with some studies exhibiting significant results while others do not. These genes illustrate the inherent difficulties in studying polygenic disorders such as ASD and highlight the issues of which any researcher in the field should be aware.

1.1. The interplay between neuroscience and genetics

The goal of human genetic studies is to define the relationship between genotype and phenotype. For a

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Fig. 1. The goal of human genetic studies is to define the relationship between observed characteristics (phenotype) and specific genetic variants (genotype). In principle, this relationship can be direct.

disorder such as autism, the primary purpose of genetics is to identify genes responsible for predisposing individuals to ASD. This common model of human genetics can be expressed in Fig. 1. The model in Fig. 1 uses a bidirectional arrow to indicate the two routes one may take to identify genes. The reverse genetics paradigm starts with a sample of phenotypically characterized families and then tests various genetic locations across the genome to detect possible linkage between the anonymous genetic markers and the phenotype. Such reverse genetics is typically carried out using some form of *linkage analysis*, a technical term referring to the statistical tests used. Alternatively, one may start with a polymorphic site in a gene and then test it for association with the phenotype (forward genetics). The forward genetics paradigm is restricted by our current knowledge of genes and their functions, which will represent only a fraction of the known and hypothetical genes in the genome. Forward genetics is commonly carried out in the form of candidate gene studies or *association studies*. For example, an association between a polymorphism in/around a gene and a disease can be established by showing that the polymorphism is more common in affected individuals than unaffected controls.

While reverse genetics may implicate a region of the genome for further analysis based on anonymous genetic markers, forward genetics provides a hypothetical role for a gene in disease pathology *before* any analysis has taken place. For this reason, it is a vital role of physiological, biochemical, or developmental studies of the CNS to identify biologically relevant candidates to test for association. However, given the genetic complexity of ASD, even if statistically significant associations are observed for proposed candidate genes, additional criteria are needed to further confirm that the locus acts as an ASD susceptibility locus. One of the goals of this review is to consider the human genetic, phenotypic and functional data *jointly*, and synthesize rational considerations based on all of the evidence as a whole. In Fig. 2, function has not been placed merely as an intermediary between the genotype and the phenotype relationship, but rather has been integrated as a key component interacting with both phenotypic and genetic data and aiding in interpretation. Under this model, the functional effects of genetic polymorphisms that are associated with ASD need to be evaluated in light of data from standard molecular techniques using autopsy samples, cell lines, and even mouse models. In this way, we are not relying solely on human genetic data to make complex decisions regarding the involvement of candidate genes in ASD, but are synthesizing a broader framework that allows the incorporation of different kinds of evidence into the process of evaluating the likely relationship between genes and disease.

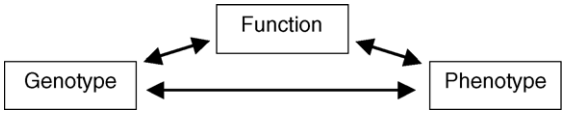

Fig. 2. Functional knowledge from molecular biological and genetic research may be incorporated into models defining the relationship between genotype and phenotype. This can help define what genotypes to examine for disease effects and what aspects of the phenotype are likely most relevant for genetic studies.

Throughout this review genetic, statistical, and neuroscience concepts will be considered jointly, rather than separately. Evidence is evaluated in terms of the overall theoretical framework and how new data change the net strength of evidence for the theory as a whole, rather than using only one type of scientific data to make complex decisions. It is our view that, in practice, scientists typically use this framework for making decisions when all of the relevant data are presented.

1.2. Clinical aspects of autism

Autism was initially defined over 60 years ago (Kanner, 1943), with the term ‘‘autism’’ specifically referring to the child’s propensity to exist in his/her own world or focus internally. Autism is clinically defined as a collection of symptoms in the areas of communication, social interaction, and stereotyped behaviors or interests that are apparent in the first 3 years of life. We use the term idiopathic autism to mean that the diagnosis cannot be accounted for by other conditions (such as Fragile X, Rett syndrome or Tuberous Sclerosis). Autism is diagnosed based on the presence of certain behaviors, either directly observed or more typically reported during parental interview. The presentation of two subjects with autism (the same diagnosis) can vary widely in the three diagnostic areas and overall level of functioning. Incidence estimates range from 1 to 15 per 10,000 with a prominent 4:1 male to female affection ratio that is unexplained at present (Fombonne, 1999).

1.3. Genetics of autism

Autism has a genetic basis evidenced by twin studies, and numerous groups have undertaken the search for susceptibility genes (Folstein and Rosen-Sheidley, 2001; Badner and Gershon, 2002). Twin studies have shown that while the MZ concordance rate for a strict diagnosis of autism is only 36%, the co-twin commonly has cognitive deficits, usually involving language delay. Using a more liberal definition of autism spectrum diagnosis that includes language delay raises the MZ concordance rate to 82% (Folstein and Rutter, 1977; Steffenburg et al., 1989; Bailey et al., 1995). Genetic linkage studies of autism have suggested several locations

where susceptibility genes may reside, including chromosomes 2q and 7q (International Molecular Genetic Study of Autism Consortium, 1998, 2001a,b; Philippe et al., 1999; Risch et al., 1999; Buxbaum et al., 2001; Collaborative Linkage Study of Autism, 2001a,b; Shao et al., 2002a,b; Gallagher et al., 2003). These loci at 2q31 and 7q21–36 have been suggested by multiple groups and convergence of these linkage results on the same chromosomal regions in independent samples suggests their involvement in the etiology of autism.

The region on chromosome 7q21–32 was first implicated in an autism linkage study of 83 sib pairs by the International Molecular Genetic Study of Autism Consortium (1998), with evidence in support of this region remaining high after adding an additional 69 sib pairs (maximum multipoint LOD score (MLS) 3.55) (International Molecular Genetic Study of Autism Consortium, 2001b). Several other groups have also suggested linkage to the same region (Ashley-Koch et al., 1999; Philippe et al., 1999; Collaborative Linkage Study of Autism, 2001b; Shao et al., 2002b). This region was initially suspected to contain a language-related autism gene by coincident linkage with the *SPCH1* locus, which has since been cloned (*FOXP2*) (Lai et al., 2001). However, studies have failed to detect defects in *FOXP2* that are associated with autism, though one group demonstrated linkage to the region (Newbury et al., 2002; Wassink et al., 2002; Gauthier et al., 2003). Some evidence for linkage to the more distal 7q36 region has also been obtained by two studies using different methodological approaches and the Autism Genetic Resource Exchange (AGRE) family samples. The Autism Genetic Resource Exchange was created by Cure Autism Now (CAN) to advance genetic research in autism spectrum disorders. Genetic biomaterials and clinical data are obtained from families that have more than one family member diagnosed with an ASD. The biological samples, along with the accompanying clinical data, are made available to AGRE-approved researchers. Liu et al. (2001) conducted fine mapping analysis of the chromosome 7q region in 110 AGRE families and reported a LOD score of 2.13 for the marker *D7S483* at 7q35–36. In a further study, Alarcon et al. used the same set of microsatellite markers and 152 AGRE families overlapping with Liu et al. (2001) to map quantitative trait loci implicated in language deficits to chromosome 7q36 ($P = 0.001$) (Alarcon et al., 2002).

Chromosome 2 is a second location with converging evidence for linkage to a language/autism phenotype. The IMGSAC sample yielded a multipoint MLS of 4.8 using a narrow diagnosis phenotype (International Molecular Genetic Study of Autism Consortium, 2001b). This region has also been examined through stratification of two independent samples based on whether both probands displayed onset of phrase speech later than 36 months (phrase speech delay or PSD) (Buxbaum et al., 2001; Shao et al., 2002a). In both samples, the evidence for linkage increased in the PSD group. In Buxbaum et al., (Buxbaum et al., 2001), the peak linkage finding overlapped the IMGSAC result (International Molecular Genetic Study of Autism Consortium, 2001a), while in Shao et al., (Shao et al., 2002a), the peak was ~15 cM away (which may be close enough to represent the same locus).

While 7q21–36 and 2q31 appear to be strong candidate regions, these loci derived from gene linkage analysis are not representative of autism linkage studies as a whole. Most of the linkage findings do not converge on the same locus, with non-replication being the rule rather than the exception. However, the non-replications seen in the majority of linkage findings do not necessarily imply that all single linkage findings are not justified. Several possible explanations for the consistent non-replication at other loci may be postulated, and we shall consider several of these complications next.

1.4. Environmental factors

Interaction between genes and the environment can take on numerous forms, ranging from binary factors that exert a fixed effect to quantitative gradations of both exposure and effect. Consider the simple case of a binary exposure (such as receiving a hit on the head) that always leads to disease when the subject has the −/− genotype, but where the −/− genotype does not lead to disease without the environmental exposure. This interaction implies that regardless of the frequency of the "−" allele in either a sample of affected or unaffected individuals, the only subjects for which this allele is relevant are those who have had a hit on the head. If hits on the head were rare, then detecting the effect of this allele would be exceedingly difficult and potentially impossible using standard association methods that fail to account for this fact. While seeking the relevant environmental information is a daunting task at best, it is to be expected that some genes are rendered undetectable by searching in samples with none or very few subjects that have the relevant environmental exposures. Thus, if environmental factors are important in expressing the disease phenotype and these environmental factors vary between populations/samples, then different linkage results would be expected between datasets.

1.5. Multiple genes

If multiple susceptibility alleles at several loci are necessary to cause the disease, then at least some of those genes must have a modest effect. Consider a simple hypothetical case of five susceptibility genes that do not interact with each other but additively contribute to disease susceptibility in a single individual. Each gene may contribute any amount of liability for the disease, but the sum total cannot be greater than 100% of the genetic contribution. If one gene were responsible for 96% of the liability, the other four genes could account for no more than 4% cumulatively, and thus would be hard to detect. Indeed,

all the genes may have a relatively low effect size unless the proper environmental exposures are present (see above) and/or the proper genetic background is present (gene–gene interactions, or epistasis). In principle, multiple genes of low effect size may require large sample sizes to detect, but even a large study is in no way assured of being successful (see next). If there are, in fact, numerous necessary autism susceptibility loci, then replication of linkage or association findings will be challenging due to low effect sizes at many of the loci.

1.6. Heterogeneity

Assuming no environmental interactions and that only one gene is required for the phenotype under study, it is still possible that the phenotype is not caused by the same gene in every family. Such genetic heterogeneity is a well-established phenomenon in simple Mendelian disorders, and may very well be a part of complex diseases. If only 10% of the families with a disorder have disease allele A′ at gene A, then on average only 10% of a random sample of affected individuals will have allele A′. Given the rather large number of unlinked families in a sample (approximately 90%), it would be very hard to detect an effect of gene A. Autism may have multiple independent genetic causes as already indicated by various cytological anomalies, such as translocations and inversion/duplication events that lead to a diagnosis of autism, as well as the existence of genetic disorders with known causes, such as Fragile X, Rett syndrome, and the tuberous sclerosis complex, each of which may produce a disorder that receives the clinical label of autism.

Autism is more complex than a simple one-gene Mendelian disorder and has several, possibly non-overlapping, genetic mechanisms. Autism does not have a simple segregation pattern indicative of either an autosomal dominant or recessive mode of inheritance. Furthermore, the 4:1 male/female affection ratio cannot be accounted for by a simple sex-linked gene.

Additionally, studies indicate possible heterogeneity within the US population, since no one genomic location has yielded even relatively uniform evidence for linkage across families. Therefore, autism appears to have at least several different susceptibility loci. However, since each family is raised in a separate shared environment, the influence of factors such as education, nutrition, and environmental exposures (to name a few) could be modulating the apparent effects (penetrance) of any given autism susceptibility locus, despite the same alleles being present across families. The gene-environment interaction of susceptibility loci cannot be directly studied until at least one gene is cloned.

The hypothesis of genetic heterogeneity is fully consistent with the available phenotypic data on children with autism. A diagnosis of autism is based on a wide range of behavioral markers that can vary tremendously between individuals. Thus, such diagnostic criteria may not be effective for producing genetically homogeneous samples. While phenotypic heterogeneity does not necessarily imply genetic heterogeneity, the breadth of phenotypic variation, which cannot solely be accounted for by any one etiological theory, and the linkage findings converge on the same conclusion—that autism is not a unitary phenomenon.

There exist several possible methods for dealing with heterogeneity. Linkage studies have statistics that allow for some families to be linked and others to be unlinked to a test locus. While these statistical methods are important, such methods cannot be used to unambiguously assign every family's linkage status without further information. Some researchers use endophenotypes, measures of a disease component or associated trait that can take on meaningful values apart from disease status, to define more homogeneous groups of subjects. As such, autism studies have typically used phenotypic characteristics to subset their sample. The Autism Diagnostic Interview—Revised (ADI-R; Lord et al., 1994) provides phenotypic data from the three domains of autism including language acquisition (such as onset of phrase speech) and stereotyped behavior. For example, as mentioned in the introduction, several groups used severe phrase speech delay (onset $>$36 months for children at least 4–5 years old) to define phenotypic subsets of their sample. Subsetting presumably creates a more homogeneous sample for analysis. Although these approaches are useful in reducing genetic heterogeneity, their utility is decreased when environmental factors and multiple disease genes are present because subsets necessarily reduce the sample size for a given test, with unrecognized environmental factors (and/or genes of low effect) acting to further reduce the ability to detect genes.

1.7. Association studies

Studies of candidate genes typically conduct association, or linkage disequilibrium (LD), analysis, using samples of either small families or unrelated individuals. Regardless of the specific sample type used, association analysis attempts to detect or quantify the correlation between two loci. Since observed recombination between two loci will decrease as genetic distance between the loci decreases, it is possible that over very short genetic distances few or no recombination events will be observed, even in a large data set. This lack of recombination gives rise to a correlation between alleles at the two loci involved. This correlation is known as linkage disequilibrium. While genetic linkage analysis requires families in order to observe recombination events, LD analysis can be performed on two sets of unrelated persons (a case–control study) or in a minimal pedigree set of parents and only 1 child (known as trios), where the child has the trait under study. The amount of LD observed between loci varies greatly between populations and genomic regions, but is generally only useful for detecting

association over very short genetic distances (Crawford et al., 2004; Stenzel et al., 2004).

In case–control studies, association is declared if the allele frequencies at a genetic polymorphism near or within the candidate locus are found to be significantly different in the cases and controls. An excess of a particular allele in the case sample is indicative of that allele being associated with increased risk for the disease. Family based studies declare association if one allele is transmitted from a parent to the affected child more often than expected by chance. Results are reported using the traditional *P*-value. No association analysis design can distinguish between an allele that actually increases risk and a benign allelic variant that is in LD with such a risk allele. Hence, evidence for an association always implies that either the associated allele is involved in the etiology of the disorder or that the associated allele is very close to the real risk allele (or perhaps that the result is a false positive).

Particularly, problematic for association studies (but not genetic linkage) is the assumption that the tested variant is in LD with the causative allele (or is the causative allele). Just as different genes may be responsible for causing the same disease in different individuals (locus heterogeneity, as described above), different alleles of the same gene may confer susceptibility to the same phenotype, which is called allelic heterogeneity. The strength of LD is a function of ancestral time since the mutation occurred. Once a mutation occurs, all the allelic variants nearby will be in strong LD with the mutation for many generations. However, if two different mutations within the same gene confer susceptibility to a disease, each in LD with a different set of allelic variants, then the observed LD between either mutation (or set of associated allelic variants) or the disease will be reduced in much the same way locus heterogeneity reduces the observed linkage between multiple non-interacting disease loci (Slager et al., 2000; Pritchard and Cox, 2002). However, in the absence of allelic and locus heterogeneity, association analysis does have the potential to detect susceptibility alleles of much smaller effect size than does linkage analysis.

The *P*-value is doubtless the most commonly used statistical measure in science. The *P*-values are typically invoked in statistical applications since they are: (1) traditionally required by journals; (2) easy to calculate exactly for a wide variety of typical scientific applications; and (3) impose a simple answer for complex decisions, which gives *P*-values a very practical use. The *P*-value is the probability of observing a test statistic as large as or larger than the observed test statistic under the null hypothesis (in this case the hypothesis of no association). However, *P*-values must be interpreted with caution. A *P*-value is not a measure of effect size as studies with very large sample sizes are almost sure to produce significant *P*-value due to very small deviations from the null hypothesis that, while statistically significant, are of little biological importance (Rosenthal and Gaito, 1963). At the other extreme, when power is very low, use of a *P*-value may be misleading because failure to reach statistical significance may be due to overall low predictive value and not the absence of a real effect (Sellke et al., 2001). Since the estimation of power requires knowledge of the true circumstances generating the data (the underlying model), which is unknown in scientific applications, one cannot assume that *P*-values are misleading due to low power only in studies with small sample sizes. This represents a major difficulty in the proper interpretation of *P*-values. Further, testing numerous candidate genes in the same sample exacerbates the inherent difficulty of using *P*-values to make decisions, as these multiple tests will increase the probability that at least one of the tests conducted will meet the criteria for significance by chance. Additionally, non-significant *P*-values cannot be interpreted as rejecting the hypothesis of association and are therefore not necessarily in conflict with significant *P*-values obtained by another study. With these caveats in mind, we shall consider in some detail data from three candidate genes for autism susceptibility.

2. *RELN* Polymorphisms

Reeler is a classical mouse mutant that perturbs the development of several laminar structures in the brain, including the cortex, cerebellum and hippocampus. *Reelin* encodes a secreted protein that binds to very low density lipoprotein (VLDL) and ApoE receptor 2 (ApoER2) complexes to coordinate neuronal migration during CNS development. The cloning of other mouse mutants and human Mendelian disorders that share phenotypic similarities with the *reeler* mutant has led to a more complete understanding of the molecular pathways that regulate neuronal migration during CNS development.

The human *REELIN* (*RELN*) gene was selected as a candidate for autism association studies for two reasons. First, *RELN* maps to 7q22, which is a region of the genome that has displayed significant linkage to autism in five separate genome scans (International Molecular Genetic Study of Autism Consortium, 1998, 2001a,b; Philippe et al., 1999; Risch et al., 1999; Collaborative Linkage Study of Autism, 2001a; Liu et al., 2001; Yonan et al., 2003). Second, some aspects of the *reeler* phenotype are similar to anatomical defects observed in individuals with autism (Bauman and Kemper, 1985a; Ritvo et al., 1986; Courchesne, 1997; Levitt et al., 1999; Fatemi, 2001; Fatemi et al., 2002a; Sparks et al., 2002; Salinger et al., 2003). For example, *rl/rl* mice display cerebellar hypoplasia and a reduction in the number of Purkinje cells, two of the more consistent phenotypes observed in individuals with autism.

The *RELN* gene is large, spanning 450 kb with 63 exons. Several polymorphisms have been characterized, but one functional variant has been thoroughly studied for association to autism. A variable length GGC repeat is located in the 5′ untranslated region just prior to the start codon. Longer

alleles have been demonstrated to produce slower rates of protein synthesis compared to shorter alleles.

An association study of this functional GGC repeat was carried out by Persico et al. (2001) using both the case–control method (95 patients and 186 controls, all from Italy) and the family-based transmission disequilibrium test (TDT) on 176 trios from the US and Italy. Both the case–control and the family-based samples displayed significant association of autism to the GGC repeat. In the case–control study, the longer alleles of the GGC repeat (11 repeats or longer) were observed more frequently in the population affected with autism (17/95, or 17.9%) compared to unaffected controls (17/186, or 9.1%) (T1 $\chi^2 = 23.91$, $P < 0.05$). In addition, the frequency of longer alleles was more than double in affected versus unaffected individuals (0.0948 versus 0.0457, T1 $\chi^2 = 22.09$, $P < 0.001$). These results were replicated in the family-based association analysis using a different sample, with the longer alleles being over-transmitted to affected individuals in trio analysis ($P < 0.05$) and a significant distortion in transmission to affected versus unaffected sibs ($P < 0.001$). These data indicate that individuals with autism inherit the longer alleles of the GGC repeat more frequently than one would expect by chance.

Zhang et al. (2002) followed-up on this finding by testing for association in 126 multiplex families. They performed linkage analysis in families with at least two siblings affected with autism and did not find evidence for linkage. When single patients were selected from each family and compared to a control group, the frequencies of the longer alleles of the *RELN* GGC repeat were not significantly different between the two groups. However, when the family based association test (FBAT, a method that uses both sibs when testing for association) was performed, there was statistically significant overtransmission of the longer alleles to affect children ($P = 0.035$). The authors suggest that the FBAT may be a more sensitive test than either linkage or case–control analyses, and concluded that *RELN* was involved in at least some cases of autism.

However, not all studies have replicated the initial findings. Krebs et al. (2002) tested 167 trios using the TDT and observed no association of the longer alleles with autism (Krebs et al., 2002). Bonora et al. (2003) tested 169 multiplex families used in the IMGSAC genome scan that displayed significant linkage to 7q22 as well as an additional 122 trios for association to *RELN* (Bonora et al., 2003). Neither sample separately nor in combination displayed any association with the longer repeat alleles. Furthermore, when patients were subset on clinical criteria defining the presence or absence of language delay the results were still non-significant.

Despite the logical choice of *RELN* as a candidate gene, the results of the human genetic analysis appear to be conflicting. Should we therefore conclude that *RELN* is not involved in autism? All of these results, including the apparent discrepancies, can be accounted for by the complex genetics of autism presented more abstractly in the introduction. For example, both Kerbs et al., and Bonora et al., noted that the frequency of the longer alleles was statistically different between their populations and the previous one studied by Persico et al., which could account for the different results.

Nevertheless, the effect size of the GGC repeat appears to be small. For example, Persico et al., noted that the long repeats are present in only 20% of their entire sample, which may indicate that this *RELN* variant plays a role in only a small percentage of cases of autism (Persico et al., 2001). In addition, Bonora et al. screened the exons, promoter, and 3′ UTR of *RELN* in individuals with autism selected from the IMGSAC families contributing to the linkage to 7q22 (Bonora et al., 2003). Nonconservative mutations in highly conserved portions of the *RELN* sequence were identified in these individuals but were not present in control families. The rather small number of cases with these presumably functional mutations makes further analysis difficult, but is suggestive that *RELN* is involved in autism, though the authors conclude that this set of variants could not account for their previously published large linkage finding to 7q22 (International Molecular Genetic Study of Autism Consortium, 1998, 2001a,b). It is this last piece of evidence that is perhaps the most informative about the role of *RELN* in their sample. If *RELN* really were the cause of the large linkage findings on 7q31–32, it would be expected to have displayed a larger effect size in the association study. However, it must be noted that only a small percentage of the total genomic extent of *RELN* was screened for mutations, so the presence of multiple different risk alleles located in non-coding portions of the gene cannot be excluded.

2.1. Biochemical studies

While the human genetic analysis is difficult to interpret, both molecular and developmental studies support the involvement of the *RELN* pathway in ASD. For example, reduced levels of RELN protein have been observed in five cerebellar cortex autopsy samples as compared to eight unaffected controls that were matched for age, sex and post-mortem interval (Fatemi et al., 2001). Several different protein species exist for RELN (410 kDa, 330 kDa, and 180 kDa) but all of them displayed a 43–44% reduction in individuals with autism. This finding was replicated in part by measuring RELN blood protein levels in families with monozygotic siblings diagnosed with autism (12 twins, 1 concordant quadruplet) from the Autism Genetic Resource Exchange and in eight controls (Fatemi et al., 2002b). A significant reduction of the 410 kDa species was found in the parents as well as siblings with and without autism ($P = 0.0402$) in all the families, while the 330 and 180 kDa isoforms displayed either no reduction (330 kDa) or a slight reduction in parents (180 kDa) ($P < 0.05$). This difference between individuals could be due to genetic heterogeneity or different genes in the same pathway affecting different populations. Nevertheless, these data provide additional

evidence that the *RELN* pathway is misregulated in families with a predisposition to autism and suggest that this same misregulation could occur during CNS development.

2.2. Neurodevelopmental studies

Phenotypic similarities between the cerebellar and cortical abnormalities of *reeler* mice and individuals with autism also support a role for the *RELN* pathway in autism. Individuals with autism consistently display cerebellar hypoplasia and a reduction in Purkinje cell number, phenotypes also observed in *reeler* mice. Further, individuals with autism also display other phenotypes in the cortex and cerebellum, such as an increased density of microcolumns in the cortex as well as an initial accelerated growth pattern in both the cortex and cerebellum.

However, there are important differences between the *reeler* mouse and autism in humans. For example, the *reeler* mouse is well known for lamination defects in both the cortex and cerebellum that are not observed in autism. In addition, human Mendelian disorders (Lissencephaly syndrome, Norman–Roberts Type; Lissencephaly, X-linked) that affect the *RELN* pathway perturb neuronal migration and cause lissencephaly, mental retardation and epileptic seizures but do not exhibit the neuropsychiatric symptoms of ASD.

Although these differences are profound, they can be easily explained by inherent genetic differences between the *reeler* mouse and individuals with ASD. For example, none of the mouse *reelin* alleles are missense or cis-regulatory mutations. Instead, they are either insertions or intragenic deletions that severely disrupt the generation of Reelin mRNA and protein. The GGC long repeats alleles seen in humans are clearly less severe variants of the gene. This difference in severity could cause the milder cortical and cerebellar phenotypes observed in individuals with autism. For example, if a mouse mutant was generated with *RELN* sequence changes associated with autism in humans, it is possible that these mice would display milder cortical and cerebellar phenotypes more similar to autism.

Alternatively, the discrepancy in anatomical phenotypes between the *reeler* mouse mutant and individuals with autism could be due to differences in monogenic (*reeler*) and polygenic (ASD) inheritance. For instance, ASD is likely to be due to several genes, each of which potentially affects CNS development. Since the *reeler* mouse is due to severe mutations in a single gene, a strict phenocopying of the autism phenotypes in these mouse mutants may not entirely be expected. Instead, only some of the autistic anatomical phenotypes like cerebellar hypoplasia might be observed.

2.3. What can be concluded about RELN?

Given the functional variant (the 5′ GGC repeat) and the neurodevelopmental function of RELN, as well as the biochemical evidence that directly links RELN levels with autism, further studies of *RELN* or other genes in the *RELN* pathway are required. For example, more variants in and around *RELN* need to be examined, perhaps in a promoter region or a long range transcription enhancer located some distance away. In addition, since the changes in brain development and RELN levels could also be due to other genes in this pathway, their possible association to ASD also needs to be investigated.

3. Serotonin transporter gene (*5HTT*) polymorphisms

The 5HTT protein is a transmembrane spanning molecule with high affinity for the neurotransmitter serotonin (5-HT) that transports 5-HT across the membrane via a sodium dependant mechanism, located on pre-synaptic terminals. The serotonergic neurotransmitter system represents a logical candidate for autism pathology based on converging evidence from diverse types of studies. Hyperserotonemia (elevated serotonin levels) has been observed in both whole blood and urine samples from patients with autism (Anderson et al., 1987, 1989; Cook et al., 1993). In addition, tryptophan depletion in individuals with autism has been found to increase autistic behaviors, and more recently brain imaging studies suggest that serotonin synthesis rates are abnormal in individuals with autism (Chugani et al., 1997, 1999; Muller et al., 1998). Further, 5HTT is targeted by serotonin specific reuptake inhibitors (SSRIs), a major class of antidepressant drugs. This class of drugs has shown some efficacy for reducing hyperactivity and compulsive and stereotyped behaviors in autism, as determined by double-blind clinical trials (Hollander et al., 2003). Further evidence of the role of 5-HT in autism is the effect of risperidone, a drug that blocks dopamine and serotonin post-synapitcally, which shows efficacy in reducing anxiety in the form of aggression and self-injury in individuals with autism (McCracken et al., 2002; Masi et al., 2003). Finally, first and second-degree relatives of individuals with autism are more likely to exhibit other psychiatric disorders that respond well to SSRIs (Leventhal et al., 1990; Veenstra-VanderWeele et al., 2000).

Located on 17q11, the *5HTT* gene (also known as the *SERT* gene) spans 31 kb of genomic sequence with 14 exons, and has at least 4 confirmed single nucleotide polymorphisms (SNPs) in the non-coding sequence. An insertion/deletion polymorphism (5HTTLPR) located in the 5′ regulatory region has been shown to alter transcription in cell lines (Sakai et al., 2002). The 5HTTLPR polymorphism has two alleles varying in length, termed long and short. The long allele shows a three-fold increase in basal activity as determined by a luciferase assay in cell lines (Heils et al., 1997; Sakai et al., 2002). Although *5HTT* was initially identified as functional candidate gene, a recent genome scan in 345 multiple incidence families from AGRE implicated 17q11 as the strongest linkage finding (Yonan

et al., 2003). This was the first genome scan to implicate this region.

3.1. Association studies

Cook et al. (1997) performed TDT analysis on 86 families and tested for association with a polymorphism in intron 2 and 5HTTLPR, finding the 5HTTLRP polymorphism TDT was significant ($P = 0.03$) (Cook et al., 1997). Examination of 5HTTLPR and the intron 2 polymorphism as a haplotype was also significant ($P = 0.018$). In both cases, the short allele was demonstrated to associate with autism. This result has recently been replicated in an Irish population ($P = 0.03$) (Conroy et al., in press). Multi-SNP haplotype analysis also yielded significant results with the short allele. However, Klauck et al. (1997) published the opposite result. TDT testing using 52 trios (with language delay) and an additional 13 trios (without language delay) indicated an over-transmission of the long allele, which reached statistical significance when the total sample (both groups) was analyzed ($P = 0.032$). The association of the long promoter allele with autism has recently been replicated by two groups (Yirmiya et al., 2001; Kim et al., 2002). In this last study, 22 polymorphisms in and around *5HTT* were investigated in 115 trios. TDT analysis of each marker yielded significant results for 11 of these markers, which included 5HTTLPR. These results suggest that 5HTTLPR may not be the most important of the polymorphisms in *5HTT*, but is merely in LD with a higher risk allele.

However, not all studies have demonstrated association of *5HTT* to ASD. The International Molecular Genetic Study of Autism Consortium (1998) conducted TDT analysis of 90 families analyzed as trios and nuclear families (using nuclear families introduces an upward bias in the test of association, see (Hodge, 1993, 1994)). No evidence for overtransmission was found in the entire sample or subsets based on gender, autism versus PDD, or the presence or absence of language delay. Similarly, Persico et al. (2000) found no evidence for association when using 98 trios (44 from the US and 54 from Italy) analyzed by the TDT and a related test, the haplotype-based haplotype relative risk test HHRR (Persico et al., 2000). These different results could reflect the variances in genetic and environmental factors described earlier that affect all these studies.

3.2. What can be concluded from the analysis of 5HTT?

Although the serotonergic system is a compelling candidate for autism, implication of such a diffuse network does not imply a pathological mechanism with any specificity. While *5HTT* appears to have a role in autism, there still remain numerous other proteins in the serotonergic system that have not yet been examined. There are at least 14 5-HT receptors (located post-synaptically), only four of which have been tested for association with autism, and none have provided compelling evidence for association (Lassig et al., 1999; Veenstra-VanderWeele et al., 2002). However, if serotonin transport/reuptake really is an etiological key, it should be noted that other proteins are capable of transporting serotonin across the membrane with lower affinity than 5HTT, such as the organic cation transporters OCT1 and OCT3, and possibly OCT2 (Schmitt et al., 2003).

It is apparent from this discussion that study of the role of the serotonergic system in the genetics of autism is far from complete. As such, serotonergic gene variants require further investigation for the rigorous evaluation of the 5-HT hypothesis of autism.

4. ENGRAILED 2 gene (*EN2*) polymorphisms

Another functional and possibly positional candidate gene of interest is the *ENGRAILED 2* gene (*EN2*). *EN2* is a homeobox transcription factor homologous to the Drosophila melanogaster *engrailed* gene. *EN2* is a developmental patterning gene that has been shown to have an essential role in both the embryonic and post-natal development of the mouse cerebellum (Millen et al., 1994, 1995; Kuemerle et al., 1997; Baader et al., 1998, 1999). There are a number of neurophysiologic lines of evidence from autopsy, histological, and imaging studies that suggest a role for genes involved in cerebellar development in the pathophysiology of autism. Cerebellar abnormalities include general hypoplasia and a reduced number of Purkinje cells. These defects occur in the absence of obvious signs of degeneration, suggesting instead that they are caused by developmental defects (Bauman and Kemper, 1985b, 1986, 1994; Ritvo et al., 1986; Kemper and Bauman, 1993; Courchesne, 1997; Bailey et al., 1998). The growth pattern of the cerebellum during early childhood in individuals with autism has also been shown to be abnormal. Initially, cerebellar growth is accelerated compared to unaffected controls, but then declines after age six (Courchesne et al., 2001, 2003). Furthermore, functional MRI studies have demonstrated that the cerebellum is active during tasks that are deficient in autism spectrum disorders, including language generation, attention, and problem solving (Courchesne et al., 1994; Kim et al., 1994; Gao et al., 1996; Akshoomoff et al., 1997; Allen et al., 1997; Courchesne and Allen, 1997; Allen and Courchesne, 2003; Corina et al., 2003; McDermott et al., 2003). Together, these studies demonstrate that cerebellar development is perturbed in autism and that these defects might contribute to the behavioral abnormalities observed in autism spectrum disorders.

A role for *EN2* in autism was proposed based on mouse genetic studies. Both an *En2* knockout (loss of function) as well as a transgenic mouse that causes the misexpression of the gene have been generated. Both types of mutants display a phenotype that is reminiscent of the cerebellar anatomical abnormalities reported for individuals with autism. Adult

mice for both mutants are non-ataxic, but their cerebella are hypoplastic, with a reduction in the number of Purkinje cells and other cell types (Millen et al., 1994, 1995; Gathercole, 1995; Kuemerle et al., 1997; Baader et al., 1998, 1999). Both mutants also disrupt the topographic mapping of spino-cerebellar mossy fibers (Vogel et al., 1996; Baader et al., 1999), which could in turn affect the electrophysiological function of the cerebellum. Together, these data suggest that functional variants in human *EN2* that affect either the level or the spatial/temporal expression of the gene during human cerebellar development might contribute to the anatomical cerebellar phenotypes observed in autism.

The *EN2* gene maps to human chromosome 7q36.3 and is composed of 2 exons separated by a single intron spanning only 8 kb of DNA. The distal localization of the *EN2* gene has meant that most genome scans have not included markers that span this locus, complicating the assessment of the evidence for linkage to this region. Although *EN2* lies outside the 7q21–32 region implicated by most studies, three studies from two independent genome scans have provided some evidence for linkage to this region (Liu et al., 2001; Alarcon et al., 2002; Auranen et al., 2002). Only one of these studies used markers that spanned the *EN2* locus. In this Finnish report, suggestive linkage to a combined phenotype of autism spectrum disorder and dysphasia was obtained at marker *D7S550* (LOD = 2.02), located about 170 kb distal of *EN2* (Auranen et al., 2002). The other two studies were carried out using a subset of the AGRE families. Liu et al. (2001) carried out fine mapping analysis of the region proximal to *EN2* in 110 AGRE families and reported a LOD score of 2.13 for *D7S483*, located approximately 5.5 Mb proximal of *EN2*. In a further study, Alarcon et al. (2002) used the same set of microsatellite markers and 152 AGRE families to map a quantitative trait locus implicated in language deficits to distal chromosome 7 at a distance of less than 1 Mb from the *EN2* locus ($P = 0.001$). Recently, Yonan et al. (2003), performed a genome scan on the complete set of 345 AGRE families and observed only minimal linkage at distal chromosome 7 (LOD < 1.3). However, the fine mapping markers used previously were not included in this latest analysis, so it appears the markers used did not span the *EN2* locus. The reduced evidence for linkage to this region could be interpreted as either suggesting that the initial findings were spurious false positive results or, once again, highlighting the complexity of the underlying model as discussed above for the *RELN* gene.

4.1. Association studies

There have been three studies to date that have investigated *EN2* as an autism susceptibility locus. The first was a French case–control study published in 1995 (Petit et al., 1995) and the two more recent reports were family-based association analyses, both conducted using overlapping subsets of the AGRE families (Zhong et al., 2003; Gharani et al., 2004). In the report by Petit et al., the allele frequency of two markers from the *EN2* gene were compared in 100 cases of infantile autism and 100 control children. Significant association ($P < 0.01$) was reported for one of these markers, a *Pvu* II polymorphism located 5′ of the *EN2* promoter. However, no association was found with the *Sst* I polymorphism located within the homeobox domain in exon 2. The LD relationship between these markers was not reported. However, there was a clear difference in the minor allele frequencies for these markers in the control group, suggesting that the lack of observed association between the *Sst* I marker and disease status may in part reflect allele frequency differences between this marker and the disease allele, a factor that is known to affect the power to successfully detect association (Garner and Slatkin, 2003). Overall, the results of this study support a role for *EN2* in autism development.

The second study involved the analysis of a single SNP located in the first exon of *EN2* in 196 multiplex families from the AGRE collection. This variant changes the amino acid specified by codon 121 from a Leu to a Phe and was reported to be approximately 2.9 kb from the *Pvu* II polymorphism studied by Petit et al. Zhong et al. used a variety of analytical methods to examine this variant for association and linkage to autism, including qualitative and quantitative measures of association, as well as non-parametric affected sibpair and QTL linkage analysis. Autism Diagnostic Interview—Revised (ADI-R; Lord et al., 1994) phenotypic data from domains measuring abilities in areas of language acquisition (such as delay in onset of phrase speech—PSD) and stereotyped behavior were also utilized, both in the QTL analyses and for sub-setting of families according to language ability. Overall, no significant evidence for association or linkage was obtained for this marker. However, some evidence for excess allele sharing of paternal alleles amongst affected sibpairs compared to discordant sibpairs was obtained in a subset of families that had developed useful phrase speech. Furthermore, multipoint linkage analysis of merged data of microsatellite markers from the Liu et al., 10cM genome scan (Liu et al., 2001) and the *EN2* marker resulted in nominal evidence for linkage at the *EN2* locus (multipoint NPL score of 1.31; $P = 0.06$), which increased to a NPL score of 1.58 when overall language ability was incorporated in the analysis.

The third and most recent report by Gharani et al. (2004) describes the analysis of four SNPs and includes the exon 1 marker analyzed by Zhong et al. In this study, family-based association analysis was carried out using the transmission/disequilibrium test, initially in 138 parent-offspring triads and subsequently extended to include 167 multiplex AGRE families. Significant evidence for association was obtained for two intronic markers under both a narrow definition of autism as well as a broad diagnosis that included Asperger's syndrome and PDD ($P < 0.002$). No evidence for association was obtained for either of two flanking exonic markers. The lack of association of the exon 1 marker was not

surprising given the dataset overlap between the Zhong et al., and Gharani et al., studies. The latter study demonstrated that the lack of association for this marker is consistent with the weaker LD observed between alleles of this marker and the autism-associated intronic markers. The 5′ *Pvu* II marker was not included in this study, although work to characterize this polymorphism in relationship to the other markers exhibiting LD is ongoing. Understanding the LD relationship between all the markers previously tested for association may clarify the understanding of these data. The study that reported LD to the 5′ *Pvu* II polymorphism was a case–control study, and so, given the nature of the test, is susceptible to a possible false association resulting from population substructure differences between the cases and controls. Alternatively, since patterns of LD across a gene may be quite complex, it is possible that the 5′ *Pvu* II polymorphism will be found to be in stronger LD with the intronic SNPs that have demonstrated association to autism than with the exon 1 SNP that has failed to demonstrate association. It is also important to consider the possible effect of differences in the underlying frequencies of the different tested polymorphisms in different populations. The 5′ *Pvu* II polymorphism may exhibit strong LD to a risk allele in the Petit et al sample, while this same polymorphism may or may not demonstrate LD to the same (or different) risk allele in the AGRE sample.

4.2. What can be concluded from EN2?

Unfortunately, despite the compelling animal model data, relatively little attention has been focused on the role of *EN2* in autism. Little or no human biochemical data is available to add support, and human genetic studies are few and are still ongoing. Although *EN2* is located adjacent to a broad autism linkage region, most linkage studies have failed to include markers that span this locus, and are therefore inconclusive, and only three studies have directly investigated this gene for a role in the etiology of autism. Of these studies, two have provided some evidence for association, though different methodological approaches were taken and different markers examined. It is therefore impossible to make direct comparisons or consider them as direct replications of the same finding. Clearly, further investigation of variants within this gene is required for both genetic as well as functional evidence of involvement in disease etiology.

5. Summary

In this review we have reviewed three candidate genes that appear to be emerging as plausible susceptibility genes in the complex etiology of autism. Our primary aim has been to highlight some important issues that plague all studies of complex genetic traits, namely, that for a given analytical approach, data variability is inevitable, and this variability may be as likely attributed to chance as to the complex underlying model of the disease. Therefore, it is our recommendation that when a candidate gene is to be genetically evaluated for a role in disease susceptibility, evidence should be gathered from complementary fields of study that include neurophysiological, biochemical, and animal model data.

5.1. Replication

Considering the complications listed above, all of which are very likely to operate to at least some degree, replication of positive association findings is not necessarily to be expected. Real and valid findings can get lost in the noise when reality is more complicated than the simple models used to examine it. It has been demonstrated for both linkage and association analysis that effect sizes are overestimated by as much as an order of magnitude when multiple testing over many locations is performed (Terwilliger, 2000; Goring et al., 2001). If the first study produces a statistically significant result, it is more likely than not an inflation of the true effect and therefore suggests an over-optimistic likelihood of replication. When one study shows a positive effect, other subsequent studies are much less likely to replicate even when the initial finding is *real* and hence valuable to the field. Ioannidis et al. (2001) examined the pattern of replication for association studies by using meta-analysis. They studied 36 disorders/phenotypes and examined the outcomes of each follow-up study compared to the first study published (median number of studies for each phenotype was nine). They found examples of non-replication, where the first study was significant but subsequent studies were not (22%), despite the fact that the overall finding was significant by meta-analysis. The reverse also occurred: candidate genes that were not initially significant reached significance when more datasets were added and considered jointly (22%). Given these two extremes in the study of candidate genes, some researchers have questioned if immediate and convincing replication is a wise requirement to impose on the field of complex disease genetics (Vieland, 2001).

Genetic studies must consider issues of heterogeneity, environmental exposures, and multiple (possibly interacting) genes, all of which make replication of real effects difficult when evaluating candidate gene association studies. *5HTT* and *EN2* highlight these difficulties but also provide insight into how we can begin to tip the scale in our favor. The most comprehensive studies of *5HTT* and *EN2* (testing the greatest number of polymorphisms in a suitably large sample) indicate that these genes have a complicated pattern of LD that is not apparent when testing only one or two polymorphisms. An initial and rather small scale study of a candidate gene may be successful in finding an association, but when a subsequent study fails to replicate, it may be due to the fact that both studies have been looking in the wrong place (i.e., the tested polymorphism is not directly involved in the disease but is simply in LD with an allele that is).

The first study may be ''lucky'' in detecting LD as a result of statistical variation or differences in the underlying population genetics of the sample studied, but other samples may not be so ''lucky.'' Only by examining numerous SNPs in a given region can one be sure of the local genetic structure. In the cases of *5HTT* and *EN2*, it appears that interesting findings would have been missed were it not for more thorough investigation. The failure to replicate a finding for one or two candidate gene polymorphisms should not rule out a gene from further investigation. The most convincing examination of a potential candidate gene is a *thorough* examination of a potential candidate gene (i.e., testing numerous variants and characterizing the genomic properties around the gene of interest where relevant). When replications do occur they are important to the field and rightfully generate interest, but failure to replicate candidate gene results do not rule out an interesting and important role for that gene, though its effect may not be large.

The genes *RELN* and *EN2* share a common neural substrate, the cerebellum. While these two genes may or may not interact in the same pathway (there is no available evidence to suggest a direct interaction), the net effect of their genetic variants on cerebellar function may be non-additive (i.e., they interact epistatically). To our knowledge no systematic investigation of this has been carried out in model systems or human genetic data. Should such epistatic interactions exist, it would be particularly informative for both neuroscience and human genetics to examine this issue and re-evaluate the role of these two genes in light of such data.

We have discussed the issues relating to replication in genetic association studies. However, given the presumed complexity of autism genetics, biochemical and molecular studies suffer from the same issues. While most researchers do recognize that autism may be a collection of mechanistically unrelated (or very loosely related) disorders, the difficulty of replicating molecular and biochemical assays using small samples may be quite large. As shown in the RELN protein studies (Fatemi et al., 2001, 2002b), the same effect was not observed in all autopsy samples and the replication results were not exactly the same in the AGRE blood samples. Because of this possible heterogeneity, pooling subjects together and performing a test of means may be quite ineffective for detecting real effects in autism. At best, the estimated effect sizes will be smaller than the real effect size; at worst, the effect will not be demonstrated. Biochemical studies may benefit from slightly larger samples and use of phenotypically well characterized subjects, ideally with some genetic analysis of the gene under consideration. Forming valid subgroups based on all three areas of information (biochemistry, genetics and behavior) in the same subjects may yield hypotheses that are well suited to additional testing. Given the complexity of autism, it may be required for such studies to examine subjects using all three types of information. As one example, it may prove productive for biochemical studies to homogenize their sample based on specific phenotypic characteristics within the autism spectrum rather than rely on the categorical diagnosis of autism. Every study mentioned in this review could potentially benefit from more data on the specific subjects under study. While it is a real challenge to acquire and use numerous sources of data effectively, given the difficulty the autism field has seen thus far, it may be necessary to consistently consider multiple disciplines simultaneously (or jointly).

In summary, we have discussed important issues surrounding the study of complex genetic disorders. Our aim has not been to diminish the value or importance of human genetic data for the understanding complex disease. Rather, we seek to inform the neuroscience and genetics communities of the advantages of synthesizing their data in a way that will help us gain insight into this complex problem. When human genetic studies appear to non-replicate, there may be valid reasons for the discrepancies that are methodological in nature. Is the data really testing the gene with the disorder, or is it merely testing one small part of the gene? Do the genetic data add to the overall hypothesized neurophysiological mechanism, or are the data less focused? In the end, *RELN*, *5HTT* and *EN2* may not be major genes in the etiology of autism, either singly or in concert, but they are important models for pointing out the difficulties in these studies so that advances in understanding the genetic and developmental basis of autism can be attained.

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