Wnt signaling, which encompasses multiple biochemical pathways that regulate neural development downstream of extracellular Wnt glycoprotein ligands, has been suggested to contribute to major psychiatric disorders including autism spectrum disorders (ASD). We used next-generation sequencing and Sequenom genotyping technologies to resequence 10 Wnt signaling pathway genes in 198 ASD patients and 240 matched controls. Results for single-nucleotide polymorphisms (SNPs) of interest were confirmed in a second set of 91 ASD and 144 control samples. We found a significantly increased burden of extremely rare missense variants predicted to be deleterious by PolyPhen-2, distributed across seven genes in the ASD sample (3.5% in ASD vs 0.8% in controls; Fisher’s exact test, odds ratio (OR) = 4.37, P = 0.04). We also found a missense variant in WNT1 (S88R) that was overrepresented in the ASD sample (8 A/T in 267 ASD (minor allele frequency (MAF) = 1.69%) vs 1 A/T in 377 controls (MAF = 0.13%), OR = 13.0, Fisher’s exact test, P = 0.0048; OR = 8.2 and P = 0.053 after correction for population stratification). Functional analysis revealed that WNT1-S88R is more active than wild-type WNT1 in assays for the Wnt/β-catenin signaling pathway. Our findings of a higher burden in ASD of rare missense variants distributed across 7 of 10 Wnt signaling pathway genes tested, and of a functional variant at the WNT1 locus associated with ASD, support that dysfunction of this pathway contributes to ASD susceptibility. Given recent findings of common molecular mechanisms in ASD, schizophrenia and affective disorders, these loci merit scrutiny in other psychiatric conditions as well.

INTRODUCTION

Autism spectrum disorders (ASD) have a strong genetic etiology. The last decade of research in psychiatric genetics has led to groundbreaking discoveries, including the identification of ASD-associated sequence and structural variants in a variety of genes implicated in brain development—particularly in synapse formation, maintenance and function. However, the mutations identified so far explain only a fraction of ASD cases, underscoring the need to continue the search for missing genetic links in ASD.

Wnt intercellular signaling refers to a set of key developmental pathways that help to organize the nervous system. Among other roles, Wnt signaling influences subdivision of the anterior neural tube into major brain regions (such as forebrain, midbrain and hindbrain), neural precursor proliferation, neural cell fate and migration, axon guidance, dendrite development and synapse formation. Given this broad spectrum of roles, it is no surprise that dysregulation of Wnt signaling causes a similarly broad spectrum of deleterious effects on neural development, and largely on this basis it has long been considered a candidate pathway in psychiatric pathogenesis. Its candidacy in this regard is supported by evidence that Wnt signaling pathway components are pharmacological targets of lithium,2,3 hallucinogenic drugs4 and antipsychotic medications,5,6 as well as by evidence derived from behavioral tests in animal models7–11 and from positive associations in scattered human genetic studies.12–17

Here we used massively parallel sequencing technology to simultaneously search for rare variants in 10 Wnt signaling pathway genes in a sample of Caucasian patients with ASD and healthy controls. We selected these 10 genes out of a much larger set of pathway molecules based primarily on empirical support for their individual relevance to psychiatry in human genetic (DKK1, DKK4, NEUROG1, CTNNB1),18–21 animal behavior (DVL1, DIXDC1),2,10 biochemical (WNT1, NEUROD1)22,23 or other types of studies (PRICKLE4, DACT2).24,25 Compared with whole exome sequencing strategies that are now underway, this very targeted approach benefited from our focus on functionally linked loci for which we have substantial expertise, allowing us to immediately follow up the most promising variant in vitro functional assays.

MATERIALS AND METHODS

ASD samples (Caucasians)

Samples were lymphoblastoid cell lines from the Simons Simplex Collection. The diagnosis in these samples was based on the ADI-R (Autism Diagnostic Interview-Revised).26

Unscreened controls (Caucasians)

Healthy control subjects were recruited by the survey research company (Knowledge Networks, Menlo Park, CA, USA) from a nationally representative internet-based panel that was selected by random digit dialing.27 Subjects completed an online version of the CIDI-SF (Composite...
International Diagnostic Interview-Short Form) for lifetime history of anxiety, mood and substance use disorders. Subjects consented to anonymization and deposition of their clinical information and DNA in the National Institute of Mental Health (NIMH) repository for use in any medical research.

Pooling samples

Concentration of individual DNA samples was measured using Pico Green. Based on previous reports using an identical pooling strategy,28,29 we expected sequencing error rates to be \( \sim 0.5\% \). To distinguish a single real mutation allele in \( 876 \) chromosomes (minor allele frequency (MAF) \( \sim 0.11\% \)) from random sequencing artifacts, we pooled samples into \( 11 \) sets of \( 38-40 \) individuals each. Therefore, the frequency of a single mutant allele in each pool was \( 1\% \), significantly above the detection threshold of \( 0.5\% \).

Target enrichment

Each pool of genomic DNA from \( 38-40 \) individuals was enriched by PCR amplification for \( 10 \) genes involved in \( WNT \) signaling (\( WNT1, DVL1, DIXDC1, PRICKLE4, DACT2, NEUROG1, DKK1, DKK4, NEUROD1, \) and \( CTNNB1 \)), plus \( 1 \) control gene, \( DACT1 \), during the validation step (Supplementary Table S1). For each gene, the entire locus was amplified in \( 1 \) or \( 2 \) ‘long-range’ PCR reactions. The total amplification length for all \( 11 \) genes was \( 71.5 \) kb.

Sample preparation and high-throughput sequencing procedure

Each pool of target-enriched genomic DNA was used to build a prepped DNA library following Illumina sample preparation protocols. In brief, each pool was ligated with adapters containing a unique index allowing us to combine \( 11 \) pools in each sequencing lane of the HiSeq flow cell (HiSeq 2000, Illumina, San Diego, CA, USA). Finished flow cells carrying \( 438 \) individuals (\( 1 \) lane containing \( 11 \) indexed pools of \( 38-40 \) individuals) were sequenced in the HiSeq 2000 for paired-end analysis.

Initial data processing (sequence assembly, SNP calling, quality assessment)

Bowtie\textsuperscript{10} was used for read alignment against the latest human genome reference. Samtools\textsuperscript{31} Picard (not published; http://picard.sourceforge.net/), BEDTools\textsuperscript{32} and the Genome Analysis Toolkit (GATK\textsuperscript{33}) were used for post-alignment processing. Multisample realignment around potential insertion/deletions (indels) and base quality score recalibration were performed before variant calling. For single-nucleotide polymorphism (SNP) calling, we used SzyQy, an algorithm specifically designed to call bases in pooled samples.\textsuperscript{34}

Analysis of conservation and prediction as deleterious

For each SNP, analysis by SeattleSeq (http://snp.gs.washington.edu/SeattleSeqAnnotation137/) provided information about base position conservation in the genome, whether it was intronic or exonic, and if exonic whether it was synonymous, missense or nonsens. Predicted deleteriousness for each SNP in exonic sequence was determined by PolyPhen-2\textsuperscript{35} version v2.1.0367 or more recent. If either version predicted that the SNP was deleterious, it was counted as deleterious.

Functional WW1 variant in autism

**Functional WT1 variant in autism**

P-M Martin \textit{et al}

Quantitative reverse transcription-PCR

Total RNA from cultured HEK293T cells transfected with an empty pcDNA3.1(\(-\) vector, or a pcDNA3.1(\(-\) vector containing either WT \( WNT1 \) or \( WNT1-S88R \), was isolated with the RNeasy Mini kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). The cDNA was synthesized from \( 2 \) \( \mu \)g of total RNA with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) using random hexamer primers. Quantitative reverse transcription PCR was performed using the PerfeCTa SYBR Green FastMix, ROX (Qianta, Byfleet, UK) with gene-specific primer pairs (\( WNT1 \) forward (Fwd): \( 5'-GACGCGACACCATGTTCACTTC-3' \), \( WNT1 \) reverse (Rev): \( 5'-GGTGAACTGACACCTG-3' \); \( AXIN2 \) Fwd: \( 5'-TTGGGGTGGG- CACTAGCCCTCTCA-3' \); \( AXIN2 \) Rev: \( 5'-GCACATTGTAACCTCCTCG-3' \)) on an ABI 7900HT real-time PCR system (ABI Advanced Technologies, Columbia, MD, USA). Data were analyzed using the comparative CT method (User Bulletin No. 2, PerkinElmer Life Sciences, Boston, MA, USA).

To normalize for loaded cDNA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control (GAPDH Fwd: \( 5'-CAAGGGTACATCGACAACTTTG-3' \); GAPDH Rev: \( 5'-GCGGATCACAGCTTCTGG-3' \)). Results shown reflect combined data from two independent experiments, each done in triplicate reactions.

Statistical analyses

Unless stated otherwise in Figure legends, the data are expressed as mean \( \pm \) s.e.m. Data were analyzed by Student’s t-test, Fisher’s exact test or Pearson’s correlation test where appropriate, using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). PUNK software\textsuperscript{17} was used for Multidimensional Scaling and logistic regression. A \( P \)-value of \( <0.05 \) was considered to be significant.

RESULTS

Using a next-generation (massively parallel Illumina-based) strategy, we sequenced promoter, 3’ untranslated, intronic and coding regions of \( 10 \) Wnt pathway genes (\( WNT1, DVL1, DIXDC1, PRICKLE4, DACT2, NEUROG1, DKK1, DKK4, NEUROD1 \) and \( CTNNB1 \)) in genomic DNA obtained from a first set of \( 198 \) Caucasian ASD patients and \( 240 \) healthy Caucasian controls, arranged in pools of \( 38-40 \) samples each. The number of reads was homogeneously distributed over the different amplicons and pools (and therefore between ASD and controls), reads mapped \( 88\% \) of the targeted regions and coverage averaged \( 865\text{-fold} \) individual. To validate the next-generation sequencing strategy employed, we compared results obtained using this strategy to results obtained using Sanger sequencing of the exons in a control locus (\( DACT1 \)) in a subset of samples (\( 40 \) ASD). Every \( DACT1 \) variant (\( n = 8 \)), including several singleton variants (\( n = 3 \)), found by Sanger sequencing in this set was also detected by the next-generation sequencing strategy, and the allele frequencies of all SNPs were identical using each methodology (Pearson’s correlation test; \( r = 0.99 \); \( P < 0.0001 \); Supplementary Table S1).

In the \( 10 \) genes under study, we identified 652 SNPs overall in this set of \( 438 \) combined ASD and control samples. We restricted most of our attention to nonsynonymous sequence variants found only in our own sample set (not referenced in dbSNP, 1000 Genomes, the Exome Variant Server (EVS) or the Exome Chip Design Consortium). To determine whether any of the putative SNPs found by this method were artifacts produced by the next-generation sequencing technology, we confirmed each of them by individual genotyping using either iPLEX Sequenom or Sanger sequencing. Using this approach we confirmed 12 novel singleton missense variants distributed across 8 of the \( 10 \) loci included in our study (Supplementary Table S2). Moreover, genotyping in a second set of \( 91 \) ASD and \( 144 \) control samples failed to identify any additional occurrences of each of these rare deleterious missense SNPs. Although each of these SNPs were found only

Luciferase reporter assays

This was performed essentially as described previously.\textsuperscript{36} Briefly, both HEK293T and SH-SYSY BAR-Luc cells were transfected with \( 1, 5, 20 \) and \( 50 \) ng pcDNA3.1(\(-\) vector containing either WT \( WNT1 \) or \( WNT1-S88R \). HEK293T cells were additionally transfected with \( 20 \) ng per well pBAR-Luc plasmid and \( 10 \) ng per well pRL-TK (Renilla luciferase control plasmid). Cells were cultured for \( 24 \) h, and then luciferase activity measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and an Envision multi-label plate reader (PerkinElmer, Waltham, MA, USA).
Once among the probands in our samples and had not been reported in any public database, genotyping of parents demonstrated that none of them had occurred de novo; all were transmitted from either the mother or father. Moreover, sequencing of family trios showed that each was also present in an unaffected sibling. This verifies that each of these variants is not an idiosyncratic artifact arising during lymphoblastoid cell line derivation, and suggests that they are extremely rare alleles (single-nucleotide variants) in the Caucasian population represented by our samples.

Although we found a greater number of extremely rare missense variants in our ASD sample compared with our control sample (4% in ASD vs 1.7% in controls; Figure 1), this difference in the ‘rare missense variant burden’ did not reach statistical significance (Fishier’s exact test, \( P = 0.11 \)). Nonetheless, when considering only those rare missense variants that were also predicted to be deleterious by PolyPhen-2, the difference in ASD versus control groups did become significant (3.5% in ASD vs 0.8% in controls; Fisher’s exact test, \( OR = 4.37, P = 0.04 \); Figure 1). We also examined the distribution of less informative variants (that is, nonsynonymous, untranslated regions and intronic variants) in each group, and found a very similar distribution for all of them (nonsynonymous: 3.5% in ASD vs 2.9% in controls; untranslated regions: 11.1% in ASD vs 9.6% in controls; intronic: 24% in ASD vs 25% in controls; Figure 1). The similarity in the incidence of less informative variants argues against the possibility that the difference in the incidence of rare deleterious variants can be attributed to differences in recent ancestry between the ASD and control sample groups.

In addition to these novel SNPs, we found one previously referenced rare (~1%) missense SNP that was overrepresented in our ASD sample versus our control group. This SNP (rs61758378) occurred in \( WNT1 \), the gene encoding the first-discovered mammalian Wnt ligand. To eliminate the possibility of a sequencing artifact we confirmed this result by genotyping all samples using IPlex Sequenom. We also genotyped an additional 91 ASD and 144 control samples to replicate and potentially extend these findings. In this way, we not only confirmed MAF results for rs61758378 obtained using the Illumina platform in the original sample set, we further found that the rs61758378 minor allele remained significantly overrepresented in the combined ASD sample compared with the combined control sample (8 A/T in 267 ASD (MAF = 1.69%) vs 1 A/T in 377 controls (MAF = 0.13%); Fisher’s exact test, allelic \( P = 0.0048 \); Table 1).

Furthermore, when we compared the rs61758378 MAF in our combined ASD sample with the reported MAF in the EVS for a much larger group of individuals, the association also remained significant (8 A/T in 267 in our combined ASD sample (MAF = 1.69%) vs 45 A/T in 4300 European/American individuals in the EVS (MAF = 0.5%), Fisher’s exact test, allelic \( P = 0.011 \)). Because different allele frequencies are reported in European populations (TSI, Tuscans in Italy, MAF = 3.0%; CEU, Northern Europeans in Utah, MAF = 0.5%), we wanted to evaluate and address possible effects of subtle stratification in ancestry between cases and controls. We therefore performed Multidimensional Scaling using genome-wide SNP genotype data for a subset of the total samples used in this analysis (235 SSCs and 329 controls) using PLINK. Multidimensional Scaling using reference CEU and TSI data showed that the ratio of individuals clustering with CEU and TSI was not significantly different between ASD and controls. Nevertheless, we noted that one of the first four dimensions was significantly different between ASD and controls, indicating possible stratification.

We sought to account for this using a logistic regression analysis including the first four dimensions as covariates. In this analysis, the odds ratio (OR) remained high (OR = 8.17) and the \( P \)-value remained close to significance (\( P = 0.053 \)). We note that our analyses have low power for SNPs with such a low MAF and in a modest number of samples.

The rs61758378 minor allele changes a conserved serine at position 88 into arginine. Although this change is not predicted to be deleterious by PolyPhen-2 (HumDiv score = 0.174), serine 88 is nonetheless a very highly conserved residue across species (PhasCons score = 0.998), and the change to arginine at this residue is predicted to be deleterious by Grantham score, which categorizes codon replacements into classes of increasing chemical dissimilarity (Grantham score = 110, moderately radical change).\(^8\) Given the potential association between the \( WNT1 \) rs61758378 minor allele and ASD in our sample set, we therefore tested for functional effects of this variant on \( WNT1 \) signaling pathway activation. To accomplish this, we engineered this variant into a human \( WNT1 \) cDNA (\( WNT1 \)-S88R) using site-directed mutagenesis and then tested it alongside WT \( WNT1 \) in several assays for \( WNT1 \) signaling activity.

First, we performed a plasmid-based \( WNT1 \) reporter assay in human embryonic kidney (HEK293T) tissue culture cells. This is a well-established method to quantify transcriptional activation downstream of the \( WNT1/\beta\)-catenin signaling pathway, based in this case on transient transfection of the pBAR-Luc reporter plasmid that expresses luciferase downstream of a \( \beta\)-catenin-responsive promoter.\(^9\) Cells were co-transfected with pBAR-Luc and a dose range (1–50 ng) of plasmids encoding \( WNT1 \)-S88R or WT \( WNT1 \) to test for the ability of these proteins to activate \( Wnt/\beta\)-catenin signaling pathway-dependent transcription. At all doses tested, \( WNT1 \)-S88R showed increased activity in this assay compared with WT \( WNT1 \) (Figure 2a). Moreover, this difference was statistically significant at the 20 ng dose (WT \( WNT1 \), 1.00 ± 0.09 vs \( WNT1 \)-S88R, 1.37 ± 0.087, \( t\)-test, \( P = 0.02 \)) and at the 50 ng dose (WT \( WNT1 \), 1.00 ± 0.18 vs \( WNT1 \)-S88R, 1.86 ± 0.26, \( t\)-test, \( P = 0.016 \); Figure 2a). Similar results were obtained when experiments were replicated in a different laboratory by a different investigator using independently prepared DNA constructs and reagents (pSuperTop; data not shown).

The aforementioned assays rely on transient transfection of reporter plasmid, and on this basis they are conceptually more variable than assays based on read-out of an endogenous \( WNT1 \) pathway target gene or of a single-copy \( Wnt \) pathway reporter integrated into the genome. Accordingly, we confirmed these results in the same (HEK293T) cell line using quantitative reverse transcription-PCR to directly measure transcriptional activation of the endogenous \( Wnt/\beta\)-catenin pathway target gene \( AXIN2 \).\(^9\) Mirroring results obtained with the \( Wnt \) reporter assay, compared with WT \( WNT1 \), \( WNT1 \)-S88R showed significantly increased
Table 1. Association of WNT1 rs61758378 with ASD

| Gene    | Reference      | Function | Illumina sequencing | Confirmation sample set | Replication sample set | Combined sample set | P-value (combined samples) |
|---------|----------------|----------|--------------------|-------------------------|------------------------|---------------------|-----------------------------|
| WNT1    | rs61758378     | S88R     | ASD Con            | ASD Con                 | ASD Con                | ASD Con             | 0.0048                      |
|         |                |          | 0 AA 0 AA          | 0 AA 0 AA               | 0 AA 0 AA              | 0 AA 0 AA          |                             |
|         |                |          | 6 AT 1 AT          | 6 AT 1 AT               | 2 AT 0 AT              | 8 AT 1 AT          |                             |
|         |                |          | 192 TT 239 TT      | 189 TT 238 TT           | 70 TT 138 TT           | 259 TT 376 TT      |                             |

Abbreviations: ASD, autism spectrum disorders; Con, controls.

**DISCUSSION**

Our finding in ASD of a higher burden of rare missense variants distributed across 7 of 10 Wnt signaling pathway genes tested, and of an ASD-associated functional variant at the WNT1 locus, supports that dysfunction of this pathway contributes to ASD susceptibility.

In functional assays for the Wnt/β-catenin pathway, the biochemical cascade physiologically activated by the WNT1 gene product,40,41 WNT1-S88R consistently showed increased activity. In animal models, hyperactivation of Wnt/β-catenin signaling can lead to dominant neurodevelopmental defects including in brain regionalization,42,43 neural precursor proliferation, cell fate specification, neuron migration,44–47 and in dendrite and synapse formation or function.48,49 A mutation in a gene, such as WNT1, that modestly increases activation of this downstream signaling pathway would therefore be expected to result in neurodevelopmental and behavioral phenotypes. Moreover, as ASD and other major psychiatric disorders are generally not associated with gross neuroanatomical defects, changes in Wnt signaling activity contributing to susceptibility for these disorders should be modest.

Anatomical changes resulting from a signaling variant such as WNT1-S88R might only be evident at a cellular level (for example, in neural subtype numbers and position, neural morphology, connectivity and so on) or at a subcellular level (for example, in dendritic spine morphology, synapse numbers, synapse strength, synapse plasticity and so on), yet could still be sufficient to cause behavioral symptoms. This of course conforms closely to the central features of major psychiatric disorders, which are distinguished from neurological disorders in part by the absence of focal or grossly evident structural brain defects. Moreover, it is not our intent to posit that this or the other rare coding variants we have identified here in WNT1 and in Wnt signaling pathway component genes are monogenic causes of psychiatric illness.50 Genetic susceptibility to these disorders is highly heterogeneous and thought to be frequently multigenic; that is, for most patients genetic loading is thought to be distributed across
many loci that affect a common pathogenic pathway. In this susceptibility model, an allelic variant at any one locus may only appreciably increase disease risk in the context of variants at other loci that affect the same pathway in the same individual. The rare nonconsensory sequence variants found in both affected and unaffected individuals within the same family may study contribute to ASD in the context of other genetic and nongenetic factors that increase susceptibility.

Several lines of evidence support that Wnt ligands (of which there are 19 family members in humans) participate in ASD susceptibility. A recent whole exome sequencing study identified copy number variants affecting both WNT3 and WNT9B in an ASD patient, whereas another study showed that WNT3 expression is increased in the prefrontal cortex of young autistic patients compared with controls. Rare variants in WNT2 have been reported to segregate with ASD. Although other groups have not always replicated this linkage, two more recent studies have found SNPs in WNT2 associated with either ASD or speech delay, in a Valproate-exposure rat model of ASD, expression of both WNT1 and WNT2 was upregulated in the developing forebrain and this was associated with increased Wnt/β-catenin pathway activity in that brain region. Other recent sequencing studies have identified several ASD-susceptibility genes that encode functionally linked components of a protein network involved in the regulation of β-catenin-dependent transcription, as occurs in Wnt/β-catenin signaling. The findings of the present study add to this evidence by suggesting that minor alleles and extremely rare variants at the WNT1 locus and other Wnt pathway loci may contribute to risk for this psychiatric disorder, a possibility that merits further attention in ongoing human genetic studies focused on ASD. Given evidence that genetic etiologies are shared across several psychiatric disorders as well as that Wnt signaling is involved more broadly in psychiatric pathogenic and therapeutic mechanisms, these loci should also be carefully scrutinized in ongoing human genetic studies of other major psychiatric disorders, such as schizophrenia and major affective disorders.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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