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A population-based study of KCNH7 p.Arg394His and bipolar spectrum disorder

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We conducted blinded psychiatric assessments of 26 Amish subjects (52 ± 11 years) from four families with prevalent bipolar spectrum disorder, identified 10 potentially pathogenic alleles by exome sequencing, tested association of these alleles with clinical diagnoses in the larger Amish Study of Major Affective Disorder (ASMAD) cohort, and studied mutant potassium channels in neurons. Fourteen of 26 Amish had bipolar spectrum disorder. The only candidate allele shared among them was rs78247304, a non-synonymous variant of *KCNH7* (c.1181G>A, p.Arg394His). *KCNH7* c.1181G>A and nine other potentially pathogenic variants were subsequently tested within the ASMAD cohort, which consisted of 340 subjects grouped into controls subjects and affected subjects from overlapping clinical categories (bipolar 1 disorder, bipolar spectrum disorder and any major affective disorder). *KCNH7* c.1181G>A had the highest enrichment among individuals with bipolar spectrum disorder ($\chi^2 = 7.3$) and the strongest family-based association with bipolar 1 ($P = 0.021$), bipolar spectrum ($P = 0.031$) and any major affective disorder ($P = 0.016$). *In vitro,* the p.Arg394His substitution allowed normal expression, trafficking, assembly and localization of HERG3/Kv11.3 channels, but altered the steady-state voltage dependence and kinetics of activation in neuronal cells. Although our genome-wide statistical results do not alone prove association, cumulative evidence from multiple independent sources (parallel genome-wide study cohorts, pharmacological studies of HERG-type potassium channels, electrophysiological data) implicates neuronal HERG3/Kv11.3 potassium channels in the pathophysiology of bipolar spectrum disorder. Such a finding, if corroborated by future studies, has implications for mental health services among the Amish, as well as development of drugs that specifically target HERG3/Kv11.3.

INTRODUCTION

Mental illness afflicts 12–49% of people worldwide (1). Mood disorders—including bipolar 1 disorder, bipolar spectrum disorder and major depressive illness—account for at least half of this global mental health burden (2). In North America, 40% of medical disability in persons aged 15–44 years is attributable to psychiatric illness (2) and in the USA, suicides outnumber homicides two to one (3). Our failure to prevent serious psychiatric morbidity results in part from insufficient understanding of

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its root causes (4). Here, the application of genetics holds promise as a means to identify individuals predisposed to psychiatric disease (5), but genetic studies of mental illness have thus far produced few specific risk alleles that help clinicians care for patients (6).

The Clinic for Special Children (CSC) is a non-profit community health center that serves uninsured Amish and Mennonite (Plain) communities of Pennsylvania (USA) and surrounding states (7). Although the CSC has historically focused on pediatric health, bipolar and other affective disorders pervade every aspect of family and community life (8) and it is increasingly apparent that adult-onset mental disorders can be associated with prodromal symptoms during childhood, including disturbances of mood, attention and thought (9). The CSC invests heavily in genetic strategies that allow prevention of disability and disease (7). This concept is germane to the diagnosis and treatment of mental disorders, for which early detection of specific risk alleles in youth could enable more timely and effective psychiatric care (5).

Endogamous populations such as the Old Order Amish provide distinct advantages for investigating the genetic bases of mental illness (10,11). The Amish Study of Major Affective Disorder (ASMAD), initiated in 1976 by Egeland and colleagues, has tracked several large, multi-generation pedigrees with high prevalence of bipolar spectrum disorders (12). Despite three decades of sustained and valuable research, the ASMAD cohort has revealed no definitive genetic risk factors for major affective disease (13). However, a recent study of ASMAD subjects ($N = 388$) that combines microsatellite and high-density single nucleotide polymorphism (SNP) genotypes with whole-genome sequence data implicates dozens of rare alleles that may interact to determine risk for bipolar disorder (14).

Traditional linkage analysis is less informative in the ASMAD cohort given multiple, unexpected lines of interrelatedness within an endogamous group such as the Amish (13). Mapping susceptibility alleles for mental disorders in any population poses additional challenges: (a) behavioral phenotypes such as bipolar disorder are, by their nature, incompletely penetrant and variable in expression both within and between individuals; (b) a single genetic variant can have pleiotropic effects on psychopathology that change over the lifespan (15,16); (c) categorization of mental illness often depends critically on self-reporting of remembered subjective experience, vulnerable to errors of both omission and commission; and (4) instruments currently used to categorize mental disorders (e.g. Diagnostic and Statistical Manual of Mental Disorders, DSM) are based on phenomenology rather than firm biological constructs (17,18), and thus do not capture the full phenotypic spectrum (i.e. endophenotypes) associated with any particular susceptibility allele (4,19,20).

These facts are especially problematic when using conventional statistical paradigms to identify rare variants of clinical significance in small, endogamous groups (11). Recognizing this, we developed a strategy that depends on multiple, converging lines of evidence to evaluate a complex phenotype within a narrow genetic context. We first applied an approach commonly used to investigate Mendelian disorders (10,21), searching whole-exome data for low-frequency alleles shared among closely related Amish individuals with bipolar spectrum disorder (11,13). We then used these findings to independently test for genetic associations within the larger ASMAD cohort (14), and finally conducted functional studies of mutant potassium channels in neuronal cells.

Based on our statistical and functional results, *KCNH7* c.1181G>A (p.Arg394His; rs78247304) emerges as a strong candidate for bipolar disease risk among the Pennsylvania Amish. This corroborates findings from a recent genome-wide association (GWA) study of an independent cohort of Taiwanese patients, which isolated *KCNH7* as one among four genes likely to be associated with bipolar 1 disorder (22). To support the genetic data, we provide functional evidence that p.Arg394His alters the electrophysiological properties of HERG3/Kv11.3-mediated potassium currents in neuronal cells. Taken together, these findings suggest that functional variation of HERG-type neuronal potassium channels (19–21), and HERG3/Kv11.3 in particular, may have a role in the pathogenesis of bipolar disorder and schizophrenia. Because our association data do not reach genome-wide significance, the main finding should be viewed as provisional until confirmed or refuted by future studies.

RESULTS

Exome variants in core Amish families A–D

We initially studied four Old Order Amish sibships with a high prevalence of bipolar disorder (Fig. 1). Families A–D consisted of 26 Amish subjects (mean age 52 ± 11 years, range 34–79 years, 58% female) who underwent independent, blinded psychiatric assessment. Phenotype was characterized on four levels (Table 1): (1) Structured Clinical Interview for DSM-IV-TR (SCID) diagnosis; (2) a sub-categorization of depressive, manic and psychotic symptom clusters; (3) a designation of *multidomain affected* if at least two of three symptom clusters (i.e. mania, depression and psychosis) were present; and (4) a detailed breakdown of specific symptoms (Supplementary Material, Table S1).

Fourteen of 26 Amish subjects from Families A–D (Fig. 1) met DSM-IV-TR criteria for at least two of three symptom clusters (mania, depression or psychosis) and were designated as *multidomain affected*. They comprised diverse Axis 1 diagnoses (Table 1 and Supplementary Material, Table S1): bipolar 1 with psychotic features ($N = 6$), bipolar 2 with psychotic features ($N = 1$), bipolar disorder not otherwise specified ($N = 3$), schizoaffective disorder ($N = 2$), schizophrenia with major depressive disorder ($N = 1$), and recurrent major depression complicated by somatoform disorder and substance-induced psychosis ($N = 1$). Seven of these 14 subjects were chosen for exome sequencing (indicated with asterisks in Fig. 1) and shared a total of 17 609 exome variants.

Because our study design lacked power to detect *common* variants associated with small or modest effects, we restricted our focus to *low-frequency* variants with potentially higher pathogenicity (Fig. 2). We first excluded alleles with minor allele frequency >10% in control Plain exomes; this narrowed the list to 35 variants. We then excluded synonymous and intronic changes which further reduced the number to 10 'candidate' variants (Table 2 and Fig. 2). To perform association analyses, all 26 subjects from Families A–D and all 340 subjects from the ASMAD cohort were genotyped for these 10 variants (Figs 1. and 2).

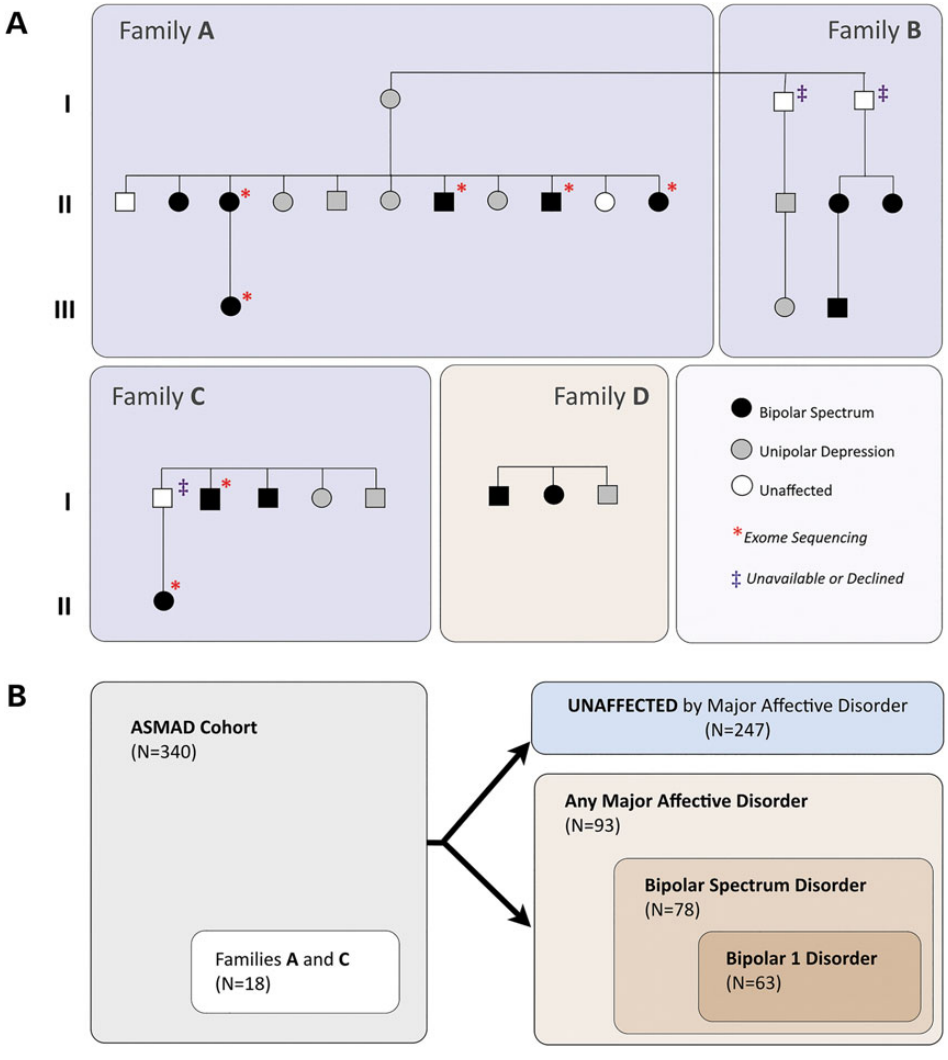

Figure 1. A (*Upper panel*): 26 individuals from four families underwent blinded, independent psychiatric assessments using the Structured Clinical Interview for DSM-IV (SCID), Research Version. Exome sequencing was done on subjects designated with a red asterisk. Families **A**–**C** (blue enclosures) were interviewed during the first phase of the study and Family **D** (green enclosure) was recruited later. Black symbols indicate individuals who met DSM-IV-TR criteria for at least two of three symptom clusters—mania, major depression, psychosis—and were considered *multidomain affected* with bipolar spectrum disorder. Gray symbols indicate individuals who met diagnostic criteria for depressive illness (recurrent or single episode) uncomplicated by mania or psychosis. The '‡'symbol indicates subjects who were unavailable for interviews or declined to participate. B (*Lower panel*): during the second phase of the study, 340 samples from the ASMAD were used to test associations of exome variants with bipolar spectrum disorder (eighteen ASMAD samples were individuals from Families A and C and thus excluded from the replication analysis). All ASMAD subjects were genotyped for 10 candidate exome variants and categorized as unaffected ($N = 247$) or affected ($N = 93$) by major affective illness; the latter category was then subdivided into the increasingly restrictive designations of bipolar spectrum disorder ($N = 78$) and bipolar 1 disorder ($N = 63$).

Candidate variants in three of 10 genes (*KRT75, UTP14C, NEK5*) had minor allele frequencies >10% in 1000 Genomes Project, European controls, or the Exome Variant Server. Among variants in the 7 genes, three (*KCNH7, MUC4, ALDH9A1*) were predicted to be pathogenic by SIFT, PolyPhen-2 and Mutation Taster, and two of these (*KCNH7, MUC4*) were absent in all three non-Plain control exome datasets (1000 Genomes Project, European controls, Exome Variant Server; Table 2). MUC4 p.Cys1309Phe was not associated with bipolar disorder in the ASMAD pedigree [family-based association test (FBAT) *P*-value = 0.965]. Moreover, mucin-4 has no known function in neurons and is not expressed in human brain (http://proteinatlas.org/). The *MUC4* variant was therefore considered an unlikely candidate.

Association of *KCNH7* c.1181G>A with psychiatric illness in the ASMAD cohort

KCNH7 c.1181G>A (rs78247304) was the only candidate exome variant carried by all 14 subjects from Families A–D who were *multidomain affected* based on the presence of at least two of three symptom clusters (i.e. mania, depression and psychosis) (Table 1 and Supplementary Material, Table S1). Moreover, *KCNH7* c.1181G>A was deemed the most likely pathogenic variant based on multiple converging lines of evidence, including: (a) results from independent GWA and whole-genome sequencing studies (14,22); (b) expression pattern of *KCNH7* in areas of the brain that are believed to mediate mood and cognition (23);

Table 1. Phenotypes and genotypes of 26 Amish Study Subjects from Families A–D

			Major Diagnoses	Symptom Clusters				Genotypes									
Subject ID	Age (yrs)	Exome	SCID DSM-IV-TR Diagnosis	Mania	Depression	Psychosis	Multidomain Affected	*ALDH9A1*	*KCNH7*	*XIRP2*	*MUC4*	*ALG10B*	*CCDC65*	*CSRNP2*	*KRT75*	*UTP14C*	*NEK5*
A-III-1	33.5	•	Schizoaffective disorder	•	•	•	•	M/+	M/+	M/M	M/+	M/+	M/+	M/+	M/+	M/+	M/+
A-II-11	42.8	•	BP1 with psychotic features; MRE mixed	•	•	•	•	M/+	M/+	M/+	M/+	M/M	M/M	M/M	M/M	M/+	M/+
C-II-1	45.1	•	BP1 with psychotic features; MRE depressed	•	•	•	•	M/M	M/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+
A-II-9	47.8	•	BP1 with psychotic features	•	•	•	•	M/M	M/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+
A-II-7	50.6	•	BP1 with psychotic features	•	•	•	•	M/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+
A-II-3	55.8	•	BP1 with psychotic features	•		•	•	M/M	M/+	M/+	M/+	M/M	M/M	M/M	M/M	M/+	M/+
C-I-2	67.9	•	BP NOS	•	•		•	M/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+
B-III-2	37.3		Schizophrenia; Major depressive disorder		•	•	•	+/+	M/+	+/+	M/+	+/+	+/+	+/+	+/+	M/+	M/+
D-I-2	44.2		BP NOS with rapid cycling	•	•		•	+/+	M/M	M/M	M/+	+/+	+/+	+/+	+/+	+/+	+/+
D-I-1	45.8		BP2; MRE depressed with psychotic features	•	•	•	•	+/+	M/M	M/M	+/+	+/+	+/+	+/+	+/+	+/+	+/+
B-II-3	49.8		Major depressive disorder, recurrent; Somatiform disorder; Substance-induced psychotic disorder	•	•	•	•	+/+	M/+	M/+	M/+	M/+	+/+	M/+	M/+	+/+	+/+
A-II-2	56.9		BP NOS	•	•		•	M/+	M/M	M/M	M/M	+/+	+/+	+/+	+/+	+/+	+/+
B-II-2	57.9		Schizoaffective disorder	•	•	•	•	+/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+	+/+	+/+
C-I-3	66.5		BP 1 with psychotic features;Cognitive disorder NOS	•	•	•	•		M/+								
B-III-1	36.9		Major depressive disorder, recurrent		•			+/+	M/+	M/+	+/+	+/+	+/+	+/+	+/+	+/+	M/+
D-I-3	41.1		Major depressive disorder, single episode		•			+/+	M/M	M/M	+/+	+/+	+/+	+/+	+/+	+/+	+/+
A-II-10	44.6		None						+/+								
A-II-8	49.3		Minor depression, single episode		•				M/+								
A-II-6	52.3		Minor depression, single episode		•			+/+	M/+	M/+	M/M	M/M	M/M	M/M	M/M	+/+	+/+
A-II-5	53.5		Major depressive disorder, recurrent		•				+/+								
A-II-4	54.5		Minor depression, single episode		•				M/+								
A-II-1	58.3		None					M/M	M/+	M/+	M/+	M/M	M/M	M/M	M/M	+/+	+/+
C-I-5	58.8		Major depressive disorder, single episode		•				+/+								
B-II-1	61.2		Major depressive disorder, recurrent		•			M/+	M/+	M/+	+/+	+/+	M/+	M/+	M/+	+/+	+/+
C-I-4	61.6		Minor depression, single episode		•				M/+								
A-I-1	79.2		Depressive disorder NOS		•			M/+	M/+	M/+	M/+	M/+	M/+	M/+	M/+	+/+	+/+

The '•' symbol indicates patients who had exome sequencing and is also used as a marker for particular symptoms clusters–i.e. mania (orange fill), depression (blue fill), and psychosis (green fill). The 14 individuals who experienced at least two of three symptom clusters are designated as 'Affected' and listed in the first 14 rows. Genotyping results: '+/+' = homozygous wild-type (no fill); 'M/+' = heterozygous for variant of interest (light purple); 'M/M' = homozygous for variant of interest (dark purple). Genotype columns are divided into subgroups based on segregation of haplotype blocks. Abbreviations: BP, bipolar disorder (Type 1, Type 2, or NOS); MRE, most recent episode; NOS, not otherwise specified; SCID DSM-IV-TR, Structured Clinical Interview for DSM-IV-TR, Research Version.

(c) evidence that antipsychotic drugs block the HERG3/Kv11.3 channels encoded by *KCNH7* (24); (d) the proposed role of other potassium channel subunits in bipolar disorder and schizophrenia (25–28); and e) the conservation of nucleotide guanine[1181], corresponding to amino acid arginine[394], across all species from *Homo sapiens* to *Caenorhabditis elegans* (PhyloP 2.61) (Table 2).

To further test this observation, we obtained de-identified DNA and clinical data for 394 ASMAD samples. Individuals from aforementioned Families A and C (Fig. 1) were represented in the ASMAD cohort, but were excluded from the replication analysis. Fifty-four ASMAD subjects had minor or incompletely characterized psychiatric phenotypes and were also excluded. We grouped the remaining 340 subjects into the following overlapping clinical categories, as depicted in Figure 1: bipolar 1 disorder ($N = 63$), bipolar spectrum disorder ($N = 78$, including bipolar 1, bipolar 2 and bipolar disorder not otherwise specified), any major affective disorder ($N = 93$, including major depressive disorder, recurrent), and unaffected by major affective illness ($N = 247$). Among these 340 individuals, we investigated association of the 10 candidate variants with psychiatric diagnoses using three complementary methods: (a) a simple χ^2 analysis of allele distribution with phenotype; (b) the FBAT, which measures transmission distortion of alternative alleles to affected and unaffected siblings in pedigrees (29) and (c) the efficient mixed-model association expedited method (EMMAX), which controls and corrects for relatedness between subjects (30).

KCNH7 c.1181G > A (rs78247304) behaved in a manner different from all other variants (Table 3 and Fig. 3). Table 3 lists nominal (uncorrected) $\chi 2$ calculations as well as FBAT and EMMAX *P*-values for the 10 candidate exome variants. *KCNH7*

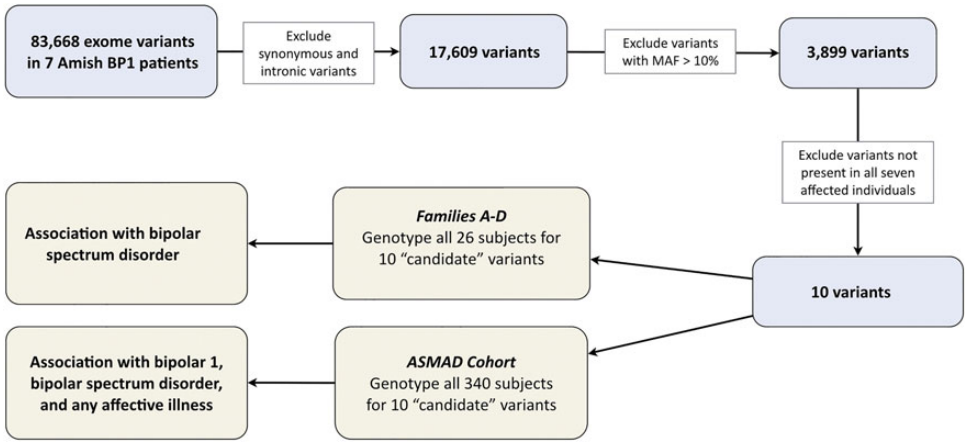

Figure 2. Among seven Amish individuals with bipolar spectrum disorder, we identified a total of 83 668 exome variants, 17 609 of which remained after filtering out synonymous and intronic changes. Focusing on low-frequency alleles with potentially high pathogenicity, we excluded exome variants with minor allele frequency (MAF) >10% among population-specific control exomes. Only 10 of these variants were present in all seven individuals. These 10 'candidate' alleles were then used to test for associations with bipolar spectrum disorder and broader diagnostic categories within the extended core pedigree (Families A–D, $N = 26$) and the larger ASMAD cohort ($N = 340$), respectively.

c.1181G>A had the highest enrichment in subjects with affective disorders (χ^2 for bipolar 1 = 4.2; bipolar spectrum = 7.3, any affective disorder = 10.2), lowest EMMAX *P*-value for bipolar 1 and bipolar spectrum disorders ($P = 0.013$) and lowest FBAT *P*-value for bipolar 1 ($P = 0.021$), bipolar spectrum ($P = 0.031$) and any major affective disorder ($P = 0.016$) (Table 3 and Fig. 3).

The statistical results presented in Table 3 do not alone provide sufficient evidence of association after correcting for multiple tests. We nevertheless pursued *KCNH7* c.1181G > A further based on (a) the weight of evidence from multiple sources (14,22–24,27,28,31–33); (b) recognition that our cohort size and study design lacked power to generate an unequivocal signal for any true positive association (discussed below); and (c) the important implications that a true positive association would have for design of preventative mental health services among Amish communities as well as future drug development for patients with bipolar disorder and related psychiatric disorders. We thus turned to studies of HERG3^{Arg394His} expression and function in neurons.

Expression and function of KCNH7 Arg394His

When overexpressed in mouse and human neuroblastoma cells, wild-type and HERG3/Kv11.3^{Arg394His} potassium channel protein subunits had similar abundance, core and mature glycosylation and localization to the plasma membrane (Fig. 4A–F and Supplementary Material, Fig. S1). Wild-type and Arg394His mixed monomers co-localized in a pattern indistinguishable from that of wild-type proteins alone, suggesting appropriate intracellular trafficking and formation of mature heteromers (Supplementary Material, Fig. S1).

Depolarization of Neuro-2a cells transfected with wild-type KCNH7 elicited outward currents that progressively diminished in size with depolarization to >20 mV (Fig. 4G), a pattern characteristic of HERG/Kv11 channels with fast C-type inactivation (34). In cells transfected with HERG3^{Arg394His}, the following differences were observed: (a) When currents were normalized to the maximal current size in each cell, fractionally smaller currents were observed through Arg394His channels at a given voltage <20 mV (Fig. 4I); (b) Greater depolarization was required to elicit currents through the Arg394His channel; the normalized conductance (G/G_{max}) curve, proportional to the probability that the channel is open, was shifted ~12 mV in the positive direction (Fig. 4J); and (c) Upon depolarization, current kinetics through the Arg394His channel were slower (Fig. 4K), but the deactivation kinetics at a negative voltage were essentially indistinguishable between the two channel types (Fig. 4L). Together, the results suggest that the p.Arg394His mutation slows the activation process of HERG3/Kv11.3 channels and thereby shifts the overall voltage dependence of activation in the positive direction.

DISCUSSION

KCNH7, HERG-type potassium channels and mental illness

By studying a few Amish families to search for low-frequency, relatively penetrant bipolar risk alleles, we discovered a specific missense variant of *KCNH7* (c.1181G>A) that appears to segregate with bipolar spectrum disorder among a subset of Pennsylvania Amish families. In our view, the most important conclusions to be drawn from our results are that the *KCNH7* c.1181G>A allele, uniquely present in all 14 affected patients among the original cohort of 26, clearly distributes in a way different from all nine other rare and potentially pathogenic exome variants tested within the larger ASMAD cohort (Table 3 and Fig. 3), and significantly alters potassium channel currents in neuronal cells. Given the relatively small sample size used and incomplete penetrance of the bipolar spectrum phenotype, the genetic evidence is alone insufficient to provide definitive proof of association. However, we believe *KCNH7* c.1181G>A warrants further investigation based on the cumulative weight of evidence from multiple sources, its high degree of specificity, and the potential public health implications for Amish communities.

The *KCNH7* c.1181G>A variant (rs78247304) was recently highlighted as one of 30 potentially pathogenic missense variants

Table 2. Ten exome variants among the seven affected Amish individuals chosen for exome sequencing

Chr	Position	Ref	Alt	dbSNP135	Gene	Class	Codon change	Amino acid change	PhyloP score	Allele frequency					Predicted effects on protein function		
										All plain exomes ($n = 84$)	Amish exomes ($n = 56$)	1000 Genomes	EVS	CEU	SIFT	PolyPhen-2	MutationTaster
1	165 648 710	G	A	rs55725612	*ALDH9A1*	Missense	gCg/gTg	A206V	2.51	0.05	0.06	0.01	0.02	.	Damaging	Probably damaging	Disease-causing
2	163 302 901	C	T	rs78247304	*KCNH7*	Missense	cGc/cAc	R394H	2.61	0.05	0.07	.	.	.	Damaging	Probably damaging	Disease-causing
2	168 115 797	G	C	rs75758327	*XIRP2*	Missense	aGa/aCa	R692T	−0.36	0.08	0.07	0.09	0.07	.	Tolerated	na	Polymorphism
3	195 492 191	C	A	.	*MUC4*	Missense	tGt/tTt	C1309F	2.19	0.02	0.03	.	.	.	Damaging	Probably damaging	Polymorphism
12	38 714 929	A	G	rs61730283	*ALG10B*	Missense	Att/Gtt	I446V	−2.44	0.03	0.04	0.01	0.02	0.10	Tolerated	Benign	Polymorphism
12	49 312 681	G	T	rs117646559	*CCDC65*	Missense	Gat/Tat	D238Y	1.24	0.03	0.05	0.01	0.01	.	Damaging; low confidence	Possibly damaging	Polymorphism
12	51 457 854	G	A	rs11542510	*CSRNP2*	Missense	aCg/aTg	T436M	0.75	0.01	0.02	0.04	0.01	0.03	Damaging; low confidence	Possibly damaging	Polymorphism
12	52 827 740	G	C	rs2232386	*KRT75*	Missense	Ccc/Gcc	P117A	0.32	0.07	0.07	0.13	0.11	0.03	Damaging	Probably damaging	Disease-causing
13	52 603 241	A	G	rs3742290	*UTP14C*	Missense	Act/Gct	T101A	−0.10	0.08	0.07	0.09	0.12	0.11	Tolerated	Benign	na
13	52 676 275	T	G	rs34756139	*NEK5*	Missense	Aaa/Caa	K255Q	1.06	0.08	0.07	0.09	0.11	0.11	Damaging	Probably damaging	Polymorphism

The highest PhyloP value is indicated by orange fill. Alleles that were not detected in non-Plain exomes are designated with green fill, and blue fill indicates alleles predicted to have damaging effects on protein function.

ASMAD, Amish Study of Major Affective Disorder; CEU, Control European Exomes; EVS, Exome Variant Server.

Table 3. Association testing of 10 exome variants with affective disorders in the ASMAD cohort ($N = 340$)[a]

Gene	Chromosome	Variant	Bipolar 1 disorder			Bipolar spectrum disorder			Any major affective disorder		
			χ^2	FBAT P	EMMAX P	χ2	FBAT P	EMMAX P	χ2	FBAT P	EMMAX P
ALDH9A1	1	A206V	0.4	0.408	0.224	0.1	0.889	0.303	0.1	0.861	0.968
KCNH7	2	R394H	**4.2**	**0.021**	**0.174**	**7.3**	**0.031**	**0.013**	**10.2**	**0.016**	0.189
XIRP2	2	R692T	1.3	0.484	0.465	2.6	0.408	0.113	1.2	0.345	0.882
MUC4	3	C1309F	0.8	0.965	0.670	0.2	0.906	0.356	0.0	0.761	0.919
ALG10B	12	I446V	1.4	0.276	0.194	0.9	0.599	0.651	0.2	0.687	0.774
CCDC65	12	D238Y	0.8	0.514	0.637	0.1	0.751	0.772	0.0	0.683	0.946
CSRNP2	12	T436M	0.2	0.824	0.939	0.1	0.654	0.606	0.5	0.940	0.867
KRT75	12	P117A	0.8	0.405	0.742	1.7	0.366	0.555	3.7	0.227	**0.081**
UTP14C	13	T101A	0.2	0.349	0.543	0.0	0.722	0.256	0.4	0.613	0.149
NEK5	13	K255Q	0.1	0.349	0.543	0.0	0.722	0.256	0.2	0.613	0.149

[a]The nominally most significant value from each column is shaded blue (bipolar 1 disorder), red (bipolar spectrum) or purple (any major affective disorder).

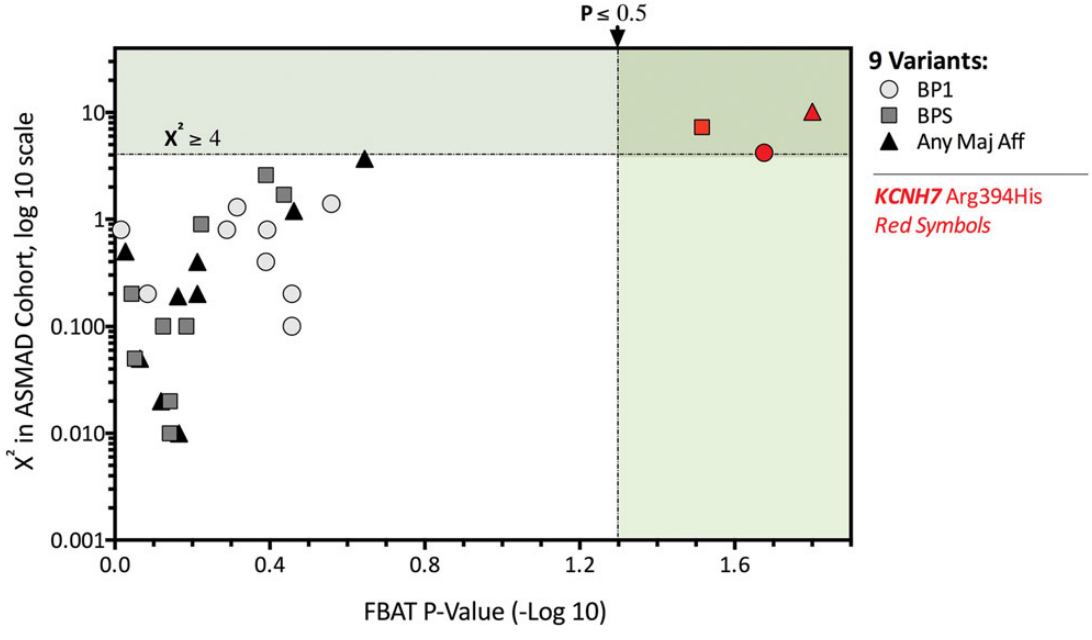

Figure 3. Testing for the association of 10 rare candidate alleles with bipolar 1 (BP1, *circles),* bipolar spectrum (BPS, *squares*), and any major affective disorder (any Aff, *triangles)* among 340 subjects from the Amish Study of Major Affective Disorder cohort. FBAT *P*-values (abscissa) and χ^2 distribution (ordinate) were calculated for each of the 10 rare candidate gene variants detected by exome sequencing. Nine of these variants (*ALDH9A1, XIRP2, MUC4, ALG10B, CCDC65, CSRNP2, KRT75, UTP14C* and *NEK5*) are plotted in gray. *KCNH7* c.1181G>A, represented with red symbols, shows the strongest association with affective disorders and shows an unusual distribution behavior among the 10 variants. For graphical clarity, FBAT is transformed to the $-\log 10$; dotted lines indicate arbitrary thresholds of $P \leq 0.5$ and $X^2 \geq 4$ for FBAT and chi-square testing, respectively.

in whole-genome sequence analysis of ASMAD extended families (14). A parallel, independent GWA study of Taiwanese patients identified a different *KCNH7* variant (rs6736615) as one of four alleles associated with bipolar 1 (empirical *P*-value = 0.0047; $N = 1555$) (22). Again, the statistical signal for rs6736615 fell short of genome-wide significance among Taiwanese patients, but this allele nevertheless behaved in a way not likely to be observed by chance. Available data also implicate other potassium channels genes (*KCNH2* and *KCNJ3*) in bipolar disorder and schizophrenia (27), localize HERG-type channels to the brain's limbic circuits (33,35), and demonstrate a role for altered potassium currents in mania and the therapeutic actions of lithium (36–38). These converging lines of evidence, combined with genetic and electrophysiological data detailed in this report, suggest that variation of neuronal HERG-type potassium channels (25–27), and specifically HERG3/Kv11.3, might contribute to mental illness in certain individuals.

KCNH7 and mechanisms of mental illness

HERG3/Kv11.3, encoded by *KCNH7,* belongs to the *ether-á-go-go*-related (ERG) family of voltage-gated potassium channels expressed throughout the mammalian brain, especially in limbic and cortical areas associated with mood and cognition (35). Heterologously expressed HERG3^{Arg394His} is processed to the plasma membrane in neuroblastoma cells, but the histidine substitution at a highly conserved cytoplasmic arginine^{394} shifts voltage dependence of activation in the positive direction and

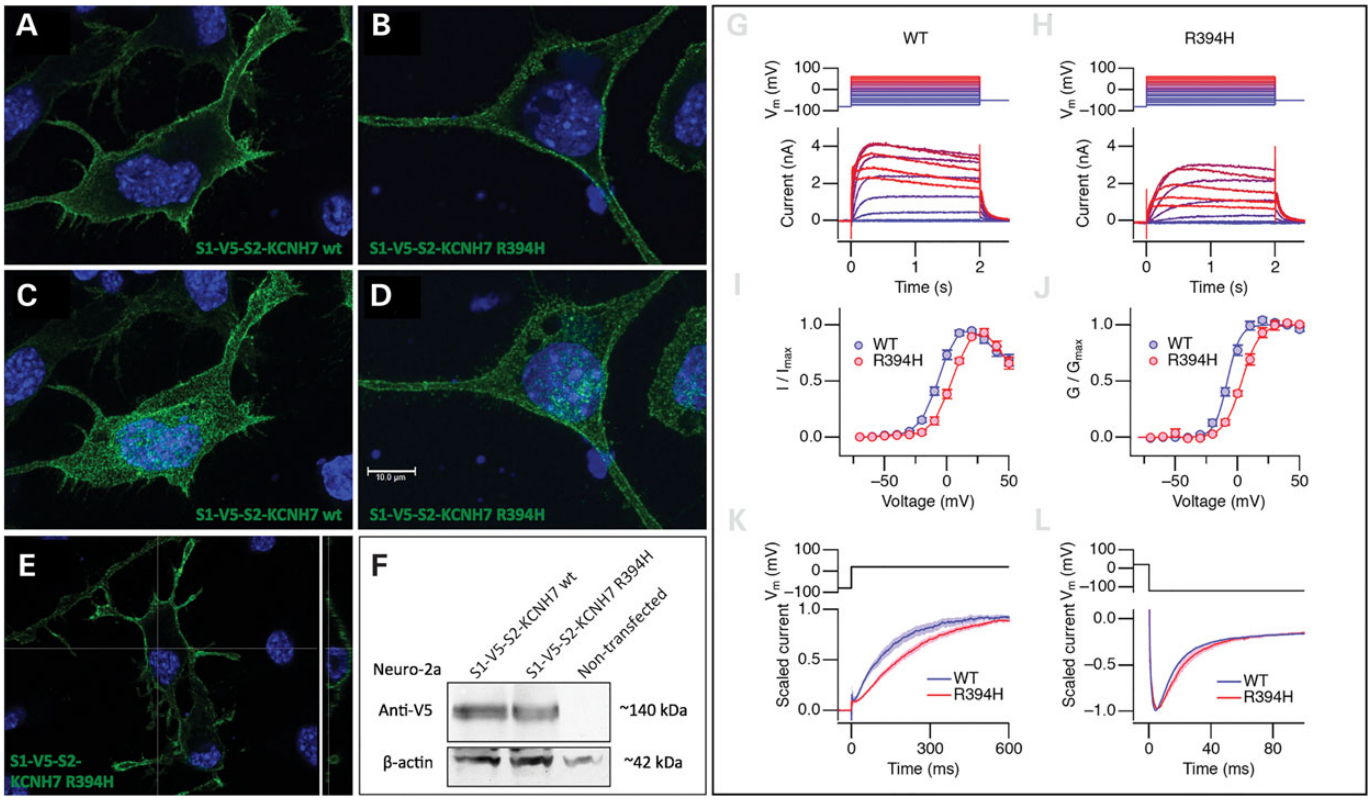

Figure 4. *Left panel:* localization of overexpressed KCNH7 wild-type and Arg394His in Neuro-2a cells immunostained under non-permeabilizing conditions (see Materials and Methods) with mouse monoclonal anti-V5 IgG_{2a} (1:500), followed by AlexaFluor 488-conjugated goat anti-mouse IgG_{2a} (1:400). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1.5 µg/ml) (blue fluorescent signal). (**A** and **B**) KCNH7 wild-type and Arg394His with the V5 epitope tag inserted in the S1–S2 extracellular loop localize to the plasma membrane in non-permeabilized Neuro-2a cells (single confocal images). (**C** and **D**) Maximum projection *z*-stack images of the cells shown in A and B. (**E**) *Left*—confocal image of Neuro-2a cells transiently overexpressing Arg394His S1-V5-S2-KCNH7. *Right*—Orthogonal projection of a section through the cell in the center of the left image demonstrating membrane localization for Arg394His S1-V5-S2-KCNH7. (**F**) Western blot of transiently overexpressed wild-type and Arg394His S1-V5-S2-KCNH7 fusion proteins in Neuro-2a cells from the same transfections used for A–D. S1-V5-S2-KCNH7 fusion proteins migrated as a core glycosylated and mature glycosylated doublet at ~140 kDa. β-Actin was labeled as a loading control. Primary antibodies: anti-V5 mouse monoclonal IgG_{2a} (1:5000) and anti-β actin (1:1 000 000). Secondary antibody: goat anti-mouse IgG HRP-conjugated (1:1500). Data are representative of four independent transfections. *Right panel:* electrophysiological characteristics of wild-type (WT) and Arg394His HERG3 (KCNH7) currents. (**G**) Representative currents from a Neuro-2a cell transiently expressing WT HERG3 channels. (**H**) Representative currents from a Neuro-2a cell transiently expressing Arg394His HERG3 channels. (**I**) Scaled peak current–voltage (I/I_{max}) curves for WT (*blue*) and Arg394His (*red*) channels. The results are normalized to the maximal current size in each cell. The data points are connected by lines for an illustrative purpose only. $n = 12$ and 9 for WT and Arg394His, respectively. (**J**) Normalized conductance (G/G_{max}) as a function of voltage for WT (*blue*) and Arg394His (*red*). The half-activation voltage ($V_{0.5}$) and the apparent equivalent charge movement were -7.5 ± 1.1 mV and $4.7 \pm 0.53e_0$ for WT and 4.5 ± 1.1 mV and $3.8 \pm 0.50e_0$ for Arg394His. $n = 12$ and 9 for WT and Arg394His, respectively. The $V_{0.5}$ values for Arg394His are statistically different from those for WT ($P < 1 \times 10^{-5}$). The equivalent charge numbers are indistinguishable between the groups ($P = 0.129$). The smooth curves are Boltzmann fits to the pooled results. Kinetics of ionic currents at 20 mV (**K**) and −120 mV (**L**). Currents are scaled to facilitate comparison. The sweep width represents the mean ± SEM. In (K), $n = 7$ and 8 for WT and Arg394His, respectively. In (L), $n = 5$ and 4 for WT and Arg394His, respectively.

slows activation kinetics; thus the mutation is predicted to increase excitability of neuronal cells *in vivo*. Penetrance and severity of mental illness were similar among *KCNH7* c.1181G>A heterozygotes and homozygotes. This may reflect the heterotetrameric nature of ERG channels (e.g. other ERG subunits may partially substitute for ERG3) and/or a high degree of potassium channel redundancy in the nervous system that attenuates the biological impact of modest functional abnormalities of any one channel subunit (39).

Potassium channel dysfunction appears mechanistically important in animal models of mania and may be relevant to the actions of lithium (32). *KCNH7* is expressed in mammalian midbrain, where its blockade prolongs plateau potentials in bursting dopaminergic neurons and may in turn alter mesolimbic dopamine release (31). Certain typical and atypical antipsychotic drugs inhibit HERG3/Kv11.3 (31) and lithium is believed to exert mood-stabilizing effects in part by modulating potassium currents, either by reducing voltage-gated potassium channel open events or by inhibiting GSK3ß kinase-mediated channel phosphorylation (37). In murine models of mania (*KCND2*/Kv4.2 knockout; *Clock*Δ19), genetic deletion or experimental manipulation of potassium currents attenuates physiological and behavioral correlates of mania and dose-dependently increases phosphorylation of GSK3β in prefrontal cortex and hippocampus (36). The latter mechanism is thought to be shared among all effective mood-stabilizing drugs (35).

A community-based approach to psychiatric genetics

Among people afflicted with serious mental disorders, conservative estimates suggest that only 50–65% in developed nations and 15–24% in less-developed nations are diagnosed and treated appropriately (1). Such is the case in Amish communities (8), where treatment for psychiatric disease may only occur in response to crises like intractable mental anguish, emergent hospitalization, violence, or the threat of suicide (8). The treatment gap (1) in Amish as well as other communities results from multiple factors, including social stigma, a dire shortage of professional resources and abiding ignorance of underlying disease mechanisms and their developmental expression (4).

For many patients, the first signs of mental illness surface during childhood or adolescence, while there remains a window for effective intervention (4). At present, identification of presymptomatic individuals who might later develop major psychiatric disease is based on a combination of family history, prodromal symptoms, and concerning patterns of behavior (5). Underlying this effort is the simple notion that recognizing a predilection for mental illness allows medical and psychosocial interventions to be implemented proactively (5). Indeed, *informed* prevention has proven the key practical benefit that genetic knowledge confers on clinical practice (10), and it is widely believed that mental health services can be improved by a firmer grounding in genetics and developmental biology (4).

Effective treatment strategies for bipolar disorder will largely depend on the identification of biological markers sufficiently specific to determine who is at risk (6). The CSC has invested heavily in the discovery of such markers—typically rare and highly penetrant alleles—that can guide the design of population-specific surveillance and prevention programs (7). Despite the presumed genetic complexity of bipolar disorder (40), we hypothesized that one or more rare alleles might exert strong pathogenic effects within certain endogamous demes (13). This strategy allowed us to identify *KCNH7* c.1181G>A as a potential risk factor for bipolar spectrum disorder within a subgroup of Pennsylvania Amish families.

Population-specific risk alleles and overlapping psychiatric phenotypes

Our observations suggest that *KCNH7* c.1181G>A, and presumably other psychiatric risk alleles, can have pleiotropic effects and do not segregate solely with a single categorical psychiatric phenotype (e.g. bipolar 1 disorder). *KCNH7* c.1181G>A carriers have prevalent psychotic symptoms and diverse, overlapping Axis 1 diagnoses (including schizoaffective disorder, schizophrenia and major depression). This is not particularly surprising; within the general population, most mental disorders are thought to arise from the combinatorial effects of multiple alleles and their interaction with epigenetic and life events (4). It is also increasingly evident that a single allele can segregate with different categorical psychiatric diagnoses (e.g. bipolar disorder or schizophrenia) (26,41). This basic model surely also applies to genetic isolates like the Amish, but within such populations it is comparatively easier to identify low-frequency alleles with stronger pathogenic effects and to document the full range of psychiatric phenotypes that segregate with a particular allele within extended families (21).

In the ASMAD cohort, *KCNH7* c.1181G>A segregates in 31 nuclear families and is found in 32% of patients with a bipolar spectrum diagnosis. However, within these families it appears to be relatively penetrant and might therefore be clinically actionable (7). Further research is needed to verify this, delineate what other alleles may predispose Amish individuals to mental illness, map their distribution among the various Amish demes, and determine how they might interact with *KCNH7* c.1181G>A to affect disease expression. Such knowledge could lead to personalized pharmacological therapies and, for the first time within this community, preventative mental health care (5).

Conclusions, limitations and future directions

Major limitations of the present study are its small size and narrow focus. By restricting our analysis to Amish cohorts, we may have identified a variant unique to this population. However, a recent independent GWA study suggests an association between bipolar illness and a different *KCNH7* variant in a cohort of ethnically homogeneous Taiwanese patients (22). Observations from Amish and Taiwanese cohorts reveal how we might advance the field of complex disease genetics through the investigation of 'common' phenotypes in relatively small, endogamous groups (13,42). An association between *KCNH7* c.1181G>A and bipolar spectrum disorder, even if limited to a few genetic isolates, informs the underlying biology of mood regulation and can suggest more widely applicable treatment strategies (i.e. new drug targets).

For certain rare pathogenic alleles discovered in small, isolated populations, conventional statistical thresholds for genome-wide significance may be difficult if not impossible to achieve. For example, a recent review suggests that studies sufficiently powered to identify rare variants of clinical significance should include discovery sets of 25 000 cases or more (11), a number representing roughly half the Amish population of Pennsylvania (42). Moreover, the Pennsylvania Old Order Amish are more accurately understood as many *separate* founder populations; the several reproductively isolated demes within the state are defined by different allele distributions (10). Germane to this point, *KCNH7* c.1181G>A only segregated in a minority of the 72 nuclear families within the ASMAD cohort, and therefore will be only one of many bipolar risk alleles within the population as a whole.

These considerations underscore the importance of using multiple or different sources of evidence to optimize investigations of complex and incompletely penetrant phenotypes within small genetic isolates. Despite limitations inherent in the genetic data, we pursued *KCNH7* c.1181G>A further for three reasons. First, this allele segregated differently from nine other rare, potentially pathogenic variants in two Amish cohorts (core families A-D and the larger ASMAD pedigree); while recognizing this result could be by chance, we were persuaded by the nominal differences represented in Table 3 and Figure 3. Second, potassium channels in general, and HERG3 channels in particular, have a plausible causative role in bipolar spectrum based on a large body of knowledge about their function in neurons (31,33), distribution within the central nervous system (23), and pharmacological interactions with lithium and antipsychotic drugs (24,36,38). Finally, our interest in *KCNH7* c.1181G>A was strengthened

by the recent finding of a potential association between *KCNH7* and bipolar 1 illness in an independent Taiwanese cohort (22), although the latter study also only demonstrated nominal, not genome-wide, significance (empirical P value = 0.0047; $N = 1555$).

Our observations, together with the evidence for genetic heterogeneity from analysis of whole-genome sequence and imputed genotypes of ASMAD extended families (14), sets the stage for a diverse genetic landscape of bipolar disease risk even within a population as seemingly 'uniform' as the Pennsylvania Amish (2,43). Moreover, this study highlights the challenges of statistical analyses using small, endogamous groups to study a phenotype that is: (a) incompletely penetrant, (b) variable in expression and (c) by its very nature, difficult to categorize with certainty. Nevertheless, efforts to link genetic variants to bipolar illness will continue at a rapid pace (4). Our experience suggests that future studies should better delineate subtypes of this complex behavioral disorder by combining systematic discovery of genetic variants with multisystem analyses of quantitative traits that more deeply and reliably characterize the psychopathology (20), and will likely rely on convergent evidence from multiple sources. Multidimensional research strategies within small founder populations could be crucial to these efforts.

MATERIALS AND METHODS

Phenotypic assessments

The study was approved by the Institutional Review Board of Lancaster General Hospital and all patients consented in writing to participate. Study subjects underwent independent, blinded psychiatric assessment using the Structured Clinical Interview for DSM-IV-TR (SCID), Research Version (http://scid4.org/) (43). For each subject, supplemental information was collected from at least two closely related individuals (e.g. parent, sibling or child) and in some cases, hospital records. Phenotype was characterized on four levels as described above, and phenotypic assessments, including final SCID DSM-IV-TR diagnoses, were determined by uniform consensus among three blinded interviewers (A.M., M.F., S.M.).

The ASMAD began in 1976 (44). A five-member psychiatric board blinded to familial ties, pre-existing diagnoses and treatment used strict Research Diagnostic and DSM-III/IV criteria to develop consensus diagnoses for each subject. Uniform assessment procedures were applied longitudinally for more than three decades of follow-up, and samples were donated to the Coriell Cell Repository (Coriell Institute for Medical Research, Camden NJ).

Genomic and statistical methods

We performed exome sequencing on a subgroup of 7 Amish subjects as previously described (Broad Institute, Boston, MA) (21). Our aim was to identify low-frequency alleles with relatively high penetrance; thus exome data were filtered to exclude synonymous and intronic changes as well as variants with minor allele frequency >10% in two different, but overlapping, sets of population control exomes (designated 'Plain' exomes: 84 control Amish and Mennonite exomes combined *and* 56 control Amish exomes). Ten candidate variants passed filtering criteria and were verified by Sanger sequencing (Table 2). For each variant, we obtained a measure of conservation (PhyloP) from the University of California Santa Cruz Genome Browser (http://genome.ucsc.edu/) and modeled potentially damaging effects on protein structure *in silico* using SIFT (http://sift.jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and MutationTaster (http://mutationtaster.org/).

ASMAD samples ($N = 340$) were genotyped using the Illumina Omni 2.5 SNP array platform. In addition, all samples were genotyped for each of the 10 candidate variants using high-resolution melt analysis (LightScanner 32, BioFire Diagnostics; LightCycler 480, Roche Diagnostics). Estimates of pair-wise relatedness of the 340 ASMAD subjects were obtained based on Illumina Omni 2.5 SNP array data. A $\chi 2$ statistic was used to assess distribution of 10 candidate alleles among individuals with and without mood disorders, and association of these variants with psychiatric diagnoses was tested using the FBAT (29) and efficient mixed-model association expedited (EMMAX) methods (30). The Bonferroni correction was applied for multiple comparisons. FBAT P-values were $-\log 10$ transformed to construct Figure 3.

Functional studies of KCNH7 Arg394His in cell lines

All cell lines were obtained from American Type Culture Collection (http://atcc.org/). We cloned wild-type *KCNH7* (also known as HERG3 or Kv11.3; NM_033272.3) from human adherent retinal pigment epithelium cells (ARPE-19), introduced c.1181G>A by site directed mutagenesis, and overexpressed verified constructs in human neuroblastoma (SH-SY5Y), mouse neuroblastoma (Neuro-2a) and transformed human embryonic kidney (HEK-293T) cell lines for immunofluorescence and western blotting (Supplementary Methods). To assess membrane localization of KCNH7 subunits, the V5 epitope tag (GKPIPNPLLGLDST) was inserted between amino acids 441 and 442 of the S1-S2 extracellular loop of KCNH7 and indirect immunofluorescence labeling was performed under non-permeabilizing conditions. Briefly, S1-V5-S2 KCNH7 fusion proteins overexpressed in Neuro-2a cells were labeled with mouse monoclonal anti-V5 (1:500) (Life Technologies) at 8°C for 25 min in DMEM with 10% fetal bovine serum and washed three times before fixation (see Supplementary Methods for details).

Neuro-2a cells overexpressing wild-type or KCNH7 Arg394His (*N*-terminal epitope tags) were tested by patch-clamp experiments using the whole-cell configuration. We recorded ionic currents at room temperature with an Axopatch 200A amplifier (Molecular Devices), elicited currents by 2 s pulses applied every 20 s from a holding potential of −80 mV, and analyzed results using custom routines implemented in Igor Pro (WaveMetrics) (Supplementary Methods).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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