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Mutation screening of *SCN2A* in schizophrenia and identification of a novel loss-of-function mutation

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Abstract

Objectives—There is a growing body of evidence suggesting a shared genetic susceptibility between many neuropsychiatric disorders, including schizophrenia, autism, intellectual disability and epilepsy. The sodium channel, voltage-gated type II alpha subunit gene *SCN2A* has been shown to exhibit loss-of-function mutations in individuals with seizure disorders, intellectual disability, autism and schizophrenia. The role of loss-of-function mutations in schizophrenia is still uncertain with only one such mutation identified to date.

Methods—To seek additional evidence for a role for loss-of-function mutations at *SCN2A* in schizophrenia we performed mutation screening of the entire coding sequence in 980 schizophrenia cases. Given an absence of LoF mutations in a public exome cohort (ESP6500, N=6503) we did not additionally sequence controls.

Results—We identify a novel, nonsense (i.e. stop-codon) mutation in one case (E169X) that is absent in 4300 European American and 2203 African-American individuals from the NHLBI Exome Sequencing Project. This is the second loss-of-function allele identified in a schizophrenia case to date. We also show a novel, missense variant, V1282F, that occurs in 2-cases and is absent in the control dataset.

Conclusion—We argue that very rare, loss-of-function mutations at *SCN2A* act in a moderately penetrant manner to increase the risk of developing several neuropsychiatric disorders including seizure disorders, intellectual disability, autism and schizophrenia.

Keywords

Schizophrenia; gene; mutation; nonsense; missense; loss-of-function

Introduction

The application of genome-wide association study (GWAS) technologies to neuropsychiatric disorders has led to the discovery of common risk-variants for schizophrenia and other brain disorders (O'Donovan et al., 2008, Ripke et al., 2013, Purcell et al., 2009, Consortium,

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2014). It is now widely accepted that these common, low-penetrance risk-alleles can impart risk of developing related neuropsychiatric disorders (Lee et al., 2013, Purcell et al., 2009), however the underlying pathological mechanisms remain unclear. The application of GWAS and Whole Exome-Sequencing (WES) technologies to the study of rare variations such as Copy Number Variants (CNVs) or loss-of-function (LoF) mutations has implicated a role for rare alleles and mutations in schizophrenia (Purcell et al., 2014, Kirov et al., 2012, Fromer et al., 2014, Li et al., 2015, Kirov et al., 2009), Autism Spectrum Disorder (ASD) (Li et al., 2015, De Rubeis et al., 2014, Guilmatre et al., 2009, Sanders et al., 2011, Sanders et al., 2012, Iossifov et al., 2012), Intellectual Disability (ID) (Cooper et al., 2011, Classen et al., 2013, Allen et al., 2013, Li et al., 2015, Gilissen et al., 2014) and seizure disorders (Carvill and Mefford, 2013, Martin et al., 2014, Allen et al., 2013). The identification of moderate-to-highly penetrant rare alleles and their enrichment in certain sub-sets of genes of known function has implicated glutamatergic post-synaptic protein complexes as one potential common pathological site for schizophrenia, ASD and ID (Fromer et al., 2014, Kirov et al., 2012).

In their WES study of individuals with schizophrenia, Fromer *et al* (Fromer et al., 2014) showed an enrichment of *de novo* loss-of-function (LoF) mutations in synapse related genes; LoF mutations are any mutation that prevents formation of a mature protein product and they encompass nonsense, frameshift and splice-site mutations. One of the LoF mutations disrupted a canonical splice-site in the gene *SCN2A* encoding an alpha subunit of voltage-gated sodium channels, a key protein involved in axon potential propagation (Meisler et al., 2010). Several loss-of-function mutations at this locus have also been shown in ASD (Sanders et al., 2012, Tavassoli et al., 2014, Fromer et al., 2014, Li et al., 2015, Codina-Solà et al., 2015, De Rubeis et al., 2014), ID (Rauch et al., 2012, de Ligt et al., 2013, Li et al., 2015) and a patient with generalised epilepsy, mental decline and autistic behaviour (Kamiya et al., 2004), as well as >20 missense alleles implicated in seizure disorders (Kwong et al., 2015, Boutry-Kryza et al., 2015, Shi et al., 2012).

The data implicating *SCN2A* in neuropsychiatric disorders is therefore compelling, regardless of the underlying pathological mechanism or specific diagnosis, however the evidence for a role for *SCN2A* in schizophrenia is weak, relying as it does on one *de novo* LoF mutation (Fromer et al., 2014, Li et al., 2015). Therefore, we undertook mutation screening of the entire coding-sequence (CDS) of *SCN2A* in sample of 980 individuals with schizophrenia, aiming to identify LoF alleles to support involvement of this gene in disease pathogenesis.

Methods

Case Samples

We acquired blood-sample genomic DNA from individuals with Treatment-Resistant Schizophrenia (TRS) via samples taken from patients taking clozapine (Clozaril™) from the United Kingdom Clozaril Monitoring Service; these sample have been employed in previous publications and further details can be found there (Hamshere et al., 2013. Strange et al., 2012). The sample consisted of 980 Caucasians with TRS (Male 705, Female 275). Anonymisation of samples from the clozapine monitoring service was maintained and

genetic studies on this sample are approved by relevant ethics panels and are in accordance with the UK Human Tissue Act.

Comparison Sample Data

Exome sequencing data were available from the NHLBI Exome Sequencing Project (ESP) via the Exome Variant Server (EVS) (http://evs.gs.washington.edu/EVS/), release ESP6500 (Exome Variant Server, [Accessed May 2015]). The dataset includes 4300 European Americans and 2203 African American individuals recruited for studies of heart, lung and blood disorders and the sample is frequently used as a comparison dataset (Zaidi et al., 2013, Lim et al., 2013, Li et al., 2015). Genotype calls for insertion and deletion variants (Indels) are less robust than SNP calls having a higher false positive rate and so are considered experimental (Tennessen et al., 2012, Project, 2014). Therefore, insertions and deletions were excluded from comparison analysis.

Polymerase Chain Reaction (PCR), High-Resolution Melting Analysis (HRMA) and Sequencing

PCR was performed using a previously published methodology (Dwyer et al., 2010, Carroll et al., 2011). The oligonucleotide primers for PCR and sequencing are given in Supplementary Table ST1. High Resolution Melting Analysis (HRMA) was performed using LightScanner™ technology (Idaho Technologies) according previously published criteria (Carroll et al., 2011, Dwyer et al., 2010). PCR products from HRMA were cleaned using AMPure™, DNA sequencing reactions used Big-Dye™ terminator chemistry and sequencing reactions were cleaned using cleanSEQ™. Samples were analysed using an Applied Biosystems™ ABI3100 genetic analyzer and then sequencing traces were inspected manually using Sequencher™ software.

Bioinformatics

Genomic positions and sequences were obtained from The UCSC Genome Browser, Feb. 2009, HG19, NCBI Build 37.1 (http://genome.ucsc.edu/) (Kent et al., 2002). Polymorphism details were extracted from dbSNP build 138 (dbSNP 135 was used during study design) (Sherry et al., 1999) and the 1000 Genomes Project (Abecasis et al., 2010). PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/) (Koressaar and Remm, 2007). Identified alleles were annotated using various software programs including SIFT and PolyPhen-2 (Kumar et al., 2009, Artimo et al., 2012, Woolfe et al., 2010, Adzhubei et al., 2013, Punta et al., 2012).

Results

Primary Analysis

The *SCN2A* locus lies at chromosome 2q24.3 and 3 mRNA transcripts code for 2 protein isoforms according to RefSeq. The 3 mRNA species of *SCN2A* have 27 exons containing coding sequence. Together with splice sites (≥2bp of adjacent intron), these were covered by 30 PCR amplimers ranging in size from 220-487bp. Oligonucleotide primers did not encompass known genetic variants >1% minor allele frequency (MAF) according to dbSNP build 135 and HapMap CEU samples (Altshuler et al., 2010).

PCR was completed and HRMA performed in all 980 individuals for all 30 PCRs. A total of 19 exonic variants (12 synonymous, 7 non-synonymous, 0 splice-site) (Table 1) were identified in this study, including all variants >1% MAF in dbSNP 138 (HapMap CEU and 1000 Genomes GBR sample (Abecasis et al., 2012, Altshuler et al., 2010)). Five novel variants were found. Of the non-synonymous variants, 6 are missense and one is a novel, nonsense variant introducing a stop-codon at amino acid 169 (169 E>X). No canonical splice-site variants were identified. When compared to the EVS dataset of 6503 sequenced individuals we identify no LoF mutations. An example of HRMA analysis where E169X was identified is shown in Supplementary Figure SF1 and the sequencing traces for the corresponding allele (chr2: 166165204 G/T) are shown in Figure 1.

The EVS database reports an indel that would introduce a frameshift (chr2:166246312, AT/A), however the genotypes for this variant are not in Hardy-Weinberg Equilibrium in both European Americans (AT/AT=1, AT/A=2, A/A=4105, HWE chi-square p<0.0001) and African Americans (AT/AT=1. AT/A=1. A/A=2089, HWE chi-square p<0.0001), in agreement with indel variants being more likely to represent false-positives (Tennessen et al., 2012) and justifying the exclusion of indel variants from analysis.

Secondary Analysis

HRMA analysis precludes the study of rare homozygote genotype calls, as only heterozygote genotypes are detected reliably (Dwyer et al., 2010). Therefore, only alleles with a MAF <0.5% have allele counts shown (Table 1). None of the 980 samples harboured >1 of these 19 rare alleles.

The 6 missense variants identified were analysed using SIFT and Polyphen-2 (Kumar et al., 2009, Adzhubei et al., 2013). Both packages highlighted the same two variants as being possibly of functional significance (damaging with low-confidence or possibly damaging). One is a novel singleton variant (chr2:166198966 G/C R850P) not present in the EVS controls. The other is also a novel variant present in two cases (chr2:166226804, G/T, V1282F) and absent in the EVS dataset and is therefore potentially interesting with the caveat that the observations are rare and the biological significance uncertain (Fisher's exact test, 2-tailed p-value=0.034). The two samples do not represent a duplicate sample as they have different LightScanner profile genotype calls at rs2121371 and rs2060198.

Mutation screening comparison

Given differences in the mutation detection platforms, we compared the frequency of rare alleles in cases and the EVS dataset. In cases we identified a small but significant deficit of the total number of variants identified when compared to the EVS sample (Pearson chi-square, two-tailed p=0.04, OR 0.61 (0.38-0.98)). There was no significant difference for synonymous variants (p=0.21, OR 0.70 (0.40-1.23)) but there was a trend for an excess of missense variants in the EVS dataset (p=0.07, OR 0.47 (0.20-1.09)). The LightScanner approach employed here may therefore be less-sensitive than WES for the identification of rare alleles, suggesting that our detection of a rare LoF mutation in the sample is not simply due to higher screening efficiency. Alternatively the EVS sample may harbour more missense variants at *SCN2A* than our case sample.

Discussion

Our understanding of the aetiology of the major psychiatric disorders remains limited, however a large part of the risk of developing these disorders is due to our individual genetic architecture (Owen, 2012). Promising insights into the molecular aetiology of neuropsychiatric diseases have come from the study of rare, moderately penetrant alleles such as inherited or *de novo* CNVs (Guilmatre et al., 2009, Kirov et al., 2012, Cooper et al., 2011, de Ligt et al., 2013, Girirajan et al., 2013) or more recently smaller LoF mutations (Rauch et al., 2012, Sanders et al., 2012, Fromer et al., 2014, Iossifov et al., 2012, Li et al., 2015, De Rubeis et al., 2014, Purcell et al., 2014). One of the genes implicated by these recent studies of ID, ASD and schizophrenia is *SCN2A* (Sanders et al., 2012, Fromer et al., 2014, Rauch et al., 2012, de Ligt et al., 2013, Li et al., 2015, Codina-Solà et al., 2015, De Rubeis et al., 2014), encoding the alpha subunit of voltage gated sodium channels. However, amongst these disorders the evidence for *SCN2A* involvement in schizophrenia is dependent upon one splice-site mutation (Fromer et al., 2014, Li et al., 2015).

We performed mutation screening of *SCN2A* in 980 individuals with schizophrenia to identify further LoF mutations and support a role for the gene in disease aetiology. Previously, nonsense loss-of-function mutations have been identified in nine unrelated individuals with ASD (Tavassoli et al., 2014, Sanders et al., 2012, Fromer et al., 2014, Codina-Solà et al., 2015, De Rubeis et al., 2014), three unrelated cases with ID (de Ligt et al., 2013, Rauch et al., 2012), an individual with epilepsy and autistic features (Kamiya et al., 2004) and an individual with schizophrenia (Fromer et al., 2014). Therefore, 14 *de novo* loss-of-function (LoF) mutations were known at *SCN2A* in individuals that have a neuropsychiatric diagnosis. Genic recurrence of *de novo* LoF alleles in unrelated individuals with a shared diagnosis unambiguously implicates LoF alleles at *SCN2A* in the pathogenesis of ASD and ID as the occurrence of ≥2 such alleles is highly-unlikely to occur by chance (Sanders et al., 2012). In 980 individuals with TRS we identify a further LoF mutation to the one identified by Fromer et al (Fromer et al., 2014). Although we cannot establish if the LoF variant identified in this study is inherited or *de novo*, a caveat of this study, we do not identify any LoF mutations in the EVS comparison dataset of 6503 individuals, which is suggestive of a pathogenic role for the variant. When taken with previous mutations identified in schizophrenia and related disorders our simple study is supportive for the involvement of *SCN2A* in the pathogenesis of schizophrenia.

Loss-of-function mutations at *SCN2A* may have a greater or lesser effect on risk of developing disease for each of the neuropsychiatric phenotypes discussed, or their higher prevalence in some samples may reflect screening in larger or smaller samples. Using data from multiple large WES studies of several neuropsychiatric phenotypes (Li et al., 2015), LoF mutations at *SCN2A* occurred in 7/5893 ASD cases (0.12%, [95% CI 0.03% to 0.21%]), 3/151 cases for ID (1.99%, [95% CI −0.24% to 4.21%]) and 2/1828 cases for schizophrenia (0.11%, 95% CI −0.04% to 0.26%) when the current study is included. These studies however do not include smaller, directed sequencing studies of the locus (Kamiya et al., 2004, Tavassoli et al., 2014). When compared to a mutation rate of 0% in a control sample of 6503 individuals this suggests a moderate-to-high penetrance for these alleles. However, particularly for schizophrenia, ID and seizure disorders much larger scale

sequencing studies are warranted to characterise the true frequency and effect-size of LoF mutations at *SCN2A* in each phenotype. A soon to be published coalition of WES studies using different methodologies, including 60,706 unrelated individuals of varying ancestry, and affection status may help to uncover the true frequency of LoF mutations at *SCN2A* in the wider population (Exome Aggregation Consortium, ExAC Browser Beta (http://exac.broadinstitute.org) [Accessed: July 2015]). Currently only two LoF mutations are observed in the >120,000 chromosomes included in this dataset confirming a low-frequency of LoF mutations at *SCN2A*. In addition, the missense variant V1282F is absent in this dataset also. Screening of such large samples will undoubtedly uncover more potentially damaging alleles at *SCN2A* although the significance of these will be difficult to interpret without accurate phenotypic information and it is likely that large, well-controlled association studies will be required to assess the true effect on disease risk of damaging mutations at this locus.

In further study of schizophrenia samples it may be pertinent to examine a wide phenotypic distribution, given that the present sample of TRS may represent a particularly severe category of schizophrenia. *In vitro* or *in vivo* analysis of the handful of loss-of-function mutations at *SCN2A* may then be justified and necessary to understand the molecular biology of these mutations. Previous studies of the SCN2A protein, NaV1.2 have implicated the protein in redistribution of channels required for neural plasticity, dependent upon scaffolding proteins (ankyrin G) (Garrido et al., 2003) and have also shown a neocortical and hippocampal expression pattern that diminishes throughout neurodevelopment (Liao et al., 2010). Given the key role for SCN2A in neuronal development and the specificity of the molecular lesions identified so far, the gene offers promise to aid understanding of the molecular pathogenesis of a complex neuropsychiatric disorder in some individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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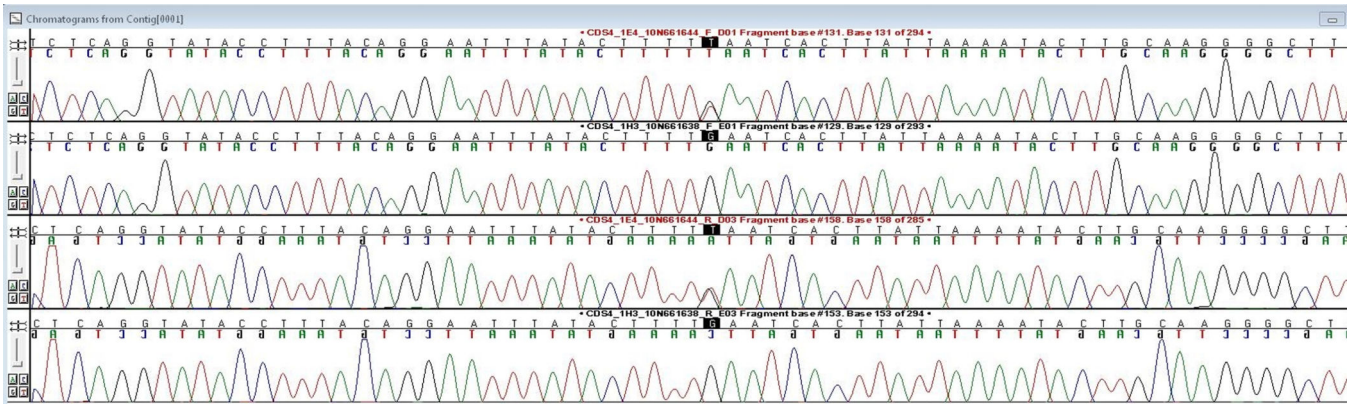

Figure 1.

Forward and reverse sequencing traces of the E169X heterozygous carrier and a non-carrier. The mutated base is central and highlighted

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Table 1

All 19 exonic variants identified by LightScanner™ HRMA and sequencing analysis of 980 schizophrenia cases. For common alleles (≥0.5%) where HRMA cannot be reliably used for accurate genotyping the HapMap CEU or 1000 Genomes project minor allele frequencies are given. The allele count refers to the number of samples showing the same LightScanner allele profile, which identifies all heterozygotes but may not identify rare homozygotes.

PCR	Flanking Sequence	rs	Hg19	Type	MAF (%)	Allele Count
CDS1	ttctttacca[G/A]ggaatccctt	rs17183814	166152389	19 R > K	8.48	-
CDS4	ttatactttt[G/T]aatcacttat	novel	166165204	169 E > X	Singleton	T=1/G=1959
CDS11	ctgaatcaag[A/G]gacttcagtg	rs200246820	166172013	Synonymous	0.10	G=2/A=1958
CDS12	actttgctga[T/C]gatgagcaca	rs141815642	166179779	Synonymous	1.02	C=20/T=1940
CDS12	tgttcgtgcc[G/T]cacagacatg	rs114315466	166179836	Synonymous	0.10	T=2/G=1958
CDS13	ctactgaaac[A/G]gaaataagaa	rs147891446	166183379	Synonymous	1.07	G=15/A=1945
CDS15	tcagttctcc[G/C]atcattccgg	novel	166198966	850 R > P	Singleton	C=1/G=1959
CDS16	aagagctaca[A/G]agaatgtgtc	rs2228980	166201225	908 K > R	0.66	G=13/A=1947
CDS17A	gtttcaggtt[C/T]tgaacctctt	rs375858093	166210705	Synonymous	Singleton	T=1/C=1959
CDS17B	ttgctgttgg[A/C]gaatctgact	rs138143967	166211112	Synonymous	0.10	C=2/A=1958
CDS18	ttgaacctga[G/A]gaatcccttg	novel	166221739	Synonymous	Singleton	A=1/G=1959
CDS20	cttcctgatt[G/T]ttgatgtgag	novel	166226804	1282 V > F	0.10	T=2/G=1958
CDS23	tacaggccac[G/A]tttaagggat	rs138241682	166234112	Synonymous	Singleton	A=1/G=1959
CDS26	gagtcaagaa[A/G]tgacaaacat	novel	166243379	1559 M > V	Singleton	G=1/A=1959
CDS27A	gaatcctacg[T/A]ctgatcaaag	rs2060198	166245230	Synonymous	26.83	-
CDS27B	ctgggatgga[T/C]tgctagcacc	rs199698414	166245471	Synonymous	Singleton	T=1/C=1959
CDS27B	agatgttcta[T/C]gaggtttggg	rs200603552	166245713	Synonymous	0.15	C=3/T=1957
CDS27C	gattttgcag[A/C]tgccctggat	rs138497939	166245784	1823 D > A	Singleton	C=1/A=1959
CDS27D	cgtctccacc[C/T]tcgtatgata	rs73025979	166246235	Synonymous	Singleton	T=1/C=1959