

1 Association between schizophrenia and both loss of function and missense mutations
2 in paralog conserved sites of voltage-gated sodium channels

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23

24

Abstract

25

26 Sequencing studies have highlighted candidate sets of genes involved in
27 schizophrenia, including activity-regulated cytoskeleton-associated protein (ARC)
28 and N-methyl-d-aspartate receptor (NMDAR) complexes. Two genes, *SETD1A* and
29 *RBM12*, have also been associated with robust statistical evidence. Larger samples
30 and novel methods for identifying disease-associated missense variants are needed to
31 reveal novel genes and biological mechanisms associated with schizophrenia. We
32 sequenced 187 genes, selected for prior evidence of association with schizophrenia, in
33 a new dataset of 5,207 cases and 4,991 controls. Included were members of ARC and
34 NMDAR post-synaptic protein complexes, as well as voltage-gated sodium and
35 calcium channels. We observed a significant case excess of rare (<0.1% in frequency)
36 loss-of-function (LoF) mutations across all 187 genes (OR = 1.36; $P_{corrected} = 0.0072$)
37 but no individual gene was associated with schizophrenia after correcting for multiple
38 testing. We found novel evidence that LoF and missense variants at paralog conserved
39 sites were enriched in sodium channels (OR = 1.26; $P = 0.0035$). Meta-analysis of our
40 new data with published sequencing data (11,319 cases, 15,854 controls and 1,136
41 trios) supported and refined this association to sodium channel alpha subunits ($P =$
42 0.0029). Meta-analysis also confirmed association between schizophrenia and rare
43 variants in ARC ($P = 4.0 \times 10^{-4}$) and NMDAR ($P = 1.7 \times 10^{-5}$) synaptic genes. No
44 association was found between rare variants in calcium channels and schizophrenia.

45 In one of the largest sequencing studies of schizophrenia to date, we provide
46 novel evidence that multiple voltage-gated sodium channels are involved in
47 schizophrenia pathogenesis, and increase the evidence for association between rare

48 variants in ARC and NMDAR post-synaptic complexes and schizophrenia. Larger
49 samples are required to identify specific genes and variants driving these associations.

50

51 **Author Summary**

52

53 Common and rare genetic variations are known to play a substantial role in the
54 development of schizophrenia. Recently, sequencing studies have started to highlight
55 specific sets of genes that are enriched for rare variation in schizophrenia, such as the
56 synaptic gene sets ARC and NMDAR, as well as voltage-gated sodium and calcium
57 channels. To confirm the role of these gene sets in schizophrenia, and identify specific
58 risk genes, we sequenced 187 genes in a new sample of 5,207 schizophrenia cases and
59 4,991 controls. We find an excess of protein truncating mutations with a frequency
60 $<0.1\%$ in all 187 targeted genes, and provide novel evidence that mutations altering
61 amino acids conserved across sodium channel proteins are risk factors for
62 schizophrenia. Through meta-analysing our new data with previously published
63 sequencing data sets, for a total of 11,319 cases, 15,854 controls and 1,136 trios, we
64 increase the evidence for association between rare coding variants and schizophrenia
65 in voltage-gated sodium channels, as well as in synaptic gene sets ARC and NMDAR.
66 Although no individual gene was associated with schizophrenia, these findings
67 suggest larger studies will identify the specific genes driving these associations.

68

69 **Introduction**

70

71 Schizophrenia is a highly heritable polygenic disorder [1]. Collectively, common
72 alleles contribute up to half of the genetic variance in schizophrenia liability [2, 3],

73 and 145 distinct loci have currently been associated with the disorder at genome-wide
74 levels of significance in the most recent genome-wide association study [4].
75 Schizophrenia risk is also conferred by rare mutations including copy number variants
76 (CNVs) [5, 6] and rare coding variants (RCVs) [7, 8], each of which sometimes occur
77 as *de novo* mutations [9, 10].

78 Studies of RCVs have the potential to inform schizophrenia pathogenesis
79 since they can pinpoint specific functional variants in individual genes. However, to
80 date, only two genes, *SETD1A* [11] and *RBM12* [12], have been strongly implicated.
81 A major limiting factor, as for studies of common variants, is that for complex
82 disorders, large samples are generally required to obtain robust results in case-control
83 [13] studies. To date, the largest published sequencing studies of schizophrenia have
84 involved around 5,000 cases, 9,000 controls and 1,000 parent-proband trios [7, 11],
85 which are an order of magnitude smaller than recently published schizophrenia SNP
86 genotyping studies of common risk alleles (e.g. 40,675 cases and 64,643 controls [4]).
87 Nevertheless, whole exome sequencing studies have provided important clues to the
88 pathophysiology of schizophrenia. For example, proband-parent trio based studies
89 have shown *de novo* RCVs to be significantly enriched among glutamatergic post-
90 synaptic proteins, in particular, the activity-regulated cytoskeleton-associated protein
91 (ARC) and N-methyl-d-aspartate receptor (NMDAR) complexes [9]. These synaptic
92 gene sets, first associated with schizophrenia through studies of *de novo* CNVs [10],
93 have also shown evidence for association in independent case-control CNV [14] and
94 sequencing datasets [7, 15]. More recently, in an extension of the Swedish sample
95 used by Purcell *et al* 2014 [15], the authors documented an elevated exome-wide
96 burden of ultra-rare, protein disruptive alleles, which was concentrated among 3,388
97 neuron-specific genes, particularly those that are expressed at synapses, including the

98 ARC and NMDAR complexes [7]. Additionally, the enrichment of RCVs in
99 schizophrenia has been shown to be concentrated among 3,488 genes that are depleted
100 for loss-of-function (LoF) mutation in large population cohorts [16, 17].

101 In the current study, we performed targeted sequencing of 187 genes, selected
102 for prior evidence for association with schizophrenia (Table S1), in 5,207 cases and
103 4,991 controls, none of which have contributed to previous schizophrenia sequencing
104 studies. Among these targeted genes, we had complete membership of 4 gene sets,
105 each of which has been postulated to be implicated in schizophrenia through rare
106 variant analysis [7, 9, 10, 15, 18]; ARC and NMDAR post-synaptic protein
107 complexes [9, 10], and voltage-gated sodium [18] and calcium channels [15].
108 Multiple voltage-gated calcium channels have also been strongly implicated through
109 common variant studies [19]. Our primary aims were to a) investigate association
110 between schizophrenia and RCVs in these gene sets, and b) identify individual genes
111 that might drive the gene set associations. The remainder of the genes targeted for
112 sequencing were selected on the basis of supportive evidence from at least two
113 sources (see methods).

114 Most recent studies of RCVs in schizophrenia have focused on LoF alleles.
115 However, it is clear that missense alleles also contribute to schizophrenia risk [7, 9],
116 but in contrast to LoF alleles, *in silico* methods cannot distinguish at high sensitivity
117 and specificity between missense alleles that alter the function of the encoded protein
118 and those that are benign. Recently, it has been shown that restricting analyses to
119 missense variants affecting amino-acids that are conserved within paralogous gene
120 families improves power for identifying pathogenic alleles [20]. Given that two of our
121 targeted gene sets consist of paralogous gene families (voltage-gated sodium and

122 calcium channels), we exploited this approach to analyse missense variants that are
123 more likely to have an adverse effect on protein function [20].

124 Finally, to maximise power, we combined the new sequencing data with
125 independent, published schizophrenia case-control (Swedish [7] and UK10K [11]
126 datasets) and trio exome-sequencing data (see methods), yielding a combined analysis
127 of RCVs in a total of 11,319 cases, 15,854 controls and 1,136 trios.

128

129 **Results**

130

131 Mutation burden

132 In the targeted sequence sample, we performed six primary tests of mutation burden
133 across all 187 targeted genes: LoF, nonsynonymous damaging and nonsynonymous
134 variants, each under two allele frequency thresholds; < 0.1% and singletons. A
135 significant ($P_{corrected} < 0.05$) excess of LoF mutations (< 0.1% in frequency) was
136 observed in cases (Table 1), who had a mean excess of 0.013 LoF mutations per
137 person across the 187 targeted genes (Supplementary Table S2). There was no
138 significant difference in the rate of any other class of allele (Table 1). Although not
139 part of our primary analysis, we note no difference between cases and controls in the
140 rate of synonymous mutations (frequency < 0.1%) (OR (95% CI) = 1.02 (0.94-1.08); P
141 = 1), suggesting the enrichment of LoF mutations in cases is unlikely to be due to
142 technical artefacts.

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147 **Table 1.** Mutation burden in 187 targeted gene

Mut. Type	MAF < 0.1%						Singletons					
	Targeted sequencing sample				Meta-analysis		Targeted sequencing sample				Meta-analysis	
	N mut. Case/Con	Rate Case/Con	<i>P</i>	OR (95% CI)	<i>P</i>	OR (95% CI)	N mut. Case/Con	Rate Case/Con	<i>P</i>	OR (95% CI)	<i>P</i>	OR (95% CI)
LoF	271/195	0.052/0.039	0.0012	1.36 (1.13-1.64)	0.00035	1.22 (1.1-1.37)	94/66	0.018/0.013	0.047	1.38 (1-1.9)	0.0043	1.31 (1.09-1.57)
NSD	5,854/5,425	1.12/1.09	0.083	1.03 (1.00-1.07)	0.93	1.00 (0.98-1.03)	1,268/1,223	0.24/0.25	0.94	1.00 (0.92-1.08)	0.98	1.00 (0.95-1.06)
NS	9,199/8,625	1.77/1.73	0.14	1.02 (0.99-1.05)	0.85	1.00 (0.98-1.02)	1,878/1,845	0.36/0.37	0.56	0.98 (0.92-1.05)	0.38	0.98 (0.94-1.02)

148 Targeted sequencing sample includes data from 5,207 cases and 4,991 controls. Meta-analysis includes data from 11,319 cases and 15,854

149 controls. Rates correspond to the average number of mutations per case/control. *P* values are 2-sided, odds ratios (OR) and 95% confidence

150 intervals (CI) were generated from logistic regression models. In the targeted sequencing sample, *P* values that survive Bonferroni correction for

151 multiple testing (6 tests) are in bold. LoF = loss of function, NSD = nonsynonymous damaging, NS = nonsynonymous, mut. = mutation, MAF =

152 minor allele frequency.

153 Meta-analysis with two previously published case-control exome sequencing
154 datasets (Sweden and UK10K, see methods for detail) strengthened the evidence for
155 an increase in LoF mutations (frequency <0.1%) in cases (Table 1 and Supplementary
156 Table S3). The additional support came entirely from the Swedish rather than the
157 UK10K dataset (Sweden OR (95% CI) = 1.27 (1.08-1.51); $P = 4.8 \times 10^{-3}$; UK10K OR
158 (95% CI) = 0.95 (0.74-1.2); $P = 0.66$). The results contributing to the meta-analysis
159 are presented in Supplementary Table S3.

160 We partitioned the 187 genes into those intolerant of LoF mutation (pLi scores
161 > 0.9 in nonpsych-ExAC data [16]) and those that are not intolerant (pLi \leq 0.9). Meta-
162 analysis of the case-control data showed association between schizophrenia and rare
163 (frequency <0.1%) LoF mutations was driven by LoF intolerant genes (106 genes
164 with pLi > 0.9: OR (95% CI) = 1.63 (1.33 – 2.0); $P = 2.9 \times 10^{-6}$. 81 genes with pLi \leq
165 0.9: OR (95% CI) = 1.09 (0.95 – 1.24); $P = 0.21$). The difference in effect size
166 between pLi and non-pLi burden tests was significant (Z-test $P = 0.0006$).

167

168 Gene set analysis

169 In the targeted sequencing analysis, as only LoF mutations with a frequency <0.1%
170 were significantly enriched in cases after correcting for multiple testing, we tested this
171 class of mutation for gene set enrichment.

172 *ARC and NMDAR*: In the targeted sequencing sample, cases had a higher rate
173 of LoF mutations (frequency <0.1%) in ARC and NMDAR sets (Fig 1). When meta-
174 analysed with published case-control datasets, we found strong evidence that LoF
175 mutations in NMDAR genes were associated with schizophrenia ($P = 1.6 \times 10^{-4}$, Fig 1
176 and Supplementary Table S4), but weaker evidence for association with ARC genes
177 ($P = 0.047$, Fig 1 and Supplementary Table S4).

178 To summarize the current status of RCVs in the above gene sets, we combined
 179 the case-control meta-analysis data with the *de novo* mutation data, selecting the class
 180 of *de novos* reported to be most strongly enriched in these gene sets (nonsynonymous
 181 *de novo* mutations in ARC and LoF *de novo* mutations NMDAR) in the previous
 182 work [9]. In the trio data, nonsynonymous and LoF *de novo* mutations were
 183 associated with ARC ($P = 0.0015$) and NMDAR ($P = 0.014$), respectively.
 184 Combining the *de novo* enrichment results with the case-control meta-analysis results
 185 (LoF, frequency $< 0.1\%$), both ARC ($P = 4.0 \times 10^{-4}$) and NMDAR ($P = 1.7 \times 10^{-5}$)
 186 were associated with schizophrenia (Table 2).

187 The ARC and NMDAR complexes share 9 overlapping genes: when excluded
 188 from the analysis, we observed independent evidence for association with both gene
 189 sets (case-control-*de novo* meta-analysis: ARC $P = 9.4 \times 10^{-4}$; NMDAR $P = 7.4 \times 10^{-5}$).
 190

191

192 **Table 2.** Synaptic gene set meta-analysis.

Gene set (N genes)	Case-Control meta-analysis			<i>De novo</i> analysis	Case-control- <i>de novo</i> combined
	N (Rate)	<i>P</i> 2-sided	OR (95% CI)	<i>P</i> (Obs/Exp)	1-side <i>P</i> (Fisher's combined)
ARC (28)	32/27 (0.0028/0.0017)	0.047	1.78 (1.01-3.13)	0.0015 (7/1.64)	4.0×10^{-4}
NMDAR (61)	114/111 (0.01/0.007)	0.00016	1.69 (1.29-2.21)	0.014 (3/0.49)	1.7×10^{-5}

193 The case-control meta-analysis tested LoF variants (frequency $< 0.1\%$) for ARC and
 194 NMDAR in 11,319 schizophrenia cases and 15,854 controls. The *de novo* analysis
 195 tested nonsynonymous and LoF variants in ARC and NMDAR, respectively, in 1,136

196 schizophrenia trios. Full details of the analysis are presented in Supplementary Table
197 S4.

198

199 *Voltage-gated sodium and calcium channels:* We found nominally significant
200 evidence for enrichment in cases for LoF mutations (<0.1% frequency) in voltage-
201 gated sodium channels (targeted sequencing sample; OR (95% CI) = 1.99 (1.11-3.71);
202 $P = 0.02$; case-control-*de novo* meta-analysis: $P = 0.025$, Supplementary Table S4),
203 but no evidence for association between schizophrenia and voltage-gated calcium
204 channels (Supplementary Table S4).

205 *Paralog conserved ion channel sites:* In the targeted sequence sample, we
206 found a significant case excess of rare (frequency <0.1%) paralog conserved missense
207 and LoF variants in sodium channels (OR (95% CI) = 1.26 (1.08 – 1.47); $P = 0.0035$)
208 but not calcium channels (Supplementary Table S5). Enrichment of rare (frequency
209 <0.1%) paralog conserved missense and LoF variants was also supported in the full
210 case-control meta-analysis (OR (95% CI) = 1.18 (1.07 – 1.31); $P = 0.0014$, Fig 2,
211 Supplementary Table S5), and was robust to exclusion of LoF mutations from the
212 analysis (OR (95% CI) = 1.16 (1.04 – 1.29); $P = 0.007$). The effect size for rare
213 (frequency <0.1%) paralog conserved missense and LoF variants was significantly
214 different to that for paralog non-conserved missense variants (Z-test $P = 0.0018$),
215 indeed there was no enrichment for missense variants at paralog non-conserved sites
216 (case-control meta-analysis $P = 0.44$, Fig 2, Supplementary Table S5).

217 We divided the voltage-gated sodium channel set into alpha (10 genes) and
218 beta (4 genes) subunits, testing these separately; only the alpha subunits were
219 significantly enriched for rare (frequency <0.1%) paralog conserved missense and
220 LoF variants (Case-control meta-analysis: Alpha subunits; OR (95% CI) = 1.2 (1.08 –

221 1.33); $P = 0.00086$; Beta subunits, OR (95% CI) = 0.92 (0.52 – 1.62); $P = 0.76$). In all
222 sodium channel genes, one nonsense *de novo* mutation was observed in *SCN2A* (*de*
223 *novo* P value for LoF and paralog conserved missense variants in sodium channel
224 alpha subunits = 0.75; case-control-*de novo* meta-analysis: $P = 0.0029$).

225 Paralog conserved analysis did not find association with schizophrenia for
226 individual voltage-gated sodium channel genes after correction for multiple testing
227 (Supplementary Table S6).

228

229 Single gene analysis

230 In the meta-analysis (LoF; frequency <0.1%) of all data, no gene was
231 associated with schizophrenia after Bonferroni correction (given this analysis
232 included exome sequence data used to select our gene targets, we corrected for
233 ~20,000 genes, Supplementary Table S7). The most significant gene was *TAFI3* ($P =$
234 1.6×10^{-5}), support coming mainly from published LoF *de novo* mutations as noted
235 before [9] (Supplementary Table S7).

236

237 **Discussion**

238

239 Sequencing studies have started to provide novel insights into the genetic architecture
240 and aetiology of schizophrenia, although these are still limited by small sample sizes
241 and low power. Seeking to increase power for a prioritized set of genes, we sequenced
242 the coding regions of 187 schizophrenia candidates in over 10,000 samples that have
243 not contributed to previous sequencing studies of schizophrenia.

244 Across all candidates, we found a significant excess of LoF alleles in the
245 independent set of schizophrenia cases, confirming our hypothesis that one or more of

246 the candidates is involved in schizophrenia pathogenesis. The strongest evidence for
247 enrichment was for LoF alleles with a frequency $<0.1\%$, suggesting that recurrent
248 rather than only singleton schizophrenia risk alleles are present among our 187
249 targeted genes. This appears to contrast with a recent Swedish exome-sequencing
250 study of schizophrenia, which reported an increased exome-wide burden in cases of
251 ultra-rare protein altering alleles observed only once in their sample and never in
252 45,376 non-psychiatric ExAC individuals [7]. Our analyses (data not shown) of the
253 same Swedish dataset confirms at an exome wide level, singleton LoF mutations to be
254 more highly enriched than those with frequency $<0.1\%$ (after excluding singletons; Z-
255 test $P = 0.00035$) although this did not hold when restricted to the 187 targeted genes
256 (Z-test $P = 0.11$).

257 In the present study, we conducted the largest schizophrenia sequencing meta-
258 analysis of RCVs in the synaptic gene sets ARC and NMDAR to date. The inclusion
259 of our new independent data in this analysis strengthened the evidence for association
260 between RCVs in ARC and NMDAR and schizophrenia. In the context of previously
261 published research, where rare and *de novo* CNVs in these gene sets have been
262 consistently associated with schizophrenia [5, 10, 14], the results provide a strong
263 body of evidence for the involvement of ARC and NMDAR proteins in the aetiology
264 of schizophrenia.

265 Of the other two comprehensively tested functional candidate sets, only
266 voltage-gated sodium channels were enriched for rare (frequency $<0.1\%$) LoF alleles
267 in cases and this was independently supported by a novel case-control analysis of
268 missense variants at paralog conserved sites. The same voltage-gated sodium channel
269 gene set was previously implicated in schizophrenia in an analysis of compound
270 heterozygous mutation [18], a model that cannot be adequately tested in the present

271 dataset given the inability to phase very low frequency variation. In addition to
272 previous genetic support in schizophrenia, we note sodium channels have high
273 biological plausibility given that mutations in this gene set have been associated with
274 other neurodevelopmental disorders, including some forms of epilepsy and
275 developmental delay [20-22]. In the current study, no single sodium channel gene was
276 significantly associated with schizophrenia (after correction for multiple testing), with
277 the most significant gene being *SCN7A* ($P_{uncorrected} = 0.0012$).

278 The sodium channel set contains 14 genes, 10 encoding alpha subunits
279 involved in generating action potentials [21], and 4 beta subunits which, in
280 association with alpha subunits, modulate their gating and cellular excitability [22]. In
281 the present study, the evidence for association derives from mutations in alpha
282 subunits, although the absence of signal in beta-subunits might simply reflect low
283 power (there are fewer beta-subunits, of which paralog conservation scores are only
284 available for *SCN2B* and *SCN4B*, whereas paralog conservation scores are available
285 for all 10 alpha subunits).

286 Despite the increased sample size, we did not observe any single-gene
287 association that remained statistically significant after correction for multiple testing.
288 However, our gene set enrichment results suggest that significant single gene
289 associations will be discovered when larger samples are combined with more
290 effective methods for identifying disease-associated missense variation.

291 A limitation of our study was the exclusion of indel mutations (see methods)
292 from the targeted sequencing. Also, given the restrictions posed by targeted
293 sequencing, we were unable to test some of the much larger gene sets that have been
294 implicated by common and rare variation in schizophrenia, for example targets of
295 FMRP [4, 7, 9, 23].

296 In conclusion, we conducted one of the largest sequencing studies of
297 schizophrenia to date, which targeted the protein coding regions of 187 putative
298 schizophrenia risk genes. We found a significant excess of LoF alleles in cases among
299 all 187 targeted genes. By leveraging information from paralog conservation, we
300 provide novel evidence that multiple voltage-gated sodium channels are involved in
301 schizophrenia pathogenesis. We provide further support for association between
302 RCVs in ARC and NMDAR post-synaptic protein complexes and schizophrenia.
303 Larger samples are required to identify the specific genes and variants driving these
304 gene set associations.

305

306 **Methods**

307

308 Sample description

309 *Targeted sequence sample:* A total of 11,493 blood-derived DNA samples were
310 selected for targeted sequencing (5,724 cases and 5,769 controls). None have been
311 included in previous schizophrenia sequencing studies. The majority of sequenced
312 cases were from the CLOZUK dataset (n=4,647), which has been described
313 previously [24] and in the Supplementary Material. We sequenced additional cases
314 from the UK (Cardiff COGS cohort; n=521), Ireland (Dublin cohort; n=335) and the
315 Netherlands (GROUP cohort [25]; n=221). The majority of sequenced controls were
316 part of the WTCCC2 consortium (1958 birth cohort n=2,860, UK blood donors n =
317 2,463) [26-28]. Additional controls were sequenced from the Dublin (n= 230) and
318 GROUP cohorts (n=216) [25]. Sample descriptions are presented in the
319 supplementary material.

320 *Additional data sets:* We acquired publically available case-control exome
321 sequencing data from two previously published schizophrenia studies, the UK10K
322 Exome-sequencing study (N=1,352 cases and 4,769 controls) [11] and a Swedish
323 study (N= 4,867 cases and 6,140 controls) [7]. Additionally, *de novo* mutations from
324 1,136 published schizophrenia-proband parent trios were derived from our own and
325 other published whole exome sequencing studies [9, 29-36] (Supplementary Material
326 Table S8) .

327

328 Targeted Sequencing

329 We designed an Ampliseq custom panel (Thermo Fisher) for targeting the exons of
330 187 genes. The panel comprised two pools of 3,094 and 3,082 amplicons each and
331 covered a total region of 750kb. Library preparation used the Ion AmpliSeq protocol,
332 using 10ng of DNA per pool, the Ion Ampliseq Kit Version 2.0 and the AmpliSeq
333 custom panel (Thermo Fisher). We barcoded the libraries using the Ion Express
334 Barcode Adapters 1-96 Kit (Thermo Fisher). Unamplified libraries were quantified by
335 qPCR using Ion TQMN Quantitation kit following manufacturer's instructions
336 (Thermo Fisher) and diluted to 100pM. Groups of 72 uniquely barcoded libraries
337 were combined onto single Ion Chips. Sequencing was performed on the Ion Proton
338 benchtop sequencing platform (Thermo Fisher) following the manufacturer's
339 protocol. Sequencing took place in two waves, corresponding to different versions of
340 the sequencing and chip kits: 2,305 cases and 2,274 controls were sequenced in wave
341 1 using the Ion PI IC 200 kit and Ion PI Chip kit v2 BC (Thermo Fisher); 3,419 cases
342 and 3,495 controls were sequenced in wave 2 using the Ion PI HiQ kit and Ion PI
343 Chip kit v3 BC (Thermo Fisher). Data processing and QC procedures were conducted
344 independently for each wave. The mean target coverage for cases and controls passing

345 QC was 158X and 160X for wave 1, and 154X and 145X for wave 2, respectively
346 (density plots of sequence coverage in Supplementary Material Fig S1). Both cases
347 and controls had at least 95% of target bases covered at $\geq 10X$.

348

349 Gene-selection

350 We sequenced the coding regions of genes belonging to the following gene sets: ARC
351 (n=28) [9], NMDAR (n=61) [9], voltage-gated calcium channels (n=26) [15] and
352 voltage-gated sodium channels (n=14) [18]. We sequenced an additional 58 genes,
353 selected for having two or more supportive lines of evidence for association with
354 schizophrenia (full criteria for gene-selection described in Supplementary Material).
355 A list of all 187 sequenced genes, and the rationale for selection is presented in
356 Supplementary Table S1.

357

358 Data processing and quality control

359 Sequence data were independently processed for each Ion Torrent wave according to
360 GATK best practice guidelines [37, 38]. Reads were aligned to the human g1k (v37)
361 reference genome using bwa [39]. Variants were called using GATK haplotype caller
362 (v3.4) and filtered using the GATK Variant Quality Score Recalibration (VQSR) tool.

363 *Sample level QC:* Individuals were excluded if they were more than 3 standard
364 deviations from their sequencing wave's mean for: proportion of variants in dbSNP;
365 number of alternative alleles; number of singletons; total number of synonymous
366 mutations; total number of nonsynonymous mutations. When available, SNP
367 genotyping array data were used to assess sequencing-array genotype concordance
368 (array genotypes used as truth set) and to identify duplicate/first degree relatives. SNP
369 genotyping array data from 3 chips (Illumina OmniExpress, Illumina ExomeChip,

370 Immunochip) were available for 96% (5,508/5,724) of cases and 72% (4,149/5,769)
371 of controls. Samples were excluded if they had a genotype concordance < 0.9 or if
372 they were found to be one member of a duplicate (kinship coefficient > 0.354) or
373 first-degree relative (kinship coefficient > 0.177) pair (identified using the KING
374 toolset [40] in the Bioconductor package SNPRelate). For samples not previously
375 genotyped using SNP arrays, we used Ion Torrent sequence data to identify and
376 exclude duplicate samples.

377 Principal component analysis (PCA) was used to identify and exclude cases
378 and controls with non-European ancestry. We performed PCA in the 1000 genomes
379 project data (phase 3), using variants found in both targeted sequence data and 1000
380 genomes data, and projected our targeted sequence samples onto these PCs using
381 EIGENSOFT smartPCA [41]. Targeted sequence samples were excluded if PCs 1 and
382 2 were more than 3 standard deviations from the mean of PCs from European 1000
383 genome samples (Supplementary Fig S2). Post sample QC, 5,207 cases and 4,991
384 controls from the targeted sequence sample were retained for analysis.

385 *Variant level QC:* Variant sites within each targeted Ion Torrent sequencing
386 wave were excluded if they failed Hardy-Weinberg equilibrium exact tests ($\chi^2 P <$
387 10^{-8}), GATK VQSR filters, had $> 20\%$ missingness, or contained an indel (sequence
388 data produced by Ion Torrent instruments has low indel specificity [42]). For targeted
389 sequencing data, individual genotypes were set to missing if they had a depth (DP) $<$
390 20, genotype quality (GQ) < 80 , allele-balance (AB) < 0.9 for non-reference
391 homozygous genotypes or an AB < 0.2 or > 0.8 for heterozygous genotypes. For
392 analysis of previously published exome sequencing data, we applied filters which
393 more closely matched those described in their original publications (DP ≤ 10 , GQ $<$
394 30, AB < 0.9 for non-reference homozygous genotypes, AB < 0.2 or > 0.8 for

395 heterozygous genotypes) [7, 11]. All variant filtering was conducted using Hail
396 software (<https://github.com/hail-is/hail>) [43].

397

398 Variant annotation

399 In primary burden tests, we analysed three classes of mutation (LoF, nonsynonymous
400 damaging, and nonsynonymous) and two allele frequency thresholds (<0.1% and
401 singletons). We defined LoF variants as those producing premature stop codons
402 (nonsense) or situated at essential splice sites (within 2 bases either side of exon
403 junctions). Damaging nonsynonymous mutations were defined as LoF alleles and
404 missense alleles with PHRED-scaled CADD score ≥ 20 (representing the predicted
405 top 1% most deleterious variants in the genome) [44]. For analysis of previously
406 published case-control data (UK10K and Swedish samples), which were exome
407 sequenced on Illumina instruments, we included frameshift indels as LoF mutations
408 and frameshift/in-frame indels as nonsynonymous mutations. We annotated and
409 filtered variants using the frequencies observed in their respective data set (Ion
410 Torrent wave 1, Ion Torrent wave 2, UK10K or Swedish) and each ExAC sub-
411 population (European (Non-Finnish), African, East Asian, European (Finnish),
412 Latino, Other, South Asian) [16]. Singletons were annotated as alleles observed once
413 in all available sequence data (targeted, UK10K and Swedish data) and never in
414 45,376 individuals without a known psychiatric diagnosis from the Exome
415 Aggregation Consortium [16].

416 To annotate sites with their paralog conservation scores, we used para_zscores
417 downloaded from <https://zenodo.org/record/817898>. In our paralog conserved
418 analysis, we followed the publication describing this metric [20] by testing the burden
419 of all LoF alleles and missense alleles at sites annotated as having a para_zscore > 0.

420 Variants were annotated using Hail's ensemble VEP method (version 86,
421 <http://oct2016.archive.ensembl.org/index.html>).

422

423 Statistics

424 **Case-control analysis:** Gene set and single gene association statistics were generated
425 using the following Firth's penalised-likelihood logistic regression model.

426

427 $\text{Logit}(\text{pr}(\text{case})) \sim N \text{ test variants} + \text{baseline synonymous count} + \text{first 10 PCs} + \text{sex} +$
428 $\text{Ion Torrent sequencing wave (targeted analysis only)}$.

429

430 N test variants refers to the number of putative risk alleles observed in each sample
431 (e.g. number of LoF singletons in the gene/gene set tested). Baseline synonymous
432 count refers to the number synonymous alleles observed in all sequenced genes, using
433 the same allele frequency threshold used for the test variant (e.g., if LoF singletons
434 are the test variant, then the overall number of synonymous singletons are corrected
435 for). This covariate was only included in tests of nonsynonymous or LoF mutation to
436 control for potential unknown technical biases [7].

437 Test statistics were generated independently for each case-control dataset
438 (targeted, UK10K and Swedish), using the `logistf` function implemented in R (version
439 3.3.1). Odds ratios (ORs) for the increased risk of schizophrenia incurred for each
440 mutation were obtained from the Firth's penalised-likelihood logistic regression
441 model described above.

442

443 **De novo mutation analysis:** Enrichment of *de novo* mutation in genes/gene sets was
444 tested using the statistical framework described in Samocha *et al* 2014 [45]. Here,

445 gene mutation rates provided in Ware *et al* 2015 [46] were used to estimate the
446 expected number of *de novo* mutations in the gene/gene set, which was then
447 compared to the observed number of *de novo* mutations using a Poisson test
448 (implemented in R). As gene mutation rates for in-frame indels are not provided in
449 [46], we adopted the method used by the Deciphering Developmental Disorders Study
450 [47], which scaled frameshift mutation rates by the ratio of in-frame to frameshift
451 mutations reported to occur in genome-wide regions under weak negative selection
452 (ratio 1:9) [47]. To estimate the expected number of LoF *de novo* mutations and
453 missense *de novo* mutations at paralog conserved sites in sodium channel alpha
454 subunits, we used the mutation rates provided in [20].

455

456 **Meta-analysis:** Coefficients and standard errors from independently analysed case-
457 control (targeted, UK10K and Swedish) regression tests were meta-analysed as fixed
458 effects using the inverse-variance method (implemented in R using the `rma.uni()`
459 function as part of the `metafor` package). To obtain a single enrichment statistic for
460 meta-analysed case-control and *de novo* tests, we followed the method described in
461 [11], which combined a 1-tail case-control *P* value with the *de novo* Poisson test *P*
462 value using Fisher's combined method. For our combined case-control-*de novo* meta-
463 analysis of nonsynonymous damaging mutations, we included all *de novo*
464 nonsynonymous mutations (i.e. not just those with a CADD score ≥ 20), given they
465 are *a priori* more likely to be deleterious than inherited variation [48] and were the
466 class of mutation most strongly associated with schizophrenia candidate genes in our
467 previous publication [9].

468

469

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542

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728

729

730 **Supporting Information Captions**

731

732 **Table S1.** Genes targeted for sequencing. Gene IDs are presented for all 187 targeted

733 genes, along with the criteria used to select them for sequencing.

734

735 **Table S2.** Targeted sequence sample case-control LoF mutations. All loss of function
736 (LoF) mutations observed in new targeted sequence data, for alleles < 0.1% in
737 frequency. Singletons are indicated in the *Is_Singleton* column.

738

739 **Table S3.** Total-burden analysis. Case-control association results for all 187 targeted
740 genes and 106 LoF intolerant genes (genes with pLi scores > 0.9). Results for variants
741 with a frequency < 0.1% are shown in tab 1, and for singleton variants in tab 2.

742

743 **Table S4.** Primary gene-set analysis. Gene set association results for all three case-
744 control datasets (Targeted sequence sample, Swedish, UK10K) and case-control-*de*
745 *novo* meta-analysis (Fisher's combined method). Results for variants with a frequency
746 < 0.1% are shown in tab 1, and for singleton variants in tab 2.

747

748 **Table S5.** Ion Channel gene set analysis of LoF and paralog conserved missense
749 variants (<0.1% frequency). Paralog conservation scores (*para_zscores*)
750 were downloaded from <https://zenodo.org/record/817898>.

751

752 **Table S6.** Single-gene meta-analysis of sodium channel genes for LoF and
753 paralog conserved missense variants (<0.1% frequency).

754

755 **Table S7.** Primary single-gene meta-analysis of LoF variants (<0.1% frequency).
756 Single-gene results for all three case-control (Targeted sequencing, Swedish, UK10K)
757 and *de novo* mutations.

758

759

Figure Captions

760

761 **Fig 1.** Case-control analysis of rare (frequency <0.1%) loss of function mutations in
762 synaptic gene sets ARC and NMDAR.

763

764 **Fig 2.** Case-control meta-analysis of rare (frequency <0.1%) variants in voltage-gated
765 sodium channels. S = synonymous; NS = nonsynonymous; NDS = nonsynonymous
766 damaging; LoF = loss-of-function.

767



