Hotspots of missense mutation identify neurodevelopmental disorder genes and functional domains

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Although de novo mutations have been predicted to account for more cases of autism than gene-truncating mutations, most research has focused on the latter. We identified the properties of de novo missense mutations in patients with neurodevelopmental disorders (NDDs) and highlight 35 genes with excess missense mutations. Additionally, 40 amino acid sites were recurrently mutated in 36 genes, and targeted sequencing of 20 sites in 17,688 patients with NDD identified 21 new patients with identical missense mutations. One recurrent site substitution (p.A636T) occurs in a glutamate receptor subunit, GRIA1. This same amino acid substitution in the homologous but distinct mouse glutamate receptor subunit Grid2 is associated with Lurcher ataxia. Phenotypic follow-up in five individuals with GRIA1 mutations shows evidence of specific learning disabilities and autism. Overall, we find significant clustering of de novo mutations in 200 genes, highlighting specific functional domains and synaptic candidate genes important in NDD pathology.

Multiple lines of evidence strongly support a genetic basis for autism spectrum disorders (ASD). De novo mutations, originating primarily in the parental germline, are individually rare but their collective risk is substantial and accounts for an estimated 30% of simplex ASD cases1,2. Most of the emphasis on identifying high-impact risk variants has focused on establishing burden for likely gene-disruptive (LGD) mutations (nonsense, frameshift or splice-site)3–5. High-impact risk genes with primarily de novo missense mutations have been understood because a much smaller fraction (13%) are thought to be pathogenic when compared to de novo LGD mutations (42%)1. Moreover, de novo missense mutations are eight times as common, making it more challenging to prove their statistical relevance. Notwithstanding, a comparison of mutation rates in individuals with ASD and their unaffected siblings reveals that missense mutations contribute to disease risk in as many, if not more, cases than LGD mutations (12% versus 9%, respectively)1. The identification of genes with a substantial burden of missense mutations, then, is likely to highlight new classes of NDD risk genes.

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In some cases, this may reflect genes with such critical functions that LGD mutations are incompatible with life\(^1,6\). In other cases, the mutation's effect on the protein may differ. For example, missense mutations are more likely to have a gain-of-function effect\(^7\) when compared to LGD mutations, which are predominantly loss-of-function. Clustering of missense mutations may highlight important and even previously unknown functional domains, providing insight into ASD pathogenesis and future downstream therapeutic targets. High-confidence ASD risk genes have been successfully identified by searching for mutation recurrence\(^3,4,8,9\). Given that missense mutations are more common and ~90% of them are thought to be incidental\(^1\), a much larger sample size is required to prove pathogenicity. We took advantage of the substantial phenotypic and genotypic overlap between ASD, developmental delay and intellectual disability, epilepsy, congenital heart disease and schizophrenia\(^10\) to study the pattern and distribution of de novo missense mutations more broadly. We focused on recurrent site and clustered mutations and tested a larger cohort of affected children to identify pathogenic events and implicate new missense 'hotspot' genes in NDD pathogenesis.

RESULTS

Properties of de novo missense mutations in NDD patients

We began by assessing the rates of de novo missense mutation in cases and controls. We identified a total of 5,807 de novo missense mutations in cases (\(n = 8,477\)) and 1,475 such events in controls (\(n = 2,178\)) (Supplementary Table 1). The fraction of probands with one or more event (50.7%) was significantly greater than the fraction of controls (47.8%; \(P = 0.016, \text{ odds ratio (OR)} = 1.12 [1.02–1.24, 95\% \text{ confidence interval (CI)}]\), two-tailed Fisher's exact test) (Fig. 1a). As there were over three times as many cases as controls, we sought to limit the possibility that the signal was driven by rare outliers in cases and thus applied a secondary test, downsampling cases to match the number of controls. This further confirmed a significant increase in the rate of de novo missense mutations in cases (one-tailed empirical \(P = 9.22 \times 10^{-4}, \text{ OR} = 1.12 [1.06–1.19, 95\% \text{ CI}, 1 \times 10^6 \text{ permutations}]\) (Fig. 1a). While the odds ratios for these two tests are nearly identical, the Fisher's exact test is considered more conservative and the hypergeometric distribution generates a wider confidence bound for the odds ratio when compared to that obtained by simulation.

Out of 4,227 genes with rare de novo missense mutations in cases, 974 (23.0%) harbored mutations in two or more unrelated cases (Supplementary Table 2). In contrast, among controls, 101 out of 1,362 genes (7.4%) were mutated recurrently (Supplementary Table 3). Matching the number of cases and controls, we observed a significant increase in the number of genes among cases with two or more (one-tailed empirical \(P = 0.011, \text{ OR} = 1.26 [1.10–1.42, 95\% \text{ CI}, 1 \times 10^6 \text{ permutations}]\) and three or more (one-tailed empirical \(P = 3.10 \times 10^{-5}, \text{ OR} = 3.13 [2.22–4.03, 95\% \text{ CI}, 1 \times 10^6 \text{ permutations}]\) de novo missense mutations (Fig. 1b). The increased recurrence rate is not explained by increased mRNA or protein length, as genes with recurrent mutations in cases were significantly shorter than those with recurrent mutations in controls (mRNA, \(P = 5.19 \times 10^{-3}\); protein, \(P = 1.47 \times 10^{-3}\); two-tailed Wilcoxon rank-sum tests). Additionally, the total number of genes with mutations was smaller among cases (1,323 in downsampled cases versus 1,362 in controls), suggesting that mutations in cases are not randomly distributed but rather cluster within fewer genes.

We next compared the severity of de novo missense mutations between cases and controls by assessing the Combined Annotation–Dependent Depletion (CADD) score\(^11\). The CADD score distribution was significantly positively skewed in cases compared to controls, consistent with an increase in deleteriousness (\(P = 2.2 \times 10^{-4}\), two-tailed Wilcoxon rank-sum test). Further, at increasing minimum CADD score thresholds, the likelihood that an observed event can be attributed to a case increased (Fig. 2a). At a CADD threshold of 28, the likelihood rose dramatically (>1.2 positive likelihood ratio). Notably, mutations in genes with higher levels of recurrence in cases also showed significantly higher CADD scores (\(P = 5.87 \times 10^{-29}, F = 45.12, 3 \text{ d.f., one-way ANOVA}\), indicating that recurrence and severity are both valuable markers of missense pathogenicity and that they are highly correlated (Fig. 2b).

Genes with recurrent missense mutations

To further assess gene-specific recurrent mutations, we applied a probabilistic model that calculates the expected number of mutations in a gene, based on locus- and base-specific relative substitution rates\(^12,13\) (see Online Methods). We identified 35 genes that had significantly more de novo missense mutations in cases than expected (false discovery rate < 5%) (Supplementary Table 2). Only two genes, YIF1A and PHKA2, reached significance in controls (Supplementary Table 3). For 17 of the genes significant in cases, an excess of loss-of-function mutations has already been established by copy number variants (CNVs) and LGD mutation (for example, GRIN2B, PTEN and SCN2A)\(^14–16\). For 13 of the remaining significant genes, no LGD mutations have been identified in the 24 cohorts studied here or in individuals with NDD in the Online Mendelian Inheritance in Man (OMIM; http://omim.org/) or ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) databases. While six of these missense-only genes are well known and associated with specific phenotypes (for example, PACS1 and Schuurs-Hoeijmakers syndrome\(^17\)), the remaining seven warrant follow-up.
As a set, the 35 genes with excess de novo missense mutations are enriched for aspects of neuronal communication such as postsynaptic membrane potential regulation (6 observed versus 0.17 expected, 35.3-fold enrichment, \(P_{adj} = 5.87 \times 10^{-29}\)). The distribution of CADD scores skews significantly as the number of de novo missense mutations per gene in cases increases (\(P = 5.87 \times 10^{-29}\), one-way ANOVA) indicating an enrichment for genes with pathogenic mutations. Boxes show IQR, with notches representing the 95% CI of the median; whiskers are 1.5 times the IQR. Circles are outliers.

In addition to recurrent mutations within the protein-coding portion of genes, we also assessed amino acids in which two or more de novo missense mutations in unrelated individuals with NDDs have been identified, hereafter referred to as sites. We identified 40 sites in 36 genes, 10 of which have a significant burden of de novo missense mutation, after excluding mutations observed in population controls (minor allele frequency (MAF) > 0.01% in the Exome Sequencing Project (ESP; NHLBI GO ESP Exome Variant Server, Seattle, WA; http://evs.gs.washington.edu/EVS/, August 2016) (\(n = 6,503\)) or the Exome Aggregation Consortium (ExAC) database v.0.3 without neuropsychiatric disorders (\(n = 45,376\)) (Supplementary Table 4)). None of these mutations were observed in unaffected controls in denovo-db v0.9. Seven sites had more than two recurrent mutations (for example, PACS1 with six mutations at residue 203) and some genes (for example, SCN2A) had more than one recurrently mutated amino acid residue. Sixteen of the amino acid sites involved adjacent mutations in the same codon. Twenty-eight of the 40 sites (36 of 56 mutations) involved CpG dinucleotides, consistent with their known association with hotspots of single-nucleotide variation. Thirty-four sites had average mutation CADD scores of 20 or greater and 17 had a score of 30, indicating that they are in the top 1% of deleterious mutations in the human genome. This observation stands in contrast to the pattern of de novo recurrent missense in controls, where only one of the three sites had a CADD score greater than 20, although the number of events compared is few.

**Targeted sequencing of missense mutations**

Using single-molecule molecular inversion probes (smMIPs), we targeted 20 of these recurrent sites for sequencing in a large cohort of 17,688 patients with a primary diagnosis of ASD or development delay (including intellectual disability; Supplementary Tables 5 and 6). The set included primarily patients with idiopathic NDDs not yet tested by exome sequencing. We also included a set of unaffected siblings and other unaffected individuals as an additional control (\(n = 3,023\)). We identified and validated 21 recurrent missense variants at 12 sites in 11 genes among cases (Fig. 3a–c and Table 1). No variants were observed at any of the 20 sites in controls. The inheritance status for only eight of the variants identified in cases could be
Table 1: New recurrent mutations at targeted missense sites

| Gene      | Mutation          | Protein Identifier | ExAC v.0.3 | Genome-wide correctiona | SmMIPs (N = 17,688) | Total genome-wide correctiona | De novo | Unknown |
|-----------|-------------------|--------------------|------------|-------------------------|---------------------|-------------------------------|---------|
| PACS1     | p.Arg203          | NP_069496.2        | 1.12 × 10\(^{-7}\) | 1                      | 8                   | 22                            | 1       |
| PPP2R5D   | p.Asp107          | NP_060936.1        | 5.26 × 10\(^{-17}\) | 1                      | 4                   | 10                            | 1       |
| ALG13     | p.Arg937          | NP_001035232.1     | 9.47 × 10\(^{-12}\) | 1                      | 3                   | 6                             | 1       |
| SCN2A     | p.Arg937          | NP_005350.1        | 1.66 × 10\(^{-11}\) | 1                      | 3                   | 6                             | 1       |
| GRIA1     | p.Ala636          | NP_000818.2        | 1.11 × 10\(^{-7}\)  | 1                      | 2                   | 4                             | 1       |
| CLCN4     | p.Arg230          | NP_004510.1        | 3.36 × 10\(^{-7}\)  | 1                      | 2                   | 4                             | 1       |
| KCNQ3     | p.Arg473          | NP_037382.2        | 6.54 × 10\(^{-7}\)  | 1                      | 3                   | 6                             | 1       |
| CUX2      | p.Arg1617         | NP_002825.2        | 9.73 × 10\(^{-7}\)  | 1                      | 3                   | 6                             | 1       |

aBonferroni family-wise error rate correction based on 1.1 × 10\(^{13}\) codons in genome. bIn two affected siblings. cAllele in denovo-db v.0.9 has 0 occurrences in ExAC; allele found with smMIPs has been seen twice.

**Table 1**: Mutations identified with different databases. The table lists mutations identified in de novo P ACMG v.0.3 (N = 8,477) and genome-wide de novo-db v.0.9 (N = 30 total genes) and sequenced this extended set (~5 kbp of coding sequence) in a subset of the NDD cohort (Supplementary Tables 5 and 6). Combined with targeted sites, we discovered a total of 139 recurrent or clustered missense variants in 137 cases compared to 7 variants in 6 unaffected controls, representing a significant enrichment (P = 1.11 × 10\(^{-4}\), OR = 3.93 [1.76–10.89, 95% CI], two-tailed Fisher's exact test) (Table 2 and Supplementary Table 7). Twelve of the clustered missense mutations in cases were confirmed to be de novo, including events in SATB2 (Fig. 3d), GRIA1 (Fig. 4a), SCN2A, KCNQ3, SCN8A, DEAF1 and PRR2R1A (Supplementary Table 8).

In addition to new variants at sites in denovo-db v.0.9, targeted sequencing established 14 new sites, although inheritance status for most variants remains unknown. The specific variants at SCN8A p.Arg1617 and STXBP1 p.Arg551 have been seen previously in NDD. While Myhre syndrome has been associated only with residue 500 of SMAD4, in silico predictions suggest that the p.Arg496Cys mutations we identified are also likely to be pathogenic as the residue is highly conserved across species and the amino acid substitution is nonconservative. Detailed phenotypic information on one patient with this mutation indicates characteristics of the syndrome, including intellectual disability, short stature and dysmorphic facial features, suggesting that Myhre syndrome is not only limited to one amino acid. Phenotypic commonalities are also present among individuals with clustered mutations, indicating the functional relevance of protein domains. For example, seven out of eight patients with a mutation in the first DNA binding domain of SATB2 (Fig. 3d) have facial dysmorphism and seven out of eight have developmental delay.

**De novo missense mutations in GRIA1**

We identified a recurrently mutated amino acid in GRIA1 (Fig. 4a), encoding GluA1, a subunit of AMPA glutamate receptors, which was originally reported in one patient with intellectual disability and another with ASD. Both patients share a de novo G>A mutation resulting in an alanine-to-threonine amino acid replacement at residue 636 (NP_000818.2). Resequencing identified the same variant in three more patients with a primary diagnosis of ASD. One newly determined due to missing parental DNA. Six of these were de novo missense mutations (Table 1): PACS1 p.Arg203 (two mutations), GRIA1 p.Ala636, SCN2A p.Arg379, and SMAD4 p.Ile500 (two mutations). Of note, one of the inherited mutations (PTPN11 p.Gly503) is adjacent to the well-known Noonan syndrome recurrent mutation (PTPN11 p.Ser502) and was transmitted paternally to two children both affected with ASD and intellectual disability. No information on the father’s phenotype is currently available. Five genes corresponding to six sites were identified with two or more recurrent missense mutations in the NDD cohort, namely GRIA1 p.Ala636, PACS1 p.Arg203, SCN2A p.Arg379, SCN2A p.Arg937, SMAD4 p.Ile500, and ZNF215 p.Arg473. Phenotypic similarities are present in patients with shared mutations, such as those in ALG13 (Fig. 3b), where all six individuals with a mutation at residue 107 have both epilepsy and developmental delay even though they were recruited from cohorts with different primary diagnostic criteria. Both individuals with newly found mutations at SMAD4 p.Ile500 have features consistent with Myhre syndrome, including intellectual disability, short stature, facial dysmorphism and hearing loss (Supplementary Clinical Case Reports).

We also observed rare, potentially disruptive, missense variants in close proximity to the original recurrent site mutations, such as that in SMAD4 (Fig. 3c), and therefore reexamined our database for regions where multiple de novo missense mutations mapped within 10 amino acids. We designed smMIPs for 17 clustered regions as well as the 20 recurrent sites (in 30 total genes) and sequenced this extended set (~5 kbp of coding sequence) in a subset of the NDD cohort (Supplementary Tables 5 and 6). Combined with targeted sites, we discovered a total of 139 recurrent or clustered missense variants in 137 cases compared to 7 variants in 6 unaffected controls, representing a significant enrichment (P = 1.11 × 10\(^{-4}\), OR = 3.93 [1.76–10.89, 95% CI], two-tailed Fisher’s exact test) (Table 2 and Supplementary Table 7). Twelve of the clustered missense mutations in cases were confirmed to be de novo, including events in SATB2 (Fig. 3d), GRIA1 (Fig. 4a), SCN2A, KCNQ3, SCN8A, DEAF1 and PRR2R1A (Supplementary Table 8).
found mutation was confirmed as de novo; paternal DNA was not available for the other two but the mutation is not present in either of the patients' mothers. Using array comparative genomic hybridization, we found no evidence for large pathogenic CNVs in any of the three patients for whom we had DNA. While this position is a CpG dinucleotide and therefore prone to recurrent mutation, this variant has not been observed in 60,706 individuals studied by ExAC18. Moreover, we identified a second de novo missense mutation in close proximity (Fig. 4a) in a patient with developmental delay. The dearth of variants in healthy controls and the observation of the same recurrent variant in six unrelated patients (three of which were de novo; $P = 5.39 \times 10^{-3}$, one-tailed binomial test, genome-wide correction) suggested that the mutation is pathogenic.

The mutated site maps to the eighth position (p.Ala636) of a highly conserved nine-amino acid motif, SYTANLA AF (Fig. 4b), present in the M3 transmembrane domain of all glutamate receptors, which contains a critical role in channel gating25. The specific alanine-to-threonine mutation observed in the five patients here has been observed following channel blockade with NBQX. No changes in current magnitude or shape were seen in cells expressing the WT channel after 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), followed by an additional voltage ramp. Subtracted current with GluA1-mediated current, inward rectification was abolished following channel blockade with NBQX. No changes in current magnitude or shape were seen in cells expressing the WT channel after NBQX application (Fig. 4c). Consistent with GluA1-mediated current, inward rectification was abolished following channel blockade with NBQX. No changes in current magnitude or shape were seen in cells expressing the WT channel after NBQX application (Fig. 4d).

**Table 2 Rare$^{a}$ clustered missense mutations identified by targeted sequencing (CADD > 20)**

| Gene | Site or cluster | Protein identifier | denovo-db v.0.9 | Controls N | Missense variants N | Cases N | Missense variants N | All cases$^{b}$ N | Missense variants N | Known de novo |
|------|----------------|-------------------|----------------|------------|-------------------|--------|-------------------|----------------|----------------|----------------|
| PACS1 | p.Arg203 | NP_0060496.2 | 6 | 3,023 | 0 | 17,688 | 4 | 26,165 | 10 | 8 |
| PPP2R5D | p.Glu198 | NP_006236.1 | 4 | 3,023 | 0 | 17,688 | 3 | 26,165 | 7 | 4 |
| SCN2A | p.Arg937 | NP_001035232.1 | 3 | 3,023 | 0 | 17,688 | 5 | 26,165 | 8 | 5 |
| DEAF1 | p.Gln264 | NP_066288.2 | 2 | 3,023 | 0 | 17,688 | 7 | 26,165 | 9 | 5 |
| ALGI3 | p.Asn107 | NP_060936.1 | 2 | 3,023 | 0 | 17,688 | 1 | 26,165 | 6 | 5 |
| GRIA1 | p.Ala636 | NP_008181.2 | 2 | 3,023 | 0 | 17,688 | 5 | 26,165 | 7 | 4 |
| COL4A3BP | p.Ser260 | NP_001123577.1 | 3 | 3,023 | 0 | 17,688 | 12 | 26,165 | 15 | 11 |
| SCN8A | p.Gly214–p.Asn215 | NP_055006.1 | 3 | 3,023 | 0 | 17,688 | 11 | 26,165 | 14 | 11 |
| SATB2 | p.Arg399–p.Glu402 | NP_001165988.1 | 2 | 3,023 | 0 | 17,688 | 3 | 26,165 | 5 | 3 |
| TRPM7 | p.Thr379–p.Glu402 | NP_001165988.1 | 2 | 3,023 | 0 | 17,688 | 3 | 26,165 | 5 | 3 |
| LAMA2 | p.Glu173 | NP_001035232.1 | 3 | 3,023 | 0 | 17,688 | 12 | 26,165 | 15 | 11 |
| DEAF1 | p.Leu19–p.Glu220 | NP_066288.2 | 2 | 3,023 | 0 | 17,688 | 2 | 26,165 | 4 | 3 |
| SATB2 | p.Arg399–p.Glu402 | NP_001165988.1 | 2 | 3,023 | 0 | 17,688 | 3 | 26,165 | 5 | 3 |
| PPP2R1A | p.Ala203 | NP_001035232.1 | 3 | 3,023 | 0 | 17,688 | 12 | 26,165 | 15 | 11 |
| SCN8A | p.Gly167–p.Glu262 | NP_055006.1 | 3 | 3,023 | 0 | 17,688 | 3 | 26,165 | 5 | 3 |
| PTPN1 | p.Ala203 | NP_006236.1 | 4 | 3,023 | 0 | 17,688 | 3 | 26,165 | 7 | 4 |
| TRPM7 | p.Thr379–p.Glu402 | NP_001165988.1 | 2 | 3,023 | 0 | 17,688 | 3 | 26,165 | 5 | 3 |
| LAMA2 | p.Glu173 | NP_001035232.1 | 3 | 3,023 | 0 | 17,688 | 12 | 26,165 | 15 | 11 |
| DEAF1 | p.Leu19–p.Glu220 | NP_066288.2 | 2 | 3,023 | 0 | 17,688 | 2 | 26,165 | 4 | 3 |
| SATB2 | p.Arg399–p.Glu402 | NP_001165988.1 | 2 | 3,023 | 0 | 17,688 | 3 | 26,165 | 5 | 3 |
| PPP2R1A | p.Ala203 | NP_001035232.1 | 3 | 3,023 | 0 | 17,688 | 12 | 26,165 | 15 | 11 |

$^{a}$MAF < 0.1% in ExAC v.0.3 and ESP v.0.0.30. $^{b}$denovo-db v.0.9 and smMIPs.

$^{*}$MAF < 0.1% in ExAC v.0.3 and ESP v.0.0.30. $^{b}$denovo-db v.0.9 and smMIPs.

The mutated site maps to the eighth position (p.Ala636) of a highly conserved nine-amino acid motif, SYTANLA AF (Fig. 4b), present in the M3 transmembrane domain of all glutamate receptors, which plays a critical role in channel gating25. The specific alanine-to-threonine mutation observed in the five patients here has been observed at the functionally equivalent site in other members of the glutamate receptor gene family. It was first identified as a spontaneous mutation in Grid2 in a mouse line at Jackson Laboratories (Lurcher) that contains homomorphic GluR8 subunits selectively expressed in cerebellar Purkinje neurons26. Mice heterozygous for this mutation in the GluR8 receptor develop severe ataxia as a consequence of neurotoxicity from excess current flux. Notably, humans with the mutation in GluR8 also suffer from ataxia27. Engineering of the A>T mutation at the homologous site in the rat isoform of the GluA1 receptor produces a similar constitutively active phenotype with altered kinetic and pharmacological properties28–30.

To confirm constitutive activity or leak current in the human isoform of GRIA1 identified in affected patients, we synthesized cDNA encoding the human wild-type (WT) and mutant (A636T) at base-pair position 1906 (G/A). Leak current was measured using whole-cell voltage-clamp recordings of HEK 293 cells heterologously expressing either WT or A636T in the absence of agonist by applying a voltage ramp from −100 mV to +80 mV. GluA1-mediated current was confirmed by application of the AMPA receptor-selective antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), followed by an additional voltage ramp. Subtracted current in the presence of NBQX revealed a notable constitutive current in A636T-expressing but not WT-expressing cells (Fig. 4d). Consistent with GluA1-mediated current, inward rectification was abolished following channel blockade with NBQX. No changes in current magnitude or shape were seen in cells expressing the WT channel after NBQX application (Fig. 4c). Affected patients with the A636T mutation were heterozygous, indicating that a majority of receptors likely contain WT and A636T receptor subunits. To assess the functional
phenotype of these heteromeric receptors, we cotransfected equal ratios of WT and A636T DNA and performed the same voltage-ramp recordings (Fig. 4e). While a noticeable constitutive current was still present, it was smaller than with the homomeric A636T channel, demonstrating that the overall effects of the mutation are mitigated by the presence of the WT subunits (Fig. 4f).

Consistent with the prevalent role of GluA1 homomeric channels in synapse development and synaptic plasticity, phenotypic analysis of four of the individuals with the A636T mutation demonstrated common features (Supplementary Table 9), including mild to moderate intellectual disability (all four individuals) and ASD (three of the four). The number of patients for whom information is available had delayed language development, with two (both with ASD)
We previously developed a tool, CLUMP, to assess the significance that clustered and recurrent missense mutations have the potential to contribute to NDD pathogenesis. Clustering missense mutations and functional domains to NDD pathogenesis.

Clustered missense mutations and functional domains

Our sequencing results as well as the GRIA1 analysis strongly suggest that clustered and recurrent missense mutations have the potential to highlight functional protein domains important in NDD pathology. We previously developed a tool, CLUMP, to assess the significance of clustered mutations, and we applied it to an updated version of denovo-db (v.1.2) to identify genes and functional domains for future investigation. Overall, we examined 8,917 de novo missense mutations in cases and calculated raw CLUMP scores for 1,699 proteins containing at least two mutations in cases. We performed case–control analyses comparing the pattern of private alleles in ExAC and separately among European individuals from the 1000 Genomes Project (see Online Methods). Twenty-eight out of 34 genes we initially identified were testable by this approach and 18 of them showed nominally significant clustering of de novo missense mutations (P < 0.05, CLUMP, one-tailed permutation test). Altogether, we identified 200 genes with significant clustering of missense mutations at the protein level (Supplementary Table 10). Once again, this set was significantly associated with aspects of neuronal communication, including regulation of the postsynaptic potential (11 observed versus 1 expected, 11.0-fold enrichment, \( P_{\text{adj}} = 6.93 \times 10^{-5} \), two-tailed binomial test) and synaptic signaling (20 observed versus 4.15 expected, 4.82-fold enrichment, \( P_{\text{adj}} = 8.38 \times 10^{-5} \), two-tailed binomial test), as well as chromatin-mediated maintenance of transcription (4 observed versus 0.1 expected, 40.7-fold enrichment, \( P_{\text{adj}} = 2.96 \times 10^{-2} \), two-tailed binomial test). Many of the genes encoded channel proteins and receptors (for example, GRIA1, GRIN1, GRIN2A, GRIN2B, KCNQ1, KCNQ2) and exhibited clustering in or near specific functional domains, such as the transmembrane, pore or voltage sensor domains (Fig. 5a–d). Other proteins, such as CTBP, were remarkable in that the clustering pattern of missense mutations in cases highlighted a subset of the C2H2 zinc finger motifs, which were never mutated in controls (Fig. 5e). These pockets of patient-only missense mutations will be increasingly important in characterizing pathogenic genes and functional domains.

DISCUSSION

The objective of this research study was twofold: define the features of likely disease-causing de novo missense mutations and identify new genes and functional domains relevant to the pathology of NDDs. To increase sample size, we broadly defined NDDs to include not only data from patients with ASD, developmental delay and intellectual disability but also patients with epilepsy and schizophrenia because of the extensive comorbidity of these diagnoses. As expected, both recurrence and severity of missense mutations were critical features. The likelihood of a pathogenic mutation rose significantly when three or more missense mutations were observed in a gene (\( P = 1.06 \times 10^{-18} \), two-tailed Wilcoxon rank-sum test) and, in particular, when the severity of the missense mutation exceeded a CADD score of 28 (>1.2 positive likelihood ratio). We use these features to identify 35 genes with an excess (false discovery rate < 5%) of de novo missense mutations (Supplementary Table 2). Targeted sequencing of specific protein-coding regions showed that recurrent and clustered amino acid replacements were more common in cases than controls (\( P = 1.11 \times 10^{-4} \), OR = 3.93 [1.76–10.89, 95% CI], two-tailed Fisher’s exact test). While many of the top-scoring genes were associated with known syndromic and nonsyndromic forms of NDD (for example, SCN2A with ASD32, PACS1 with Schuurs-Hoeijmakers syndrome17 and ALGI3 with epilepsy33), seven of these candidates have not been previously reported in ClinVar or OMIM. We also identified 200 genes with patterns of de novo missense mutations that were more clustered in cases than in population controls (Supplementary Table 10), 79% (\( n = 157 \)) of which have not yet been associated with an NDD in OMIM or ClinVar databases.

Among the 35 genes with a significant excess of recurrent missense mutations, 37% (\( n = 13 \)) have not yet been associated with a de novo LGD mutation (for example, COL4A3BP, PPP2R5D), suggesting that LGD events either are not tolerated or are associated with a different diagnostic outcome. In support of this observation, 71% (\( n = 25 \)) of genes were also recently highlighted as likely pathogenic in an exome sequencing study of 3,287 individuals with developmental delay15. Of the 200 genes with significant clustering of missense mutations, 67% (\( n = 134 \)) did not show any evidence of LGD mutation in NDDs in denovo-db (v.1.2, OMIM or ClinVar; 45% (\( n = 89 \)) have been shown to be loss-of-function intolerant in the ExAC database18, suggesting that LGD mutations in them may be genetically lethal (for example, cause embryonic lethality or infertility), although more experiments will be required to make this determination. In many cases, the clustering of de novo mutations highlights protein functional domains (Fig. 5), such as specific zinc-finger motifs (for example, in CTBP), transmembrane domains (for example, in GRIN1) and voltage sensors and channel pores (for example, in KCNQ2). As the number of exomes increases, these hotspots of pathogenic missense mutation will become more transparent and may be better understood in the context of protein structure. PTPN11, associated with Noonan syndrome30, is predicted, for example, to have three clusters by CLUMP, and three-dimensional protein structure analysis revealed that these three clusters define the cleft of the ligand binding site34 (Supplementary Fig. 1).

Genes associated with hotspots of missense mutation (Supplementary Table 10) were particularly enriched for presynaptic active zone proteins, FMRF-binding targets and covalent chromatin modification, although not CHD8 target genes. Accumulating evidence supports a link between the development and function of excitatory synapses in NDD and ASD35. Consistent with this, we found 35-fold and 11-fold enrichments of genes regulating postsynaptic membrane potential in genes that carried a significant burden and genes with significant clustering of de novo missense events, respectively. While several scaffolding and intracellular signaling proteins have been associated with ASD and disruption of synaptic function, including SH3 and multiple ankyrin repeat domain (SHANK) proteins36, synaptic Ras GTPase-activating (SYNGAP) proteins37, neurexins38, neureligins39 and others35, a functional mutation in an essential pore-forming subunit of an excitatory ionotropic glutamate receptor has not been described to our knowledge.

The fact that five patients with phenotypic similarity were identified with a gain-of-function A636T mutation strongly supports a role for GRIA1 in ASD and related NDDs. This specific de novo missense mutation has been observed before28 at the homologous position in a highly conserved motif in a different glutamate receptor, GluRδ2. The mutation has a gain-of-function effect, causing constitutive channel opening, neurotoxicity, and degeneration of the cerebellar Purkinje cells in which GluRδ2 is selectively expressed40. Both mice and humans with this mutation in GluRδ2 develop ataxia as a direct
consequence. This mutation in rodent GluA1 (the product of Gria1) has the same effect on channel gating, and here we have replicated this finding in human GluA1. As GluA1 is important in learning and memory, this is a biologically plausible link between this de novo missense mutation in GRIA1 and intellectual disability.

GRIA1 has been demonstrated to play a key role in early synapse development, with GluA1 homomeric channels being inserted into nascent synapses to provide a calcium-permeable, high-conductance channel before being replaced by GluA2-containing channels that mediate long-term synaptic connectivity. Continuing into adulthood, long-term potentiation of excitatory synapses, associated with learning and memory, requires initial insertion of GluA2-absent, calcium-permeable AMPA receptors followed by replacement with GluA2-containing receptors. The developmental and adult function of GluA1 in these contexts likely contributes to the intellectual disability associated with this mutation. Loss-of-function of GluA1 in Gria1 knockout mice leads to impaired synaptic function and behavioral phenotypes, including social behavior deficits and impulsivity, which suggests that bidirectional aberration in excitatory signaling can result in similar ASD and NDD phenotypes. Future studies investigating the impact of the gain-of-function, Lurcher-like A636T mutation in synapse development and function will shed further light on how alterations in excitatory synaptic function contribute to ASD.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

E.E.E., L.S.Z., M.R.G., G.H., T.N.T., B.P.C., H.A.F.S. and K.X. designed the study; M.R.G., G.H., T.N.T., B.P.C., T.W. and K.H. performed the experiments; B.P.C. and T.N.T. helped with MIP design and data analysis; M.K., M.N., M.S., J.G., C.R., E.M.T., G.V., R.F.K., T.P., S.N., H.P., C.R., R.A.B., K.X. and H.H. tested inheritance and provided clinical follow-up on select patients; other authors participated in the sample collection and DNA extraction and/or preparation. M.R.G., E.E.E., L.S.Z., G.H., B.P.C. and T.N.T. wrote the manuscript with input from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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Online Methods

Exome data sets and missense mutation annotation. We initially analyzed all de novo missense mutations available from 24 published cohorts\(^1\)–\(^7\), Supplementary Table 1\(^8\). The NDD set included 8,477 individuals diagnosed with ASD, developmental delay, intellectual disability, epilepsy, schizophrenia or congenital heart disease (CHD), as well as four cohorts of unaffected controls\(^1\)–\(^7\), (n = 2,178) (Supplementary Table 1). Only patients with CHD from Homzy et al.\(^9\) with a secondary diagnosis of NDD were included in this study; we also excluded unaffected siblings of ASD patients as controls if they had a Social Responsiveness Scale (SRS) score ≥60 to remove controls on the autism spectrum\(^6\). Variants were annotated with SeattleSeq\(^5\) version 138, which provides annotation for all available RefSeq transcripts in GRCh37/hg19. In the case of multiple transcripts, we selected the transcript for which the majority of missense mutations were annotated in both cases and controls. All de novo missense mutations either were previously validated or investigators relied on a high (>95%) validation rate in a subset of mutations to ensure specificity. As some individuals with ASD were assayed as part of multiple cohorts, we took care to remove any duplicate entries. When possible, we compared the global identifier given to the samples that were housed at Rutgers (RUID). Three duplicate entries were found in this manner. For other shared mutations in ASD cohorts, we performed PCR amplification and Sanger sequencing on in-house DNA samples to confirm secondary variants. Five out of six pairs tested—two ASC (Autism Sequencing Consortium)–SCS (Simons Simplex Collection) pairs and three ASC–TASC (The Autism Simplex Collection) pairs—shared a second variant and we therefore assumed them to be duplicates.

The presence of uniquely identifying secondary-site mutations was also used to eliminate potential duplicates for globally dispersed samples. We excluded high-frequency mutations (MAF > 0.1%) observed in NHLBI GO ESP Exome Variant Server (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA; http://evs.gs.washington.edu/EVS/; August 2016).

Statistical analyses. Wherever possible, non-parametric tests were used. Data collection and analysis were not performed blind to the conditions of the experiments. Burden was compared between cases and controls for rare (MAF < 0.1%) in ESP de novo missense mutations. Comparisons in rate of mutation and gene recurrence were made using two-sided Fisher’s exact tests. For comparisons of mutation rate and recurrence that depended on identical numbers of cases and controls, we performed 1 million downsamplings and used permutation tests, reporting the empirical P-values. Data distribution was assumed to be normal but was not formally tested. To identify significant enrichments for missense mutations within genes and genomic regions, we applied a probabilistic model that incorporates sequence context and human–chimpanzee fixed differences to generate a null model for the distribution of missense variation across the genome and applied a one-tailed binomial test to test for enrichment\(^13\). For examination of individual codons and specific target regions, we applied the same method but restricted it to the sequence context of the target region and normalized by the gene-specific human–chimpanzee divergence. For all tests we assumed a mutation rate of 1.8 de novo coding variants per generation\(^12\). Multiple-testing corrections were applied using two procedures based on the analysis type. For significance calculations of whole genes, we used the Benjamini-Hochberg false discovery rate correction based on an estimated 19,000 genes in the human genome\(^6\) and report the empirical P-values for each test. For codon analysis we applied the conservative Bonferroni family-wise error rate correction based on the number of amino acids in the genome (n = 1.1 × 10\(^3\)) to generate genome-wide significance estimates and report the adjusted P-value (P\(_{adj}\)). Gene ontology enrichment was assessed using PANTHER (database 2017-04-13) for GO biological process annotation and corrected for multiple testing (Bonferroni, reported as P\(_{adj}\)). We also applied a one-tailed Fisher’s exact test for testing the enrichment of specific gene sets, including neuronal compartments such as the postsynaptic density\(^9\) and targets of CHD8 (ref. 70) and FMRP in brain tissue. To verify channel expression, a saturating concentration of glutamate (1 mM) was applied with 100 μM CX614, and only cells with detectable current were included. NBOX and CX614 were acquired from Tocris Biosciences. Sample size was chosen based on previous literature and variance observed in ion channel studies of similar nature.

Array comparative genomic hybridization. Array labeling and hybridization was performed as previously described\(^7\). Briefly, 250 ng of sample DNA was labeled with Cy5 using a NimbleGen labeling kit (Roche). Reference DNA (NA12878) was labeled in a pooled reaction for four arrays with Cy5 using 1 μg of DNA. Hybridization was performed using the Agilent 2x480K array platform using standard reagents, imaged using an Agilent Scanner, and processed using Agilent Feature Extraction. CNV calls were generated using Agilent Cytogenomics 4.03.12 and the ADM2 calling algorithm with default parameters. For samples passing standard Agilent quality control parameters (DLRSD < 0.2), all CNVs over 100 kb were visually inspected, filtered for known reference sample artifacts, and compared to those seen among 29,085 cases of intellectual disability or developmental delay and 19,584 controls\(^7\) to identify rare CNVs that may contribute to pathogenicity in these cases.
Missense clustering. Genes with significant clustering of missense mutations were identified by CLUMP² (clustering by mutation position; https://github.com/karchinlab/clump/), which applies an unsupervised clustering algorithm based on partitioning around medoid distances between mutations. We implemented the permutation (r = 1000) and minimum mutation options (-m 2) and calculated a P-value based on the null distribution of case and control CLUMP score differences. The case set included individuals with an NDD primary phenotype (ASD, developmental delay, intellectual disability, or epilepsy) from denovo-db⁶⁴ v.1.2 (Supplementary Table 1) and consisted of 22 studies⁴²,⁴³,⁴⁷,⁴⁸,⁵³,⁵⁳-⁵⁵,⁵⁷-⁶²,⁷⁸ with 9,997 affected individuals (8,917 de novo missense variants). We compared against two control missense data sets: (i) missense mutations (MAF > 0.01) from the 1000 Genomes Project⁷⁹ and (ii) private missense mutations present in individuals from ExAC v.0.3 without neuropsychiatric disorders (n = 45,376; 1,466,439 mutations)⁸⁰. All variants were reannotated using the CRAVAT software to enable exact transcript comparisons⁹⁰.

Data availability. De novo mutations used for discovery may be obtained from http://denovo-db.gs.washington.edu/denovo-db/. Data from smMIP targeted sequencing is available through the National Database of Autism Research (NDAR) under the project “Sporadic Mutations and Autism Spectrum Disorders” (NDAR) and the Simons Simplex Collection by applying at https://base.sfari.org/. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

A Supplementary Methods Checklist is available.

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