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Network Topologies and Convergent Aetiologies Arising from Deletions and Duplications Observed in Individuals with Autism

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Abstract

Autism Spectrum Disorders (ASD) are highly heritable and characterised by impairments in social interaction and communication, and restricted and repetitive behaviours. Considering four sets of de novo copy number variants (CNVs) identified in 181 individuals with autism and exploiting mouse functional genomics and known protein-protein interactions, we identified a large and significantly interconnected interaction network. This network contains 187 genes affected by CNVs drawn from 45% of the patients we considered and 22 genes previously implicated in ASD, of which 192 form a single interconnected cluster. On average, those patients with copy number changed genes from this network possess changes in 3 network genes, suggesting that epistasis mediated through the network is extensive. Correspondingly, genes that are highly connected within the network, and thus whose copy number change is predicted by the network to be more phenotypically consequential, are significantly enriched among patients that possess only a single ASD-associated network copy number changed gene \(p = 0.002\). Strikingly, deleted or disrupted genes from the network are significantly enriched in GO-annotated positive regulators (2.3-fold enrichment, corrected \(p = 2 \times 10^{-5}\)), whereas duplicated genes are significantly enriched in GO-annotated negative regulators (2.2-fold enrichment, corrected \(p = 0.005\)). The direction of copy change is highly informative in the context of the network, providing the means through which perturbations arising from distinct deletions or duplications can yield a common outcome. These findings reveal an extensive ASD-associated molecular network, whose topology indicates ASD-relevant mutational deleteriousness and that mechanistically details how convergent aetiologies can result extensively from CNVs affecting pathways causally implicated in ASD.

Introduction

Autism Spectrum Disorders (ASD) form a group of complex disorders affecting ~1% of individuals [1]. ASD are characterised by impairments in social interaction, communication, and restricted and repetitive interests and behaviours [2], although other symptoms such as intellectual disability, seizures or auditory abnormalities frequently co-occur [3]. Despite the high estimates of heritability for ASD found from monozygotic twin studies (~90%) [4], the genetic cause is recognized in only ~20% of cases suggesting that there are many causal variants yet to be identified [5,6]. ASD-causative alleles are likely to be rare as (i) they are under strong purifying selection from the population due to the low fertility (~5%) of individuals with ASD [7], and (ii) there is a strong positive correlation between paternal age and ASD risk which suggests that ASD-contributing mutations frequently may be arising de novo in the continuously-replicating paternal germ line [8]. Thus, in this study we examine de novo variants, specifically de novo copy number variants (CNVs), found in individuals with ASD as a set of variants likely enriched in causal mutations [6].

By contrast to methods that require either recurrent or common genetic variation to identify disease-associated loci, functional enrichment analysis (FEA) approaches gain considerable power by simultaneously examining the contributions of many disparate variants across many individuals’ genomes and thus may be particularly appropriate for investigating the many rare and distributed variants underlying autism [9,10]. FEA approaches hypothesise that dispersed variants observed in patients with...
that participate in that function as candidate disease genes [9].
with the disorder and nominates those copy number variable genes
overlapped by dispersed CNVs identified in the genomes of
symptoms [11,12]. Thus, FEA considers whether there is a
process within each of these patients that underlies their common
shared symptoms may be affecting genes that participate in a
common biological process and it is the disruption of the same
process within each of these patients that underlies their common
symptoms [11,12]. Thus, FEA considers whether there is a
functional category that is exceptionally common for genes
overlapped by dispersed CNVs identified in the genomes of
patients that present the same disorder. It thus associates function
with the disorder and nominates those copy number variable genes
that participate in that function as candidate disease genes [9].
The functional category types used in FEA approaches are key
to the biological insights that they can provide. Different
approaches have been applied to investigate the genetics
underlying autism, including literature annotations [6,13],
protein-protein interactions [14,15], mouse model phenotypes
[13], gene co-expression [16] and functional linkage networks
[6,17]. As the application of these different approaches in autism
studies often accompanies the publication of a novel genetic
dataset, each method has highlighted many, usually novel,
candidate genes that add to a rapidly growing list [18] and
replication of significant functional enrichments has only rarely
been attempted, let alone achieved [13,19]. Synaptic functioning
has been recurrently associated with ASD by many of the recent
studies [13,17] but the small proportions of genes that form these
associations along with the functional diversity broadly exhibited
by genes implicated in ASD has led authors to question specific
associations [20]. However, given that it appears likely that the
variants of several hundred genes contribute to autism [6],
identifying those biological process(es) that are commonly
disrupted may provide a more explanatory approach than to
collate individual causative genes. In particular, FEA, when
applied to ASD CNVs, should not just aim to identify unifying
functional themes but should also provide a framework for
interpreting how these variants exert their proposed phenotypic
effects.
In this study, we examined the genes affected by four
previously-published sets of rare, de novo CNVs identified in
autistic patients. Given that ASD is a behavioural disorder, we
initially considered the phenotype-level gene associations provid-
ed by mouse gene models before moving on to consider more
molecular gene descriptions. We identified a significant enrich-
ment of genes whose orthologues’ disruption in mouse yields an
abnormal synaptic transmission phenotype in 3 of 4 sets. We show
that the protein products of the genes contributing to these
enrichments form an extensive physical interaction network with
genes previously implicated in autism and that extends to many
other genes located in CNVs (herein termed CNV genes). We
show that many of the autistic individuals considered here possess
multiple CNV genes that reside within the network, suggesting
extensive extasisis, and provide evidence that the number of
interactions a gene has within the network is related to the
propensity of its copy change to cause autism. Finally, within this
network we find that whereas genes deleted in ASD are
significantly enriched in those that positively regulate biological
processes, the converse is also true: genes that are duplicated are
enriched in negative regulators of biological processes. We
provide several examples of how the direction of copy number
change reinforces the biological interpretation of the ASD-
associated physical interaction network.

Results
Initially, we sought to objectively identify autism-related mouse
behaviours among phenotypes that are over-represented among de
novo CNV genes for individuals with ASD (herein termed ASD dn
CNVs). For this, we obtained 4 sets of ASD dn CNVs (Table S1
and Materials and Methods). The set collated by the AGP is likely
to be most powerful due both to its higher number of CNVs and
their generally smaller sizes, although all methodologies employed
in this study account for variations in the numbers of genes
affected by each CNV set (Table S2). We considered Gain or Loss
CNVs separately, in addition to the set formed from their union
(All).

Many mouse model phenotypes are associated with ASD
dn CNVs
The Mammalian Phenotype Ontology, the set of terms by
which the MGI annotates the phenotypes of mouse models, is
organised at its highest level into 30 over-arching phenotypes [21].
Of these, three categories [Behaviour/Neurological, Hearing/Vestibular/
Ear and Lethality-Postnatal] were significantly over-represented by
AGP ASD dn CNV genes compared to expectation by random
chance, thus associated with AGP ASD dn CNV genes (BH-
adjusted one-sided Fisher’s test \(p<0.05\); Table S3). Importantly,
these significant associations are all specific to Gain CNVs (2.0–2.7-
fold increases) whilst observed counts for Los CNVs differ little
from expected values (data not shown).
The significant enrichments of these three overarching catego-
ries with the AGP ASD dn CNVs then provided the rationale
necessary for testing of all their finer-scale phenotypic terms for
association [162, 218 and 2 terms, for Behaviour/Neurological,
Hearing/Vestibular/Ear and Lethality-Postnatal categories, respective-
ly; see Materials and Methods]. Although the Nervous System
phenotypic category was not significantly over-represented among
ASD dn CNV genes (All AGP ASD dn CNVs: 1.3-fold increase,
\(p=0.03\), BH-adjusted \(p>0.05\)), the behavioural presentations of
ASD are likely to be manifestations of nervous system abnorma-
lities. Consequently, we also tested for significant enrichments of
finer-scale phenotypes within this category (282 terms).
Subse-
quently, 23 behavioural, 21 nervous system, 27 hearing and 1
postnatal-lethality phenotypes were identified as being significantly
enriched among the AGP ASD dn CNV genes (BH-adjusted
For 3 CNV sets, namely AGP, Marshall et al. and Levy et al., we also identified a significant excess of genes associated with abnormal CNS synaptic transmission phenotypes in mice, thereby triplicating this association (AGP 3.0-fold enrichment, \( p = 7 \times 10^{-5} \), BH-adjusted \( p < 5\% \); Marshall et al. 2.1-fold enrichment, \( p = 1 \times 10^{-4} \), BH-adjusted \( p < 5\% \); Levy et al. AGP 2.2-fold enrichment, \( p = 5 \times 10^{-5} \), BH-adjusted \( p < 5\% \); Sanders et al. 1.6-fold enrichment, \( p = 0.008 \), BH-adjusted \( p > 5\% \); Table 1, Table S3).

Genes previously implicated in ASD are also associated with these model phenotypes

We next sought to determine whether model phenotypes enriched among the mouse orthologues of genes previously

| MGI mouse model phenotype | Mammalian Phenotype Ontology identifier | Phenotype definition | ASD de novo CNV sets significantly enriched | Enrichment (rank within category) among AGP-Implicated genes | Enrichment among AGP control CNVs |
|--------------------------|----------------------------------------|---------------------|--------------------------------------------|-------------------------------------------------------------|---------------------------------|
| Abnormal Synaptic Transmission | MP:0003635 | Defect in the communication from a neuron to a target across a synapse | AGP-AI (2.5-fold), Marshall-AI (2-fold), Levy-AI (2.1-fold) | 5.5-fold (3) | 1.2-fold |
| Abnormal CNS synaptic transmission | MP:0002206 | Defect in the communication from a neuron to a target across a synapse in the central nervous system | AGP-AI (3-fold), Marshall-AI (2.1-fold), Levy-AI (2.2-fold) | 6.5-fold (1) | 1.3-fold |

Enrichments are given as the fold change over that expected by chance (see Materials and Methods).

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implicated in ASD are equivalent to those we now associate with ASD dn CNVs. Of 36 genes that had been causally-implicated in ASD by previous studies, as defined previously [6], phenotypic annotations were available for the unique mouse orthologues of 26 (see Materials and Methods). We removed 4 genes that were also overlapped by an ASD dn CNV to form a wholly independent set of 22 genes herein termed ASD-Implicated genes (Table S4).

We observed a striking concordance between the model phenotypes associated with the ASD-Implicated genes and those associated with the ASD dn CNV genes despite these sets’ complete independence: the two abnormal synaptic phenotypes with triplicated associations to ASD dn CNVs ranked 1st and 3rd among those Nervous System-category phenotypes that were most significantly associated with ASD-Implicated genes, while 15 of the top 18 behavioural-category phenotypic associations among ASD-Implicated genes were among those independently associated with the AGP dn CNVs (Figure 1, Table 1, Table S4).

The protein products of genes that contribute to these phenotypic associations interact

Given the repeated enrichment within independent CNV sets of genes whose mouse orthologues are associated with abnormal synaptic transmission phenotypes, we asked whether the protein products of the 59 synaptic phenotype CNV genes taken from across all sets might interact within common processes or pathways. Even after correcting for the increased likelihood that the products of genes with behavioural or neurological associations interact, our analysis showed that the number of these proteins’ interactions is unexpectedly high (3.75-fold over-representation, p = 0.0066; Figure 2, Table 2 and Table S5; see Materials and Methods). When then added the set of 36 ASD-Implicated genes, the number of direct protein interactions increased yet further (3.2-fold over-representation, p<0.002). Cumulatively, our results show that many of the 59 synaptic phenotype CNV genes and 36 ASD-Implicated genes function in concert and yield similar consequences when disrupted (Figure 2, Table 2).

Mouse model phenotypic information is available only for the orthologues of fewer than a quarter of human genes (see Materials and Methods). It is thus expected that not all genes within CNVs that are causally associated with synaptic abnormalities can be identified using this resource alone. To identify additional ASD candidate genes, we sought all those ASD dn CNV genes whose protein products were known to directly interact with the products of any of the 59 synaptic phenotype CNV genes or 36 ASD-Implicated genes. This identified an additional 174 CNV genes that form an expanded network with a 5.4-fold interaction over-representation (p<0.002; herein termed the ASD-associated network; Figure 2, Table S5). Of these 174 additional interacting proteins, the mouse orthologues of 74 (43%) do not yet have phenotypic information. Of the 100 additional interacting genes with mouse model phenotypes, 44 are known to exhibit behavioural phenotypes, and of these 35 exhibit one or more of the significantly associated behavioural phenotypes identified above (Figure 1 and Table S5). Examining the more general functional annotations of genes within the ASD-associated network using Gene Ontology (GO) identifies convergent functional themes that are consistent with broad synaptic functioning, organisation and maintenance (Table S6; Summarised using REVIGO in Figures S1, S2 and S3 [22]). This functional coherence is supported by the observation that 192 of the 210 (91%) proteins within the ASD-associated network reside in a single inter-connected cluster, thereby also providing known interactions that provide pathways through which effects originating from distinct mutations can aetiologicaly converge (Figure 2). Despite their known functional interconnections, the vast majority of these ASD candidate genes are novel (Table S5).

The 203 CNV genes singled-out through the synaptic mouse phenotype associations and the ASD-associated network provide a causal hypothesis for 81 (45%) of the patients considered. The median number of candidate genes per patient is 3 (mean 3.8, s.d. 3.2) suggesting a substantial role for epistasis in ASD. The network identified here provides not only the means for mediating epistatic interactions but is also indicative of the deleteriousness of copy change: Among the 22 patients that have only a single copy change, candidate gene, that candidate gene has on average 3 times the number of interaction partners in the network as compared to the candidate genes from patients with multiple candidate genes (medians 3 vs. 1, respectively, Mann-Whitney U test p = 0.002). Thus, given the known deleteriousness of disrupting highly interacting “hub” genes within biological networks [23], we propose that the disruption of multiple non-hub genes within the autism network may be required to elicit an autistic phenotype comparable to the singular disruption of a hub gene within the same network.

Duplicated and deleted ASD candidate genes converge on common aetiologies

Of the 203 CNV genes identified through the synaptic mouse phenotype associations and the ASD-associated network, 110 (54%) are found only in duplications while 91 (45%) are only in deletions. We next investigated how the two directions – duplications or deletions – of copy number change might reflect common or divergent aetiologies. To achieve this we analysed the GO biological process annotations assigned to duplicated and, separately, to deleted genes for significantly over-represented terms (Table S6). While many of the over-represented annotation terms are shared between the deleted and duplicated gene sets, we noted a striking difference: The deleted candidate genes are significantly enriched only in genes that are positive regulators of biological processes (GO:0048518, 35/82 annotated genes, 2.4-fold enrichment, BH-adjusted p = 3 x 10^{-4}) while, conversely, an enrichment of genes that are negative regulators of biological processes is only observed amongst the duplicated candidate gene set (GO:0048519, 34/105 annotated genes, 2-fold enrichment, BH-adjusted p = 0.006; Table 3). Each of the 4 CNV set’s candidate genes contribute to each of these enrichments with many sets nominally significant individually (Table S7). Furthermore, reclassifying the partially duplicated, and therefore likely-disrupted, genes as deletions enhances these enrichments further (Table S8). These enrichments are complementary and thus immediately suggest a convergent model of action in which the duplication of negative regulator genes or the deletion of positive regulator genes both act to perturb a common target process and affect the same outcome. The unusually frequent deletions of positive regulators and duplications of negative regulators enable specific and biologically-meaningful interpretations of the ASD-associated network (see Figure 3 and Discussion).

Discussion

This study has (i) identified among 3 independent sets of ASD dn CNVs, and therefore triplicated, an enrichment of genes whose mouse orthologues, when disrupted, yield an abnormal synaptic transmission phenotype; (ii) shown that these genes’ protein products exhibit a significantly high number of interactions between themselves and to the products of genes previously implicated in ASD; (iii) that this interaction network extends directly to include many more proteins of genes affected by the ASD dn CNVs of
Figure 2. An ASD-associated interaction network. The network is formed from direct protein-protein interactions between the products of ASD dn CNV genes that are associated with synaptic phenotypes (shown with thicker grey border), genes previously implicated in ASD (ASD-implicated genes), and other ASD dn CNV genes whose products directly interact with these gene's products. Physical interactions between two proteins are shown as an edge connecting two circles representing each gene. Genes found to be duplicated in autistic patients in this study are shown in green, deleted genes in blue, and ASD-implicated genes in red. An alternative and more detailed view of this network is shown in Figure S6.
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Table 2. Protein-Protein Interaction (PPI) network enrichments.

| Gene Set                                           | Genes | Direct PPIs | P-value | Enrichment |
|----------------------------------------------------|-------|-------------|---------|------------|
| Abnormal Synaptic Transmission (AST) ASD dn CNV genes | 59    | 22          | 0.006   | 3.8-fold   |
| AST ASD dn CNV genes + ASD-Implicated genes        | 90    | 43          | <0.002  | 3.2-fold   |
| AST ASD dn CNV genes + ASD-Implicated genes-other directly-interacting ASD dn CNV genes | 233   | 452         | <0.002  | 5.4-fold   |

Enrichments are given as the fold change over that expected by chance (see Materials and Methods).

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Figure 3. Distinct duplications and deletions of genes whose proteins interact within the ASD-associated network perturb pathways in the same direction. Genes duplicated within ASD dn CNVs are indicated with green upwards arrows while those deleted are denoted by blue downwards arrows. Previously identified ASD-Implicated genes found to be disrupted in autism patients are denoted with red downwards arrows. The nature of the interactions/regulations between proteins/molecules are shown with different edge types (see in-figure legend). The ASD-associated network (Figure 2) identifies several deletion/duplication pathway cascades, for example the MAPK3 pathway (see Discussion for additional examples). Here, deletions of the MAPK3 pathway components (i.e. SYNGAP1, SHANK2, KRAS, MAPK3, PAK2, and CREBBP) and duplications of their negative regulators (i.e. FMR1, GDI1, ARHGDIA, CAMK2B, and CAMKK2) found in autistic patients identify converging effects on the MAPK pathway, specifically reduced CREB-dependent transcription [9,62,63,64]. CREB-dependent transcription has been implicated in neuroadaptation [20]. In addition, increased NO* production leads to the inhibition of MAPK1/3 activity [65], which fits well with the observed CNV duplications of both NOS1 and DLG4, the latter gene promoting recruitment of NOS1 [66]. Similarly, duplication of PRKG1, which is up-regulated by NO* and expresses a product that inhibits IP3 production, is predicted to reduced activation of the calcium-releasing IP3-receptor ITPR1 [67], which is in turn found to be deleted.

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almost half of the cohort; (ii) that the gene products in this ASD-associated network possess roles in synaptic function, organisation and maintenance; (iii) that many individuals with ASD possess multiple copy changed genes from the ASD-associated network; (iv) that genes that are highly connected within the network (“hub genes”) are significantly enriched among patients that possess only a single ASD-associated network gene; and, finally (v) that this network's genes that are deleted are significantly enriched in genes that act to positively regulate biological processes while those that are duplicated are significantly enriched in negative regulators.

An association of ASD CNVs with genes that yield synaptic phenotypes when disrupted in mice has been reported before in rare CNVs but replication was not achieved [13]. Here, despite little overlap between the 3 CNV sets involved (Table S2E), we are able to triplicate this association. These synaptic associations provide aetiological insights that accord well with the emerging neurophysiological view of a strong role for synaptic dysfunction in autism [24]. It is also further strengthened by the over-represented functions among genes within the broader ASD-associated network, whose functions include vesicle transport, cell junction organisation and calcium transport (Figures S1, S2 and S3, Table S6). However, the breadth of dysfunction suggested by the roles of these physically-interacting proteins implicate other, more intra-cellular processes, such as the cytoskeletal and cellular transport processes, that may affect synapse formation, structure and/or maintenance (Figure S1, Table S6).

The known physical interactions between these genes’ products provide pathways through which separate genetic perturbations can converge functionally (Figure 2, Table S5) while the importance of a gene within the ASD-associated network, as specified by the degree of connectivity, appears to be an indicator of ASD-relevant deleteriousness (see Results). Recently, two large-scale studies examining the exomes of autistic patients also identified an excess of protein-protein interactions between genes harbouring suspected causative mutations, reporting smaller networks with 49 [15] and 45 [14] participating genes of which 3 genes and 2 genes, respectively, are also identified through our network. After excluding overlapping genes and compared to random gene sets of equivalent size, the number of connections between gene products in each of the O’Roak *et al.* and Neale *et al.* reported networks to the network we identify here are 12-fold and 38-fold over-represented, respectively (*p*<0.002 for both). Thus, despite little overlap in genes, the strong interconnectedness between these networks identifies pathways through which cellular perturbations arising from distinct mutations identified in separate studies may converge. The single nucleotide variants (SNVs) detected in these two published exome studies are largely predicted to be harmful to the function of the encoded proteins, and therefore comparable to the copy number deletion events in our study. Corroborating our finding of an enrichment of genes that positively regulate among deletions, we also observe a highly significant enrichment of positive regulators among the more strongly-interconnected set of genes identified by Neale *et al.* ([2.7-fold enrichment, *p* = 3.8 × 10⁻⁶, BH-adjusted *p*<0.05] which, while enriched, is not significant among the less well-connected genes reported by O’Roak *et al.* ([1.4-fold enrichment, *p*>0.05]).

Despite chronologically limiting our mouse phenotypic dataset to avoid bias (see Materials and Methods), the similarities between the behavioural mouse phenotypes associated with the AGP dn CNVs and human ASD presentations appear clear, with abnormal social/conspecific interaction, stereotypic behaviour and abnormal memory/learning/conditioning phenotypes all over-represented (Figure 1). Many of our study’s ASD-associated phenotypes bear a striking resemblance to other frequently co-occurring symptoms, such as impaired coordination [25,26,27], anxiety-related phenotypes [28], and absence and tonic-clonic seizures [29,30,31] (Figure 1). Finally, we observe a strong enrichment of genes whose disruption yields hearing phenotypes in mice. This observation accords well with estimates in the literature that hearing abnormalities (including sensorineural hearing disorders) affect between 33–46% of ASD cases (Figure 1G) [32,33]. Many of the associated hearing, and some nervous system, mouse phenotypes are related to peripheral hearing abnormalities, particularly concerning the cochlea and mecanoreception (Figure S4 and Table S3). Inner ear mecanoreception abnormalities appear to have received little attention compared to other regions involved in auditory reception and processing [33]. This is despite improvements in hearing following cochlear implants in individuals with ASD [34] and the knowledge that rare mutations in several genes implicated in ASD (including *CHD7*, *NIPBL*, *PTPN11* and *TBX1*) can cause inner ear abnormalities in humans [35,36,37,38].

The enrichment of deleted genes in the network that positively regulate biological processes and a complementary enrichment of duplicated genes that negatively regulate biological processes suggest the occurrence of convergent aetiologies whereby both deletions and duplication act to perturb biological processes relevant to autism in the same direction (Figures 2 and 3). Indeed, the interactions within the ASD-associated network reveal this proposition to be consistent with the experimental literature. For example, considering the STX1A/CFTR/SLC6A3 (aka. DAT) interactions (Figure 3), over-expression of *STX1A* decreases DAT dopamine transport activity [39], and reduces the CFTR-mediated chloride current by inhibiting trafficking of CFTR to the cell surface [40]. These findings predict that over-expression of *STX1A* yields an effect similar to the deletion of *DAT* or *CFTR* and, conversely, whereas *STX1A* lies within a duplication, *CFTR* and *DAT* are each deleted. Furthermore, STX1A also interacts with SYP (also duplicated), which negatively regulates

### Table 3. Regulatory GO enrichments amongst ASD dn CNVs candidate genes.

| Copy Change Direction | Total GO genes | Positive regulators (GO:0048518) | Negative Regulators (GO:0048519) |
|-----------------------|---------------|---------------------------------|---------------------------------|
|                       |               | Genes | BH-adjusted *p*-value | Enrichment | Genes | BH-adjusted *p*-value | Enrichment |
| Duplications          | 105           | 24 (23%) | >1                 | 1.3-fold   | 34 (32%) | **0.006** | 2.0-fold   |
| Deletions             | 82            | 35 (43%) | 0.0003             | 2.4-fold   | 24 (29%) | 0.08       | 1.8-fold   |

The set of candidate genes was defined as those CNV genes associated with Abnormal Synaptic Transmission mouse phenotypes (Table 1) and those CNV genes identified through direct protein-protein interactions (Table 2). Enrichments are given as the fold change over that expected by chance (see Materials and Methods). doi:10.1371/journal.pgen.1003523.t003
SNAP proteins (SNAP29 is deleted) [41,42]. SNAP proteins are key to presynaptic exocytosis, a process also likely to be disturbed by altered calcium homeostasis resulting from the array of deleted voltage-dependent calcium channels (CACNA1B [deletion], CACNA1C [ASD-implicated deletion], and CACNA1H [deletion]) [43] (Figure 3).

Another example of apparent convergence in aetiology and outcome are the copy number changes affecting the P83K/Wnt pathways (Figure 3). Here, many copy number changes are predicted to converge to reduce or disrupt the action of the β-catenin destruction complex in the Wnt/β-catenin signalling pathway; the deletion of AXIN1, the increased ubiquitination of AXIN1 by duplication of UBC [44] or the disruption of the PI3K pathway due to mutations in PTEN, TSC1, or TSC2 [45] (Figure 3). Perturbations affecting AXIN1 in ASD include the duplication of DVL2 which inhibits AXIN1 (deleted) function [46]. Furthermore, as NKD family proteins promote the degradation of DVL proteins [47,48], the deletion of NKD2 may increase the activities of DVL2 and thereby also inhibit AXIN1. Concordant with a decrease in β-catenin degradation, an increase in β-catenin stabilization could result from LRPAP1, NKD2 and DVL2 copy number changes. LRPAP1 is thought to have protective roles in LRP1 trafficking and its duplication may therefore increase LRP1 availability [49]. The outcome of the copy number change and disruption of each of these genes is likely to up-regulate the Wnt-stimulated TCF/LEF-dependent transcription, a pathway whose down-regulation has been proposed to have therapeutic benefits in ASD models [50,51,52].

Given the ever-increasing number of genetic variants that thus far have been implicated in ASD, the focus will inevitably shift from enumeration towards understanding how these variants contribute to the common pathways and processes underlying this complex disease. Here we have identified a large network of interacting proteins affected by copy number variants identified in patients with ASD, and shown how the network topology and direction of copy number change can be used to interpret these variants’ pathway perturbations. Therapeutically targeting molecules at the ends of pathologically-perturbed regulatory cascades may provide more broadly-applicable treatments, while pathological gene duplications may identify attractive targets for knockdown therapeutics as a means of ameliorating perturbed pathways.

Materials and Methods

De novo CNVs identified in individuals with ASD

Four sets of de novo CNVs were employed in this study (Table S1), of which two are drawn from the Simons Simplex Collection and thus overlap [53]. The largest set consists of 73 de novo CNVs identified in 54 (out of 996) individuals with strict autism by the Autism Genome Project (AGP; Table S1) [6]. Of these, 39 CNVs had been confirmed as de novo by independent methods while 34 were considered likely to be de novo by the CNV calling algorithms. The second set consisted of 28 de novo CNVs identified in 24 patients reported in a study by Marshall et al. [54]; two patients who had been reanalysed by the AGP have been removed; Table S2. The third set consisted of 94 de novo CNVs identified in 82 patients reported in a study by Levy et al. [20] and the fourth set consisted of 67 de novo CNVs identified in 63 patients reported in a study by Sanders et al. [55]. Forty two patients examined by Levy et al. were also present in the study by Sanders et al. but this does not affect our findings; As the synaptic phenotype associations that we report and take forward in the Results were identified amongst both the AGP and the Marshall et al. sets, neither the Levy et al. nor Sanders et al. sets were required for replication and thus these latter sets non-independence from each other does not undermine this association. For all sets, contributing patients have been evaluated as having ASD according to ADI-R and/or ADOS criteria. Herein, de novo CNVs identified in patients with ASD is termed “ASD dn CNVs”.

Assigning genes to CNVs

Human genes were assigned to ASD dn CNVs according to Ensembl Ensmart54 [56]. To be confident that the expressed coding sequence of a gene is affected by the copy number change, we conservatively required at least one coding exon of every known transcript of a gene to be overlapped by a CNV for that gene to be deemed overlapped (Table S2). Particular consideration was given to showing that our gene assignment procedure and statistical over-representation analyses did not yield any functional bias under the null hypothesis (see Figure S3 and Methods S1). Genes observed to be copy number variable in the same direction (gain/loss) within a set of CNVs employed by the AGP as a control, i.e. identified from individuals with no obvious psychiatric history in a previous study were removed from the ASD dn CNV gene lists because these are less likely to be associated with ASD [6]. Although it remains possible that common variants contribute to ASDs, our study focuses on genes affected by rare, de novo variants (see Introduction).

Mouse Genome Informatics (MGI) phenotypes

Annotations of phenotypes resulting from disruptions of mouse orthologues of these affected genes were obtained from the Mouse Genome Informatics (MGI) resource [http://www.informatics.jax.org] and interpolated as described previously [12,57,58,59]. Using simple, unambiguous, 1:1 gene orthology relationships from the MGI resource, 5,283 distinct MGI phenotypic terms were mapped to 5,671 human genes. Each phenotype belongs to one or more of 33 over-arching categories. We considered only 4,055 reasonably populated phenotypes, defined as those with at least 1% of all genes associated with the relevant phenotypic category, thereby reducing uninformative results and improving methodological power. As an unrelicated association between genetic variants in autism patients and a mouse model phenotype was reported in April 2010 [13], we employed only those phenotypes reported in the MGI resource prior to this date, thereby reducing any subsequent phenotyping bias or consequential circularity in discovery. However, our findings remain, or are strengthened by, those more recently reported mouse model phenotypes (data not shown).

Protein-protein interactions

We employed DAPPLE: Disease Association Protein-Protein Link Evaluator [60] to identify direct protein-protein interactions among the protein products of the genes contributing to functional enrichments. A protein-protein interaction network’s connectivity was calculated as published previously [60]. Enrichment analysis was carried out by comparing the number of identified direct protein interactions with the average of those identified from 500 gene sets, in which genes were randomly sampled while matched in set size. To account for the increased likelihood that genes that share behavioural associations are more likely to interact than randomly selected genes, we randomly selected sets of orthologues from 1,766 genes annotated with behaviour and neurological phenotypes in the MGI.

Statistical analysis

Due to the small numbers of de novo CNVs considered here and a lack of a control set of de novo CNVs, performing a case-control
comparison is not possible. Thus, employing the one-sided Fisher’s exact test, we tested the null hypothesis that a (mouse) phenotype associated with (human) Ensembl genes overlapping a set of ASD-associated CNV genomic intervals occurs at a frequency that is no different from that expected from the genome as a whole. Randomisations confirmed that this approach did not yield artefactual bias (see Methods S1 and Figure S5). A multiple testing correction, BH-adjusted \( p < 0.05 \), was applied to account for number of functional terms (phenotypes or GO terms) tested when examining a given gene set [61].

Supporting Information

Figure S1 REVIGO-summarised Biological Process Gene Ontology terms enriched within the combined set of ASD dn CNV genes that are associated either with an abnormal synaptic transmission phenotype in the mouse or whose protein product directly interacts with an ASD dn CNV gene that is associated with this phenotype (Supck et al., 2011; The Gene Ontology Consortium et al., 2000). The summarised GO terms are given in full and separately for deletions and duplications in Table S6. (TIFF)

Figure S2 REVIGO-summarised Cellular Location Gene Ontology terms enriched within the combined set of ASD dn CNV genes that are associated either with an abnormal synaptic transmission phenotype in the mouse or whose protein product directly interacts with an ASD dn CNV gene that is associated with this phenotype (Supck et al., 2011; The Gene Ontology Consortium et al., 2000). The summarised GO terms are given in full and separately for deletions and duplications in Table S6. (TIFF)

Figure S3 REVIGO-summarised Molecular Function Gene Ontology terms enriched within the combined set of ASD dn CNV genes that are associated either with an abnormal synaptic transmission phenotype in the mouse or whose protein product directly interacts with an ASD dn CNV gene that is associated with this phenotype (Supck et al., 2011; The Gene Ontology Consortium et al., 2000). The summarised GO terms are given in full and separately for deletions and duplications in Table S6. (TIFF)

Figure S4 Enriched cochlear and mechanoreception-associated mouse model phenotypes among orthologues of genes overlapped by Gain de novo CNVs from the AGP set. Panel A shows the relevant terms that were identified among Nervous System category phenotypes while Panel B shows the relevant terms that were identified among Hearing/Vestibular/Ear category phenotypes. Relationships between phenotypic terms within the Mammalian Phenotype Ontology are indicated by a blue arrow running from the child term to the parent term. Terms are significantly enriched (BH-adjusted \( p < 0.05 \)) if they are shown with a blue border. (TIFF)

Figure S5 Distributions of \( p \)-values for the surfeits of genes associated with 3 phenotypic categories obtained for 500 randomised sets of case-matched CNVRs. 500 sets of genomic regions matched in size and number to each of the Losu and the Gain CNVRs were obtained (see Materials and Methods). For each set, the likelihoods for the observed surfeit of genes associated with a particular phenotypic category, namely Behaviour/Neurological, Nervous System and Hearing/Vestibular/Ear, were recorded and a histogram charted. See also Methods S1. (TIFF)

Figure S6 An alternative view of the ASD-associated interaction network shown also in Figure 2. The network is formed from direct protein-protein interactions between the products of ASD dn CNV genes that are associated with synaptic phenotypes (shown in with thicker grey border), genes previously implicated in ASD (ASD-implicated genes), and other ASD dn CNV genes whose products directly interact with these gene’s products. Physical interactions between two proteins are shown as an edge connecting two circles representing each gene. Genes found to be duplicated in autistic patients in this study are shown in green, deleted genes in blue, and ASD-implicated genes in red. (TIFF)

Methods S1 Statistical analysis and gene assignment. Supporting materials for our method of gene assignment and confirming the null of statistical analysis. See also Figure S3. (DOC)

Table S1 Genomic extent and ENSEMBL gene content for ASD-associated de novo CNVs and AGP control CNVs. In CNV datasets, the genes considered are those remaining after excluding genes also overlapped by benign CNVs in the same direction of copy change (see Materials and Methods). Sets termed All are formed by combining both Gain and Loss CNVs. (XLS)

Table S2 5 (sheets): De novo CNVs identified in individuals with ASD employed in this study: Sheet A lists the de novo CNVs reported by the Autism Genome Project (AGP), Sheet B lists the de novo CNVs reported in the Marshall et al. study, Sheet C lists the de novo CNVs reported in the Levy et al study and Sheet D lists the de novo CNVs reported in the Sanders et al. study. Sheets A–D provide the genomic location (NCBI36) and copy change for the AGP set of CNVs employed in this study. Sheet E details the overlapping sequence between the three sets of de novo CNVs considered in the study. The total amount of sequence covered by each set is given next to the set name in the row headings. The percentage shown in brackets in the main body gives the proportion of the set listed in the row heading that is overlapped by the set listed in the column headings. (XLSX)

Table S3 (11 sheets): Significantly enriched mouse model phenotypes among the orthologues of genes overlapped by 3 independent sets of de novo CNVs identified in individuals with ASD: Sheets (A–E) AGP de novo CNV enrichments, sheets (F–H) Marshall et al. de novo CNV enrichments, and sheets (I–K) Levy et al. de novo CNV enrichments. For AGP enrichments (sheets A–E), the attached excel spreadsheet provides 5 spreadsheets/tables: one sheet/table each for enrichments in three phenotypic categories Behaviour/Neurological (sheet A), Hearing/Vestibular/Ear (sheet B) and Nervous System (sheet C); The AGP candidate genes identified through these enrichments (sheet D); and the AGP CNVs for which candidate genes have been identified (sheet E). For the Marshall et al. enrichments, 3 spreadsheets/tables are provided: the enrichments in the phenotypic category Nervous system (sheet F); The Marshall et al. candidate genes identified through these enrichments (sheet G); and the Marshall et al. CNVs for which candidate genes have been identified (sheet H). For the Levy et al. enrichments, 3 spreadsheets/tables are provided: the enrichments in the phenotypic category Nervous system (sheet I); The Levy et al. candidate genes identified through these enrichments (sheet J); and the Levy et al. CNVs for which candidate genes have been identified (sheet K). Phenotype tables: PhenotypeID is the Mammalian Phenotype Ontology identifier. PhenotypeName (Abbreviation: Total Genes in Genome with Phenotype) provides...
the phenotype name. Within the brackets, the phenotype abbreviation for use in other tables is given first, while the second number gives the total number of human orthologues with a mouse orthologue that yields this phenotype when disrupted. CopyChangeSet = ALL/[GAIN]/LOSS gives the direction of copy change of the CNVs whose overlapping genes possess this enrichment. Percentage Increase is the enrichment of genes associated with this phenotype over that expected from the genome by chance (see Materials and Methods). P-value is the associated probability for this enrichment (hypergeometric probability, BH-adjusted \( p<0.05 \)). Number of Genes forming Enrichment is the number of overlapped genes that contribute to this enrichment. Total Genes in CNV Set gives the number of genes with this CNV set for which phenotypic information is available. Number of ASD implicated genes hit by enrichment gives the number of genes within this enrichment that also belong to a set of 26 genes that have both been previously implicated in ASD as defined by the AGP Consortium and for which phenotypic information is available. Number of CNV hits (unique CNVs, percentage of CNVs) gives the total number of genes from this enrichment that overlap a CNV within this set. In parenthesis, the first number is the number of unique CNVs from this set that are overlapped by at least one gene from this enrichment, while the second number gives the proportion of all CNVs within this set that are overlapped. Number of patients hits (unique patients, percentage of patients) gives the total number of genes from this enrichment that overlap a de novo CNV within a patient from this cohort. In brackets, the first number is the number of unique patients from this set that possess a de novo CNV that is overlapped by at least one gene from this enrichment, while the second number gives the proportion of all patients within this cohort that possess a CNV that is overlapped. Genes hit lists the genes that contribute to this enrichment. Ensgs hit gives the ENSEMBL ids for the contributing genes. CNVs hit gives the CNVs that are overlapped by genes from this enrichment. The CNV format is [cnv_identifier]_chr[chromosome]_[gain/loss]_[chromosome start position]. Candidate Gene Table: GENE is the gene name, ENSG is the ENSEMBL identifier, Phenotypic enrichments contributed to lists the enriched phenotypes for this set to which this gene contributes. The phenotypic abbreviations are listed in the phenotypic enrichment table(s) for this CNV set in the previous spreadsheet/table within this excel workbook. Number of CNVs gives the number of CNVs in this set that candidate gene overlaps. CNVs lists the particular CNVs overlapping this gene (see phenotype sheet/table for format description). Patients lists the patients possessing these CNVs. CNVs hit Table: CNV gives the CNV from this set that is overlapped by a candidate gene (see phenotype sheet/table for format description). Number of Candidate Genes hit is the number of genes that contribute to the significant enrichments observed for this set of CNVs that are overlapped by this particular CNV. Genes (enriched phenotypes) lists the overlapped candidate genes and gives abbreviations for the enriched phenotypes that this gene contributes to - see phenotype tables/sheets for abbreviation listings.

### Table S3 (6 sheets): Candidate ASD CNV genes identified in this study. (A) All CNV candidate genes are listed, along with the ASD \( dn \) CNVs that harbour them. PPI partner(s) lists other candidate genes whose proteins directly interact with the protein product of the listed gene. (B) 59 ASD \( dn \) CNV genes whose mouse orthologues’ disruption yield a synaptic phenotype. (C) 144 ASD \( dn \) CNV genes whose protein products interact with the protein products of either the ASD \( dn \) CNV genes whose mouse orthologues’ disruption yield a synaptic phenotype listed in sheet B or the 22 ASD-Implicated genes listed in Table S4. (D) Each ASD \( dn \) CNV with a candidate gene is listed along with the candidate genes proposed by this study. Total Genes Considered lists the number of protein-coding genes within that CNV. (E) Each patient with autism for whom a candidate gene is proposed in this study is listed. (F) 1:1 PPI partners and DAPPLE network parameters.

### Table S6 Gene Ontology terms significantly enriched among 204 candidate genes identified in this study. Probability is the associated \( P \)-value for this enrichment (hypergeometric test, BH-adjusted \( p<0.05 \)). Total Genes in Genome with annotation gives the total number of human genes annotated with this GO term. Sample Genes with annotation is the total number genes within the set annotated with this GO term. % difference over expected is that obtained when compared with the genome when sampled randomly.

### Table S7 Genes annotated with GO terms “positive regulation of biological process” (GO:0048518) and/or “negative regulation of biological process” (GO:0048519) within each set of de novo CNVs considered in this study. The \( P \)-value is the raw uncorrected value derived from comparison to all genes. “%Change” gives the percent change over or under that expected against the background of all genes.

### Table S8 Regulatory GO enrichments amongst ASD \( dn \) CNVs candidate genes after reclassifying partially-duplicated genes as deleted. The candidate genes considered here are the subset of CNV genes whose orthologues have synaptic phenotypes in mice plus other CNV genes whose protein products directly interact with the synaptic genes’ proteins. If the outcomes of many of these genes’ duplications result in the increased down-regulation of their targets, then each such gene would be expected to be duplicated largely in its entirety for the duplication to be functional. Indeed, after removing partially-duplicated genes, we find that the enrichment of duplicated genes that negatively regulate biological processes increases (32/92 genes, +119% enrichment, BH-adjusted \( p=0.005 \); Table 1). Concomitantly, including the partially-duplicated genes that would be expected to functionally resemble deletions, with the deleted genes maintains the...
enrichment of positive regulators (40/95 genes, +134% enrichment, BH-adjusted p = 2 × 10⁻⁵; Table 1). Corroboratively, the proportions of genes that regulate in the opposite direction to the proposed general model (i.e. the opposite being positively associated) are reduced when considering partially duplicated genes as deleted genes. (XLSX)

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Author Contributions

Conceived and designed the experiments: HJN SWS CW. Performed the experiments: HJN CW. Analyzed the data: HJN CW. Contributed reagents/materials/analysis tools: HJN HCB SM CB DP CRM ACL SWS CW. Wrote the paper: HJN CJP JDB CW.

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