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Investigating the role of common *cis*-regulatory variants in modifying penetrance of putatively damaging, inherited variants in severe neurodevelopmental disorders

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Recent work has revealed an important role for rare, incompletely penetrant inherited coding variants in neurodevelopmental disorders (NDDs). Additionally, we have previously shown that common variants contribute to risk for rare NDDs. Here, we investigate whether common variants exert their effects by modifying gene expression, using multi-*cis*-expression quantitative trait loci (*cis*-eQTL) prediction models. We first performed a transcriptome-wide association study for NDDs using 6987 probands from the Deciphering Developmental Disorders (DDD) study and 9720 controls, and found one gene, *RAB2A*, that passed multiple testing correction ($p = 6.7 \times 10^{-7}$). We then investigated whether *cis*-eQTLs modify the penetrance of putatively damaging, rare coding variants inherited by NDD probands from their unaffected parents in a set of 1700 trios. We found no evidence that unaffected parents transmitting putatively damaging coding variants had higher genetically-predicted expression of the variant-harboring gene than their child. In probands carrying putatively damaging variants in constrained genes, the genetically-predicted expression of these genes in blood was lower than in controls ($p = 2.7 \times 10^{-3}$). However, results for proband-control comparisons were inconsistent across different sets of genes, variant filters and tissues. We find limited evidence that common *cis*-eQTLs modify penetrance of rare coding variants in a large cohort of NDD probands.

Neurodevelopmental disorders (NDDs) such as intellectual disability, epilepsy and autism have a large genetic component¹. One of the largest studies of NDD patients, the Deciphering Developmental Disorders (DDD) study^{2,3}, consists of 13,451 undiagnosed probands, ~85% of whom have at least one abnormality of the nervous system, who underwent exome sequencing and exon-resolution microarray analysis. Exome-wide burden analysis has shown that around 42% of the cases within the cohort are attributable to de novo coding mutations in either known or undiscovered developmental disorder (DD)-associated genes⁴, with smaller contributions from coding variants following other Mendelian inheritance modes such as X-linked (~7%)⁵ and autosomal recessive variants (~3%)⁶. To date, around 41% of probands have received a genetic diagnosis⁷.

Most parents in the DDD study are unaffected; amongst the 1230 trio probands with an affected father and/or mother, inherited autosomal dominant causes have been identified in 257 (20.9%), which is 2.6% of the 9859 trio probands⁷. However, there is increasing evidence that incompletely penetrant, inherited, rare coding variants

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contribute to risk of NDDs. Firstly, burden analyses have demonstrated that probands with autism have an increased rate of rare deleterious coding variants compared to neurotypical individuals, particularly in a set of ~3000 ‘constrained’ genes that are intolerant of loss-of-function (LoF) variation in the general population⁸, and that they over-inherit such variants from unaffected parents^{9,10}. Indeed, we find similar signals in our undiagnosed DDD probands and evidence that these variants contribute to risk in a large fraction of probands (Samocha et al. manuscript in preparation). Secondly, a small number (N = 22) of DDD probands have been diagnosed with known pathogenic variants in autosomal dominant conditions that were inherited from clinically unaffected parents¹¹. In parallel, there is emerging evidence from population-based cohorts that rare, deleterious coding variants in known DD-associated genes¹² or constrained genes^{13,14} are associated with reduced cognitive function and mental health conditions in the general population. Why these variants are incompletely penetrant represents a major gap in our understanding of DDs and these related phenotypes. Stochastic environmental and genetic modifiers of penetrance likely exist. We previously showed that genome-wide common variants contribute to risk of NDDs¹⁵; we hypothesize that at least some of these common variants may act by modifying penetrance of rare coding variants in these disorders.

Castel et al. previously presented evidence that *cis*-expression quantitative trait loci (*cis*-eQTLs) modify penetrance of rare coding variants in healthy and disease cohorts¹⁶. Specifically, they found evidence in a healthy cohort (N = 620) for a depletion of haplotype configurations that should increase penetrance of pathogenic variants (implying a role for negative selection), but that cancer patients (N = 615) and autistic individuals (N = 2600) were enriched for penetrance-increasing haplotype configurations of pathogenic variants in disease-linked genes. Michaud et al. found an example of a similar mechanism in albinism, whereby a common regulatory variant modified the penetrance of two common coding variants in *TYR*¹⁷. We set out to test whether this mechanism is contributing to the incomplete penetrance of rare, inherited coding variants in DD-associated and constrained genes in the DDD study.

We build on the work of Castel et al.¹⁶ in four main ways. Firstly, we apply more stringent filtering of rare coding variants to focus on those most likely to be damaging. Secondly, we use a cross-tissue, multiple *cis*-eQTL method (UTMOST¹⁸) to predict gene expression, rather than using a single *cis*-eQTL per gene. Thirdly, we consider genetically-predicted expression in a disease-relevant tissue (cortex) as well as in whole blood, rather than taking the most significant *cis*-eQTL in any tissue for each gene. Finally, we use a within-family design which allows us to avoid potential false positive associations due to population stratification, comparing predicted expression between probands with an inherited rare coding variant to their variant-transmitting parents. Our analysis finds limited evidence to support the hypothesis that *cis*-eQTLs are modifying the penetrance of inherited, putatively damaging coding variants in DDs.

Results

Datasets

Individuals from the DDD study were exome-sequenced and genotyped on three different SNP arrays, with some individuals genotyped on more than one array (Supplementary Fig. 1). In this work, we used two different array datasets from DDD (see Methods). Analyses on individual NDD probands were based on the dataset used in Niemi et al.¹⁵, comprising 6987 unrelated NDD cases from DDD with ancestry similar to the 1000 Genomes^{19,20} Great British samples (henceforth referred to as ‘GBR ancestries’) and 9270 ancestry-matched controls from the UK Household Longitudinal Study (UKHLS)²¹. These had been genotyped on the Illumina CoreExome chip and imputed to the Haplotype Reference Consortium (HRC) panel²². Analyses based on trios used a dataset of 1700 undiagnosed NDD probands with unaffected parents (of which 1352 probands were also in the aforementioned CoreExome dataset), all with GBR ancestries, genotyped on either the Illumina OmniExpress chip or the Illumina Global Screening Array and imputed to TOPMed^{23–25}.

Predicting genetically-determined gene expression

To predict the genetically-determined component of gene expression, we used UTMOST¹⁸, a cross-tissue multi-eQTL method that jointly models multiple tissues when estimating the SNP weights. This has been shown to increase imputation accuracy, particularly for tissues with small sample sizes in the training data, and to generate effective imputation models for an average of 120% more genes than single-tissue methods¹⁸. We used UTMOST¹⁸ weights generated from GTEx v6p training data²⁶ for two tissues: cortex and whole blood. We chose cortex because it is implicated in various cognitive functions relevant to global developmental delay and intellectual disability^{27,28}. We also used weights based on GTEx v6p whole blood (N = 338 vs. N = 96 for cortex) in an attempt to balance statistical power with likely physiological relevance to NDDs. While brain tissues may be the most relevant to NDDs, work by Qi et al. has shown a gain of power in gene discovery for brain-related phenotypes using blood *cis*-eQTL data on larger sample sizes²⁹. We restricted our analyses to genes with cross-validation adjusted *p* value < 0.05: 11,103 genes for whole blood, and 11,338 in cortex, with an overlap of 9476 genes. To see if our results differed by method used to predict gene expression, we also predicted gene expression in cortex and whole blood using two other methods: PrediXcan³⁰ and JTI³¹. The number of overlapping genes with predicted expression using each method in both tissues can be found in Supplementary Figs. 2 (whole blood) and 3 (cortex). Predicted gene expression was highly correlated among these three methods (Supplementary Fig. 4).

Testing the effect of genetically-predicted gene expression on NDD risk

We first tested whether genetically-predicted expression of any given gene was associated with being an NDD case, regardless of the presence of rare variants, to assess whether *cis*-eQTLs play a role in risk of NDDs when considering average predicted expression. We conducted a transcriptome-wide association study (TWAS) comparing 6987 unrelated NDD cases with GBR ancestries with 9270 ancestry-matched UKHLS controls. TWAS

have been widely used to try to prioritize likely causal genes underlying complex disease risk³². While the GWAS for NDDs in DDD did not identify any genome-wide significant SNPs¹⁵, a TWAS is generally better powered than a GWAS^{33,34}. In our TWAS for NDDs using predicted gene expression from whole blood using UTMOST, we identified one gene passing Bonferroni correction ($p = 6.7 \times 10^{-7}$), *RAB2A* (Fig. 1). Predicted expression of *RAB2A* was also significantly ($p = 4.88 \times 10^{-7}$) associated with NDD case status in cortex, a more phenotypically relevant tissue, using JTI³¹ (Supplementary Fig. 5), although not when using UTMOST (Fig. 1). This gene encodes a protein belonging to the Rab family which is required for protein transport from the endoplasmic reticulum (ER) to the Golgi complex³⁵. Mutations in multiple other genes in the Rab family are known to cause NDDs^{36–39}. No other genes pass Bonferroni correction (Fig. 1). While *RAB2A* is an interesting candidate for involvement in NDDs, it requires replication in another cohort, and would be more compelling if coding variants in *RAB2A* were associated with NDDs. Broadly, TWAS results were similar using JTI³³ (Supplementary Fig. 5) and PrediXcan⁴² (Supplementary Fig. 6), with no additional genome-wide significant genes identified. A comparison of the p values from TWAS using the three different methods for predicting gene expression can be found in Supplementary Fig. 7. Summary statistics for both TWAS can be found in Supplementary Data Tables S1 (cortex) and S2 (whole blood).

The TWAS was intended to assess whether *cis*-eQTLs play a role in risk of NDDs when considering average predicted expression for cases versus controls. We next hypothesized that a small subset of NDD probands might be explained by having an unusual configuration of *cis*-eQTLs for a gene such that it was expressed at an extremely low level (for a LoF mechanism gene) or an extremely high level (for a gain-of-function mechanism gene). Thus, we tested DD-associated genes in the Developmental Disorder Genotype–Phenotype database (DDG2P)⁴⁰ to see whether undiagnosed NDD probands were enriched for extreme genetically-predicted gene expression compared to controls (± 3 standard deviations from the mean for controls), using a Fisher's exact test. None of the genes passed Bonferroni correction ($p > 0.05/1321$ DD-associated protein-coding genes = 3.8×10^{-5} in cortex; $p > 0.05/1202 = 4.2 \times 10^{-5}$ in whole blood).

Testing whether *cis*-QTLs modify penetrance of rare coding variants in NDDs

We next used the genetically-predicted expression values from NDD probands to test whether *cis*-eQTLs modify the penetrance of putatively damaging, heterozygous, rare coding variants that had been inherited by these probands from their unaffected parents (hereafter: 'putatively damaging variants'). We focused on rare, inherited heterozygous variants (single nucleotide variants (SNVs) and insertions and deletions (indels); minor allele frequency (MAF) $< 1.0 \times 10^{-5}$ in gnomAD⁸, and $\leq 1.0 \times 10^{-4}$ in DDD) predicted to be damaging, in three categories: (1) protein-truncating variants (PTVs) and missense variants (with missense badness, PolyPhen-2, and constraint (MPC) ≥ 2 ⁴¹) in constrained genes (probability of LoF intolerance (pLI) > 0.9)⁴², (2) PTVs or missense variants in dominant DDG2P genes with a LoF mechanism, and (3) PTVs or missense variants in recessive DDG2P genes with a LoF mechanism. We focused on constrained genes and DD-associated genes with a LoF mechanism because the effect of PTVs and missense variants in such genes is more interpretable (i.e. we assume they result in LoF), whereas identifying which missense variants have an activating or gain-of-function effect in genes for which this is the pathogenic mechanism is more difficult. For the first two categories (constrained genes and dominant DD-associated genes), we hypothesized that the penetrance of the putatively damaging variant is increased by low expression of the other wild-type haplotype (Fig. 2A). For the third category (recessive DD-associated genes), we hypothesized that lower expression of the non-variant-carrying haplotype constitutes a 'second hit' to the gene, such that, combined with the putatively damaging variant on the other haplotype, gene activity is reduced to a level that is below the pathogenic threshold (Fig. 2A).

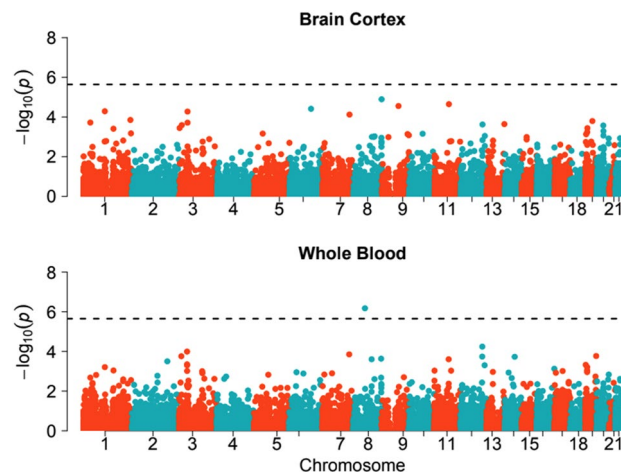


Figure 1. Gene-based p values from a TWAS comparing genetically-predicted gene expression between 6987 NDD cases and 9270 UKHLS controls. The *cis*-eQTL weights are from GTEx v6p cortex ($N = 11,338$ genes) and whole blood ($N = 11,103$ genes). Black dotted line represents the significance threshold after Bonferroni correction [$p = 0.05/(11,338 + 11,103)$].

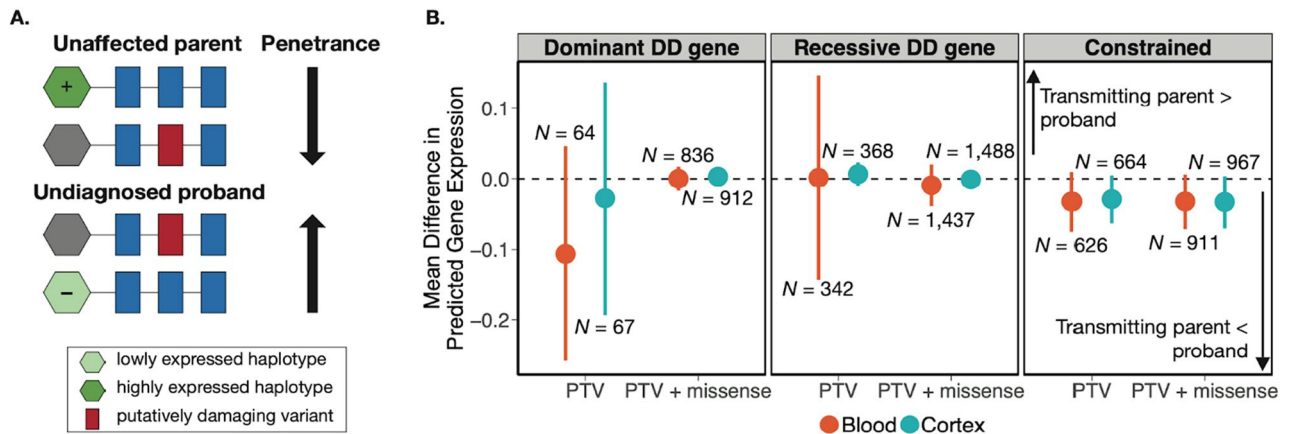


Figure 2. Comparison of predicted gene expression between unaffected variant-transmitting parents and their undiagnosed children with an NDD. **(A)** Schematic figure depicting how *cis*-eQTLs may modify the penetrance of a putatively damaging variant transmitted from an unaffected parent to their undiagnosed child in a gene with a LoF mechanism. The haplotype with the '+' symbol has higher predicted expression based on its *cis*-eQTLs, whereas the one with the '-' symbol has lower predicted expression. **(B)** Mean difference (parent-child) in predicted gene expression between parents transmitting putatively damaging variants and their children with an undiagnosed NDD, with lines indicating 95% confidence intervals. *N* denotes the number of unique child-parent pairs. Predicted gene expression can be interpreted as the inverse quantile-normalised number of reads per kilobase of transcript per million mapped reads (RPKM). The three panels show results for putatively damaging variants in three different sets of genes: dominant DD-associated genes with a LoF mechanism (left), recessive DD-associated genes with a LoF mechanism (middle) or constrained genes ($pLI > 0.9$) (right). Red and blue dots represent results from genetically-predicted gene expression imputed from whole blood and cortex, respectively. We show estimates considering only PTVs, as well as PTVs and missense variants (with $MPC \geq 2$) together.

We began with a within-family test on 1700 undiagnosed NDD trios to assess whether unaffected parents transmitting a putatively damaging variant were protected by higher genetically-predicted expression of the gene compared to their affected child. Specifically, we ran a one-sided paired *t*-test to compare genetically-predicted gene expression in cortex and whole blood between unaffected parents transmitting a putatively damaging variant and their affected children, with the hypothesis that transmitting parents had higher genetically-predicted expression of the relevant gene. This within-family test controls for population stratification, and allows for a direct comparison of predicted expression of the wild-type haplotype while controlling for both the same putatively damaging variant, and the haplotype wherein it lies (the shared haplotype). We saw no significant difference in genetically-predicted expression between putatively damaging variant-transmitting parents and their children (Fig. 2B). We repeated this analysis with a more lenient MAF threshold ($MAF < 0.1\%$) and also saw no significant difference in genetically-predicted expression (Supplementary Fig. 8). Further, we conducted sensitivity analysis using two other gene expression prediction methods (PrediXcan³⁰ and JTI³¹) (Supplementary Fig. 9). Results from PrediXcan³⁰ (in blood) supported our hypothesis that parents would have higher predicted gene expression than their children when testing dominant and recessive DD genes containing putatively damaging PTVs, but this result was only nominally significant and did not pass multiple testing correction ($p < 0.05$) (Supplementary Fig. 9). Counter to our expectation, we observed higher predicted expression in children compared to parents in constrained genes containing putatively damaging PTVs, using both PrediXcan³⁰ (nominally significant in a two-sided test; $p = 0.026$ in blood) and JTI³¹ (two-sided $p = 1.62 \times 10^{-4}$ in blood, two-sided $p = 1.25 \times 10^{-3}$ in cortex) (Supplementary Fig. 9). Similarly, when considering both PTVs and missense variants in constrained genes, we observed higher predicted expression in children compared to parents using PrediXcan³⁰ (two-sided $p = 0.0022$ in blood) and JTI³¹ (nominally significant; two-sided $p = 0.038$ in blood) (Supplementary Fig. 9). As these results were inconsistent between the gene sets considered and methods used for gene expression prediction, we did not find them persuasive in either direction.

Importantly, we have low power to detect experiment-wide significant differences in predicted gene expression between variant-transmitting parents and their children at current sample sizes (Table S6). Assuming the true effect is at the upper end of the 95% confidence intervals shown in Fig. 2B, our power to detect a significant effect ranges from 2% to 48%. Assuming these same effect sizes, future studies would require 2–53 fold more parent-child pairs (depending on variant/gene set) to detect a significant positive difference in predicted expression between variant-transmitting parents and their children with 80% power.

We next compared genetically-predicted expression between NDD probands carrying a putatively damaging variant in a given gene with the predicted expression for the same gene in 9720 UKHLS controls. Specifically, we calculated the percentile ranks of genetically-predicted gene expression values, per gene, in both cortex and whole blood, across undiagnosed NDD probands with putatively damaging variants in the gene and UKHLS controls. We then aggregated these percentile ranks across genes and ran a one-sided Wilcoxon rank test to compare the average ranking of variant-carrying probands with controls. We hypothesized that the probands' ranked predicted expression values would be lower than in controls. We found that genetically-predicted expression from

whole blood of constrained genes harboring putatively damaging variants is lower in variant-carrying probands than in controls (PTVs: $p = 1.0 \times 10^{-4}$; PTVs + missense: $p = 2.7 \times 10^{-3}$, which pass Bonferroni correction for 12 tests) (top right panel of Fig. 3; Table 1). We also found nominally significant evidence to suggest that, in cortex, genetically-predicted expression of recessive ($p = 0.03$) DD-associated genes harboring putatively damaging PTVs is lower in variant-carrying probands than controls (bottom middle panel of Fig. 3; Table 1). Similarly, we found nominally significant evidence ($p = 0.04$) to suggest that, in whole blood, genetically-predicted expression of dominant DD-associated genes harboring putatively damaging PTVs and/or missense variants is lower in variant-carrying probands than controls (top left panel of Fig. 3; Table 1). These findings are consistent with our hypothesis that the haplotype with the wild-type allele may be expressed at a lower level, thus increasing the penetrance of putatively damaging variants in undiagnosed NDD cases compared to controls. However, results were inconsistent across the two tissues and gene sets tested. Furthermore, this analysis does not take into account whether any controls carry a rare, potentially damaging variant in the same gene as the cases (since sequence data are not available for controls), and is thus less robust than the within-family analysis mentioned above. We repeated this analysis with a more lenient MAF threshold (MAF < 0.1%) and found similarly inconsistent results (Supplementary Fig. 10).

A limitation of these analyses is that, while these variants were filtered to be rare and predicted to be damaging by in silico predictors, many of the variants are likely not damaging, or only have mild effects. Thus, we investigated differences in predicted gene expression for specific cases in which the proband had a diagnostic variant that was inherited from an unaffected parent, and consequently incompletely penetrant. We focused on a set of

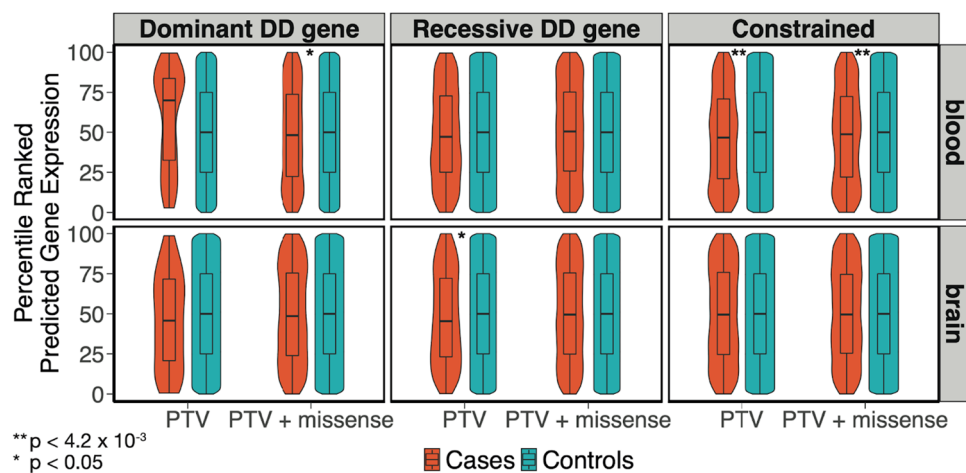


Figure 3. Violin and box plots of percentile-ranked genetically-predicted expression values of genes harboring putatively damaging variants in undiagnosed NDD cases, compared to controls. Vertical lines of the box plot indicate the range and horizontal lines indicate the lower quartile, median and upper quartile. The p-value is from a one-sided Wilcoxon test assessing whether cases are lower than controls. The Bonferroni multiple testing threshold is $0.05/12 = 4.2 \times 10^{-3}$.

Tissue	Variant type	Gene type	N genes	N cases with variant	Mean case rank	Mean control rank	P value
Blood	PTV	dominant DD	43	85	57.82	50.00	0.99
Blood	PTV + missense	dominant DD	156	1055	48.68	50.00	0.04
Cortex	PTV	dominant DD	50	90	45.85	50.00	0.08
Cortex	PTV + missense	dominant DD	186	1168	49.54	50.00	0.26
Blood	PTV	recessive DD	297	469	48.40	50.00	0.10
Blood	PTV + missense	recessive DD	590	1895	50.38	50.00	0.80
Cortex	PTV	recessive DD	316	501	47.72	50.00	0.03
Cortex	PTV + missense	recessive DD	627	1950	50.11	50.00	0.61
Blood	PTV	constrained	386	901	46.79	50.00	1.0×10^{-4}
Blood	PTV + missense	constrained	731	1242	48.11	50.00	2.7×10^{-3}
Cortex	PTV	constrained	440	951	49.61	50.00	0.32
Cortex	PTV + missense	constrained	839	1327	49.85	50.00	0.41

Table 1. Results of one-sided Wilcoxon rank test for percentile-ranked predicted expression of genes harboring putatively damaging variants in undiagnosed NDD probands compared to controls. The sample size for each test is the (number of probands with a putatively damaging variant) + [number of unique genes in which a proband has a putatively damaging variant \times N controls (9270)].

twenty-two variants in DDG2P genes that were known to be pathogenic based on their ClinVar annotation and that were deemed pathogenic/likely pathogenic by the proband's clinician, despite having been inherited from an unaffected parent¹¹. We postulated that this set of variants is the most likely to show evidence of this mode of modified penetrance. In Table 2, we show the results for the five variants that fell in genes with a predicted loss-of-function consequence and whose expression was predicted by UTMOST¹⁸ with FDR-adjusted p value < 0.05 in blood and/or cortex. For three of these variants, our hypothesis was supported by the results based on the one tissue for which predicted expression was available (*RORA*, plus two variants in *EBF3*). However, there were two variants (those in *ANKRD11* and *NF1*) for which results were inconsistent between genetically-predicted gene expression values from cortex versus from whole blood. This is either because one or more *cis*-eQTLs have different predicted directions of effect in the two tissues, or different *cis*-eQTLs are used to predict expression in the two tissues, or some combination of the two. Thus, even in this small set of variants for which we most expected to see some signal of this mode of modified penetrance, the evidence for it is inconsistent.

Discussion

In this work, we evaluated whether levels of gene expression predicted based on common variants modulated NDD risk and penetrance of rare, inherited damaging variants in a large sample of probands from the DDD study. In a TWAS comparing NDD cases with controls, we found one gene passing multiple testing correction in whole blood, *RAB2A*. We are cautious in interpreting this result for several reasons: there is no additional supporting evidence in the literature, it has not yet been replicated in an independent sample, and TWAS hits may not reflect the true causal gene^{32,44}. In evaluating the role of *cis*-eQTL-mediated gene expression in modifying penetrance of rare, inherited, damaging variants, our within-family test found no consistent evidence of this, with results differing depending on which method was used for predicting gene expression, and which gene set was used (Supplementary Fig. 9). Results from a case/control analysis were likewise equivocal, supporting our hypothesis for some gene set-tissue-variant type combinations but not others (Table 1). Analysis of a small set of known pathogenic, incompletely penetrant variants also failed to provide consistent evidence that their penetrance was being modified by *cis*-eQTLs (Table 2).

There are several limitations to our analysis. A major one is that, in an attempt to boost power, we aggregated evidence across rare variants in many genes, many of which are likely not deleterious. We used stricter filtering of rare coding variants than Castel et al.¹⁶, focusing on a set that is over-transmitted from unaffected parents to probands in DDD (Samocha et al. manuscript in preparation). For example, we used a more stringent MAF filter of < 0.001% in gnomAD⁸ rather than MAF < 1%. Castel et al. considered all missense variants with CADD > 15 and, at least for part of their analysis, assumed that penetrance would be increased by higher expression of the variant-containing haplotype. This is not what one would expect if the variant results in loss-of-function, and hence, we restricted to variants that seemed more likely to have a LoF consequence (PTVs or missense variants in genes constrained against LoF variation and/or with a known LoF disease-causing mechanism), and considered predicted expression of the other haplotype. Despite our more stringent filtering, many of the rare variants we included still likely do not result in true LoF, which undoubtedly reduces our power.

Another limitation is that our set of NDD probands is phenotypically heterogeneous; 88% of recruited DDD probands also had abnormalities in at least one other organ system¹⁵. This makes it challenging to choose an appropriate tissue in which to predict gene expression, since this may differ between probands. Furthermore, eQTLs can be cell-, state- and time-dependent^{32,45–51}. The more relevant cell type and developmental stage to consider is even more difficult to pinpoint, and likely will differ between probands. Genetically-predicted expression models using single-cell data instead of bulk RNA-seq data may also improve results, however identifying relevant cell types for such a heterogeneous phenotype would be difficult. It may be that selecting *cis*-eQTLs ascertained in fetal brain would be more physiologically relevant for neurodevelopmental disorders than those from adult brain.

A fundamental problem is that common *cis*-eQTLs only explain about 10% of the genetic variance in real gene expression³², which limits their predictive accuracy. For example, using a single tissue method, PrediXcan³⁰, the average Pearson correlation between predicted gene expression and real gene expression across tissues is around 0.14³⁰. UTMOST¹⁸ modestly improves average imputation r^2 across tissues over PrediXcan³⁰ by 36.8%¹⁸. Moreover, genes associated with Mendelian diseases are likely depleted for common *cis*-eQTLs^{42,52–56}. Future

Gene	Location (GRCh37) (chr:pos:ref:alt)	Gene consequence	Variant consequence	Child versus parent (Blood)	Child versus parent (Cortex)
<i>EBF3</i>	chr10:131665510:G:A	Loss-of-function	NM_001005463.3:p.Arg303*	Child lower	NA
<i>EBF3</i>	chr10:131665510:G:A	Loss-of-function	NM_001005463.3:p.Arg303*	Child lower	NA
<i>RORA</i>	chr15:60789728:G:A	Loss-of-function	NM_134260.2:p.Arg533*	Child lower	NA
<i>ANKRD11</i>	chr16:89348863:G:A	Loss-of-function	NM_001256182.2:p.Arg1363*	Child higher	Child lower
<i>NF1</i>	chr17:29560229:T:C	Loss-of-function	NM_001042492.3:p.Trp1236Arg	Child higher	Child lower

Table 2. Comparison of genetically-predicted gene expression in cortex and whole blood between variant-transmitting parents and their children, for a set of known pathogenic variants from ClinVar that were deemed pathogenic/likely pathogenic in the proband by their clinician, despite being inherited from an unaffected parent¹¹. These genes are listed in DECIPHER⁴³ as causing DDs via a LoF⁴³ mechanism. In the two rightmost columns, NA indicates that the gene's expression was not sufficiently well predicted in that tissue to be considered.

studies could potentially incorporate methods that use rare eQTLs⁵⁴, *trans*-eQTLs, and epigenetic information⁵⁷ to predict gene expression. Alternatively, they could measure gene expression directly using RNA sequencing, to assess whether expression patterns (whatever their causes) are modifying penetrance of rare variants. To have sufficient power, such studies would either need to be very large or targeted at individuals with putatively pathogenic transmitted variants.

In conclusion, we did not find strong evidence to support the hypothesis that common *cis*-eQTL-mediated gene expression modifies NDD risk or penetrance of rare coding variants in NDDs. Despite addressing this in one of the largest available datasets of NDD probands, our power was still limited by the phenotypic heterogeneity of the cohort, uncertainty about which variants have true effects, and the low accuracy of gene expression prediction models. Future studies should consider this hypothesis in larger datasets with direct measurements of expression and genome sequencing data to evaluate rare variants that could alter gene expression. They should also consider alternative explanations for this apparent incomplete penetrance of rare inherited variants, such as a modifying role of polygenic background⁵⁸, epistasis, stochastic effects, alternative splicing, changing effects of these rare variants with age, or environmental factors.

Methods

Preparation of DDD cases and UKHLS controls on the CoreExome chip

We focused the case–control analyses (Figs. 1, 3, Table 1) on the DDD and UKHLS data that were used in Niemi et al.¹⁵. These included 6987 unrelated NDD cases from DDD with GBR ancestries (defined based on their clustering around the 1000 Genomes Great British samples) and 9720 ancestry-matched controls from UKHLS. These samples were genotyped on the Illumina HumanCoreExome chip¹⁵. Pre-imputation quality control of these genotype data and imputation to the HRC panel are described in Niemi et al.¹⁵. Post-imputation, genotype data were filtered to SNPs with an imputation $r^2 \geq 0.8$ and MAF > 1%.

Preparation of genotype data from DDD trios

The analyses in Fig. 2 and Table 2 are based on a set of DDD trios that had been genotyped on either the Illumina Infinium Global Screening Array (GSA) or the Illumina OmniChipExpress chip. The preparation of those data is detailed below, with a summary of the filtering steps in Supplementary Fig. 11.

Quality control and imputation of the GSA data

9850 DDD samples were genotyped on the GSA at King's College London in March 2020. Samples were genotyped in a pilot batch (N = 1152), and a second, larger batch (N = 8698). Tables S1 and S2 show the results of the quality control steps applied to samples and SNPs before and after merging the batches, respectively.

Samples were checked for concordance with whole-exome sequencing (WES) data previously generated and cleaned on all DDD individuals, described in previous publications²; discordant samples were removed, as were sample swaps and duplicate samples. Individuals with $\geq 5\%$ SNPs missing genotyped data were removed. After examining the heterozygosity rate per individual versus the proportion of missing genotypes per individual⁵⁹, we removed individuals with a heterozygosity rate below 0.158 and above 0.17. Trios for which the offspring had > 200 Mendelian errors ($\sim 0.03\%$ error rate) were removed.

Palindromic, duplicated and multiallelic markers were removed, as well as indels. Markers with either a call rate < 5%, a MAF < 1%, or with significant deviation from Hardy–Weinberg Equilibrium ($p < 1.0 \times 10^{-6}$) were also removed. Markers with a significantly different non-missing rate ($p < 1.0 \times 10^{-50}$) or marked allele frequency difference between the pilot batch and second batch of GSA data were removed. SNPs with Mendelian errors in > 1% of trios were removed. This left 9534 individuals and 474,926 genotyped SNPs before imputation.

After this SNP-level QC, we identified individuals of GBR ancestries. (See Supplementary Fig. 12 and Supplementary Methods for further detail). This left 8879 individuals.

Imputation was carried out using the TOPMed imputation server. After removing variants with imputation $r^2 \geq 0.8$, 35,901,148 autosomal SNPs remained.

Preparation of DDD trios genotyped on the Omni chip

Niemi et al.¹⁵ also made use of a set of 3504 individuals from DDD who had been genotyped on the Illumina OmniChipExpress chip. The pre-imputation quality control of these genotype data has been described previously¹⁵. The prior study used the Haplotype Reference Consortium (64,976 low-coverage genomes) as an imputation panel. We re-imputed the post-QC genotype data using the TOPMed reference panel (97,256 high-coverage genomes) and imputation server, which uses Eagle2 for phasing and minimac4²⁴ for genotype imputation^{23–25,60}. We removed SNPs with imputation $r^2 < 0.8$, leaving 36,904,864 SNPs.

Merging and checking ancestry of the DDD trios genotyped on the Omni and GSA chips

We merged the data from the GSA and Omni chips (11,227 individuals) and verified that the individuals were well-matched for ancestry (Supplementary Fig. 13). See Supplementary Methods for further details. This merged dataset contained 3344 trios of which all three individuals were inferred to have GBR ancestries.

Subsetting DDD trios for analyses

We then removed trios in which probands or parents were related to individuals in other trios up to three degrees of relatedness. To identify related individuals across trios, we ran the `-genome` command in PLINK v1.9⁶¹. Pairs of individuals with $\hat{\pi} \leq 0.2$ were considered unrelated. After filtering, we retained a set of 3170 unrelated trios with GBR ancestries. Among these, 2422 probands were considered undiagnosed (see section

below on ‘Identifying undiagnosed probands’), and 2002 had unaffected parents. Finally, of those, 1700 had a neurodevelopmental disorder, defined as having one of the following HPO terms⁶²: abnormal metabolic brain imaging by MRS (HP:0012705), abnormal brain positron emission tomography (HP:0012657), abnormal synaptic transmission (HP:0012535), abnormal nervous system electrophysiology (HP:0001311), behavioural abnormality (HP:0000708), seizures (HP:0001250), encephalopathy (HP:001298), abnormality of higher mental function (HP:0011446), neurodevelopmental abnormality (HP:0012759), abnormality of the nervous system morphology (HP:0012639). This filtering process is depicted in Supplementary Fig. 11.

Identifying undiagnosed probands

The DDD exome analysis team identified potentially clinically relevant variants from the WES and arrayCGH data as described in Wright et al.³. The clinical filtering procedure focuses on identifying rare damaging variants in a set of genes known to cause developmental disorders (DDG2P) (<https://www.deciphergenomics.org/ddd/ddgenes>), that fit an appropriate inheritance mode. Variants that pass clinical filtering are uploaded to DECIPHER⁴³, where the probands’ clinicians classify them as either ‘definitely pathogenic’, ‘likely pathogenic’, ‘uncertain’, ‘likely benign’ or ‘benign’. We downloaded all DDD variants from DECIPHER⁴³, on July 30, 2021. Of these, 23.5% had not yet been classified by clinicians. Thus, to better differentiate between diagnosed and undiagnosed probands, we estimated positive predictive values (PPV) for different classes of variants and used this to identify probands for whom the variants that passed clinical filtering seemed likely to contain the true diagnosis. We estimated positive predictive values as the proportion of variants in that class (e.g. de novo PTV in dominant gene with a loss-of-function mechanism) that clinicians had rated as ‘pathogenic’ or ‘likely pathogenic’. The classes of variants considered and their positive predictive values are shown in Supplementary Table 3.

We defined ‘undiagnosed probands’ as those that did not fulfill at least one of the following criteria:

- (i) The proband was amongst the diagnosed set in a thorough reanalysis of the first 1133 trios⁶³,
- (ii) The proband had at least one variant (or pair of compound heterozygous variants) rated as pathogenic’ or ‘likely pathogenic’ by a clinician,
- (iii) The proband had at least one variant (or pair of compound heterozygous variants) in a class with a high or medium PPV (i.e. $PPV > 50\%$; see Supplementary Table 3) that passed clinical filtering but had not yet been rated by clinicians,
- (iv) The proband had a de novo PTV in a gene with a $pLI > 0.9$ ⁴².

Predicting gene expression

We used SNP weights from UTMOST¹⁸ to genetically predict gene expression based on the imputed genotype dosage files. UTMOST¹⁸ is a cross-tissue gene expression imputation model¹⁸. Genetically-predicted expression was only generated for genes which had a cross-validation FDR-adjusted p value < 0.05 in the dataset used to build the models. We used *cis*-eQTL SNP weights generated from two datasets: GTEx v6p brain cortex and whole blood. For sensitivity analyses, we used SNP weights estimated from the same tissues using two other gene expression imputation models: PrediXcan³⁰ and JTI³¹. Genetically-predicted expression using these methods was only generated for genes with a Pearson correlation between predicted and observed expression of $r > 0.1$ and $p < 0.05$.

Transcriptome-wide association study (TWAS) for NDDs

We ran two TWASs using predicted gene expression with weights derived from the GTEx v6p brain cortex ($N = 96$) and GTEx v6p whole blood ($N = 338$). We predicted gene expression using estimated SNP weights from UTMOST¹⁸, then ran logistic regression of predicted expression for each gene on case status ($N = 6987$ cases, $N = 9270$ controls), controlling for the first 10 genotype PCs. We set a Bonferroni significance threshold of p value $< 2.23 \times 10^{-6}$ for the two TWAS ($0.05 / (11,338$ genes in cortex + $11,103$ genes in whole blood)).

Quality control of whole-exome sequencing data

A brief overview of the quality control carried out on the DDD whole-exome sequencing data can be found in Supplementary Table 5. We focused on SNVs and indels. Coding consequences are defined by the worst annotation across transcripts using the Variant Effect Predictor⁶⁴.

When multiple indels are found nearby in the same individual, this frequently indicates a complex mutational event. Properly resolving these complex mutational events would require haplotype-aware annotation, which was beyond the scope of this work. Consequently, we removed instances in which a sample had more than one indel in a given gene. This filter removed fewer than 4% of all indels with a $MAF < 1\%$ in our dataset.

Investigating role of genetically-predicted gene expression in modifying penetrance of rare variants

Amongst the undiagnosed, NDD probands of GBR ancestries with unaffected parents, we identified those with at least one rare ($MAF < 0.001\%$ in gnomAD⁵ and $< 0.01\%$ in DDD, inherited, heterozygous variant that was either (1) a PTV or missense variant in a dominant DDG2P gene with a LoF mechanism, (2) a PTV or missense variant in a recessive DDG2P gene with a LoF mechanism or (3) a PTV or missense variant ($MPC \geq 2$) in a constrained gene ($pLI > 0.9$). We used the DDG2P list downloaded on August 20, 2020, and focused on genes that were confirmed/probable DD genes.

In the first analysis (Fig. 2), we compared genetically-predicted expression between probands with a putatively damaging variant in one of the aforementioned categories with their transmitting parent. Specifically, we tested (using a one-sided paired t -test) whether undiagnosed NDD cases carrying a variant in a given class had lower

predicted gene expression than their parent who transmitted the variant. We only compared gene expression for one proband with one parent for one gene with a putatively damaging variant. If a proband inherited more than one putatively damaging variant, either from the same or both parents, a unique proband-parent-gene combination was selected at random with an equal probability of selection.

We conducted a power analysis to assess our ability to detect these effects using a one-sided paired *t*-test, considering both a nominal significance level ($p < 0.05$) and a Bonferroni-corrected threshold ($p < 0.0042$) (Table S6). Additionally, we determined the required sample size to achieve 80% power for detecting effects at both nominal and Bonferroni-corrected significance levels, based on current point estimates and upper bounds of the 95% confidence interval for effect sizes (Table S6). We used the `pwr.t.test` function from the “pwr” package in R (based on Cohen et al.⁶⁵).

In the second analysis (Fig. 3; Table 1), we calculated the percentile ranks of genetically-predicted gene expression values, per gene, in both cortex and whole blood, across undiagnosed NDD probands with putatively damaging variants in the gene and UKHLS controls. For each gene, we extracted the rank of genetically-predicted expression for cases carrying a variant in a given class, as well as the controls’ ranks. We then aggregated the ranks across genes and conducted a one-sided Wilcoxon rank test to test whether these ranks were lower in the variant-carrying cases compared to controls.

Identifying undiagnosed probands with outlier expression in DDG2P genes

For each DDG2P gene, we identified undiagnosed NDD probands that had predicted gene expression at least three standard deviations above or below the mean predicted gene expression in controls from brain cortex or whole blood. We then conducted a Fisher’s exact test to test whether the number of cases with extreme levels of predicted gene expression was significantly different from that in controls.

Ethical approval

The DDD study has UK Research Ethics Committee approval (10/H0305/83, granted by the Cambridge South Research Ethics Committee and GEN/284/12, granted by the Republic of Ireland Research Ethics Committee). The University of Essex Ethics Committee has approved all data collection on Understanding Society main study, COVID-19 surveys and innovation panel waves, including asking consent for all data linkages except to health records. Approval for the collection of biosocial data by trained nurses in Waves 2 and 3 of the main survey was obtained from the National Research Ethics Service (Understanding Society – UK Household Longitudinal Study: A Biosocial Component, Oxfordshire A REC, Reference: 10/H0604/2).

Data availability

The DDD data are available in the European Genome-Phenome Archive (EGA). These include the exome sequence data (EGAD00001004389), phenotypic and family descriptions (EGAD00001004388), CoreExome array data (EGAD00010001598, EGAD00010001600, EGAD00010001604) and Global Screening Array data (first batch raw data: EGAD00010002567, second batch raw data, EGAD00010002569 and QCed data: EGAD00010002568). The UKHLS genotype data are also available on EGA (EGAS00001001232).

Received: 19 April 2023; Accepted: 4 April 2024

Published online: 15 April 2024

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Acknowledgements

We thank the families and their clinicians for their participation and engagement, and our colleagues at the Wellcome Sanger Institute who assisted in the generation and processing of data, including the Human Genetics Informatics core. The DDD study presents independent research commissioned by the Health Innovation Challenge Fund (grant number HICF-1009-003), a parallel funding partnership between Wellcome and the Department of Health, and the Wellcome Sanger Institute (grant number WT098051). This study makes use of DECIPHER⁴³, which is funded by the Wellcome Trust. The full acknowledgements can be found at www.ddduk.org/access.html. Sanger investigators are currently funded by Wellcome grant 220540/Z/20/A. We used data from UKHLS (Understanding Society). Understanding Society is an initiative funded by the Economic and Social Research Council and various Government Departments, with scientific leadership by the Institute for Social and Economic Research, University of Essex, and survey delivery by the National Centre for Social Research (NatCen) and Verian (formerly Kantar Public). The research data are distributed by the UK Data Service.

Author contributions

Conceptualization, E.M.W., K.E.S., and H.C.M.; Formal Analysis, E.M.W.; Investigation, E.M.W., V.K.C., K.E.S., H.C.M., M.E.H.; Resources, C.F.W., H.V.F., M.E.H.; Data Curation, R.Y.E.; Writing—Original Draft, E.M.W. and H.C.M.; Visualization, E.M.W.; Supervision, H.C.M. and M.E.H.

Competing interests

Dr. Firth is an author of Oxford Desk Reference: Clinical Genetics & Genomics. Professor Hurles is a consultant for AstraZeneca, and is a non-executive director of, consultant for, and stock-holder in Congenica. All the other authors do not hold any competing interest.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-58894-y>.

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