BRAIN
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Integrative genomics reveals pathogenic mediator of valproate-induced neurodevelopmental disability

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Prenatal exposure to the anti-seizure medication sodium valproate (VPA) is associated with an increased risk of adverse postnatal neurodevelopmental outcomes, including lowered intellectual ability, autism spectrum disorder and attention-deficit hyperactivity disorder. In this study, we aimed to clarify the molecular mechanisms underpinning the neurodevelopmental consequences of gestational VPA exposure using integrative genomics. We assessed the effect of gestational VPA on foetal brain gene expression using a validated rat model of valproate teratogenicity that mimics the human scenario of chronic oral valproate treatment during pregnancy at doses that are therapeutically relevant to the treatment of epilepsy. Two different rat strains were studied—inbred Genetic Absence Epilepsy Rats from Strasbourg, a model of genetic generalized epilepsy, and inbred non-epileptic control rats. Female rats were fed standard chow or VPA mixed in standard chow for 2 weeks prior to conception and then mated with same-strain males. In the VPA-exposed rats maternal oral treatment was continued throughout pregnancy. Foetuses were extracted via C-section on gestational Day 21 (1 day prior to birth) and foetal brains were snap-frozen and genome-wide gene expression data generated. We found that gestational VPA exposure via chronic maternal oral dosing was associated with substantial drug-induced differential gene expression in the pup brains, including dysregulated splicing, and observed that this occurred in the absence of evidence for significant neuronal gain or loss. The functional consequences of VPA-induced gene expression were explored using pathway analysis and integration with genetic risk data for psychiatric disease and behavioural traits. The set of genes downregulated by VPA in the pup brains were significantly enriched for pathways related to neurodevelopment and synaptic function and significantly enriched for heritability to human intelligence, schizophrenia and bipolar disorder. Our results provide a mechanistic link between chronic foetal VPA exposure and neurodevelopmental disability mediated by VPA-induced transcriptional dysregulation.

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Introduction

Sodium valproate (VPA) is an anti-seizure medication (ASM) widely used in the treatment of epilepsy, in particular genetic generalized epilepsy (GGE) for which it is the most effective ASM,[1,2] as well as being used to treat non-epileptic conditions such as migraine and bipolar disorder (BD).[3] In addition to the well-established approximate 10% risk of major congenital malformations associated with prenatal VPA exposure,[4,5] children born to mothers taking VPA are at an increased risk of adverse neurobehavioural outcomes including lowered intellectual ability, neurodevelopmental delay, autism spectrum disorder (ASD) and attention-deficit hyperactivity disorder (ADHD).[6–8]

We have previously reported the development and validation of a translational animal model of VPA-induced teratogenicity that mimics a number of the clinical features of human gestational VPA exposure, including chronic oral treatment of maternal rats before and after conception at therapeutically relevant blood levels (i.e. dosed orally to match VPA blood levels observed in humans).[9] This model was shown to recapitulate many of the developmental consequences of gestational VPA exposure, including brain maldevelopment, altered intravertebral distances and a significant developmental delay of vertebral arches. Here, we consider that the model also offers an opportunity to evaluate the molecular mechanisms underpinning VPA-associated neurodevelopmental disability.

In addition to its known effects on neuronal membrane excitability, VPA is an established histone deacetylase (HDAC) inhibitor and can induce gene expression changes both *in vitro* and *in vivo*.[10,11] Although these studies have established that VPA can induce gene expression changes in the CNS and neurons, their relevance to human neurodevelopmental outcomes is unclear due either to the use of non-therapeutically relevant VPA concentrations or non-clinically utilized drug delivery systems (e.g. the use of subcutaneous or intraperitoneal delivery), as well as the use of gestational exposures that are not representative of the human condition, such as acute exposures restricted to specific gestational time points.

We therefore aimed to advance insights into the mechanistic underpinnings of valproate-associated neurodevelopmental disability by evaluating brain gene expression changes of rat pups chronically exposed to VPA *in utero* and by considering the consequences of these VPA-induced brain gene expression changes using integrative genomics. The influence of epilepsy and genetic background on VPA-induced changes in brain gene expression was examined by investigating both epileptic [Genetic Absence Epilepsy Rats from Strasbourg (GAERS)—a model of GGE] and non-epileptic control (NEC) dams.

Materials and methods

Experimental design

Female rats from two different strains, inbred NEC and GAERS, were obtained from the Department of Medicine, Royal Melbourne Hospital, University of Melbourne Biological Research Facility.[12] VPA was administered via food chronically in a clinically relevant manner. Rats were fed standard rodent diet pre-mixed with VPA 20 g/kg or standard chow, as previously described.[9] Day 0 of pregnancy was marked by the presence of a plug. C-section was used to extract foetuses from the uterus on the 21st day of gestation. Pup brain tissues were then extracted using the QIAGEN Simultaneous Purification of Genomic DNA and total RNA from Animal Tissues protocol. External assessment, including spinal measurements, was used to examine the foetuses for any form of birth defects. The brain samples were extracted and immediately frozen and stored a –80°C without reference to the malformation score.

Transcriptome sequencing and gene expression data processing

The mRNA Isolation Kit was used to extract total RNA from brain tissue samples following the manufacturer's instructions. Total RNA was examined for quantity and quality using the TruSeq Stranded Total RNA Library Prep Gold. Sequencing libraries were quantified and sequence reads per sample generated. Quality control (QC) of RNA-seq reads was conducted using FastQC.[13] Low-quality reads and ribosome RNA reads were removed. Trimmed reads were then mapped to the *Rattus norvegicus* reference genome (rno6) using STAR (version 2.5).[14]

Foetal sex determination

Using a list of prenatally expressed Y-chromosome-encoded genes from a recently published study,[15] we were able to categorize the pup brains into male or female. The study identified these genes by comparing mRNA levels in different brain regions from human prenatal brain samples. In the current study, we assessed the expression of those genes using the rodent orthologues: USP9Y (Ubiquitin Specific Peptidase 9 Y-Linked), DDX3Y (DEAD-Box Helicase 3 Y-Linked), and UTY (Ubiquitously Transcribed Tetratricopeptide Repeat Containing, Y-Linked) and classified 14 samples as female and 16 samples as male (Table 1).

Differential gene expression analysis

R-package edgeR[16] was used to perform differential expression analysis and a threshold of false discovery rate (FDR) < 0.05 was applied to identify significantly differentially expressed genes (DEGs). To explore mRNA expression differences between VPA-exposed and non-exposed pup brains (after regressing out any technical differences due to sex), the following comparisons were undertaken: (i) E-NEC versus N-NEC; (ii) E-GAERS versus N-GAERS; and (iii) all exposed pups (i.e. E-GAERS + E-NEC) versus all non-exposed pups (N-GAERS + N-NEC). To inform gene expression changes in the rat pup brain relating to epilepsy we also considered the following two comparisons: (iv) E-GAERS versus E-NEC; and (v) N-GAERS versus N-NEC. We report all changes, but the primary aim of the work was to interrogate transcriptional changes following gestational VPA exposure.

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Table 1 Number of pups per group and exposure status

Strain	VPA exposure	Number of pups	Abbreviation	Male	Female
Generalized Epileptic Rats from Strasbourg (GAERS)	Exposed	7	E-GAERS	4	3
	Non-Exposed	8	N-GAERS	3	5
Non-epileptic control (NEC)	Exposed	7	E-NEC	4	3
	Non-Exposed	8	N-NEC	5	3

Functional enrichment and pathway analysis

The R package WEB-based Gene SeT AnaLysis Toolkit (WebGestaltR, v 0.4.4),[17] which uses databases from the Gene Ontology (GO) Consortium, was used to perform functional enrichment analysis of DEGs from each pairwise comparison. Default values for WebGestalt parameters were used, including overlap of 10 for minimum and maximum 500 genes and FDR-correction for multiple testing was performed using Benjamini and Hochberg method and significant pathways identified using a threshold of FDR < 0.05. In order to reduce redundancy across significant GO terms the following steps were taken. First, for each of the analyses GO terms were filtered to exclude terms with <20 genes or >1000 genes. Second, using the go_reduce() function from the r-utils R package (https://rhreynolds.github.io/rutils/articles/rutils.html), semantic similarity of GO terms were calculated and a threshold of 0.9 was applied. This threshold was applied to the hierarchical tree generated by reduceSimMatric () function from the rrvgo R package (v 1.1.4), which uses a bottom-up clustering method.

Linkage disequilibrium score regression

Linkage disequilibrium score regression (LDSC) was used to test for enrichment of heritability using published genome-wide association studies (GWAS). First, annotation files were generated for each set of DEGs with rows corresponding to a single nucleotide polymorphism (SNP) and a column for each subannotation. SNPs were then mapped to genes using dsSNP file NCBI Build 37 coordinates (build 147 and hg19), and values of 0 were given to SNPs not present in the file. Overall, 15 annotation files were generated for all the comparisons (separate files for all-dysregulated, upregulated and downregulated DEGs from each comparison). Second, LDSC was run using data files from phase 3 of the 1000 Genome Project Phase 3 European population. As per LDSC Github Wiki recommendation, linkage disequilibrium (LD) scores were calculated for the annotations using 1cM window and the analysis was restricted to Hapmap3 SNPs. Third, LDSC python scripts ('munge_sumstats.py') were used to format the summary statistic files. Fourth, for the regression weights, LD calculated for HapMap3 SNPs were downloaded from the LDSC Github page (https://github.com/bulik/ldsc). Additionally, for the LD score files used for the LD score regression, the full baseline model was used. For all the LDSC analyses, an enrichment score and its associated P-value was calculated based on the proportion of total SNPs per annotations (column), after taking into account all other annotation. Annotation categories with a significant positive enrichment of SNP heritability are then reported as a final result (a one-tailed test). GWAS summary statistics were downloaded from The European Bioinformatics Institute (EMBL-EBI) and Psychiatric Genomic Consortium (PGC) Cross-Disorder Group. All subjects were of European ancestry. A full detailed list of all summary statistics used in these analyses can be found in Supplementary Table 4.

Deconvolution

To deconvolute the bulk RNA-seq signature into its component single cell-type expression profiles, we anchored the analysis in a single-cell RNA-seq dataset from a recently published study that characterized the molecular architecture of the developing mouse brain.[18] Here, using cells extracted on E(18), a gene by cell matrix was constructed and cell types were identified, followed by further analysis using Seurat functions.[19] First, the dataset was normalized using the NormalizeData () function. This global scaling normalization method normalizes the gene expression values by multiplying the total expression of the cell by the number (n) of cells and then log transforms the result. A size factor of 10 000 was used for each cell. Second, cells were clustered by: (a) calculating the distance between two cells with similar expression using Euclidean algorithm and drawing edges. (b) Then using the FindClusters() function the cells were clustered (parameters used: 30 principal components (PCs) and a resolution parameter of 2). Third, the clusters were then visualized using non-linear dimensionality reduction algorithm known as Uniform Manifold Approximation and Projection (UMAP, v 0.1.10). Finally, Wilcoxon rank sum test [FDR < 0.05; implemented in the FindAllMarkers() function] was the used to identify genes differentially expressed (DE) in one cluster compared with all other clusters. Cell types were annotated by testing DEGs in a particular cell type for enrichment (Fisher's exact test) for cell-type markers from mouse datasets.[20] Next, the relative proportions of cell types in rat pups brain tissue samples were determined using weighted least squares-based deconvolution algorithms (DWLS). First, the expression matrix from all rat pup samples was normalized using count per million-based normalization methods. Second, using the biomaRt R-package (v4.1), the orthologous mouse gene was inferred (Ensembl Genes 104). Finally, cells were deconvolved using a weighted least-squares approach.

Data and code availability

The raw and processed rat sequencing data generated in this study have been deposited in NCBI Gene Expression Omnibus database under accession number GSE198756. All scripts used in this study can be found in GitHub (https://github.com/rahfel/VPA). Supplementary materials including full tables can also be found in GitHub (https://github.com/rahfel/VPA).

Results

Study workflow and data collection

A summary of the study workflow is shown in Fig. 1. Female GAERS and NEC rats were both derived from the same original Wistar rat colony but selectively inbred to express, or not to express, absence seizures.[12,21] The animal facility was maintained on a light dark cycle of 12 hours light, 12 hours dark at a temperature range of 19–22°C. All females were fed a standard rodent diet as reported by Senn and colleagues,[22] either pre-mixed with VPA 20 g/kg of food or not (controls) for 2 weeks

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before mating with males of the same strain and continued throughout pregnancy. We have previously demonstrated that this dose of VPA administered orally results in significant seizure suppression in adult GAERS and achieves blood serum levels broadly equivalent to human therapeutic levels of VPA; across VPA-exposed GAERs and NEC dams, VPA levels ranged 120–380 μmol/l and 250–460 μmol/l, respectively (*t*-test $P = 0.197$).[9] The presence of a copulatory plug indicated Day 0 of pregnancy. Once the plug was present, females were separated from the males for the course of their pregnancy. The following day was classified as Day 1. The foetuses were extracted from the uterus via C-section on Day 21, 1 day before expected birth. The dams were sacrificed and the foetus brains removed, snap-frozen and stored at −8 °C. We have previously reported the results of the teratology studies on rat pups exposed to valproate *in utero*.[9] Pup brain samples were then chosen without reference to morphological score and were not significantly different in weight between the VPA-exposed and unexposed groups (mean weights ± SEM: NEC control 0.151 g ± 0.017; NEC VPA 0.155 g ± 0.014; GAERS control 0.180 g ± 0.012; GAERS VPA 0.137 g ± 0.011, two-way ANOVA (drug) $P = 0.15$; (strain) $P = 0.71$). Six litters were generated for each group and one or two pup brains from each litter were randomly processed. Total RNA was extracted from 30 pup brains (Table 1) and genome-wide gene expression data were generated using RNA-sequencing (RNA-seq; see the 'Materials and methods' section).

Deconvolution analysis reveals no difference in cell-type composition between VPA-exposed and non-exposed pup brains

Previous preclinical studies on the effects of intraperitoneal administration of VPA have demonstrated VPA-induced apoptotic neurodegeneration in the developing rat brain and this has been suggested as a mechanism to explain VPA-associated cognitive impairment.[23,24] We therefore first sought to identify whether chronic oral VPA exposure used in our model was associated with significant changes in the proportion of cell types in the rat pup brain. To this end, we applied a weighted least-squares-based deconvolution algorithm, DWLS,[25] which estimates cell-type composition in a bulk tissue RNA-seq dataset using prior information from an unrelated single-cell RNA-sequencing (scRNA-seq) signature from an analogous tissue. Taking this approach, we observed no significant difference in cell-type composition between valproate-exposed and unexposed pup brains, for either GAERS or NEC rats, suggesting chronic orally delivered gestational VPA exposure does not have a major effect on cell loss or gain (FDR-corrected Wilcoxon rank sum test > 0.05; Supplementary Fig. 1 and Supplementary Table 1).

Differential gene expression analysis highlights transcriptional changes in VPA-exposed pup brains

Prior to undertaking differential gene expression analysis, we explored the principal components of variation between epileptic and non-epileptic pups and between VPA-exposed and non-exposed pups (Supplementary Fig. 2). Using genome-wide gene expression profiles for each brain, we observed that epileptic and non-epileptic pups were broadly separated by the first principal component (PC1) and VPA-exposed and non-exposed pups by PC2. We therefore undertook differential gene expression analyses for the 'case control' comparisons listed in Table 2.

We observed significantly (FDR < 0.05) differentially expressed genes in all five pairwise comparisons (summarized in Table 2; see Supplementary Table 2 for a full list of DEGs for each comparison). Considering VPA-exposed versus non-exposed pup brains, we observed the largest number of DEGs when comparing all VPA-exposed pups (All-E) consisting of VPA-exposed GAERS pups (E-GAERS) and VPA-exposed NEC (E-NEC) pups combined versus all non-exposed pups (All-N) consisting of non-exposed GAERS (N-GAERS) and non-exposed NEC (N-NEC) pups combined, where 3470 genes were significantly (FDR < 0.05) differentially expressed. Of these, 1632 genes were significantly (FDR < 0.05) upregulated and 1838 downregulated.

Figure 2 shows the overlaps in genes differentially expressed for each of the three pairwise VPA-exposed versus non-exposed comparisons (i.e. All-E versus All-N, E-NEC versus N-NEC and E-GAERS versus N-GAERS). We observed that the majority of genes differentially expressed by VPA in GAERS pups were also differentially expressed in NEC pups, suggesting that genetic epilepsy status is not a major determinant of the pattern of differential expression induced by VPA, as was previously the case for VPA-induced birth defects in this model.[9]

To investigate the functional consequences of VPA-induced differential gene expression in the pup brain, we first undertook pathway enrichment analysis. We found that genes downregulated following VPA exposure were highly significantly enriched for functional processes related to modulation of synaptic function and neuronal processes (see Fig. 3 for summary of biological processes dysregulated by VPA and Supplementary Tables 3a–3c for full details of pathway enrichment analyses). For example, when considering all VPA-exposed pups versus unexposed pups (All-E versus All-N), among the set of genes downregulated by VPA we observed highly significant enrichment for terms relating to glutamate receptor complex (FDR = 9.74×10^{-12}), regulation of membrane potential (FDR = 0), synapse assembly (FDR = 2.17×10^{-11}), post-synaptic membrane (FDR = 2.11×10^{-4}), neurotransmitter receptor activity (FDR = 2.33×10^{-4}) and axon guidance (FDR = 0). Interestingly, also among the pathways enriched in the downregulated set of genes was regulation of insulin secretion (FDR = 9.5×10^{-3}), which if replicated in the pancreas (not examined in this study) might suggest a possible drug-induced transcriptional mechanism for the increased incidence of impaired glucose control in patients treated with VPA.[26,27]

In contrast to the substantial enrichment of neuronal functions in genes downregulated by VPA, the genes upregulated by VPA were generally enriched for functional terms not directly related to neural processes, namely mRNA splicing (FDR = 7.29×10^{-14}), translation (FDR = 1.40×10^{-13}), extracellular matrix organization (FDR = 2.41×10^{-12}) and cell cycle (FDR = 2.40×10^{-9}). Consistent with VPA's known activity as an HDAC inhibitor, we also saw enrichment of biological processes related to chromatin organization (FDR = 7.37×10^{-7}) and chromatin assembly/disassembly (FDR = 1.45×10^{-6}) among the genes upregulated by VPA.

In summary, genes up- and downregulated by chronic *in utero* VPA exposure in the developing rat brain were represented by divergent functional categories, with genes downregulated by VPA predominantly characterized by pathways relating to nervous system function.

Genes downregulated by gestational VPA exposure are highly enriched for heritability for neurodevelopmental traits and disease

We next sought to investigate the relationship between genes differentially expressed in pup brains following gestational VPA and genetic risk for neurodevelopmental disease and behavioural traits. Because, in the context of rare variant genetic

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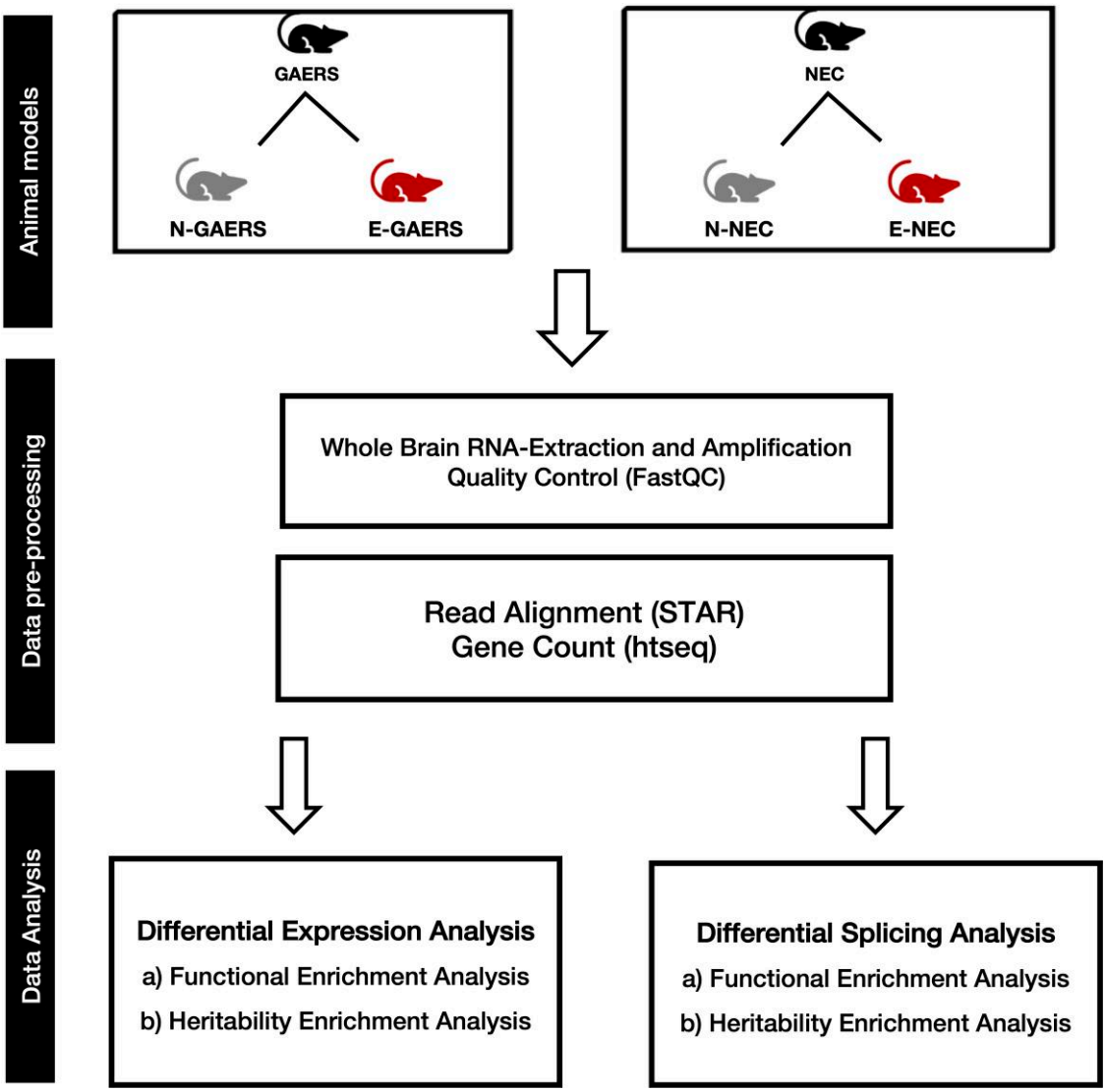

Figure 1 Overview of experimental design and data processing. Two different genetic strains of rat were studied: GAERS and NEC. Whole-brain samples were extracted from valproate-exposed (E-GAERS and E-NEC) and non-exposed (N-GAERS and N-NEC) pups (total $n = 30$). Pup brains were snap-frozen in liquid nitrogen and genome-wide gene expression assayed using RNA-sequencing (RNA-seq). Data preprocessing steps included quality control for sequence reads alignment to the rat genome and library size normalization. Differential gene expression and differential splicing analyses were carried out with downstream pathway and heritability enrichment analyses.

analyses, neurodevelopmental disability primarily arises from loss-of-function and deleterious mutations,[28] we hypothesized that genetic risk for neurodevelopmental disease may be enriched in the set of genes significantly downregulated by VPA in the developing brain.

To test for enrichment of genetic association in sets of genes, we used stratified LDSC.[29] Given the broad range of adverse neurodevelopmental outcomes in children gestationally exposed to VPA, we considered GWAS for a broad range of psychiatric diseases and behavioural traits: ADHD,[30] BD,[31] ASD,[32] schizophrenia (SCZ),[33] full-scale intelligence quotient (IQ),[34] epilepsy (EPI)[35] and 'cross disorders group' (CDG), the latter representing a meta-analysis of eight individual psychiatric disorder GWAS, namely anorexia nervosa, ADHD, ASD, BD, major depression, obsessive-compulsive disorder, SCZ and Tourette syndrome.[36] As a negative control GWAS dataset not expected to be enriched in the set of genes differentially expressed in pup brains we used a GWAS for waist/hip ratio (WHR).[37]

Enrichment of genetic association analyses were run for all sets of genes significantly (FDR < 0.05) differentially expressed in the VPA-exposed pup brain (results are summarized in Fig. 4; full details are provided in Supplementary Table 4). For genes downregulated by VPA, we observed highly significant enrichment of heritability with bipolar disorder (All-E versus All-N, $FDR_{LDSC} = 1.16 \times 10^{-8}$; E-NEC versus N-NEC, $FDR_{LDSC} = 2.93 \times 10^{-9}$; E-GAERS versus N-GAERS, $FDR_{LDSC} = 1.55 \times 10^{-3}$), SCZ (All-E versus All-N, $FDR_{LDSC} = 6.04 \times 10^{-8}$; E-NEC versus N-NEC, $FDR_{LDSC} = 6.40 \times 10^{-8}$; E-GAERS versus N-GAERS, $FDR_{LDSC} = 2.91 \times 10^{-3}$), IQ (All-E versus All-N, $FDR_{LDSC} = 1.17 \times 10^{-3}$; E-NEC versus N-NEC, $FDR_{LDSC} = 7.03 \times 10^{-4}$; E-GAERS versus N-GAERS, $FDR_{LDSC} = 1.62 \times 10^{-2}$) and CDG (All-E versus All-N, $FDR_{LDSC} = 3.89 \times 10^{-4}$; E-NEC versus N-NEC, $FDR_{LDSC} = 1.82 \times 10^{-4}$; E-GAERS versus N-GAERS, $FDR_{LDSC} = 5.44 \times 10^{-2}$). No significant enrichments of heritability were observed for these traits in unexposed GAERS pups compared to unexposed NECs, and enrichments of association for WHR were non-significant across all comparisons (Supplementary Table 4).

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Table 2 Differential gene expression analysis: case control comparisons

Comparisons	Sample size	Number of DEGs (FDR < 0.05)	Upregulated genes (FDR < 0.05)	Downregulated genes (FDR < 0.05)
E-NEC versus N-NEC	15	2973	1307	1666
E-GAERS versus N-GAERS	15	553	248	305
All-E (E-GAERS + E-NEC) versus All-N (N-GAERS + N-NEC)	30	3470	1632	1838
E-GAERS versus E-NEC	14	1885	1039	846
N-GAERS versus N-NEC	16	1066	548	518

NEC = non-epileptic control; GAERS = Genetic Absence Epilepsy Rats from Strasbourg; E-NEC = VPA-exposed NEC pups; E-GAERS = VPA-exposed GAERS pups; N-NEC = non-exposed NEC pups; N-GAERS = non-exposed GAERS pups; All-E = all exposed pups (E-NEC + E-GAERS); All-N = all non-exposed pups (N-NEC + N-GAERS).

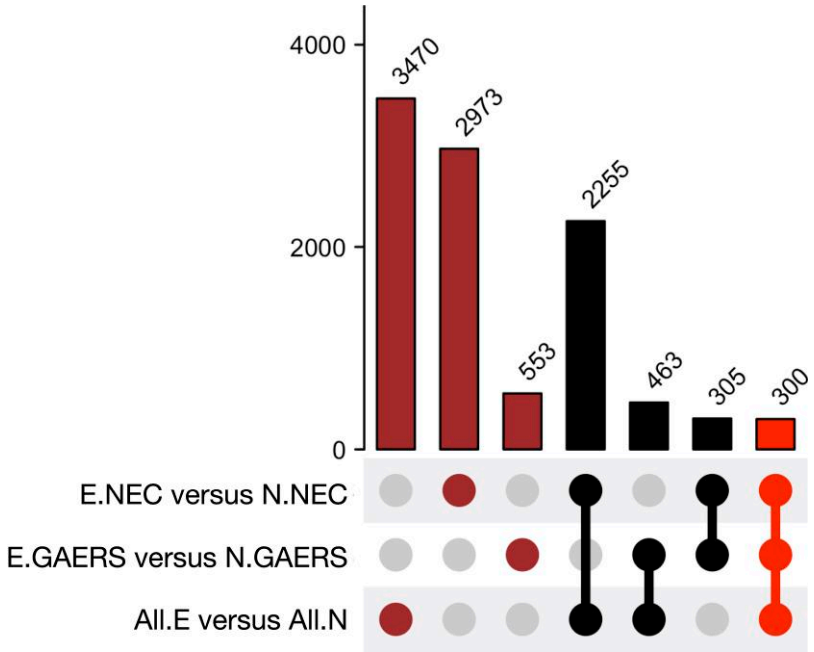

Figure 2 Summary of differential gene expression results for the three valproate-exposed versus non-exposed comparisons. Total number of significantly (FDR < 0.05) DEGs for each comparison are shown (single dot corresponding to the first three histograms). The number of genes overlapping between each of the case versus control comparisons is shown by the 2-way and 3-way comparisons (*right* four histograms).

There was no significant enrichment of heritability in the set of genes downregulated in VPA-exposed pup brains to ADHD (All-E versus All-N, $FDR_{LDSC} = 0.69$; E-NEC versus N-NEC, $FDR_{LDSC} = 0.24$; E-GAERS versus N-GAERS, $FDR_{LDSC} = 0.88$), ASD (All-E versus All-N, $FDR_{LDSC} = 0.66$; E-NEC versus N-NEC, $FDR_{LDSC} = 0.68$; E-GAERS versus N-GAERS, $FDR_{LDSC} = 0.68$) or EPI (All-E versus All-N, $FDR_{LDSC} = 0.77$; E-NEC versus N-NEC, $FDR_{LDSC} = 0.57$; E-GAERS versus N-GAERS, $FDR_{LDSC} = 0.77$).

For genes upregulated by gestational VPA exposure, we did not observe a significant enrichment of genetic association to any neurodevelopmental disease or trait, other than a marginal enrichment for BD (All-E versus All-N, $FDR_{LDSC} = 2.7 \times 10^{-2}$; E-NEC versus N-NEC, $FDR_{LDSC} = 3.6 \times 10^{-3}$; E-GAERS versus N-GAERS, $FDR_{LDSC} = 0.84$) and SCZ (All-E versus All-N, $FDR_{LDSC} = 4.3 \times 10^{-2}$; E-NEC versus N-NEC, $FDR_{LDSC} = 0.32$; E-GAERS versus N-GAERS, $FDR_{LDSC} = 0.56$).

Taken together, these results suggest VPA exerts its adverse effects on foetal neurodevelopment predominantly via drug-induced downregulation of genes highly relevant to neurodevelopment and nervous system function. The directionality of the effect is consistent with that observed from rare-variant analyses of genetic risk to neurodevelopmental disability where the predominant mechanism is a dominant negative effect from deleterious gene mutations.[38] Given the functional enrichment of chromatin assembly/disassembly terms among the genes upregulated by VPA, along with VPA's known activity as an HDAC inhibitor, it seems likely that this transcriptional dysregulation is epigenetically encoded with potentially long-lasting consequences for human brain function and health even in the absence of persisting valproate exposure postnatally.

Alternatively spliced genes in VPA exposed pups compared to non-exposed pups

Among the pathways enriched in genes upregulated by VPA we observed a highly significant (FDR = 0) enrichment for genes involved in splicing function. We therefore evaluated the role of VPA on differential splicing by first quantifying the read counts to individual gene exons and then comparing differential exon usage using EdgeR (see the 'Materials and methods' section). Overall, there were 57 significantly (FDR < 0.05) alternatively spliced genes, among which 21 were also differentially expressed following VPA exposure (14 were downregulated, seven upregulated; Supplementary

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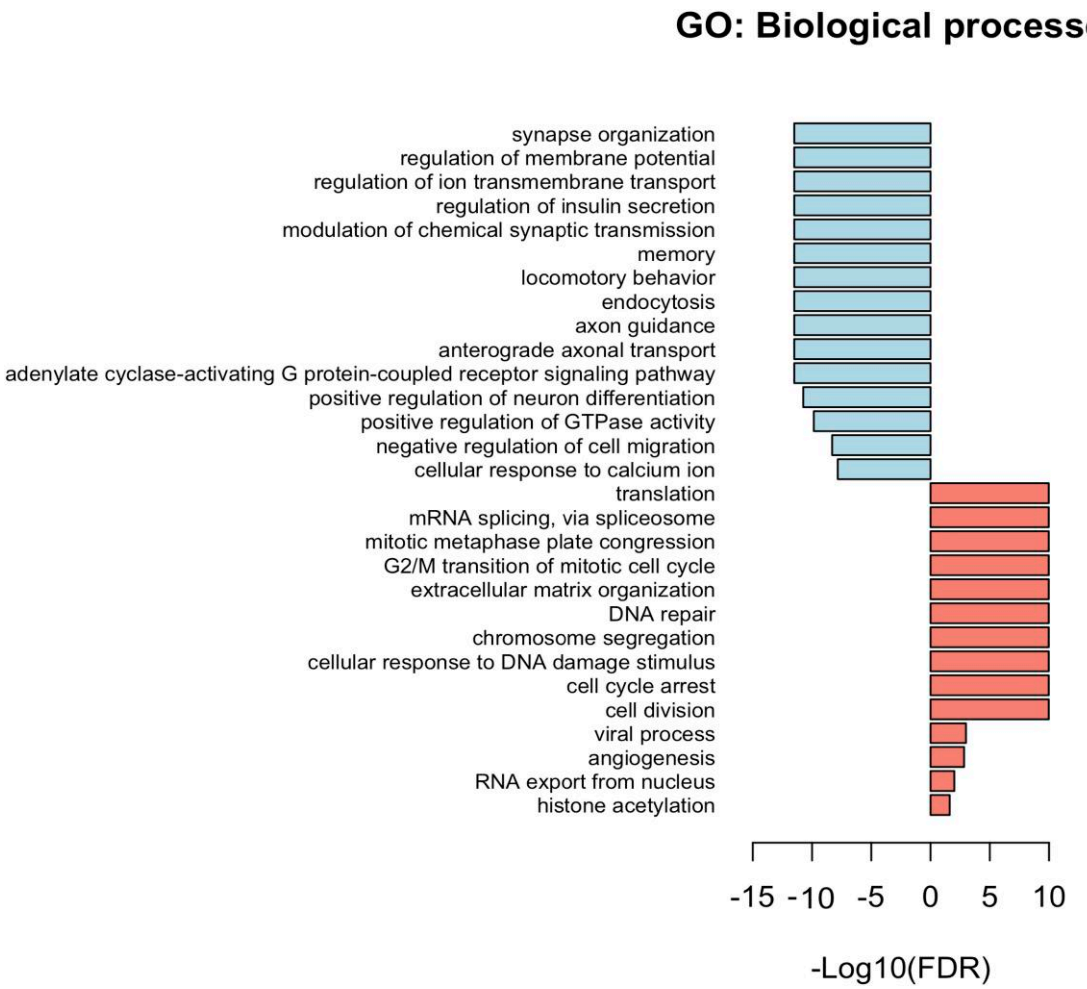

Figure 3 Pathway enrichment for valproate-exposed versus non-exposed pups. Biological process (BP) GO parental terms enriched in genes significantly (FDR < 0.05) differentially expressed between All-E versus All-N pups for downregulated (*top* 15 bars) and upregulated (*bottom* 14 bars) genes. For full details including enrichment FDR-values for parental and child terms including Cellular Compartment and Molecular Function GO pathway enrichments see Supplementary Tables 3a–c. *–Log_{10}FDR values have been signed to indicate enrichment in the down- or upregulated genes.

Table 5). While there were no significant enrichments for known functional pathways or enrichment of genetic association to neurodevelopmental disease among the set of differentially spliced genes (data not shown), individual differentially spliced genes included genes for neuronal proteins such as Calmodulin Binding Transcription Activator 1 (CAMATA1; FDR = 3.69×10^{-26}), which is known to play a role in the regulation of glutamate levels and neuronal excitability; glutamate decarboxylase 2 (GAD2; FDR = 9.03×10^{-25}), which plays a role GABA-synthesis in neurons[39]; and Forkhead box P4 (FOXP4; FDR = 4.62×10^{-3}), which is known to regulate neurogenesis and in which mutations are associated with speech delay and congenital abnormalities.[40] These results suggest differential splicing as a potential further mechanism influencing behavioural outcomes following foetal VPA exposure.

Discussion

The adverse neurodevelopmental consequences of chronic foetal valproate exposure have been well-documented and remain a significant limitation on the use of VPA in women who could become pregnant, with particular implications for the treatment of generalized epilepsy, where it remains the most effective treatment,[1,2] as well as other diseases in women of child-bearing potential such as BD, where it is an important therapy. The mechanisms by which VPA contribute to behavioural and cognitive disability in children following gestational exposure remain poorly defined, with existing studies limited by non-physiological drug administration and dosing schedules and relatively few studies having examined transcriptome-wide alterations in brain gene expression.[11] In this study, we utilized an established rat model of VPA-induced teratogenicity[9] that recapitulates human prenatal valproate exposure and chronicity of oral dosing during pregnancy.

Among the proposed mechanisms for VPA-associated neurodevelopmental disability is VPA-induced apoptosis.[41] In the present study, using deconvolution analysis anchored in scRNA-seq, we found no evidence for substantial shifts in the composition of the major cell types of the brain (i.e. excitatory neurons, inhibitory neurons, astrocytes, oligodendrocytes, oligodendrocyte precursor cells, microglia) following VPA exposure. While our results suggest the adverse functional consequences of VPA exposure reside in altered synaptic function, they do not exclude the possibility of VPA-induced variation in the proportion of cellular subtypes, which will require scRNA-seq of large numbers of cells from VPA-exposed and non-exposed foetal brains to identify.

In contrast to our finding of no measurable difference in the proportion of the major cell types of the mammalian brain, we

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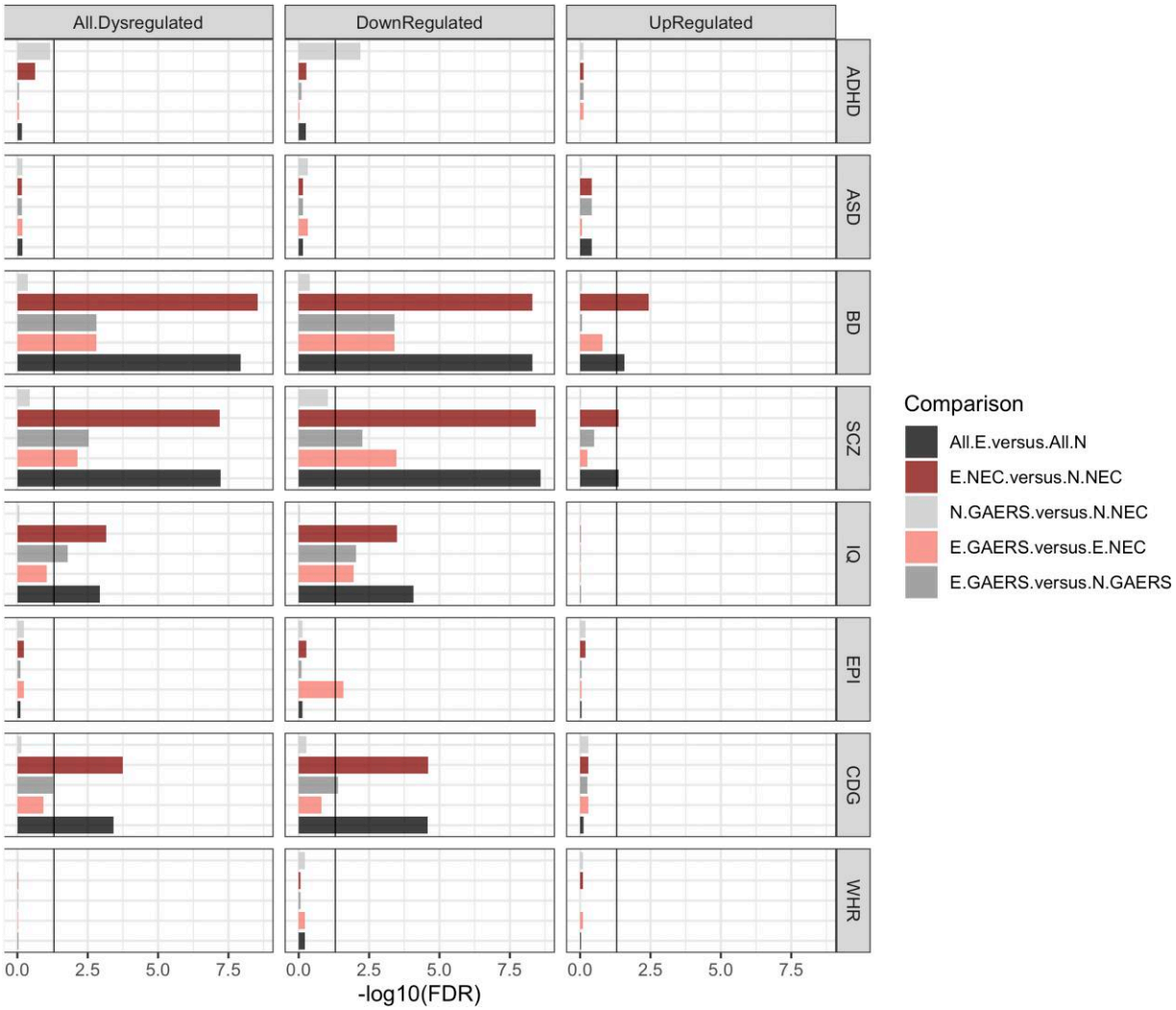

Figure 4 Heritability enrichment in DEGs. LDSC was used to test for enrichment of heritability in genes significantly differentially expressed for each of the five case control comparisons shown. Enrichment $-\log_{10}$(FDR) for the enrichment of genetic association for each trait is indicated by the horizontal bars coloured by the comparison group from which the DEGs were identified. Vertical line indicates FDR values at 0.05. GWAS used for the enrichment analysis are indicated on the vertical axis.

observed significant VPA-induced differential gene expression in gestationally exposed pup brains. Pathway enrichment analysis showed a striking separation of enriched terms between the up- and downregulated genes, with upregulated genes relating predominantly to cell division, mRNA splicing, translation and extracellular matrix organization and downregulated genes highly enriched for functional processes directly relevant to neurodevelopment including synapse assembly, post-synaptic membrane, regulation of neuronal membrane activity and synaptic transmission.

To further investigate the functional consequences of VPA-induced differential gene expression we integrated DEGs with GWAS summary statistics from a range of clinically relevant neurodevelopmental diseases and behavioural traits. We observed significant enrichments of heritability in the set of genes downregulated by VPA for BD, SCZ, IQ and CDG. We did not see an enrichment for ASD, which was surprising given that some of the features of VPA-associated neurodevelopmental disability have been likened to autism, and maternal valproate exposure has been associated with autism-like behaviours in non-human primates.[42] This absence of a genetic association with ASD was in stark contrast to the enrichment for BD, SCZ and IQ, and suggests that VPA-associated neurodevelopmental disability may have specific clinical characteristics unique to VPA exposure and points to a requirement for more research to continue to define the clinical phenotype. Notably, the directionality of the effect of VPA-induced gene expression on brain function is consistent with that observed from rare-variant analyses of genetic risk to neurodevelopmental disease where the predominant mechanism is a dominant negative effect from deleterious mutation.[38] The functional enrichment of chromatin assembly/disassembly terms among the genes upregulated by VPA suggests the VPA-induced transcriptional dysregulation is epigenetically encoded and therefore with potential long-lasting consequences for human brain function and behaviour even following birth, prompting the need for further clinical research on the long-term outcomes of children born following foetal VPA exposure.

In addition to substantial differential gene expression, we found that chronic prenatal VPA exposure is associated with differential mRNA splicing in the brain. Interestingly, recent studies

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have shown evidence that implicates the regulatory role of neuron-specific alternative splicing in neurodevelopmental disorders[43,44] and alternative splicing in the brain is important for several neurological processes including cell differentiation, neurogenesis, synaptogenesis and in the generation of functional neuronal networks.[45] Among the genes observed to be significantly differentially spliced in the prenatal brain following VPA exposure were GAD2, which plays a role GABA-synthesis in neurons,[39] and Foxp4, which is known to regulate neurogenesis and is associated with speech delay and congenital abnormalities.[40] These results suggest that alternative splicing may be an additional mechanism for adverse neurodevelopment in VPA-exposed foetal brains.

Apart from valproate, some studies have suggested a risk of neurodevelopmental complications from foetal exposure to other ASMs, although such risks remain to be fully characterized.[46–48] Additionally, studies have suggested that anti-depressant use during pregnancy may be associated with an increased risk of neurodevelopmental disorders including ASD and ADHD,[49] although not following exposure to anti-psychotics.[50] These studies highlight the concern that exists for effects on neurodevelopment for several drugs and classes of drugs commonly prescribed during pregnancy. The research presented here, which demonstrates a robust brain transcriptional response to VPA that is both functionally and genetically associated with relevant cognitive (IQ) and psychiatric (BD, SCZ, CDG) outcomes suggests that the rat model of chronic dosing followed by transcriptional assay in pup brains might provide a general approach to screening for drug-induced adverse neurodevelopmental effects. Moreover, the inference that the adverse behavioural and cognitive outcomes from gestational VPA exposure arise from transcriptional dysregulation provides a potential system for testing drugs capable of reversing or ameliorating these changes. For example, as previously highlighted, VPA is a well-recognized HDAC inhibitor, and pretreatment with methionine has been shown to significantly reduce the incidence of spina bifida and other VPA-associated defects in mice, albeit at the expense of significantly increased embryo lethality.[51]

There are several limitations to our study. First, we did not measure serum VPA levels in the rats although we did follow the same oral dosing schedule previously described,[9] which has been shown to achieve a blood level of 180–280 μmol/l and to have anti-seizure effects.[52] Second, we did not undertake a dose–response curve across VPA dosages, which has the theoretical potential to establish if there is a 'safe' dosage below which the transcriptional effects of VPA do not occur. This is an important clinical unknown highly relevant to women whose seizures are not controlled by any medication other than VPA. We note, however, that the blood levels achieved by oral dosing used in our study are below the standard therapeutic range for human epilepsy of 346–693 μmol/l, suggesting that our results are not an artefact of artificially high VPA levels and also pointing to effects on foetal brain transcription even at relatively low serum levels of valproate. This observation is in keeping with reports of impaired cognition and a 6-fold increase in educational intervention in children born to mothers taking low-dose valproate (<800 mg/day) during pregnancy.[53] Third, we did not perform behavioural testing in exposed and unexposed offspring in this model so we cannot confirm if the transcriptional effects of VPA on the brain directly correlate with changes in behaviour. However, previous research on this animal model has established that chronic oral dosing of valproate at the dosages employed in the present study do induce the expected developmental and morphological abnormalities in pups.[9]

Our study did not address the question of whether a genetically defined subpopulation of women can be identified who are at risk of having children with VPA-associated neurodevelopmental disability. Previous research has observed that women who had given birth to a malformed baby in their first VPA pregnancy are more likely to have a malformed child in their next compared to those who had taken VPA without foetal abnormalities.[4] This suggests that maternal factors, perhaps genetic factors, contribute to VPA-associated congenital malformations. However, we are not aware of similar studies that have examined the offspring recurrence risk with respect to VPA-associated neurodevelopmental disability. Additionally, the extent to which variation in risk of VPA-associated neurodevelopmental disability (independent of VPA dosage) is explained by maternal genetic variation (e.g. using GWAS methodology or exome-sequencing) is, to date, unexamined. As well as potential maternal genetic effects on risk (for example, perhaps mediated by genetic variation in valproate clearance), one can hypothesize a potential effect from foetal genotype on risk as well, for example via genetic effects on VPA-induced neuronal gene expression (so-called 'response eQTLs'). Determining if maternal or foetal genetic factors play a role in VPA-associated neurodevelopmental disability is therefore likely to require substantial further research.

In conclusion, the data presented here provide a mechanistic explanation for VPA-induced adverse neurodevelopment anchored in drug-induced transcriptional dysregulation. The extent to which these transcriptional effects are related to irreversible brain development and/or fixed epigenetically encoded changes, or which might be associated with a gradual restoration of normal brain transcription and function over time postnatally is unknown, but could potentially be explored using the experimental paradigm described in this study. Our research prompts the need for longer-term follow-up of children born following gestational VPA exposure and the evaluation of other ASMs as well as dose–response curves in this model using transcriptional readouts.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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