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Title:

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Date:

2022-11-01

Citation:

Feleke, R., Jazayeri, D., Abouzeid, M., Powell, K. L., Srivastava, P. K., O'Brien, T. J., Jones, N. C. & Johnson, M. R. (2022). Integrative genomics reveals pathogenic mediator of valproate-induced neurodevelopmental disability. *Brain*, 145 (11), pp.3832-3842. <https://doi.org/10.1093/brain/awac296>.

Persistent Link:

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Integrative genomics reveals pathogenic mediator of valproate-induced neurodevelopmental disability

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Running title: Valproate-induced neurodevelopment

Keywords: valproate; gene expression; neurodevelopment; disability

Abbreviations: ASM = Anti-seizure medication; ADHD = Attention Deficit Hyperactivity Disorder; All-E = All valproate exposed pups; All-N = All non-exposed pups; ASD = Autism Spectrum Disorder; BD = Bipolar Disorder; BP = Biological Process; CDG = Cross Disorders Group; DE = Differentially Expressed; DEG = Differentially Expressed Genes; DWLS = Dampened Weighted Least Squares; EPI = Epilepsy; GAERS = Genetic Absence Epilepsy Rats from Strasbourg; GGE = Genetic

1 Generalized Epilepsy; GO = Gene Ontology; GWAS = Genome-Wide Association Studies; HDAC =
2 Histone DeAcetylase; IQ = Intelligence Quotient; LDSC = Linkage Disequilibrium Score Regression;
3 MCMs = Major Congenital Malformations; NEC = Non-Epileptic Control; PC = Principal Component;
4 QC = Quality control; rno6 = Rattus norvegicus reference genome; scRNA-seq = Single-cell RNA-
5 sequencing; SCZ = Schizophrenia; VPA = Sodium valproate; WebGestaltR =WEB-based Gene SeT
6 AnaLysis Toolkit; WHR = Waist/Hip Ratio

7

8

ACCEPTED MANUSCRIPT

1 **ABSTRACT**

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

25
26

1 **INTRODUCTION**

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

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

18 In addition to its known effects on neuronal membrane excitability, VPA is an established
19 histone deacetylase inhibitor and can induce gene expression changes both *in vitro* and *in vivo*.^{10,11}
20 Although these studies have established that VPA can induce gene expression changes in the central
21 nervous system and neurons, their relevance to human neurodevelopmental outcomes is unclear due
22 either to the use of non-therapeutically-relevant VPA concentrations or non-clinically utilized drug
23 delivery systems (e.g., the use of sub-cutaneous or intraperitoneal delivery), as well as the use of
24 gestational exposures that are not representative of the human condition such as acute exposures
25 restricted to specific gestational time points.

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

MATERIALS AND METHODS

Experimental Design

Female rats from two different strains, inbred Non-Epileptic Controls (NEC) and Genetic Absence Epilepsy Rats from Strasbourg (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 20g/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 were used to examine the foetuses for any form of birth defects. The brain samples were extracted and immediately frozen and stored at -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 FastQC⁴⁶. 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).⁴⁷

Fetal sex determination

Using a list of prenatally expressed Y-chromosome-encoded genes from a recently published study,⁴⁸ we were able to categorise 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 orthologs: 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 (refer to **Table 1**).

1 **Differential gene expression analysis**

2 R-package edgeR⁴⁹ was used to perform differential expression analysis and a threshold of FDR
3 < 0.05 was applied to identify significantly differentially expressed genes (DEGs). To explore mRNA
4 expression differences between VPA-exposed and non-exposed pup brains (after regressing out any
5 technical differences due to sex), the following comparisons were undertaken: i) E-NEC vs N-NEC; ii)
6 E-GAERS vs N-GAERS; iii) All exposed pups (i.e., E-GAERS + E-NEC) vs all non-exposed pups (N-
7 NEC + N-NEC). To inform gene expression changes in the rat pup brain relating to epilepsy we also
8 considered the following two comparisons: iv) E-GAERS vs E-NEC; v) N-GAERS vs N-NEC. We
9 report all changes but the primary aim of the work was to interrogate transcriptional changes following
10 gestational VPA exposure.

11 **Functional enrichment and pathway analysis**

12 The R package WEB-based Gene SeT AnaLysis Toolkit (WebGestaltR, v 0.4.4),⁵⁰ which uses
13 databases from the Gene Ontology Consortium, was used to perform functional term enrichment
14 analysis of DEGs from each pairwise comparison. Default values for WebGestalt parameters were
15 used, including overlap of 10 for minimum and maximum 500 genes and FDR-correction for multiple
16 testing was performed using Benjamini and Hochberg (BH) method and significant pathways identified
17 using a threshold of FDR < 0.05. In order to reduce redundancy across significant GO-terms the
18 following steps were taken. First, for each of the analyses GO terms were filtered to exclude terms with
19 ≥ 20 genes or ≤ 1000 genes. Second, using `go_reduce()` function from the `r-utils` R package
20 (<https://rhreynolds.github.io/rutils/articles/rutils.html>) semantic similarity of GO terms were calculated.
21 Third, a threshold of 0.9 was applied to get fewer GO-terms. This threshold was applied to the
22 hierarchical tree generated by `reduceSimMatric ()` function from the `rrvgo` R package (v 1.1.4), which
23 uses a bottom-up clustering method.

24 **LD score regression**

25
26 LDSC was used to test for enrichment of heritability using published GWAS studies. First,
27 annotation files were generated for each set of DEGs with rows corresponding to a SNP and a column
28 for each sub-annotation. SNPs were then mapped to genes using `dsSNP` file NCBI Build 37 co-
29 ordinates (build 147 and hg19), and values of 0 were given to SNPs not present in the file. Overall,
30 fifteen annotation files were generated for all the comparisons (separate files for all-dysregulated, up-
31 regulated and down-regulated DEGs from each comparison). Second, LDSC was run using data files
32 from phase 3 of the 1000 Genome Project Phase 3 European population. As per LDSC Github Wiki
33

1 recommendation, LD scores were calculated for the annotations using 1cM window and the analysis
2 was restricted to Hapmap3 SNPs. Third, LDSC python scripts (“munge_sumstats.py”) were used to
3 format the summary statistic files. Fourth, for the regression weights, LD calculated for HapMap3
4 SNPs were downloaded from the LDSC Github page (<https://github.com/bulik/ldsc>). Additionally, for
5 the LD score files used for the LD score regression, the full baseline model was used. For all the LDSC
6 analyses, an enrichment score and its associated P-value was calculated based on the proportion of total
7 SNPs per annotations (column), after taking into account all other annotation. Annotation categories
8 with a significant positive enrichment of SNP-heritability are then reported as a final result (a one tailed
9 test). GWAS summary statistics were downloaded from The European Bioinformatics Institute
10 (EMBL-EBI) and Psychiatric Genomic Consortium (PGC) Cross-Disorder Group. All subjects were of
11 European ancestry. A full detailed list of all the summary statistic used in these analyses can be found
12 in **Supplementary Table 4**.

14 **Deconvolution**

15 To deconvolute the bulk RNA-seq signature into its component single cell-type expression
16 profiles, we anchored the analysis in a single-cell RNA-seq dataset from a recently published study that
17 characterised the molecular architecture of the developing mouse brain.⁵¹ Using this dataset, we were
18 able to characterise 93 mouse brain tissues at single-cell resolution and generate transcriptomic atlas of
19 the embryonic mouse brain from gastrulation up until birth. Here, using cells extracted on E(18), gene
20 by cell matrix was constructed and cell types were identified, followed by further analysis using Seurat
21 functions.⁵² First, the dataset was normalised using the `NormalizeData()` function. This global scaling
22 normalisation method normalises the gene expression values by multiplying the total expression of the
23 cell by the number (n) of cells and then log transforms the result. A size factor of 10,000 was used for
24 each cell. Second, cells were clustered by: a) Calculating the distance between two cells with similar
25 expression using Euclidean algorithm and drawing edges. b) Then using the `FindClusters()` function the
26 cells were clustered (parameters used: 30 principal components (PCs) and a resolution parameter of 2).
27 Third, the clusters were then visualised using non-linear dimensionality reduction algorithm known as
28 Uniform Manifold Approximation and Projection (UMAP, v 0.1.10). Finally, Wilcoxon rank sum test
29 (FDR < 0.05) (implemented in the `FindAllMarkers()` function) was the used to identify genes
30 differentially expressed (DE) in one cluster compared with all other clusters. Cell types were allocated
31 by testing DEGs in a particular cell type for enrichment (Fisher’s exact test) for cell-type markers from
32 mouse datasets.⁵³ Next, the relative proportions of cell types in rat pups brain tissue samples were
33 determined using weighted least squares-based deconvolution algorithms, DWLS. First, expression

1 matrix from all rat pup samples was normalised using count per million based normalisation methods.
2 Second, using biomaRt R-package (v4.1), the orthologous mouse gene was inferred (Ensembl Genes
3 104). Finally, cells were deconvolved using weighted least squares approach.
4

5 **Data and code availability**

6 The raw and processed rat sequencing data generated in this study have been deposited in NCBI
7 Gene Expression Omnibus database under the accession number GSE198756. All scripts used in this
8 study can be found in GitHub (<https://github.com/rahfel/VPA>). Supplementary materials including full
9 tables can also be found in GitHub (<https://github.com/rahfel/VPA>).
10

11 **RESULTS**

13 **Study workflow and data collection**

14 A summary of the study workflow is shown in **Figure 1**. Female GAERS and NEC rats were both
15 derived from the same original Wistar rat colony but selectively inbred to express, or not to express,
16 absence seizures.^{12,13} The animal facility was maintained on a light dark cycle of 12 hours light, 12
17 hours dark at a temperature range of 19 to 22 degrees. All females were fed a standard rodent diet as
18 reported by Senn and colleagues¹⁴ either pre-mixed with VPA 20g/kg of food or not (controls) for 2
19 weeks before mating with males of the same strain and continued throughout pregnancy. We have
20 previously demonstrated that this dose of VPA administered orally results in significant seizure
21 suppression in adult GAERS and achieves blood serum levels broadly equivalent to human therapeutic
22 levels of VPA; across VPA-exposed GAERS and NEC dams, VPA levels ranged 120-380 µmol/L and
23 250-460 µmol/L respectively (t-test $P=0.197$).⁹ The presence of a copulatory plug indicated day 0 of
24 pregnancy. Once the plug was present, females were separated from the males for the course of their
25 pregnancy. The following day was classified as day 1. The fetuses were extracted from the uterus via
26 C-section on day 21, one day before expected birth. The dams were sacrificed and the fetus brains
27 removed, snap frozen and stored at -80C. We have previously reported the results of the teratology
28 studies on rat pups exposed to valproate *in utero*.⁹ Pup brain samples were then chosen without
29 reference to morphological score and were not significantly different in weight between the VPA-
30 exposed and unexposed groups (Mean weights +/- SEM: NEC control 0.151g +/- 0.017; NEC VPA
31 0.155g +/- 0.014; GAERS Control 0.180g +/- 0.012; GAERS VPA 0.137g +/- 0.011, 2 WAY ANOVA
32 (drug) $p=0.15$; (strain) $p=0.71$). Six litters were generated for each group and one or two pup brains

1 from each litter were randomly processed. Total RNA was extracted from 30 pup brains (**Table 1**) and
2 genome-wide gene expression data was generated using RNA-sequencing (RNA-seq) (**Methods**).

4 **Deconvolution analysis reveals no difference in cell-type composition between VPA-exposed and** 5 **non-exposed pup brains**

6 Previous pre-clinical studies on the effects of intraperitoneal administration of VPA have
7 demonstrated VPA-induced apoptotic neurodegeneration in the developing rat brain and this has been
8 suggested as a mechanism to explain VPA-associated cognitive impairment.^{15,16} We therefore first
9 sought to identify whether chronic oral VPA exposure used in our model was associated with
10 significant changes in the proportion of cell-types in the rat pup brain. To this end, we applied a
11 weighted least squares-based deconvolution algorithm *Dampened Weighted Least Squares* (DWLS)¹⁷
12 which estimates cell-type composition in a bulk tissue RNA-seq dataset using prior information from
13 an unrelated single-cell RNA-sequencing (scRNA-seq) signature from an analogous tissue. Taking this
14 approach, we observed no significant difference in cell-type composition between valproate-exposed
15 and unexposed pup brains, for either GAERS or NEC rats, suggesting chronic orally delivered
16 gestational VPA exposure does not have a major effect on cell loss or gain (FDR-corrected Wilcoxon
17 rank sum test >0.05; **Supplementary Figure 1; Supplementary Table 1**).

19 **Differential gene expression analysis highlights transcriptional changes in VPA-exposed pup** 20 **brains**

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

27 We observed significantly (FDR < 0.05) differentially expressed genes in all five pairwise
28 comparisons (summarised in **Table 2**; see **Supplementary Table 2** for a full list of differentially
29 expressed genes for each comparison). Considering VPA-exposed vs non-exposed pup brains, we
30 observed the largest number of differentially expressed genes (DEG) when comparing all VPA-
31 exposed pups (All-E) consisting of VPA-exposed GAERS pups (E-GAERS) and VPA-exposed NEC
32 (E-NEC) pups combined vs all non-exposed pups (All-N) consisting of non-exposed GAERS (N-
33 GAERS) and non-exposed NEC (N-NEC) pups combined, where 3,470 genes were significantly (FDR

1 < 0.05) differentially expressed. Of these, 1,632 genes were significantly (FDR < 0.05) up-regulated
 2 and 1,838 down-regulated.

3 **Figure 2** shows the overlaps in genes differentially expressed for each of the 3 pairwise VPA-
 4 exposed vs non-exposed comparisons (i.e., ALL-E vs ALL-N, E-NEC vs N-NEC and E-GAERS vs N-
 5 GAERS). We observed that the majority of genes differentially expressed by VPA in GAERS pups
 6 were also differentially expressed in NEC pups, suggesting that genetic epilepsy status is not a major
 7 determinant of the pattern of differential expression induced by VPA, as was previously the case for
 8 VPA-induced birth defects in this model.⁹

9 To investigate the functional consequences of VPA-induced differential gene expression in the
 10 pup brain, we first undertook pathway enrichment analysis. We found that genes down-regulated
 11 following VPA exposure were highly significantly enriched for functional processes related to
 12 modulation of synaptic function and neuronal processes (see **Figure 3** for summary of Biological
 13 Processes dysregulated by VPA and **Supplementary Tables 3a-3c** for full details of pathway
 14 enrichment analyses). For example, when considering all VPA-exposed pups vs unexposed pups (All-E
 15 vs All-N), among the set of genes down-regulated by VPA we observed highly significant enrichment
 16 for terms relating to glutamate receptor complex (FDR= 9.74×10^{-12}), regulation of membrane potential
 17 (FDR= 0), synapse assembly (FDR= 2.17×10^{-11}), regulation of actin cytoskeleton organisation (FDR=
 18 1.48×10^{-02}), post-synaptic membrane (FDR= 2.11×10^{-04}), neurotransmitter receptor activity (FDR=
 19 2.33×10^{-04}) and axon guidance (FDR= 0). Interestingly, also among the pathways enriched in the down-
 20 regulated set of genes was regulation of insulin secretion (FDR= 9.5×10^{-03}), which if replicated in the
 21 pancreas (not examined in this study) might suggest a possible drug-induced transcriptional mechanism
 22 for the increased incidence of impaired glucose control in patients treated with VPA.^{18,19}

23 In contrast to the substantial enrichment of neuronal functions in genes down-regulated by
 24 VPA, the genes up-regulated by VPA were generally enriched for functional terms not directly related
 25 to neural processes namely mRNA splicing (FDR= 7.29×10^{-14}), translation (FDR= 1.40×10^{-13}), extra-
 26 cellular matrix organisation (FDR= 2.41×10^{-12}) and cell cycle (FDR= 2.40×10^{-09}). Consistent with
 27 VPA's known activity as a histone deacetylase inhibitor, we also saw enrichment of biological
 28 processes related to chromatin organization (FDR= 7.37×10^{-07}) and chromatin assembly/disassembly
 29 (FDR = 1.45×10^{-6}) among the genes upregulated by VPA.

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

33

1 Genes down-regulated by gestational VPA exposure are highly enriched for heritability for 2 neurodevelopmental traits and disease

3 We next sought to investigate the relationship between genes differentially expressed in pup
4 brains following gestational VPA and genetic risk for neurodevelopmental disease and behavioural
5 traits. Since, in the context of rare variant genetic analyses, neurodevelopmental disability primarily
6 arises from loss-of-function and deleterious mutations,²⁰ we hypothesised that genetic risk for
7 neurodevelopmental disease may be enriched in the set of genes significantly down-regulated by VPA
8 in the developing brain.

9 To test for enrichment of genetic association in sets of genes, we used stratified LD score
10 regression (LDSC).²¹ Given the broad range of adverse neurodevelopmental outcomes in children
11 gestationally exposed to VPA, we considered genome-wide association studies (GWAS) for a broad
12 range of psychiatric diseases and behavioural traits: attention deficit hyperactivity disorder (ADHD),²²
13 bipolar disorder (BD),²³ autism spectrum disorder (ASD),²⁴ schizophrenia (SCZ),²⁵ full-scale
14 intelligence quotient (IQ),²⁶ epilepsy (EPI)²⁷ and “cross disorders group” (CDG), the latter representing
15 a meta-analysis of eight individual psychiatric disorder GWAS namely anorexia nervosa, attention-
16 deficit/hyperactivity disorder, autism spectrum disorder, bipolar disorder, major depression, obsessive-
17 compulsive disorder, schizophrenia and Tourette syndrome²⁸. As a negative control GWAS dataset not
18 expected to be enriched in the set of genes differentially expressed in pup brains we used a GWAS for
19 waist/hip ratio (WHR).²⁹

20 Enrichment of genetic association analyses were run for all sets of genes significantly (FDR <
21 0.05) differentially expressed in the VPA-exposed pup brain (results summarised in **Figure 4**; full
22 details in **Supplementary Table 4**). For genes down-regulated by VPA, we observed highly significant
23 enrichment of heritability with bipolar disorder (All-E vs All-N, $FDR_{LDSC} = 1.16 \times 10^{-08}$; E-NEC vs N-
24 NEC, $FDR_{LDSC} = 2.93 \times 10^{-09}$; E-GAERS vs N-GAERS, $FDR_{LDSC} = 1.55 \times 10^{-03}$), schizophrenia (All-E vs
25 All-N, $FDR_{LDSC} = 6.04 \times 10^{-08}$; E-NEC vs N-NEC, $FDR_{LDSC} = 6.40 \times 10^{-08}$; E-GAERS vs N-GAERS,
26 $FDR_{LDSC} = 2.91 \times 10^{-03}$), IQ (All-E vs All-N, $FDR_{LDSC} = 1.17 \times 10^{-03}$; E-NEC vs N-NEC, $FDR_{LDSC} =$
27 7.03×10^{-04} ; E-GAERS vs N-GAERS, $FDR_{LDSC} = 1.62 \times 10^{-02}$) and cross disorders group (All-E vs All-
28 N, $FDR_{LDSC} = 3.89 \times 10^{-04}$; E-NEC vs N-NEC, $FDR_{LDSC} = 1.82 \times 10^{-04}$; E-GAERS vs N-GAERS,
29 $FDR_{LDSC} = 5.44 \times 10^{-02}$). No significant enrichments of heritability were observed for these traits in
30 unexposed GAERS pups compared to unexposed non-epileptic controls, and enrichments of association
31 for WHR were non-significant across all comparisons (**Supplementary Table 4**).

32 There was no significant enrichment of heritability in the set of genes down-regulated in VPA-
33 exposed pup brains to ADHD (All-E vs All-N, $FDR_{LDSC} = 0.69$; E-NEC vs N-NEC, $FDR_{LDSC} = 0.24$;

1 E-GAERS vs N-GAERS, $FDR_{LDSC} = 0.88$) autism spectrum disorder (All-E vs All-N, $FDR_{LDSC} = 0.66$;
 2 E-NEC vs N-NEC, $FDR_{LDSC} = 0.68$; E-GAERS vs N-GAERS, $FDR_{LDSC} = 0.68$) or epilepsy (All-E vs
 3 All-N, $FDR_{LDSC} = 0.77$; E-NEC vs N-NEC, $FDR_{LDSC} = 0.57$; E-GAERS vs N-GAERS, $FDR_{LDSC} =$
 4 0.77).

5 For genes up-regulated by gestational VPA exposure, we did not observe a significant
 6 enrichment of genetic association to any neurodevelopmental disease or trait, other than a marginal
 7 enrichment to bipolar disorder (All-E vs All-N, $FDR_{LDSC} = 2.7 \times 10^{-02}$; E-NEC vs N-NEC, $FDR_{LDSC} =$
 8 3.6×10^{-03} ; E-GAERS vs N-GAERS, $FDR_{LDSC} = 0.84$) and schizophrenia (All-E vs All-N, $FDR_{LDSC} =$
 9 4.3×10^{-02} ; E-NEC vs N-NEC, $FDR_{LDSC} = 0.32$; E-GAERS vs N-GAERS, $FDR_{LDSC} = 0.56$).

10 Taken together, these results suggest VPA exerts its adverse effects on fetal neurodevelopment
 11 predominantly via drug-induced down-regulation of genes highly relevant to neurodevelopment and
 12 nervous system function. The directionality of the effect is consistent with that observed from rare-
 13 variant analyses of genetic risk to neurodevelopmental disability where the predominant mechanism is
 14 a dominant negative effect from deleterious gene mutations.³⁰ Given the functional enrichment of
 15 chromatin assembly/disassembly terms among the genes upregulated by VPA, along with VPA's
 16 known activity as a histone deacetylase inhibitor, it seems likely that this transcriptional dysregulation
 17 is epigenetically encoded with potentially long-lasting consequences for human brain function and
 18 health even in the absence of persisting valproate exposure postnatally.

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

21 Among the pathways enriched in genes up-regulated by VPA we observed a highly significant
 22 ($FDR = 0$) enrichment for genes involved in splicing function. We therefore evaluated the role of VPA
 23 on differential splicing by first quantifying the read counts to individual gene exons and then
 24 comparing differential exon usage using EdgeR (**Methods**). Overall, there were 57 significantly (FDR
 25 < 0.05) alternatively spliced genes, among which 21 were also differentially expressed following VPA
 26 exposure (14 were down-regulated, 7 up-regulated) (**Supplementary Table 5**). Whilst there were no
 27 significant enrichments for known functional pathways or enrichment of genetic association to
 28 neurodevelopmental disease among the set of differentially spliced genes (data not shown), individual
 29 differentially spliced genes included genes for neuronal proteins such as Calmodulin Binding
 30 Transcription Activator 1 (CAMATA1; $FDR = 3.69 \times 10^{-26}$) which is known to play a role in the
 31 regulation of glutamate levels and neuronal excitability, glutamate decarboxylase 2 (GAD2; $FDR =$
 32 9.03×10^{-25}) which plays a role GABA-synthesis in neurons³¹ and Forkhead box P4 (FOXP4; $FDR =$
 33 4.62×10^{-3}) which is known to regulate neurogenesis and in which mutations are associated with speech

1 delay and congenital abnormalities.³² These results suggest differential splicing as a potential further
2 mechanism influencing behavioural outcomes following fetal VPA exposure.

4 **DISCUSSION**

5 The adverse neurodevelopmental consequences of chronic fetal valproate exposure have been
6 well-documented and remain a significant limitation on the use of VPA in women who could become
7 pregnant, with particular implications for the treatment of generalized epilepsy, where it remains the
8 most effective treatment,^{1,2} as well as other diseases in women of child-bearing potential such as
9 bipolar disorder where it is an important therapy. The mechanisms by which VPA contributes to
10 behavioural and cognitive disability in children following gestational exposure remain poorly defined,
11 with existing studies limited by non-physiological drug administration and dosing schedules and
12 relatively few studies having examined transcriptome-wide alterations in brain gene expression.¹¹ In
13 this study, we utilized an established rat model of VPA-induced teratogenicity⁹ that recapitulates
14 human pre-natal valproate exposure and chronicity of oral dosing during pregnancy.

15 Among the proposed mechanisms for VPA-associated neurodevelopmental disability is VPA-
16 induced apoptosis.³³ In the present study, using deconvolution analysis anchored in single-cell RNA-
17 seq, we found no evidence for substantial shifts in the composition of the major cell-types of the brain
18 (i.e., excitatory neurons, inhibitory neurons, astrocytes, oligodendrocytes, oligodendrocyte precursor
19 cells, microglia) following VPA-exposure. Whilst our results suggest the adverse functional
20 consequences of VPA-exposure reside in altered synaptic function, they do not exclude the possibility
21 of VPA-induced variation in the proportion of cellular sub-types, which will require single-cell RNA-
22 sequencing of large numbers of cells from VPA-exposed and non-exposed fetal brains to identify.

23 In contrast to our finding of no measurable difference in the proportion of the major cell-types
24 of the mammalian brain, we observed significant VPA-induced differential gene expression in
25 gestationally exposed pup brains. Pathway enrichment analysis showed a striking separation of
26 enriched terms between the up-and down-regulated genes, with up-regulated genes relating
27 predominantly to cell division, mRNA splicing, translation and extracellular matrix organisation, and
28 down-regulated genes highly enriched for functional processes directly relevant to neurodevelopment
29 including synapse assembly, post-synaptic membrane, regulation of neuronal membrane activity and
30 synaptic transmission.

31 To further investigate the functional consequences of VPA-induced differential gene expression
32 we integrated differentially expressed genes with GWAS summary statistics from a range of clinically
33 relevant neurodevelopmental diseases and behavioural traits. We observed significant enrichments of

1 heritability in the set of genes down-regulated by VPA for bipolar disease, schizophrenia, IQ and cross
2 disorder group. We did not see an enrichment for autism spectrum disorder (ASD) which was
3 surprising given that some of the features of VPA-associated neurodevelopmental disability have been
4 likened to autism, and maternal valproate exposure has been associated with autism-like behaviours in
5 non-human primates.³⁴ This absence of a genetic association with ASD was in stark contrast to the
6 enrichment for bipolar disorder, schizophrenia and IQ and suggests that VPA-associated
7 neurodevelopmental disability may have specific clinical characteristics unique to VPA exposure and
8 points to a requirement for more research to continue to define the clinical phenotype. Notably, the
9 directionality of the effect of VPA-induced gene expression on brain function is consistent with that
10 observed from rare-variant analyses of genetic risk to neurodevelopmental disease where the
11 predominant mechanism is a dominant negative effect from deleterious mutation.³⁰ The functional
12 enrichment of chromatin assembly/disassembly terms among the genes upregulated by VPA suggests
13 the VPA-induced transcriptional dysregulation is epigenetically encoded and therefore with potential
14 long-lasting consequences for human brain function and behaviour even following birth, prompting the
15 need for further clinical research on the long-term outcomes of children born following fetal VPA
16 exposure.

17 In addition to substantial differential gene expression, we found that chronic prenatal VPA
18 exposure is associated with differential mRNA splicing in the brain. Interestingly, recent studies have
19 shown evidence that implicates the regulatory role of neuron-specific alternative splicing in
20 neurodevelopmental disorders^{35,36} and alternative splicing in the brain is important for several
21 neurological processes including cell differentiation, neurogenesis, synaptogenesis and in the
22 generation of functional neuronal networks.³⁷ Among the genes observed to be significantly
23 differentially spliced in the prenatal brain following VPA exposure were GAD2, which plays a role
24 GABA-synthesis in neurons³¹ and Foxp4, which is known to regulate neurogenesis and is associated
25 with speech delay and congenital abnormalities.³² These results suggest that alternative splicing may be
26 an additional mechanism for adverse neurodevelopment in VPA-exposed fetal brains.

27 Apart from valproate, some studies have suggested a risk of neurodevelopmental complications
28 from fetal exposure to other anti-seizure medications, although such risks remain to be fully
29 characterised.^{38,39} Additionally, studies have suggested that anti-depressant use during pregnancy may
30 be associated with an increased risk of neurodevelopmental disorders including ASD and ADHD,⁴¹
31 although not following exposure to antipsychotics.⁴² These studies highlight the concern that exists for
32 effects on neurodevelopment for several drugs and classes of drugs commonly prescribed during
33 pregnancy. The research presented here, which demonstrates a robust brain transcriptional response to

1 VPA that is both functionally and genetically associated with relevant cognitive (IQ) and psychiatric
2 (BP, SCZ, CDG) outcomes suggests that the rat model of chronic dosing followed by transcriptional
3 assay in pup brains might provide a general approach to screening for drug-induced adverse
4 neurodevelopmental effects. Moreover, the inference that the adverse behavioural and cognitive
5 outcomes from gestational VPA exposure arise from transcriptional dysregulation, provides a potential
6 system for testing drugs capable of reversing or ameliorating these changes. For example, as previously
7 highlighted, VPA is a well-recognised histone deacetylase (HDAC) inhibitor, and pre-treatment with
8 methionine has been shown to significantly reduce the incidence of spina bifida and other VPA-
9 associated defects in mice, albeit at the expense of significantly increased embryo lethality.⁴³

10 There are several limitations to our study. First, we did not measure serum VPA levels in the
11 rats although we did follow the same oral dosing schedule previously described⁹ which has been shown
12 to achieve a blood level of 180-280 $\mu\text{mol/L}$ and to have antiseizure effects.⁴⁴ Second, we did not
13 undertake a dose-response curve across VPA dosages, which has the theoretical potential to establish if
14 there is a “safe” dosage below which the transcriptional effects of VPA do not occur. This is an
15 important clinical unknown highly relevant to women whose seizures are not controlled by any
16 medication other than VPA. We note however, that the blood level achieved by oral dosing used in our
17 study are below the standard therapeutic range for human epilepsy of 346 to 693 $\mu\text{mol/L}$, suggesting
18 that our results are not an artefact of artificially high VPA levels and also pointing to effects on fetal
19 brain transcription even at relatively low serum levels of valproate. This observation is in keeping with
20 reports of impaired cognition and a 6-fold increase in educational intervention in children born to
21 mothers taking low-dose valproate (<800mg per day) during pregnancy.⁴⁵ Third, we did not perform
22 behavioural testing in exposed and un-exposed offspring in this model so we cannot confirm if the
23 transcriptional effects of VPA on the brain directly correlate with changes in behaviour. However,
24 previous research on this animal model has established that chronic oral dosing of valproate at the
25 dosages employed in the present study do induce the expected developmental and morphological
26 abnormalities in pups.⁹

27 Our study did not address the question of whether a genetically defined subpopulation of
28 women can be identified who are at risk of having children with VPA-associated neurodevelopmental
29 disability. Previous research has observed that women who had given birth to a malformed baby in
30 their first VPA pregnancy are more likely to have a malformed child in their next compared to those
31 who had taken VPA without fetal abnormalities.⁴ This suggests that maternal factors, perhaps genetic
32 factors, contribute to VPA-associated congenital malformations. However, we are not aware of similar

1 studies that have examined the offspring recurrence risk with respect to VPA-associated
2 neurodevelopmental disability. Additionally, the extent to which variation in risk of VPA-associated
3 neurodevelopmental disability (independent of VPA dosage) is explained by maternal genetic variation
4 (e.g., using genome-wide association study methodology or exome-sequencing) is, to date,
5 unexamined. As well as potential maternal genetic effects on risk (for example perhaps mediated by
6 genetic variation in valproate clearance), one can hypothesise a potential effect from fetal genotype on
7 risk as well, for example via genetic effects on VPA-induced neuronal gene expression (so-called
8 “response eQTLs”). Determining if maternal or fetal genetic factors play a role in VPA-associated
9 neurodevelopmental disability is therefore likely to require substantial further research.

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

18 **ACKNOWLEDGEMENTS**

19 This work was supported by the UKRI MRC (Award No: MR/S02638X/1) and by the NIHR Imperial
20 Biomedical Research Centre (BRC) to MR Johnson, and a NHMRC Program (#APP1091593) Grant
21 and NHMRC Investigator Grant (APP1176426) to TJ O’Brien. We acknowledge Ms. Emma Brain for
22 technical assistances with the rat surgeries.
23

24 **Supplementary material**

25 Supplementary material is available at *Brain* online.
26
27
28

1 **References**

- 2
- 1.3 Marson AG, Al-Kharusi AM, Alwaidh M, et al. The SANAD study of effectiveness of valproate,
4 lamotrigine, or topiramate for generalised and unclassifiable epilepsy: an unblinded randomised
5 controlled trial. *The Lancet*. 2007;369(9566):1016-1026. doi:10.1016/S0140-6736(07)60461-9
- 2.6 Marson A, Burnside G, Appleton R, et al. The SANAD II study of the effectiveness and cost-
7 effectiveness of valproate versus levetiracetam for newly diagnosed generalised and unclassifiable
8 epilepsy: an open-label, non-inferiority, multicentre, phase 4, randomised controlled trial. *The Lancet*.
9 2021;397(10282):1375-1386. doi:10.1016/S0140-6736(21)00246-4
- 3.0 Perucca E. Pharmacological and Therapeutic Properties of Valproate. *CNS Drugs*. 2002;16(10):695-
11 714. doi:10.2165/00023210-200216100-00004
- 4.2 Vajda FJ, O'Brien TJ, Graham JE, Lander CM, Eadie MJ. Dose dependence of fetal malformations
13 associated with valproate. *Neurology*. 2013;81(11):999-1003. doi:10.1212/WNL.0b013e3182a43e81
- 5.4 Tomson T, Battino D, Bonizzoni E, et al. Comparative risk of major congenital malformations with
15 eight different antiepileptic drugs: a prospective cohort study of the EURAP registry. *The Lancet*
16 *Neurology*. 2018;17(6):530-538. doi:10.1016/S1474-4422(18)30107-8
- 6.7 Bromley R, Weston J, Adab N, et al. Treatment for epilepsy in pregnancy: neurodevelopmental
18 outcomes in the child. *Cochrane Database of Systematic Reviews*. 2014;2020(6).
19 doi:10.1002/14651858.CD010236.pub2
- 7.0 Mill J, Petronis A. Pre- and peri-natal environmental risks for attention-deficit hyperactivity disorder
21 (ADHD): the potential role of epigenetic processes in mediating susceptibility. *Journal of Child*
22 *Psychology and Psychiatry*. 2008;49(10). doi:10.1111/j.1469-7610.2008.01909.x
- 8.3 Christensen J, Grønberg TK, Sørensen MJ, et al. Prenatal Valproate Exposure and Risk of Autism
24 Spectrum Disorders and Childhood Autism. *JAMA*. 2013;309(16):1696. doi:10.1001/jama.2013.2270
- 9.5 Jazayeri D, Braine E, McDonald S, et al. A rat model of valproate teratogenicity from chronic oral
26 treatment during pregnancy. *Epilepsia*. 2020;61(6):1291-1300. doi:10.1111/epi.16536
- 10.7 Chau DKF, Choi AYT, Yang W, Leung WN, Chan CW. Downregulation of glutamatergic and
28 GABAergic proteins in valproic acid associated social impairment during adolescence in mice.
29 *Behavioural Brain Research*. 2017;316. doi:10.1016/j.bbr.2016.09.003
- 11.0 Cui K, Wang Y, Zhu Y, et al. Neurodevelopmental impairment induced by prenatal valproic acid
31 exposure shown with the human cortical organoid-on-a-chip model. *Microsystems & Nanoengineering*.
32 2020;6(1). doi:10.1038/s41378-020-0165-z

- 12 Casillas-Espinosa PM, Powell KL, Zhu M, et al. Evaluating whole genome sequence data from the
2 Genetic Absence Epilepsy Rat from Strasbourg and its related non-epileptic strain. *PLOS ONE*.
3 2017;12(7). doi:10.1371/journal.pone.0179924
- 13 Marescaux C, Vergnes M, Depaulis A. Genetic absence epilepsy in rats from Strasbourg — A review.
4 In: *Generalized Non-Convulsive Epilepsy: Focus on GABA-B Receptors*. Springer Vienna; 1992.
5 doi:10.1007/978-3-7091-9206-1_4
- 14 Senn SM, Kantor S, Poulton IJ, et al. Adverse effects of valproate on bone: Defining a model to
6 investigate the pathophysiology. *Epilepsia*. 2010;51(6). doi:10.1111/j.1528-1167.2009.02516.x
- 15 BITTIGAU P, SIFRINGER M, IKONOMIDOU C. Antiepileptic Drugs and Apoptosis in the
7 Developing Brain. *Ann N Y Acad Sci*. 2003;993(1):103-114. doi:10.1111/j.1749-6632.2003.tb07517.x
- 16 Bittigau P, Sifringer M, Genz K, et al. Antiepileptic drugs and apoptotic neurodegeneration in the
8 developing brain. *Proceedings of the National Academy of Sciences*. 2002;99(23):15089-15094.
9 doi:10.1073/pnas.222550499
- 17 Tsoucas D, Dong R, Chen H, Zhu Q, Guo G, Yuan GC. Accurate estimation of cell-type composition
10 from gene expression data. *Nature Communications*. 2019;10(1). doi:10.1038/s41467-019-10802-z
- 18 Avery LB, Bumpus NN. Valproic Acid Is a Novel Activator of AMP-Activated Protein Kinase and
19 Decreases Liver Mass, Hepatic Fat Accumulation, and Serum Glucose in Obese Mice. *Molecular*
20 *Pharmacology*. 2014;85(1). doi:10.1124/mol.113.089755
- 21 Sidhu HS, Srinivas R, Sadhotra A. Evaluate the effects of long-term valproic acid treatment on
22 metabolic profiles in newly diagnosed or untreated female epileptic patients: A prospective study.
23 *Seizure*. 2017;48. doi:10.1016/j.seizure.2017.03.007
- 24 Parenti I, Rabaneda LG, Schoen H, Novarino G. Neurodevelopmental Disorders: From Genetics to
25 Functional Pathways. *Trends in Neurosciences*. 2020;43(8). doi:10.1016/j.tins.2020.05.004
- 26 Finucane HK, Bulik-Sullivan B, Gusev A, et al. Partitioning heritability by functional annotation using
27 genome-wide association summary statistics. *Nature Genetics*. 2015;47(11). doi:10.1038/ng.3404
- 28 Demontis D, Walters RK, Martin J, et al. Discovery of the first genome-wide significant risk loci for
29 attention deficit/hyperactivity disorder. *Nature Genetics*. 2019;51(1). doi:10.1038/s41588-018-0269-7
- 30 Stahl EA, Breen G, Forstner AJ, et al. Genome-wide association study identifies 30 loci associated with
31 bipolar disorder. *Nature Genetics*. 2019;51(5). doi:10.1038/s41588-019-0397-8
- 32 Grove J, Ripke S, Als TD, et al. Identification of common genetic risk variants for autism spectrum
33 disorder. *Nature Genetics*. 2019;51(3). doi:10.1038/s41588-019-0344-8

- 25 Ripke S, Walters JT, O'Donovan MC, Schizophrenia Working Group of the Psychiatric Genomics
2 Consortium. Mapping genomic loci prioritises genes and implicates synaptic biology in schizophrenia.
3 *MedRxiv*. Published online 2020.
- 26 Savage JE, Jansen PR, Stringer S, et al. Genome-wide association meta-analysis in 269,867 individuals
5 identifies new genetic and functional links to intelligence. *Nature Genetics*. 2018;50(7).
6 doi:10.1038/s41588-018-0152-6
- 27 Genome-wide mega-analysis identifies 16 loci and highlights diverse biological mechanisms in the
8 common epilepsies. *Nature Communications*. 2018;9(1). doi:10.1038/s41467-018-07524-z
- 28 Lee PH, Anttila V, Won H, et al. Genomic Relationships, Novel Loci, and Pleiotropic Mechanisms
10 across Eight Psychiatric Disorders. *Cell*. 2019;179(7). doi:10.1016/j.cell.2019.11.020
- 29 Pulit SL, Stoneman C, Morris AP, et al. Meta-analysis of genome-wide association studies for body fat
12 distribution in 694 649 individuals of European ancestry. *Human Molecular Genetics*. 2019;28(1).
13 doi:10.1093/hmg/ddy327
- 30 Johnson MR, Shkura K, Langley SR, et al. Systems genetics identifies a convergent gene network for
15 cognition and neurodevelopmental disease. *Nature Neuroscience*. 2016;19(2):223-232.
16 doi:10.1038/nn.4205
- 31 Walls AB, Nilsen LH, Eyjolfsson EM, et al. GAD65 is essential for synthesis of GABA destined for
18 tonic inhibition regulating epileptiform activity. *Journal of Neurochemistry*. 2010;115(6).
19 doi:10.1111/j.1471-4159.2010.07043.x
- 32 Rousso DL, Pearson CA, Gaber ZB, et al. Foxp-Mediated Suppression of N-Cadherin Regulates
21 Neuroepithelial Character and Progenitor Maintenance in the CNS. *Neuron*. 2012;74(2).
22 doi:10.1016/j.neuron.2012.02.024
- 33 Velez-Ruiz NJ, Meador KJ. Neurodevelopmental Effects of Fetal Antiepileptic Drug Exposure. *Drug*
24 *Safety*. 2015;38(3). doi:10.1007/s40264-015-0269-9
- 34 Zhao H, Wang Q, Yan T, et al. Maternal valproic acid exposure leads to neurogenesis defects and
26 autism-like behaviors in non-human primates. *Translational Psychiatry*. 2019;9(1).
27 doi:10.1038/s41398-019-0608-1
- 35 Porter RS, Jaamour F, Iwase S. Neuron-specific alternative splicing of transcriptional machineries:
29 Implications for neurodevelopmental disorders. *Molecular and Cellular Neuroscience*. 2018;87.
30 doi:10.1016/j.mcn.2017.10.006
- 36 WANG Y, LIU J, HUANG B, et al. Mechanism of alternative splicing and its regulation. *Biomedical*
32 *Reports*. 2015;3(2). doi:10.3892/br.2014.407

- 37 Su CH, D D, Tarn WY. Alternative Splicing in Neurogenesis and Brain Development. *Frontiers in*
2 *Molecular Biosciences*. 2018;5. doi:10.3389/fmolb.2018.00012
- 38 Vossler DG. Comparative Risk of Major Congenital Malformations With 8 Different Antiepileptic
4 Drugs: A Prospective Cohort Study of the EURAP Registry. *Epilepsy Currents*. 2019;19(2).
5 doi:10.1177/1535759719835353
- 39 Meador KJ, Loring DW. Developmental effects of antiepileptic drugs and the need for improved
7 regulations. *Neurology*. 2016;86(3):297-306. doi:10.1212/WNL.0000000000002119
- 40 Meador KJ, Cohen MJ, Loring DW, et al. Two-Year-Old Cognitive Outcomes in Children of Pregnant
9 Women With Epilepsy in the Maternal Outcomes and Neurodevelopmental Effects of Antiepileptic
10 Drugs Study. *JAMA Neurology*. 2021;78(8):927. doi:10.1001/jamaneurol.2021.1583
- 41 Boukhris T, Sheehy O, Mottron L, Bérard A. Antidepressant Use During Pregnancy and the Risk of
12 Autism Spectrum Disorder in Children. *JAMA Pediatrics*. 2016;170(2).
13 doi:10.1001/jamapediatrics.2015.3356
- 42 Wang Z, Chan AYL, Coghill D, et al. Association Between Prenatal Exposure to Antipsychotics and
15 Attention-Deficit/Hyperactivity Disorder, Autism Spectrum Disorder, Preterm Birth, and Small for
16 Gestational Age. *JAMA Internal Medicine*. 2021;181(10). doi:10.1001/jamainternmed.2021.4571
- 43 Ehlers K, Elmazar MMA, Nau H. Methionine Reduces the Valproic Acid-Induced Spina Bifida Rate in
18 Mice without Altering Valproic Acid Kinetics. *The Journal of Nutrition*. 1996;126(1):67-75.
19 doi:10.1093/jn/126.1.67
- 44 Al-Roubaie Z, Guadagno E, Ramanakumar A v., Khan AQ, Myers KA. Clinical utility of therapeutic
21 drug monitoring of antiepileptic drugs. *Neurology: Clinical Practice*. 2020;10(4):344-355.
22 doi:10.1212/CPJ.0000000000000722
- 45 Baker GA, Bromley RL, Briggs M, et al. IQ at 6 years after in utero exposure to antiepileptic drugs: A
24 controlled cohort study. *Neurology*. 2015;84(4):382-390. doi:10.1212/WNL.0000000000001182
- 46 Andrews S. FastQC: a quality control tool for high throughput sequence data. . Published online 2010.
- 47 Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*.
27 2013;29(1). doi:10.1093/bioinformatics/bts635
- 48 Reinius B, Jazin E. Prenatal sex differences in the human brain. *Molecular Psychiatry*.
29 2009;14(11):988-989. doi:10.1038/mp.2009.79
- 49 Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression
31 analysis of digital gene expression data. *Bioinformatics*. 2010;26(1). doi:10.1093/bioinformatics/btp616
- 50 Liao Y, Wang J, Jaehnig EJ, Shi Z, Zhang B. WebGestalt 2019: gene set analysis toolkit with
33 revamped UIs and APIs. *Nucleic Acids Research*. 2019;47(W1):W199-W205. doi:10.1093/nar/gkz401

- 51 la Manno G, Siletti K, Furlan A, et al. Molecular architecture of the developing mouse brain. *Nature*.
2 2021;596(7870). doi:10.1038/s41586-021-03775-x
- 52 Satija R, Farrell JA, Gennert D, Schier AF, Regev A. Spatial reconstruction of single-cell gene
4 expression data. *Nature Biotechnology*. 2015;33(5). doi:10.1038/nbt.3192
- 5 53. Zeisel A, Hochgerner H, Lönnerberg P, et al. Molecular Architecture of the Mouse Nervous
6 System. *Cell*. 2018;174(4). doi:10.1016/j.cell.2018.06.021
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1 **FIGURE LEGENDS**

2

3 **Figure 1 Overview of experimental design and data processing.** Two different genetic strains of rat
 4 were studied, Genetic Absence Epilepsy Rats from Strasbourg (GAERS) rats and Non-Epileptic (NEC)
 5 control rats. Whole brain samples were extracted from valproate-exposed (E-GAERS and E-NEC) and
 6 non-exposed (N-GAERS and N-NEC) pups (total n=30). Pup brains were snap frozen in liquid nitrogen
 7 and genome-wide gene expression assayed using RNA-sequencing (RNA-seq). Data pre-processing
 8 steps included quality control for sequence reads alignment to the rat genome and library size
 9 normalization. Differential gene expression and differential splicing analyses were carried out with
 10 downstream pathway and heritability enrichment analyses.

11

12 **Figure 2 Summary of differential gene expression results for the three valproate-exposed vs non-**
 13 **exposed comparisons.** Total number of significantly (FDR <0.05) differentially expressed genes
 14 (DEGs) for each comparison are shown (single brown dot corresponding to the first three histograms).
 15 The number of genes overlapping between each of the case vs control comparisons are shown in black
 16 (2-way) or red (3-way). The bars are arranged left to right based on the highest to least number of
 17 overlaps respectively.

18

19 **Figure 3 Pathway enrichment for valproate-exposed vs non-exposed pups.** Biological process (BP)
 20 gene ontology (GO) parental terms enriched in genes significantly (FDR <0.05) differentially
 21 expressed between All-exposed (All-E) vs All non-exposed (All-N) pups for down-regulated (blue
 22 bars) and up-regulated (red-bars) genes. For full details including enrichment FDR-values for parental
 23 and child terms including Cellular Compartment and Molecular Function GO pathway enrichments see
 24 **Supplementary Tables 3a-c.**

25 **-log₁₀FDR* values have been signed to indicate enrichment in the down regulated or up-regulated
 26 genes.

27

28 **Figure 4 Heritability enrichment in differentially expressed genes.** Linkage Disequilibrium Score
 29 Regression (LDSC) was used to test for enrichment of heritability in genes significantly differentially
 30 expressed for each of the 5 case control comparisons shown. Enrichment -log₁₀(FDR) for the
 31 enrichment of genetic association for each trait is indicated by the horizontal bars coloured by the
 32 comparison group from which the differentially expressed genes were identified. Vertical line indicates
 33 FDR values at 0.05. Genome-wide association studies used for the enrichment analysis are indicated on

1 the vertical axis. Abbreviations: ASD: Autism Spectrum Disorder, ADHD: Attention Deficit
2 Hyperactivity Disorder, BD: Bipolar Disorder, SCZ: Schizophrenia, IQ: Intelligence Quotient, EPI:
3 Epilepsy, WHR: Waist-to-Hip-Ratio, CDG: Cross-Disorder Group.

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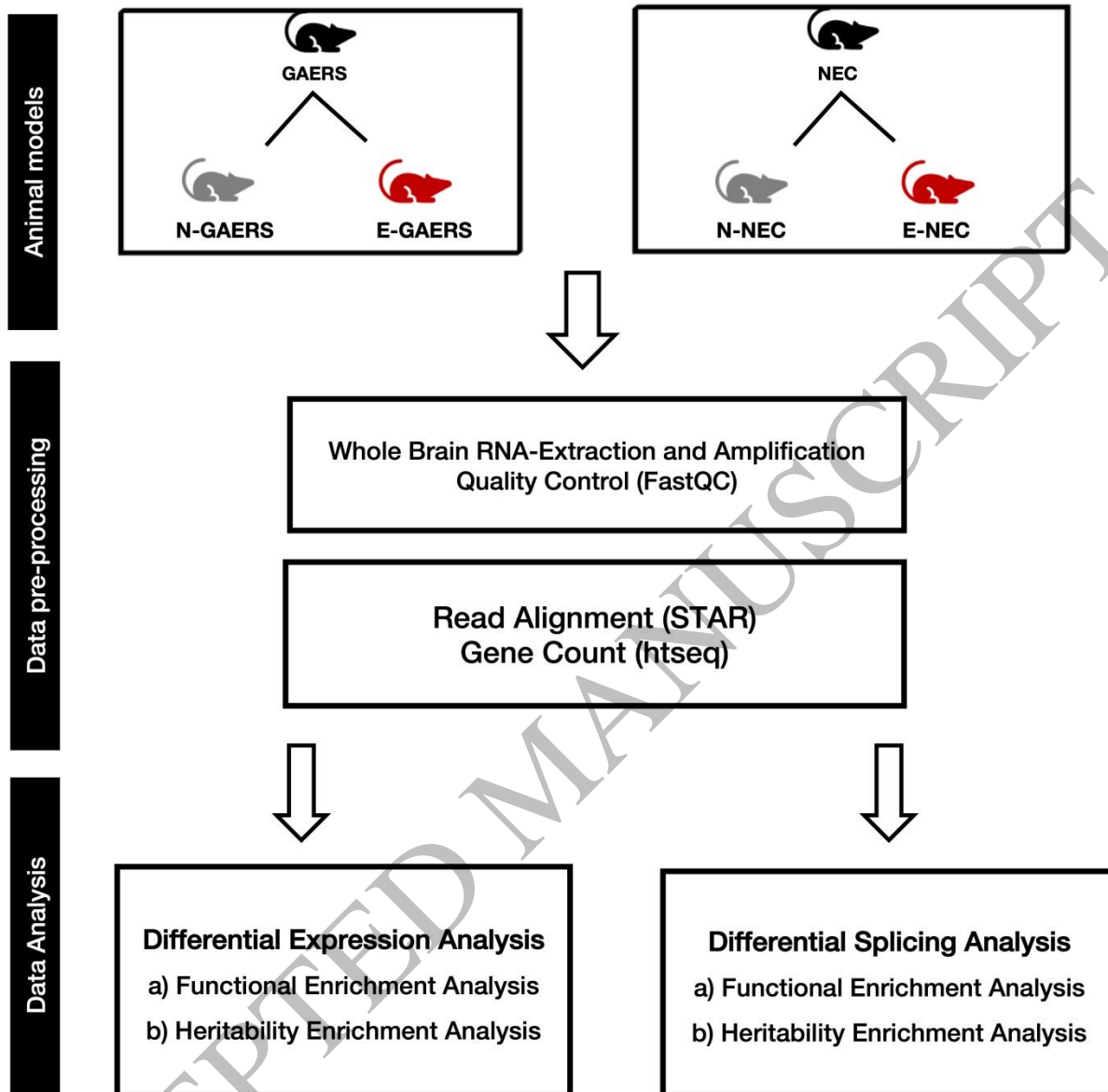


Figure 1
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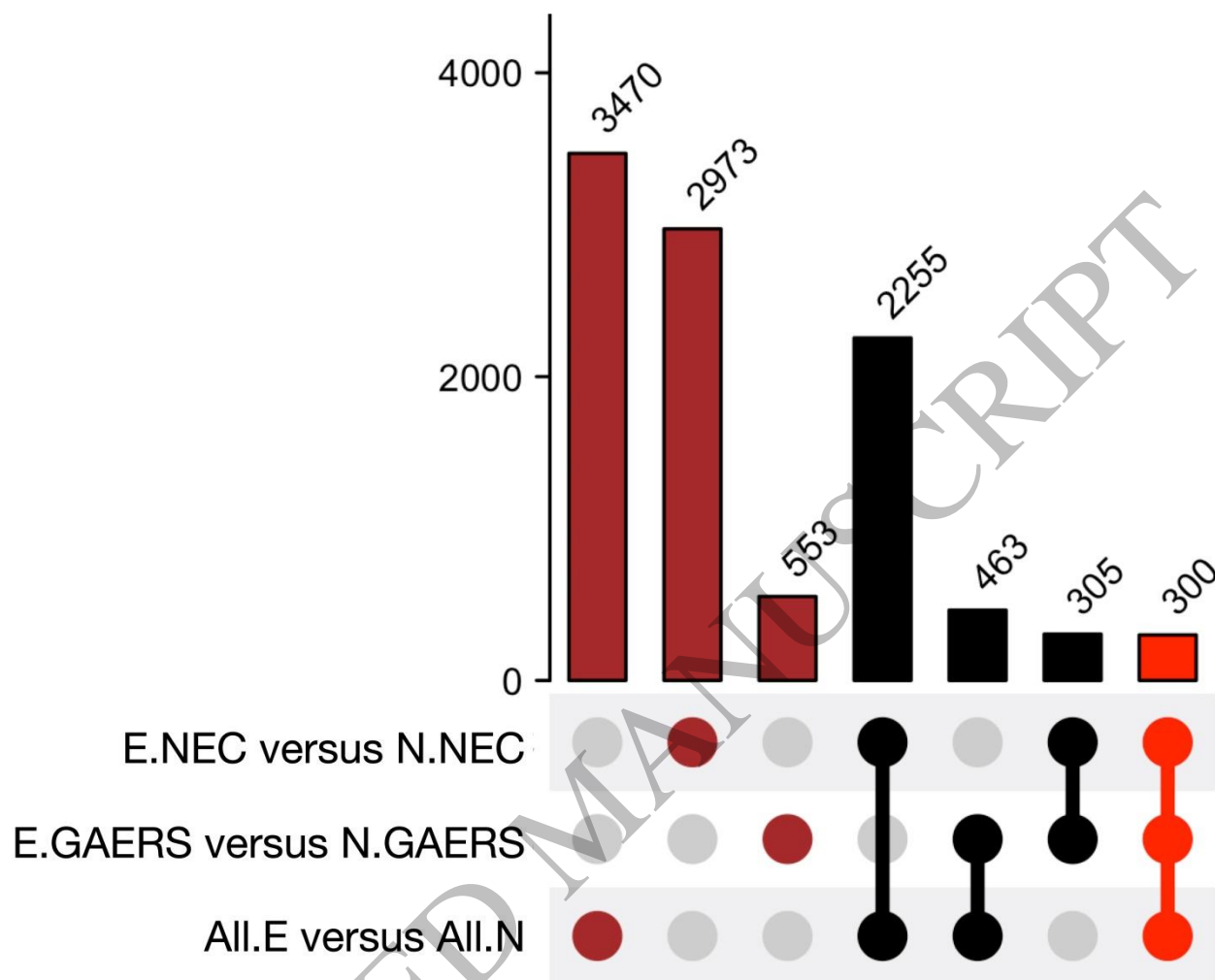


Figure 2
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GO: Biological processes

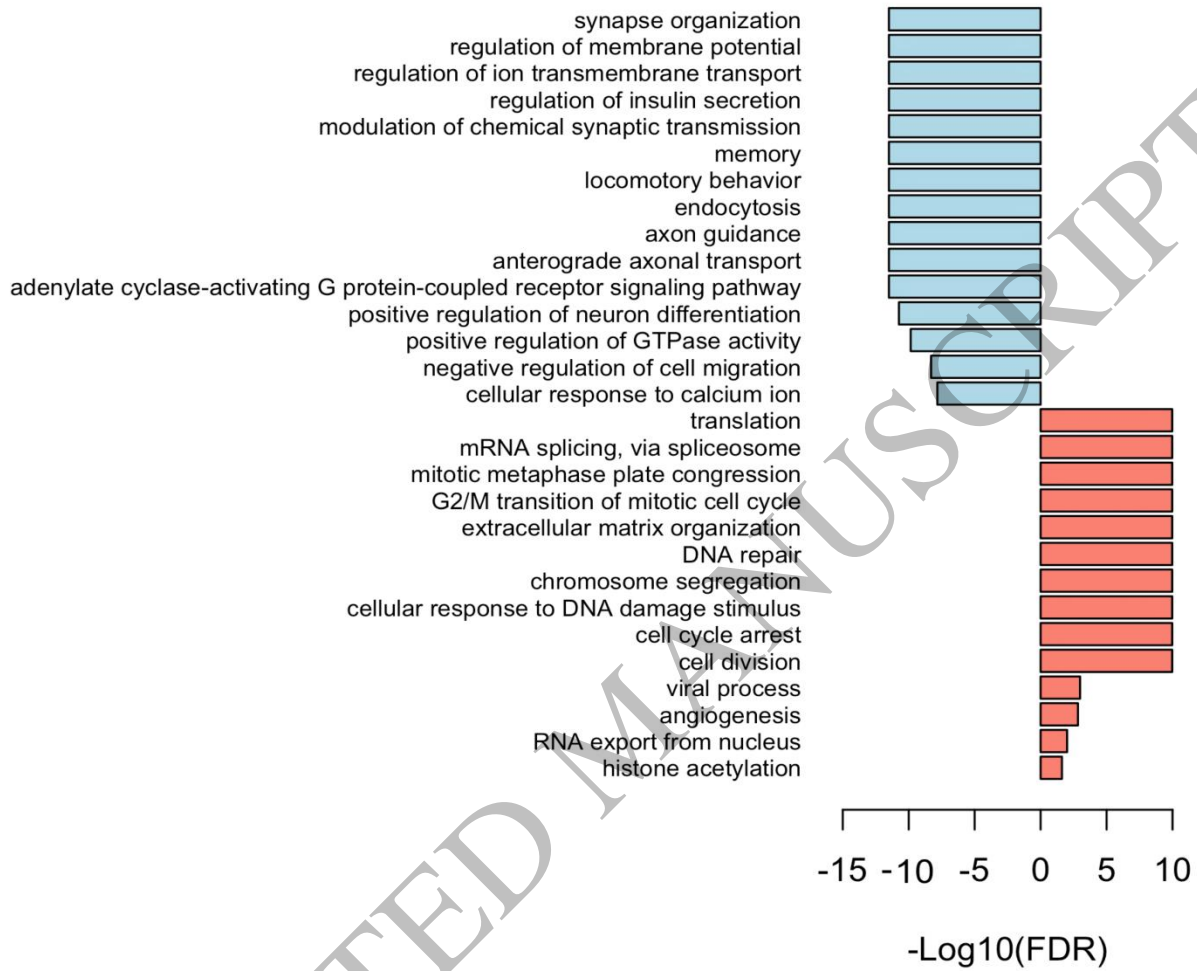


Figure 3
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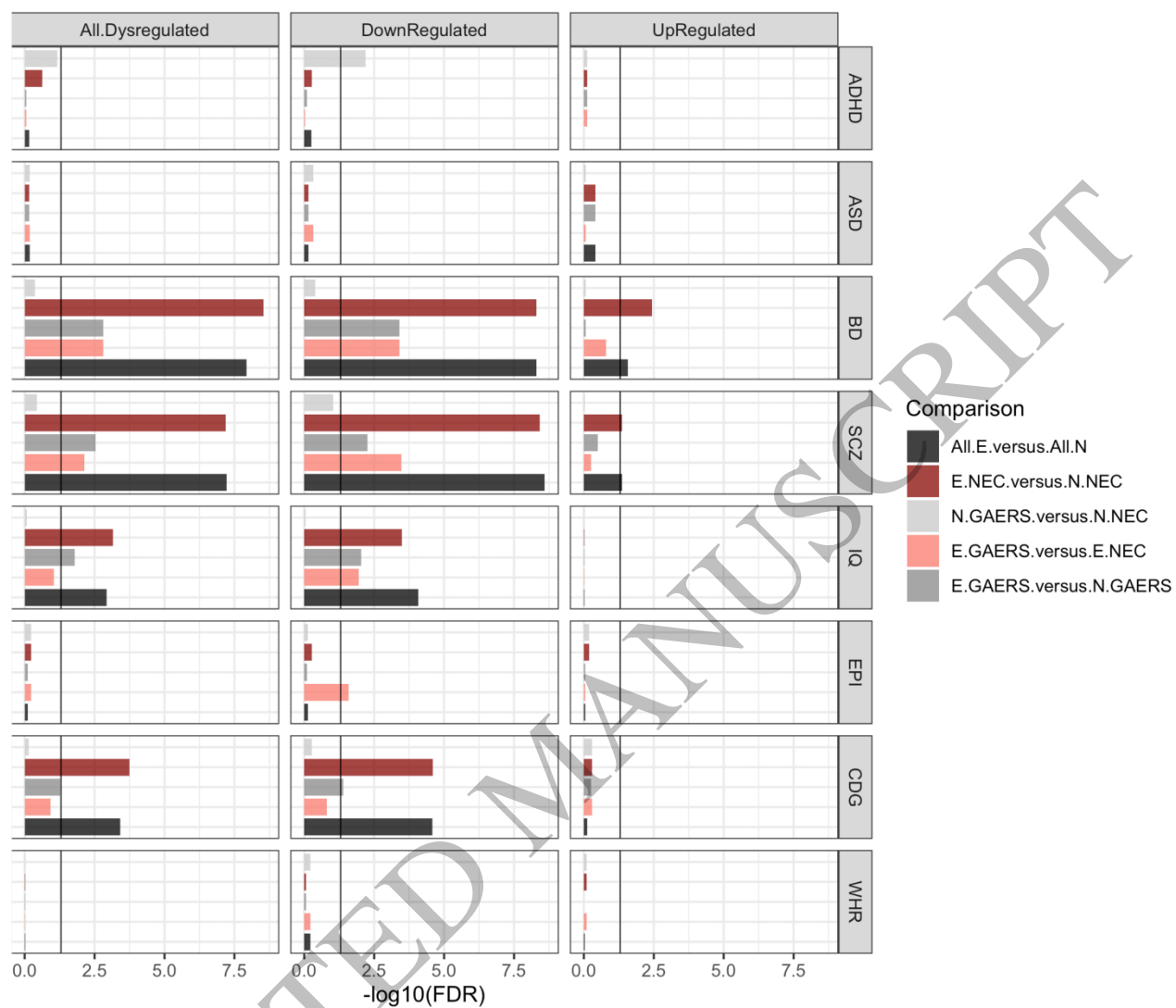


Figure 4
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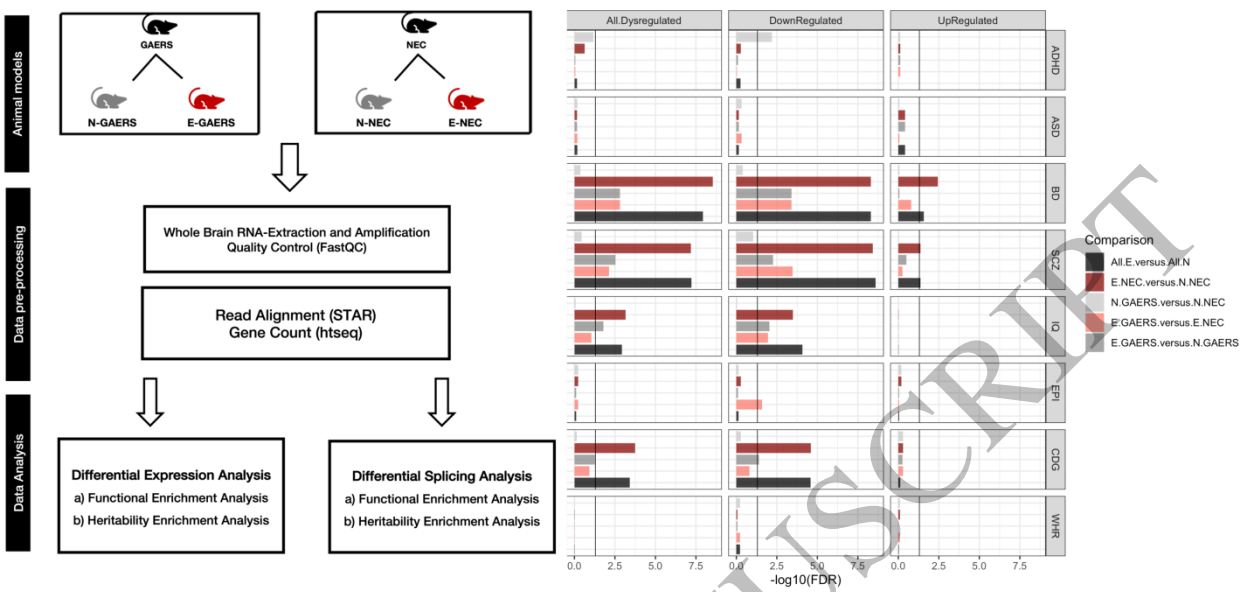


Figure 5
428x348 mm (x DPI)

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