Rapid effects of valproic acid on the fetal brain transcriptome: Implications for brain development and autism

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ABSTRACT:

There is an increased incidence of autism among the children of women who take the anti-epileptic, mood stabilizing drug, valproic acid (VPA) during pregnancy; moreover, exposure to VPA *in utero* causes autistic-like symptoms in rodents and non-human primates. Analysis of RNA-seq data obtained from E12.5 fetal mouse brains 3 hours after VPA administration revealed that VPA significantly increased or decreased the expression of approximately 7,300 genes. No significant sex differences in VPA-induced gene expression were observed. Expression of genes associated with neurodevelopmental disorders (NDDs) such as autism as well as neurogenesis, axon growth and synaptogenesis, GABAergic, glutaminergic and dopaminergic synaptic transmission, perineuronal nets, and circadian rhythms was dysregulated by VPA. Moreover, expression of 399 autism risk genes was significantly altered by VPA as was expression of 252 genes that have been reported to play fundamental roles in the development of the nervous system but are not otherwise linked to autism. The goal of this study was to identify mouse genes that are: **(a) significantly up- or down-regulated by VPA in the fetal brain** and **(b) known to be associated with autism and/or to play a role in embryonic neurodevelopmental processes, perturbation of which has the potential to alter brain connectivity in the postnatal and adult brain.** The set of genes meeting these criteria provides potential targets for future hypothesis-driven approaches to elucidating the proximal underlying causes of defective brain connectivity in NDDs such as autism.

INTRODUCTION:

Autism is a neurodevelopmental disorder (NDD) characterized by social interaction deficits including language, and repetitive, stereotyped behavior with restricted interests (American Psychiatric Association, 2022). Autistic individuals may also display an increased incidence of anxiety, intellectual disability and seizures. The reported incidence of autism has increased dramatically over the last two decades (Zeidan et al., 2022) with some estimates now as high as 1:44 (Maenner et al., 2021). The severity of autism varies widely, ranging from high-functioning with minimal disability, to severely afflicted, where aggressive, self-destructive repetitive behaviors pose a threat to the safety of the patient, caregivers, and family. The term “autism spectrum disorders” (ASDs) reflects this symptomatic variability. The neurological mechanisms underlying ASDs are not understood nor is it known whether autism is a single disorder or multiple disorders sharing common core features.

Twin studies have revealed that 60 to 88% of autism cases are inherited (Hallmayer et al., 2011; Rosenberg et al., 2009), however, many cases have been linked to *in utero* exposure to environmental

factors such as pharmaceuticals, air pollution, chemical insecticides, and maternal infection (Landrigan, 2010; Rosenberg et al., 2009; Karimi et al., 2017).

The diagnosis of autism typically is made at around two years of age when the child fails to meet normal milestones for social development and language. However, it has become increasingly evident that autism arises before birth, with infants failing to show normal attention to faces (Macari et al., 2021). Epidemiological studies indicate that the onset of autism occurs during fetal development; one such study found that folate supplementation for pregnant women reduced the incidence of autism, but only when administered between two weeks before and four weeks after conception (Surén et al., 2013). Exposure to a variety of environmental factors, notably the anti-epileptic, mood stabilizing drug, valproic acid (VPA; Depakote®) (Christensen et al., 2013) and the organophosphate insecticide, chlorpyrifos (Burke et al., 2017), increases the incidence of autism and other NDDs in the offspring of women who are exposed during pregnancy. Another environmental cause of autism is maternal immune activation (MIA) in which pregnant women who have systemic bacterial or viral infections with a hyper-immune response have an increased incidence of autism in their children (Bilbo et al., 2018; Careaga et al., 2017; Zawadzka et al., 2021).

Studying the etiology of autism in humans is limited to epidemiological approaches implicating genetic variants (single nucleotide polymorphisms, copy number variants, and single-gene syndromic mutations). The Simons Foundation Autism Research Initiative (SFARI) has compiled a list of 1,115 human genes (https://gene.sfari.org/database/human-gene/) for which there is evidence for association with autism based on genome-wide association studies (GWAS), referred to as the "SFARI List" below.

Hypothesis-driven experiments to determine cause and effect need to be done in animal models, which display a range of behaviors that are remarkably similar to the core symptoms of autism (Silverman et al., 2010; Lan et al., 2017; Nicolini and Fahnestock, 2018). These autistic-like behaviors have been reported in transgenic animals lacking ASD-linked genes such as *Cntnap2* and *Shank2* (Peñagarikano and Geschwind, 2012; Eltokhi et al., 2018), supporting the conclusion that pathogenic mutations in these genes are causative for syndromic autism and leading to the widespread use of these transgenic mice as animal models (Bey and Jiang, 2014; Kazdoba et al., 2016). Systemic administration of VPA (Nicolini and Fahnestock, 2018), chlorpyrifos (Lan et al., 2017) or induction of MIA (Careaga et al., 2017) in genetically normal pregnant rodents also leads to increased autistic-like behaviors in their offspring. Unlike transgenic mouse models, these environmental toxicity models of autism provide the opportunity to control the timing of exposure of the fetus to the toxic stressor, as will be discussed below. Gene expression analyses of the brains of mice in the VPA and MIA models have been

conducted using RNA microarray analysis or next-generation RNA sequencing (RNA-seq). In the majority of those animal studies, the fetus was exposed to the drug during mid-gestation, but RNA expression was analyzed in postnatal animals, usually following behavioral testing (e.g., see Qian et al., 2018; Zhao et al., 2019; Matsuo et al., 2022; Zhang et al., 2018; Guerra et al., 2023). This approach has the potential to explain behavior in terms of contemporaneous gene expression but does not inform as to the initial molecular events occurring at the time the environmental stressor is present.

There are two competing, but not mutually exclusive, theories about the role of differential gene expression in autism. In one, abnormal gene expression in the child or adult at the time of behavioral testing, is responsible for autistic-like behavior. The second posits that abnormal gene expression, due to genomic variants or *in utero* environmental stressors, interferes with one or more critical steps in the "program" controlling fetal brain development. Such errors could carry forward throughout life leading to a brain with subtle anatomical or connectivity defects that underlie the abnormal development of an autistic brain. This has been referred to as a developmental "signature" (Ben-Ari, 2008). The present study focused on the second hypothesis by examining altered gene expression in fetal brains when the environmental stressor (VPA) is still present; these genes need not be continuously dysregulated throughout life. Consequently, genes associated with critical steps in early brain development such as neurogenesis, neuron fate specification, axon and dendrite growth, and synaptogenesis are of particular interest.

The incidence of autism is about 4-times higher in males than in females (Ferri et al., 2018). Sex differences in neural function are generally considered to be mediated by sex hormones acting from late prenatal brain development through to the adult. Since the time of VPA administration in the present study (E12.5) is just prior to the maturation of gonads and production of sex hormones (Ross and Capel, 2005), any sex differences in gene expression observed likely would be due to sexually-dimorphic gene expression rather than to the effects of sex hormones.

The complexity and variability of the behavioral symptoms of autism together with identification of over 1,100 ASD-associated gene variants make a mechanistic understanding of the causes of the disorder a daunting task. One approach to this problem is to compare the results of multiple studies using GWAS results from human patients and gene expression results from animal models of autism, looking for common differentially expressed genes. The subset of genes in common from both types of studies can provide a short(er) list of candidate genes that could be subjected to future hypothesis-driven studies to establish causal relationships between gene dysregulation and ASD symptoms.

The goal of the present study was to address this question using the VPA mouse model in which pregnant mice receive a single i.p. injection of VPA at gestational day 12.5 (E12.5). Pharmacokinetic studies have demonstrated that injected VPA dissipates within 3−5 hours due to metabolism of the drug by the maternal liver (Nau and Löscher, 1982; Nau, 1986a); VPA levels in the fetal brain follow a similar trajectory (Nau, 1986b; Dickinson et al., 1980). Fetal brains were processed for RNA-seq three hours after VPA administration to the pregnant dam; male and female fetuses were analyzed separately. We propose that VPA-induced molecular events occurring on E12.5 are both necessary and sufficient for the autistic-like behaviors in VPA-treated animals assessed 5−6 weeks later. Analysis of these data revealed that VPA induced a significant increase or decrease in the expression of approximately 7,300 genes, of which 399 are among the 1,115 genes on the SFARI List and at least 252 additional genes are known to play fundamental roles in the development and function of the nervous system.

METHODS:

Timed-pregnant C57BL6 mice were generated by the University of Maryland School of Medicine Division of Veterinary Resources. All animal procedures were approved by the Institutional Animal Care and Use Committee. VPA was obtained from Sigma.

One male was paired overnight with two females; the day of separation was designated E0.5. On E12.5 pregnant females received i.p injections of VPA (400 mg/kg) in sterile saline or saline alone. 7 pregnant females received VPA and 7 received saline. Three hours after VPA administration, pregnant females were euthanized by cervical dislocation and decapitation. Fetuses were transferred to ice-cold saline, decapitated and the entire brain was removed to Trizol and disrupted for 60 sec with a Bead Beater using 0.2 mm beads. The 105 individual fetal brain samples were frozen on dry ice and stored at −80ºC prior to RNA extraction and quality control (RIN = 10 for all samples).

Sex was determined by analyzing *Sry* RNA derived from the torso of each fetus by PCR. Males were identified by the presence of both *Sry* and *Gapdh* RNA determined; *Gapdh* but not *Sry* RNA was expressed in females (Konopko et al., 2017). One male and one female brain from each of the 7 pregnancies was processed for RNA-seq by the University of Maryland Institute for Genome Sciences.

Libraries were prepared from 25ng of RNA using the NEB Ultra II Directional RNA kit. Samples were sequenced on an Illumina NovaSeq 6000 with a 150bp paired-end read configuration. The quality of sequences was evaluated by using FastQC (Wingett and Andrews, 2018). The alignment was performed using HiSat (version HISAT2-2.0.4) (Kim et al., 2019) and *Mus musculus* GRCm38 as reference genome and annotation (version 102). Aligned bam files were used to determine number of reads by gene using HTSeq (Anders et al., 2015). On average, we sequenced 187,000,000 reads. 96.6% of them properly mapped the reference: 87% mapped exons, 2.4% mapped introns and the remaining 10.6% mapped intergenic regions.

Differential gene expression between mice treated with VPA and saline controls was conducted using DESeq2 (Love et al., 2014), which models gene counts using the negative binomial distribution. P-values were corrected for false discovery rate (FDR) using the Benjamini-Hochberg procedure; $p_{FDR} \leq 0.025$ and $\log_2$-fold change ≥ 0.32 was used as the criterion for significance. Differentially expressed genes due to VPA treatment were tested for enrichment in SFARI autism risk genes (https://gene.sfari.org/database/human-gene/) using Fisher's Exact Test.

Significance of sex-differences in FC was analyzed by 2-way ANOVA using the Limma-Voom tool (Law et al., 2014).

RESULTS:

RNA-seq. 19,721 individual genes were analyzed in fetal mouse brain by RNA-seq. Male and female brains were analyzed separately. The raw data showing base counts (mean of male and female counts) for each gene in each of seven independent fetal brain samples of each sex, each from a different pregnancy, are shown in Supplemental Table 1 (S1), i.e., n = 7 fetal brains from 7 different pregnancies for each condition. Only genes with base counts ≥100 were analyzed further. Genes that increased or decreased in response to VPA by < 1.25-fold and > −1.25-fold and those with adjusted p-values (p_{FDR}) > 0.025 were also filtered out; a gene was included if VPA increased or decreased its expression by ≤ 1.25-fold and ≥ −1.25-fold in only one sex. Shown in Supplemental Table 4 (S2) are the 6516 genes significantly affected by VPA ($p_{FDR} \leq 0.025$) in both sexes, 498 in females only and 280 in males only (listed in three separate sections in Table S2). These apparent sex differences were due to variability in the replicates of one of the sexes (i.e., $p_{FDR} > 0.025$).

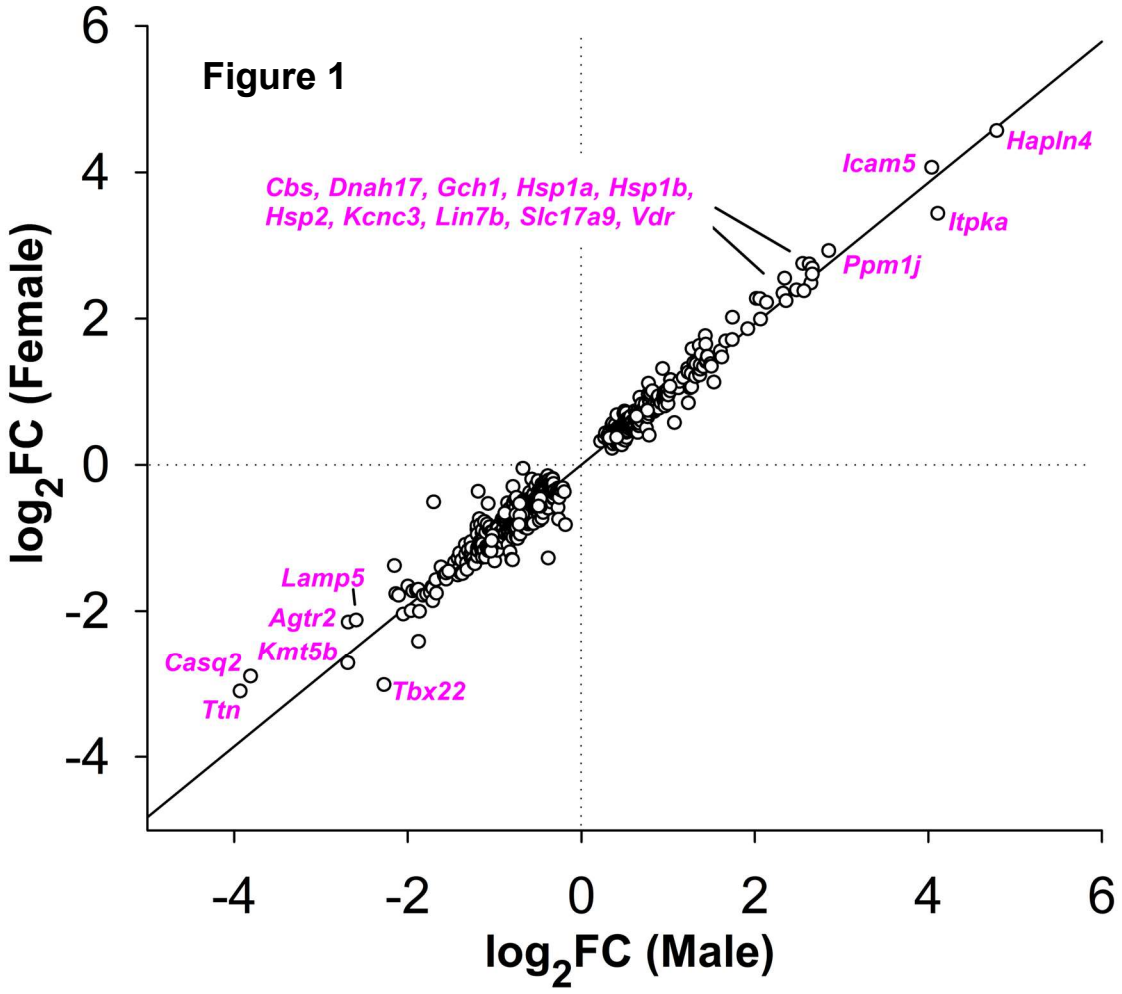
Throughout this report, a positive fold-change (FC) indicates that VPA increased gene expression; a negative FC indicates that VPA decreased expression such that the FC is the ratio of control to VPA gene expression. For example, FC = −3.0 indicates that V/C = 0.33, a 67% reduction by VPA.

Curating the mouse genes dysregulated by VPA in the fetal brain. The 7294 genes that were significantly altered by VPA (Table S2) were further analyzed in two ways. First, they were merged with the genes on the SFARI List identifying 399 common genes (Table S3). It should be noted that inclusion on the SFARI List is based on GWAS; in many cases, a mechanistic role in neither brain development nor the etiology of autism is known.

Second, we identified 252 genes (Table S4) significantly altered by VPA not on the list of SFARI risk genes but having a documented role in brain development including neurogenesis, synaptogenesis, and axon or dendrite growth. As will be discussed below, although these latter genes have not been associated with autism in GWAS, it is plausible that changes (either positive or negative) in the expression of their gene products in the fetal brain might adversely affect the trajectory of brain development leaving a permanent “signature” (Ben-Ari, 2008) on brain structure and connectivity leading to autistic-like behavior in the adult.

No significant sexually-dimorphic effects of VPA. Figure 1 is a plot of the $\log_2$ fold-change (FC) of males versus females for the 651 genes in Tables S3 and S4. The gap in the data reflects the ±1.25-fold FC cut-off. Deviations from the regression line (r^2=0.973) indicate possible sex differences; however, in no case was the effect of VPA found to be significantly different between males and females.

20 genes (magenta) were upregulated by > 5-fold or downregulated > 80% by VPA (average of males and females



Gene synonyms/protein products:

Arc, *Arg3.1*
Arntl, Bmal1
Cacna1c, Cav1.2 Ca^{2+} channel
Cacnb4, Cav2.2 Ca^{2+} Ch β-4 subunit
Calm1, calmodulin
Chrna7, α7 nAChR
Cnr1, CB1 eCB receptor
Dop1b, Dopey2
Eomes, Tbr2
Gad1, GAD67
Kcnc3, Kv3.3 K channel
Kcnmb2, BK channel β-subunit
Map2k1, Mek
Mapk1, Erk
Pik3cb, PI3 kinase β subunit
Pik3cg, PI3 kinase γ subunit
Ppp1r1b, DARPP-32
Ptk2b, *Pyk2*
Ptpn5, STEP
Rcan1, *Dscr1*
Slc17a6/7/8, VGLUT2/1/3
Slc17a9, NaHX9
Slc8a1/2, NCX1/2
Syn2, synapsin II

DISCUSSION:

The overarching hypothesis for this research is that NDDs involving intellectual disability (ID) such as ASDs are the result of abnormal connections among multiple brain regions. Normal connectivity is established beginning during fetal brain development as the various brain regions become populated with specific classes of neurons that subsequently connect with other neurons to establish the complex neuronal networks that underlie cognition and behavior. Although these networks are refined during late pre- and postnatal brain development, it is likely that certain early prenatal developmental epochs are particularly vulnerable to alterations in the neurodevelopmental "program" (Ben-Ari, 2008). Although it is by no means clear that the fetal rodent brain accurately recapitulates the autistic human brain during prenatal development, the VPA model enables experimental control of the timing of exposure to an environmental factor that causes abnormal behavior resembling autism. In the present experimental paradigm, VPA is administered to the pregnant dam at E12.5, a time when multiple early neurodevelopmental events critical for brain organization and connectivity are occurring. These include proliferation of neural progenitors (NPs), determination of the neuronal fate of these NPs, migration of newborn neurons away from the proliferative ventricular zones, differentiation of NPs to establish a mature neuronal phenotype, extension and branching of axons and dendrites and establishment of synaptic connections.

Approximately 7,300 genes were significantly up- or downregulated in the E12.5 fetal brain 3 hr after exposure to VPA (Table S2). 399 of those genes have been linked to autism in GWAS (Table S3), although in many cases the mechanism of action is unknown. In addition, at least 252 of the genes dysregulated by VPA but not on the SFARI Autism Risk list (Table S4), are known to play a mechanistic role in mediating one or more of the neurodevelopmental processes discussed above. Therefore, the goal of this study was to identify genes that are:

a. significantly up- or down-regulated by VPA in the fetal mouse brain (Table S2) AND

b. known to be linked to autism in GWAS (Table S3) or to play a role in embryonic neurodevelopmental processes, perturbation of which has the potential to alter brain connectivity in the postnatal and adult brain (Table S4).

The set of genes meeting these criteria would provide potential targets for future hypothesis-driven approaches to understanding the underlying proximal causes of defective brain connectivity in NDDs such as autism.

It was not practical to vet each of the nearly 7,300 VPA-dysregulated genes for a potential role in abnormal brain development. However, as described below, the 252 genes in Table S4 and some of

the genes in Table S3 have been reported to play substantial roles in specific aspects of fetal brain development. Consequently, it is plausible that the up- or downregulation of one or more of those genes could contribute to the abnormal connectivity in subjects with NDDs. The validity of this potential contribution could be tested in animals by determining the effect of manipulating the expression of individual (or combinations of) genes in the fetal brain on subsequent behavior.

A common subset of genes in GWAS and VPA-fetal mouse brain studies. Several GWAS have identified genes linked to autism (see SFARI Autism list). Here we tabulated the findings of four GWAS studies (Satterstrom et al., 2020; Yuen et al., 2017; Stessman et al., 2017; Ruzzo et al., 2019) and one report categorizing autism risk genes potentially involved in neurogenesis (Garcia-Forn et al., 2020). As shown in Table 1, many of the genes identified in these reports are also dysregulated by VPA in the fetal mouse brain (Tables S3 and S4). Genes discussed in the respective papers are listed in each column in Table 1; genes that are also dysregulated by VPA in the present study are shown in **bold**. Five VPA-regulated genes in Tables S3 and S4 that also appear in all five of the published reports are in **red**. (*Arid1b*, *Dyrk1a*, *Pogz*, *Pten*, *Tbr1*). Six genes in Tables S3 and S4 that also appear in four of the five other studies are in **blue**. (*Adnp*, *Ash1l*, *Chd2*, *Kmt5b*, *Tcf7l2*, *Wac*). There were four genes (yellow) identified in all five previously published studies that were not affected by VPA in the fetal mouse brain. *Chd2*, which is listed in three of the four published studies, and *Chd3*, (50% reduced by VPA), are both SFARI genes and have been reported to have similar functions to *Chd8*. Of the 11 **red** and **blue** genes in Table 1, three (*Adnp*, *Dyrk1a*, *Pten*) appear in Table 2 as VPA-regulated, autism-associated genes involved with the structural stability of neurons (Lin et al., 2016).

VPA dysregulation of high-confidence (hc)ASD genes in mid-fetal layer 5/6 cortical projection neurons. (Willsey et al., 2013) identified 9 hcASD genes that are expressed in layer 5/6 projection neurons in the fetal human brain. Three of these genes (*Dyrk1a*, *Pogz*, *Tbr1*) are downregulated in fetal mouse brain following VPA administration to the pregnant dam (Table S3). Willsey et al. (2013) used the nine hcASD genes as seed genes for co-expression network analysis which revealed 10 probable ASD genes, of which two (*Bcl11a*, *Nfia*) were down-regulated and one (*Aph1a*) was up-regulated by VPA (Table S4). All of these genes are on the SFARI autism risk gene list (Table S3) and the three seed genes were identified in all 5 reports analyzed in this study (Table 1). These findings raise the possibility that dysregulation of one or more of these genes in developing layer 5/6 projection neurons in cortical plate of the fetal brain contributes to permanent connectivity defects underlying autistic-like behaviors.

Table 1

Satter-strom	Garcia-Forn	Yuen	Stessman	Ruzzo	
41%	**50%**	**37%**	**41%**	**44%**	
		ADCY3	ADCY5		
ADNP		**ADNP**	**ADNP**	**ADNP**	
		AFF2			
		AGAP2		**AKAP9**	
ANK2	ANK2	ANK2	ANK2	ANK2	*
ANKRD11		ANKRD11	ANKRD11		
AP2S1					
ARID1B	**ARID1B**	**ARID1B**	**ARID1B**	**ARID1B**	
ASH1L		**ASH1L**	**ASH1L**	**ASH1L**	
ASXL3		**ASXL3**	**ASXL3**		
BABRB3					
BCL11A	**BCL11A**			**BCL11A**	
BRIN2B				BTRC	
CACNA1E				C16orf13	
CELF4		CACNA2D3	CACNA2D3	CACNA2D3	
			CASK	CAPN12	
			CDC42BPB	CCSER1	
CHD2		**CHD2**	**CHD2**	**CHD2**	
CHD8	CHD8	CHD8	CHD8	CHD8	*
		CIC		**CMPK2**	
		CLASP1	COL4A3BP		
		CNOT3			
CREBBP					
CTNNB1	CTNNB1		CTNNB1	CTTNBP2	
		CUL3	CUL3	CUL3	
DEAF1	**DDX3X**	**DIP2C**	**DDX3X**	**DDX3X**	
DNMT3A		**DNMT3A**	DIP2A	**DNMT3A**	
DPYSL2			DLG4		
			DLGAP1		
DSCAM		DSCAM	**DOCK8**	DSCAM	
		DYNC1H1	DSCAM		
DYRK1A	**DYRK1A**	**DYRK1A**	**DYRK1A**	**DYRK1A**	
EIF3G	FMR1	FAM47A		**ERBIN**	
				ETFB	
				FAM98C	
FOXP1	FOXP1	FOXP1	FOXP1	FOXP1	*
FOXP2				GABRB3	
GFAP					
GIGYF1		GIGYF1	GIGYF2	GIGYF1	
GNAI1		GRIN2B	GRIN2B	GRIA1	
IRF2BPL		KDM6A	HIVEP3	GRIN2B	
KCNQ3			ILF2	ILF2	
KDM5B			ITPR1	**INTS6**	
KDM6B		**KDM6B**		**KDM6B**	
KIAA0232		KIAA2022	KATNAL2	KATNAL2	
		KMT2A	KMT2A	KDM5B	
KMT2C		KMT2C	KMT2C	KMT2C	
			KMT2E	**KMT2E**	
KMT5B		**KMT5B**	**KMT5B**	**KMT5B**	
LDB1			LAMC3	MFRP	
MAP1A		**MECP2**	**MECP2**	MLANA	
MBD5					
MED13L		MED13	MED13L		

Satter-strom	Garcia-Forn	Yuen	Stessman	Ruzzo	
MKX		**MYO5A**		**MYO5A**	
MYT1L		MYT1L	MYT1L	MYT1L	
NRXN1		NAA15	NAA15	NCKAP1	
NSD1		NLGN3	NCKAP1	NRXN1	
PAX5		NLGN4X	NRXN1	P2RX5	
PHF12		PAX5	PARD3B	PCM1	
PHF21A		PCDH11X	PAX5		
		PHF2	**PHF2**	**PHF2**	
		PHF3	**PHIP**		
			PLXNB1		
POGZ	**POGZ**	**POGZ**	**POGZ**	**POGZ**	
			PPM1D	PRKAR1B	
PPP2R5D			**PPP2R5D**		
PRR12					
PTEN	**PTEN**	**PTEN**	**PTEN**	**PTEN**	
RAI1	SCN2A	SCN2A	**PTK7**	**RANBP17**	
RFX3			**PTPN11**	RAPGEF4	
RORB			RELN	SCN2A	
SATB1			RIMS1		
			SCN2A		
SCN2A			**SETBP1**		
			SETD2		
SETD5			SETD5	SETD5	
SHANK2		**SHANK2**		**SHANK2**	
SHANK3	SHANK3	SHANK3		SHANK3	
SIN3A			SLC6A1	SLC6A1	
SKI		SLC6A1	SMC3	SMURF1	
SLC6A1					
SMARCC2		**SMARCC2**			
SPAST		SPAST	SPAST	SPAST	
SRPRA					
		SRSF11	**SRCAP**		
			SRGAP3		
STXBP1			STXBP1		
SYNGAP1	SYNGAP1	SYNGAP1	SYNGAP1	SYNGAP1	*
			TANC2		
TBL1XR1		**TAF6**	**TBL1XR1**		
TBR1	**TBR1**	**TBR1**	**TBR1**	**TBR1**	
TCF20					
TCF4	**TCF4**		**TCF4**		
TCF7L2		**TCF7L2**	**TCF7L2**	**TCF7L2**	
TLK2				**TMEM39B**	
TM9SF4		**TNRC6B**	**TNRC6B**	**TNRC6B**	
		UBN2	**TRIO**		
TRIP12			TRIP12	TRIP12	
VEZF1		UPF3B	UNC80	**TSPAN4**	
				UIMC1	
				USP45	
WAC		**WAC**	**WAC**	**WAC**	
ZYMND8		**WDFY3**	**WDFY3**	**WDFY3**	
			WDR45	ZNF559	
			ZC3H4		
			ZNF292		

*Not affected by VPA

Table 1 (previous page). Autism-risk genes identified in five published studies (Satterstrom, et al., 2020; Garcia-Forn, et al., 2020; Yuen et. al., 2017; Stessman et al., 2017; Ruzzo et al., 2019). Genes dysregulated by VPA in fetal brain in the present study (Table S3 and 2) are indicated in ***bold***. The percentage of VPA-regulated genes is shown for each study. ***Red***: genes identified in all five published papers and **were** affected by VPA the present study; ***blue***: genes identified in four of the five published papers and **were** affected by VPA the present study; ***yellow***: autism-associated genes identified in all five published papers but **were not** affected by VPA in the present study.

Autism-associated genes and structural stability of neurons. Lin et al. (2016) tabulated genes linked to autism in GWAS that have also been reported to be involved in the structural stability of neurons. These genes were further sorted into three categories, viz., "neurite outgrowth", "spine/synapse formation" and "synaptic plasticity". 61 genes were assigned to these three categories although some genes appeared in two or all three categories, resulting in 29 different genes among the three categories (Table 2). Shown in red ***bold*** are 10 of these genes (35%) that were significantly up- or downregulated by VPA (i.e., in Table S3). As discussed in Lin et al. (2016), dysregulation of one or more of these genes (which could be due to gene variants or fetal exposure to VPA) could alter the structure and function of synapses throughout brain development.

Examination of Tables 1 and 2 revealed that *Pten* and *Dyrk1a* are common to the Lin et al., (2016) study and to all five of the GWAS. Consequently, these two genes may be good candidates for investigating the molecular basis for the proximal causes of autism.

Table 2

Neurites	Synapse	Plasticity
CTTNBP2	***ADNP***	***CDH(S)***
CDH(S)	***CDH(S)***	***CDKL5***
CDKL5	***CDKL5***	*DLGAP2*
CNTN	*CNTN*	*FMR1*
CNTNAP2	***CNTNAP2***	*GRIK2*
DYRK1A	*CTTNBP2*	***GRIK4***
ELMO1	*DLGAP2*	*GRIN2B*
FMR1	***DYRK1A***	***MECP2***
GRIN2B	*ELMO1*	*NLGN*
MECP2	*FMR1*	*NRXN*
MYO16	*GRIK2*	***PTEN***
NCAM2	***GRIK4***	*SHANK3*
NLGN	*GRIN2B*	***STXBP5***
NRXN	***MECP2***	*SYNGAP1*
NTRK2	*NLGN*	*UBE3A*
PCDH	*NRXN*	
PTEN	*NTRK2*	
SPRICKLE1	*PCDH*	
STXBP5	***PTEN***	
TSC1	*SHANK3*	
TSC2	*SYNGAP1*	
UBE3A	*TSC1*	
	TSC2	
	UBE3A	

Table 2. Genes linked to autism in GWAS that have also been reported to regulate "neurite outgrowth", "synapse/spine formation" or "synaptic plasticity" (Lin et al., 2016). Genes in red ***bold*** are also up- or down-regulated by VPA in the fetal brain (Table S3). Lin et al. (2016) did not distinguish among the multiple known isoforms of cadherins (*Cdh's*); *Cdh11* is downregulated 60% by VPA in the fetal brain (c.f., Table S3).

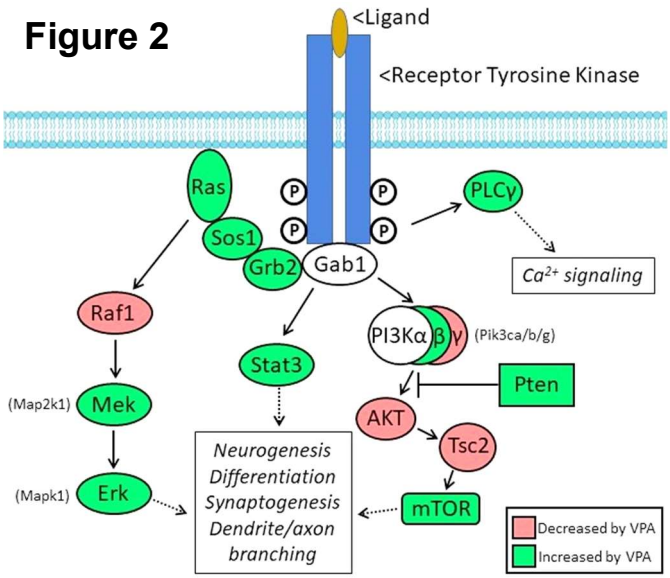
Biological roles of genes dysregulated by VPA. Table 3 (next page) shows the biological function of genes that were significantly dysregulated by VPA in the E12.5 fetal brain. One or more developmental errors caused by these changes could induce a "signature" of abnormal brain circuitry resulting in abnormal behavior in juveniles and adults. In this discussion, the change in gene expression induced by VPA at E12.5 (expressed as fold-change) is shown in parentheses (mean of males and females). Following Table 3, the rationale for inclusion of the genes in the 18 categories is provided together with references to the literature.

Table 3

Intracellular signaling	***Ak2*** **(+1.3)**, ***Akap8*** **(−1.3)**, ***Akap8l*** **(+1.6)**, ***Akt1*** **(−1.4)**, ***Akt2*** **(−2.1)**, ***Col6a6*** **(−4.1)**, ***Dyrk1a*** **(−1.5)**, ***Dyrk1b*** **(−2.2)**, ***Grb2*** **(+1.4)**, ***Kras*** **(+1.4)**, ***Map2k1*** **(+1.7)**, ***Mapk1*** **(+1.3)**, ***mTor*** **(+1.4)**, ***Ndst3*** **(−1.6)**, ***Pik3cb*** **(+1.8)**, ***Pik3cg*** **(−2.3)**, ***Pik3r4*** **(+1.3)**, ***Plcg2*** **(+1.8)**, ***Pten*** **(+1.4)**, ***Raf1*** **(−1.6)**, ***Rmnd5b*** **(+1.3)**, ***Rnf19b*** **(+1.4)**, ***Sos1*** **(+1.3)**, ***Stat3*** **(+2.7)**, ***Tsc2*** **(−1.3)**
Calcium signaling	***Calm1*** **(+1.4)**, ***Cacna1b*** **(+1.4)**, ***Cacna1c*** **(−1.4)**, ***Cacna2d1*** **(−1.7)**, ***Cacnb2*** **(−1.9)**, ***Camk1*** **(−1.6)**, ***Camk2a*** **(+1.9)**, ***Camkk1*** **(+2.6)**, ***Camkv*** **(+2.4)**, ***Ppp3cb*** **(−1.3)**, ***PPP3cc*** **(−1.3)**
Retinoic acid signaling	***Megf10*** **(−1.9)**, ***Cntnap2*** **(+1.3)**, ***Meis2*** **(−1.4)**, ***Cbln2*** **(−1.8)**, ***Rxrb*** **(−1.3)**, ***Rarb*** **(−2.2)**
Neurogenesis	***Cdk5rap2*** **(−1.3)**, ***Ebf1*** **(−1.4)**, ***Eomes*** **(*Tbr2*) (−1.8)**, ***Fgf17*** **(−1.6)**, ***Foxc1*** **(−1.9)**, ***Hnrnpk*** **(−1.3)**, ***Hnrnph1*** **(−1.6)**, ***Hnrnpu*** **(−1.6)**, ***Lrrk1*** **(−2.9)**, ***Neurog1*** **(−2.2)**, ***Plk1*** **(−1.7)**, ***Pogz*** **(−2.2)**, ***Trnp1*** **(+1.5)**, ***Wnt3a*** **(−3.8)**
Neuronal fate specification	***Ebf3*** **(−1.6)**, ***Neurod1*** **(−1.5)**, ***Nkx2-2*** **(−1.8)**, ***Nr2f1*** **(*Coup-tf1*), (−1.8)**, ***Olig1*** **(+2.1)**, ***Tbr1*** **(−2.8)**
Axon growth/guidance	***Draxin*** **(−1.5)**, ***Hnrnpab*** **(−1.6)**, ***Marcks*** **(−2.2)**, ***Slitrk1*** **(−1.5)**, ***Syn2*** **(+1.7)**
Neuronal migration	***Apc2*** **(−1.7)**, ***Mllt11*** **(−1.5)**, ***Neurod1*** **(−1.5)**, ***Nsd1*** **(−1.4)**, ***Otp*** **(−2.5)**, ***Ptk2b*** **(−1.7)**, ***Robo4*** **(−2.2)**
Synaptogenesis	***Arc*** **(+1.6)**, ***Cbln2*** **(−1.8)**, ***Csde1*** **(−2.0)**, ***Ddx3x*** **(−1.6)**, ***Hapln1*** **(−3.9)**, ***Hapln4*** **(+25.7)**, ***Pogz*** **(−2.2)**, ***Slc17a7*** **(+1.6)**, ***Slitrk5*** **(−1.6)**, ***Slitrk4*** **(+1.7)**, ***Syncrip*** **(−1.4)**, ***Top3b*** **(−1.5)**
Dendrite development	***Cc2d1a*** **(−1.4)**, ***Csde1*** **(−2.0)**, ***Disc1*** **(−1.9)**, ***Dlg5*** **(−3.0)**, ***Gas7*** **(−1.7)**, ***Mef2d*** **(+1.5)**, ***P2rx7*** **(−2.0)**, ***Slitrk1*** **(−1.5)**, ***Tmem163*** **(−1.7)**, ***Twf2*** **(+1.6)**
Postsynaptic cytoskeleton	***Homer1*** **(+1.6)**, ***Homer3*** **(−1.4)**, ***Shank2*** **(−2.3)**
Excitation-Inhibition	***Arid1b*** **(−2.1)**, ***Camk2a*** **(+1.9)**, ***Cc2d1a*** **(−1.4)**, ***Gad1*** **(−2.5)**, ***Gad2*** **(−3.3)**, ***Gabra1*** **(−1.7)**, ***Gabra2*** **(−1.7)**, ***Gabra4*** **(+2.5)**, ***Grin2a*** **(−2.3)**, ***Insyn1*** **(−1.4)**, ***Kmt5b*** **(−6.5)**, ***Maf*** **(−1.6)**, ***Mafb*** **(−1.6)**, ***Nf1*** **(−1.3)**, ***Trpm4*** **(+2.1)**
Dopaminergic system	***Adora2a*** **(−1.3)**, ***Bdnf*** **(+2.8)**, ***C1qtnf1*** **(−1.4)**, ***Csf1r*** **(−1.4)**, ***Drd2*** **(+2.0)**, ***Emp2*** **(−1.6)**, ***Foxa1*** **(−1.6)**, ***Klf4*** **(−1.6)**, ***Lmx1a*** **(−1.7)**, ***Mcam*** **(+1.7)**, ***Palm3*** **(+1.9)**, ***Ppp1r1b*** **(+3.7)**, ***Robo4*** **(−2.2)**, ***Rtn4rl1*** **(+2.0)**, ***S1pr1*** **(−1.3)**, ***Slc9a3r1*** **(+3.0)**, ***Smyd2*** **(−1.3)**, ***Sparc*** **(−1.4)**, ***Srebf1*** **(+1.4)**, ***Th*** **(−1.4)**, ***Tmem108*** **(+1.4)**, ***Wnt7b*** **(−1.8)**,
Cholinergic system	***Chrna7*** **(+1.4)**, ***Gbx1*** **(−2.3)**, ***Isl1*** **(−1.5)**, ***Lhx8*** **(−2.7)**, ***Nkx2-1*** **(−2.5*)**,
Endocannabinoid system	***Cnr1*** **(−2.0)**
Autism and Down syndrome	***Brwd1*** **(−1.3)**, ***Cbs*** **(+5.0)**, ***Dop1b*** **(+1.4)**, ***Dyrk1a*** **(−1.5)**, ***Erg*** **(−2.4)**, ***Ets2*** **(−1.4)**, ***Pigp*** **(−1.6)**, ***Setd4*** **(−1.9)**, ***Shebgr*** **(+1.8)**, ***Sim2*** **(−2.2)**, ***Wdr4*** **(−1.4)**
Circadian rhythms	***Arntl*** **(+2.1)**, ***Cry1*** **(+2.5)**, ***Cry 2*** **(+2.0)**, ***Fbxl21*** **(+3.2)**, ***Fbxl3*** **(−1.8)**, ***Gabra4*** **(+2.5)**, ***Gad1*** **(−2.5)**, ***Gabbr2*** **(−2.4)**, ***Mef2d*** **(+1.5)**, ***Per1*** **(+1.9)**, ***Per2*** **(+1.7)**
Neuroinflammation	***Cd200*** **(−2.0)**, ***Dnajc12*** **(+4.5)**, ***Hsp1a*** **(+5.9)**, ***Hsp1b*** **(+5.6)**, ***Hsp2*** **(+6.2)**, ***Igf1*** **(−3.2)**, ***Igf2*** **(−2.7)**, ***Tgfb2*** **(−2.4)**, ***Tgfb3*** **(−2.0)**, ***Tgfbr2*** **(−2.5)**
Epigenetic regulation	***Ash1l*** **(−1.5)**, ***Brd1*** **(−1.4)**, ***Brd2*** **(−1.3)**, ***Ehmt1*** **(−1.4)**, ***Kmt5b*** **(−6.5)**, ***Mecp2*** **(−1.6)**, ***Setd1b*** **(−2.2)**, ***Smarcb1*** **(−1.7)**

Table 3. The biological roles of genes that are dysregulated by VPA in the fetal brain and are associated with autism or prenatal neurodevelopmental processes (from Tables S3 and S4). The number in parentheses shows the FC induced by VPA (mean of males and females); **red** text indicates that VPA increased gene expression. Note that some genes are included in more than one category. The basis for this categorization, with references, is given below.

Intracellular signaling. As summarized in Figure 2, the genes encoding 10 members of the canonical signaling pathways downstream from receptor tyrosine kinase receptors are significantly up-regulated by VPA in the fetal brain (green); four are down-regulated (red). ***Pten* (+1.4)** (Phosphatase and tensin homolog), a negative regulator of the PI3K-AKT-mTor signaling pathway, has a particularly strong association with autism, appearing in all GWAS (Tables 1, 3) and associated with the structural stability of neurons (Table 2) (Lin et al., 2016).



Twelve additional genes associated with intracellular signaling are included in Table 3. Thus, abnormal signaling via these pathways is a potential mechanism by which VPA could interfere with the establishment of normal brain connectivity. Meta-analysis of GWAS and copy number variant studies of autism-related genes revealed that three signaling networks, regulating steroidogenesis, neurite outgrowth and excitatory synaptic function, were enriched (Poelmans et al., 2013). A-kinase anchoring proteins (Akaps) functionally integrate signaling cascades within and among these networks. ***Akap8* (−1.3)** and ***Akap8l* (+1.6)** was decreased and increased, respectively, in fetal mouse brains exposed to VPA. ***Dyrk1b* (−2.2)** (which encodes dual specificity tyrosine phosphorylation regulated kinase 1B) is an early mediator of double-stranded DNA break repair (Dong et al., 2020) and regulates hedgehog signaling through activation of the mTor/AKT pathway (Singh et al., 2017; Upadhyay et al., 2021; Singh and Lauth, 2017).

Dyrk1b has been linked to metabolic syndrome and autism (Orenstein et al., 2022). The 55% reduction in *Dyrk1b* expression induced by VPA could contribute to autistic-like behavior by dysregulating hedgehog or mTor/AKT signaling. ***Ppp1r1b* (+3.7)**, encodes the dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32). DARPP-32 amplifies and/or mediates many actions of cyclic AMP-dependent protein kinase at the plasma membrane and in the cytoplasm, with a broad spectrum of potential targets and functions (Yger and Girault, 2011).

Calcium signaling. Ca^{2+} is an intracellular messenger in a wide variety of cellular responses; calmodulin, encoded by ***Calm1* (+1.4)**, acts as the Ca^{2+}-sensor in many responses including Ca^{2+}-calmodulin dependent kinases [***Camk1* (−1.6)**, ***Camk2a* (+1.9)**, ***Camkk1* (+2.6)**, ***Camkv* (+2.4)**], calcineurin, [***Ppp3cb* (−1.3)** and ***PPP3cc* (−1.3)**]. Four genes encoding subunits of voltage-gated Ca^{2+} channels on the SFARI list (Liao and Li, 2020; Breitenkamp et al., 2014) (Table S3) were dysregulated by VPA in the fetal brain: ***Cacna1b* (+1.4)**, ***Cacna1c* (−1.4)**, ***Cacna2d1* (−1.7)** and ***Cacnb2* (−1.9)**. Like *Cacnb2*,

***Cacnb4* (−1.5)** is an auxiliary subunit, however, it is not linked to autism by GWAS. *Cacna1c* is associated with Timothy syndrome, an ASD characterized by ID as well as long-QT syndrome, a frequently lethal heart rhythm defect (Herold et al., 2023; Endres et al., 2020).

Retinoic acid signaling. Retinoic acid (RA) is a diffusible derivative of vitamin A that has been reported to regulate the fetal development of the prefrontal cortex and the dopaminergic system in the striatum; dysregulation of RA signaling in mice can lead to autistic-like behavior. ***Rarb* (−2.2)**, which encodes the RA receptor, RARβ, is downregulated by more than 50% by VPA in the E12.5 mouse brain. In addition, four additional genes dysregulated by VPA **[*Megf10* (−1.9)**, ***Cntnap2***, **(+1.3) *Meis2* (−1.4)**, ***Cbln2* (−1.8)]** are involved in RA signaling; three of these (*Megf10*, *Cntnap2*, *Meis2*) are on the SFARI list. RA-dependent developmental processes in the fetal brain generation of GABAergic striatal projection neurons and interneurons (Chatzi et al., 2013), expression of D2 dopamine receptors in the ventral striatum (Krezel et al., 1998) and regulation of cortical neurogenesis and cortical plate thickness (Haushalter et al., 2017). Shibata et al. (2021) reported that genetic deletion of *Rarb* disrupts proper molecular patterning of prefrontal and motor areas, development of prefrontal cortex (PFC)-mediodorsal thalamus connectivity and development of intra-PFC dendritic spinogenesis. RA signaling occurs through the RA receptors, RXRB and RARB, and genetic deletions of ***Rxrb* (−1.3)** and *Rarb* demonstrated that these molecules are involved in the proper molecular patterning of the prefrontal and motor cortices, the development of cortico-thalamic connectivity and cortical spinogenesis (Shibata et al., 2021). RA signaling may also regulate the development of the dopamine (DA) system (see below), (Krezel et al., 1998); deletion of *Rxrb* and *Rarb* resulted in locomotor defects related to dysfunction of the mesolimbic DA signaling pathway, as well as expression of D1 and D2 DA receptors in the ventral striatum. VPA exposure reduced expression of *Rarb* (55%) and *Rxrb* (23%) in the fetal mouse brain. Interestingly, VPA reduced the expression the D2 DA receptor, ***Drd2* (−1.9)** by nearly half. Thus, these findings suggest that VPA could reduce the strength of RA signaling in the fetal brain, particularly in the dopaminergic system, resulting in altered numbers and/or connectivity of cortical or striatal neurons.

Neurogenesis. Excitatory neurons in the mammalian cortex are generated from proliferating radial glia cells (RGs) which undergo mitosis at the ventricular surface to expand the pool of neuroblasts and consequently determine the final number of neurons (Silbereis et al., 2016). Neurogenesis proceeds according to a “program” that is tightly-regulated by intercellular signals from a variety of brain cells as well as the meninges. Disturbances in this regulation, for example by environmental factors such as VPA or gene mutations that disrupt the expression of the genes encoding the relevant intercellular signals, have the potential to alter the configuration and neuronal population of the developing brain leading to permanent defects in connectivity that could lead to altered behavior.

As discussed above, RA plays a number of roles in the regulation of cortical neurogenesis (Haushalter et al., 2017; Shibata et al., 2021). The RA has been reported to be produced in the dorsal forebrain meninges and to act at receptors on the end-feet of RGs (Siegenthaler et al., 2009). This group used a transgenic mouse with reduced expression of ***Foxc1* (−1.9)** leading to defects in forebrain meningeal formation and loss of neuron progenitor production due to failure of proliferating RGs to exit the cell cycle. This would be predicted to lead to a delay in the generation of postmitotic neurons and consequently, an expansion of the proliferating RG pool neuroprogenitor pool. ***Eomes* (*Tbr2*) (−1.8)** is also implicated in the regulation of cortical neurogenesis (Englund et al., 2005; Baala et al., 2007; Arnold et al., 2008; Elsen et al., 2013; Sessa et al., 2017), while ***Neurog1* (−2.2)** has been reported to play a role as a negative regulator of cortical neurogenesis (Han et al., 2018).

The conversion from symmetrical to asymmetrical mitoses associated with the generation of postmitotic neurons is regulated by the ***Plk1* (−1.7)** – ***Lrrk1* (−2.9)** – ***Cdk5rap2* (−1.3)** cascade (Hanafusa et al., 2015). Suliman-Lavie et al. (2020) reported that ***Pogz* (−2.2)** is a negative regulator of transcription and *Pogz* deficiency upregulates expression of genes associated with ASD resulting in disruption of embryonic neurogenesis; *Pogz* was found to be associated with autism in all five GWAS (Table 1). Several members of the heterogeneous nuclear ribonucleoprotein family have been linked to NDDs including ***Hnrnph1* (−1.6), *Hnrnpk* (−1.3), *Hnrnpu* (−1.6);** expression of these genes in radial glia has been reported to be critical during neurodevelopment (Gillentine et al., 2021).

Comparison of the mechanisms controlling neurogenesis in rodents and humans has revealed that gyrification of the human brain is driven by changes in the expression of regulatory genes and growth factors (Borrell and Götz, 2014) . Stahl et al. (2013) reported that, over the period E12 to E16, decreased expression of the DNA-associated protein, ***Trnp1* (+1.5)**, leads to radial expansion of the cortex and the appearance of gyri-like folding of the mouse brain (Stahl et al., 2013). In the present study, VPA induced a 50% increase in the expression of *Trnp1* in the E12.5 fetal mouse brain; this would be predicted to inhibit or delay the shift to radial growth of the cortex. Fibroblast growth factor 2 (FGF2) has also been reported to induce gyrification in the mouse brain, having an effect opposite to that of *Trnp1* (Rash et al., 2013). In the present study, VPA caused a 65% reduction on the expression of ***Fgf2* (−2.9)** (Table S4). This would also be predicted to bias cortical neurogenesis toward tangential and away from radial growth that would lead to gyrification. These findings together with the present results lead to a hypothesis that increased *Trnp1* and decreased *Fgf2* expression, induced by VPA in the fetal brain at E12.5, cause a delayed shift in radial growth resulting in autism-like behavioral defects. This hypothesis could be tested by experimentally decreasing *Trnp1* and increasing *Fgf2* expression in the mouse fetal brain exposed to VPA; these maneuvers would be predicted to normalize behavior.

WNT/β-catenin signaling plays a number of roles in regulating neurogenesis in the developing neocortex (Da Silva et al., 2021). ***Wnt3a* (−3.8)** regulates the timing of differentiation of neocortical intermediate progenitors into neurons (Munji et al., 2011). The 74% reduction in *Wnt3a* expression induced by VPA has the potential to disrupt the normal timing of cortical neurogenesis in the fetal brain.

Neuronal fate specification. ***Tbr1* (−2.8)** encodes T-brain-1, a brain-specific T-box transcription factor that has been linked to autism in multiple GWAS (see Table 1). VPA reduced *Tbr1* expression by 63% in the fetal mouse brain (Table S3). *Tbr1* has been described as a "master regulator of ASDs" because it regulates the expression of multiple genes with links to autism (Chuang et al., 2015), thereby controlling brain connectivity in ASDs (Huang and Hsueh, 2015; Notwell et al., 2016). *Tbr1* has been reported to regulate differentiation of the preplate and layer 6 of the fetal cortex (Hevner et al., 2001) as well as the connectivity of cortical layer 6 (Fazel Darbandi et al., 2018). Recently, different *Tbr1* mutations found in individuals with ASD, intellectual disability and developmental delay had differing effects on cortical development (Co et al., 2022); one such mutation (K228E) caused significant upregulation of *Tbr1* but similar behavioral phenotypes to those of *Tbr1* KO mice (Yook et al., 2019). Thus, it appears that both positive and negative deviations from an optimal level of *Tbr1* expression are associated with an autism-like behavioral phenotype. Considering its links to autism in GWAS and its central role in regulating fetal brain development, VPA-induced changes in *Tbr1* expression in the fetal brain could contribute to the behavioral abnormalities observed in animals exposed to VPA *in utero*.

***Nr2f1* (*Coup-tf1*) (−1.8)** encodes an orphan nuclear receptor that has been implicated in a range of neurodevelopmental functions including coordinating cortical patterning, neurogenesis, and laminar fate (Armentano et al., 2007; Faedo et al., 2008; Tocco et al., 2021). *Nr2f1* has also been reported to control subtype and laminar identity of cortical interneurons derived from the medial ganglionic eminence (Hu et al., 2017) as well as regional dynamics of neuroprogenitors in the cortex, possibly being responsible for abnormal gyrification (Bertacchi et al., 2020). *Nr2f1*, together with *Nr1f2*, regulates cell migration in the basal forebrain (Tripodi et al., 2004) and is required for forebrain commissural projections (Armentano et al., 2006). As discussed below, Engeln et al. (2021) reported that *Nr2f1* is a common regulator for multiple genes involved in spine morphology of D1-MSNs in the striatum.

Neuronal differentiation and regional specification in the CNS are regulated by the *Ebf* family of genes including ***Ebf3* (−1.6)** (Garel et al., 1997). Variants of *EBF3* are associated with hypotonia, developmental delay, intellectual disability, and autism (Padhi et al., 2021; Tanaka et al., 2017). The *Olig* genes (*Olig1/2/3*), which encode members of the basic helix-loop-helix (bHLH) family of transcription factors, were originally identified as regulators of oligodendrocyte production in the CNS. More recently, the *Olig* genes have been found to regulate the specification and differentiation of neuronal phenotype

during fetal brain development (Chakrabarti et al., 2010; Szu et al., 2021). ***Olig1* (+2.1**, significant in females only**)** expression was more than doubled in fetal brains exposed to VPA. The basic Helix-loop-Helix gene, ***Neurod1* (−1.5)**, regulates multiple steps in cortical development including neuronal fate specification, differentiation, and migration (Tutukova et al., 2021).

Axon growth/guidance. Myristoylated Alanine-Rich C-kinase Substrate, encoded by ***Marcks* (−2.2),** plays a wide variety of roles in brain development and function (El Amri et al., 2018; Stumpo et al., 1995). These may be due at least in part to the regulation of axon growth in which MARCKS mediates membrane targeting of plasmalemmal precursor vesicles during axon development (Xu et al., 2014). Although total deletion of *Marcks* in mice is embryonic lethal, the 55% reduction in *Marcks* expression induced by VPA may be less severe and cause subtle changes in brain connectivity that could lead to autistic-like behavior. ***Syn2* (+1.7)** (synapsin-2) has been reported to be required for normal axon formation (Ferreira et al., 1998). ***Draxin* (−1.5)** (dorsal inhibitory axon guidance protein) is essential for the axon guidance underlying development of the corpus callosum and thalamocortical projections (Liu et al., 2018; Ahmed and Shinmyo, 2021; Morcom et al., 2021). Heterogeneous nuclear ribonucleoprotein A/B encoded by ***Hnrnpab* (−1.6)** facilitates olfactory sensory neuron maturation and axon projections by regulating the local expression of its target genes at axon terminals (Fukuda et al., 2023).

Neuronal migration. ***Robo4* (−2.2)** has been reported to regulate the radial migration of newborn neurons in the developing neocortex (Zheng et al., 2012; Gonda et al., 2020); *Robo4* expression was reduced by more than half in the fetal brain by VPA. Dysregulation of *Robo4* expression at E12.5 could affect the developing connectivity of the fetal brain leading to behavioral abnormalities in the adult. ***Mllt11* (−1.5)** regulates migration and neurite outgrowth of cortical projection neurons during development (Stanton-Turcotte et al., 2022). ***Ptk2b* (−1.7)** (*Pyk2*) is a non-receptor cell-adhesion kinase and scaffold protein which, if either overexpressed or knocked down, impairs cortical neuron migration by altering growth cone dynamics (Fan et al., 2018). *Ptk2b* is regulated by ***Ptpn5* (−1.5)** (STEP), a tyrosine phosphatase (Chatterjee et al., 2020). None of these three genes has been linked to autism in GWAS but downregulation of *Ptk2b*, *Ptpn5* and/or *Mllt11a* by VPA has the potential to alter neuronal migration at a critical time when circuits are forming in the fetal brain. ***Otp* (−2.5)** encodes a homeodomain transcription factor that is associated with the development of the hypothalamus in vertebrates. *Otp* is necessary for the migration of diencephalic neurons to the amygdala (García-Moreno et al., 2010) and affects neuropeptide switching in oxytocin neurons (Wircer et al., 2017; Acampora et al., 1999; Ryu et al., 2007). A loss of function mutation in ***Apc2* (−1.7)** causes Sotos syndrome which is characterized by ID and characteristic facial features; this is because *Apc2* is a downstream effector of ***Nsd1* (−1.4)**

in regulating the migration and laminar positioning of cortical neurons (Almuriekhi et al., 2015). Thus, VPA inhibits this critical developmental pathway at two steps.

Synaptogenesis. ***Slc17a7* (+1.6)**, which encodes the vesicular glutamate transporter VGLUT1, promotes the development of cortical presynaptic terminals (Berry et al., 2012). The VPA-induced increase in *Slc17a7* would be consistent with the abnormal acceleration or premature initiation of this process. *SLTRKs* are single-pass transmembrane proteins of the leucine-rich repeat (LRR) superfamily that have been proposed as candidate genes for neuropsychiatric disorders (Proenca et al., 2011). There are 6 members of this family in humans and mice, encoded by *Slitrk1−6*. ***Slitrk1* (−1.5)** has been shown to modulate neurite outgrowth (Aruga and Mikoshiba, 2003) and to promote the development of excitatory synapses (Beaubien et al., 2016), *Slitrk2* (not significantly affected by VPA) is on the SFARI list and has been linked to schizophrenia and X-linked NDDs (El Chehadeh et al., 2022) and ***Slitrk5* (−1.6)** deficiency impairs corticostriatal circuitry (Shmelkov et al., 2010) and synaptogenesis (Song et al., 2017). *Slitrk1* and *Slitrk5* expression in the fetal brain was reduced 33% and 37% by VPA; ***Slitrk4* (+1.7)** expression was increased 67% by VPA (Table S4).

Activity-dependent immediate early genes (IEGs) have been shown to regulate synaptogenesis (Kim et al., 2018). The IEG, ***Arc* (+1.6)** (*Arg 3.1*), has been described as a flexible hub for synaptic plasticity and cognition (Nikolaienko et al., 2018) and has been reported to mediate activity dependent synapse elimination in the developing cerebellum (Mikuni et al., 2013).The cerebellins (CBLNs) are secreted glycoproteins that link presynaptic neurexins with postsynaptic δ1 glutamate receptors to form trans-synaptic adhesion complexes and promote the formation and stability of excitatory synapses (Seigneur et al., 2021). The formation of these complexes is deficient in the ***Cbln2* (−1.8)** knockout mouse (Tao et al., 2018) resulting in the destabilization of excitatory synapses. Consequently, the 45% reduction in *Cbln* expression induced by VPA in the fetal mouse brain would be predicted to compromise excitatory synaptogenesis.

***Top3b* (−1.5)** encodes an RNA topoisomerase that works with FMRP to promote synapse formation (Xu et al., 2013) and is a high-confidence gene on the SFARI list (Table S3). *Top* family members have been reported to facilitate transcription of long genes linked to autism (King et al., 2013). ***Syncrip* (−1.4)** (SFARI list) (also known as *Hnrnpg*) encodes the synaptotagmin-binding cytoplasmic RNA-interacting protein, which is a candidate gene for ASD and ID (Rauch et al., 2012; Lelieveld et al., 2016; Semino et al., 2021). Bannai et al. (2004) reported that *Syncrip* is a component of mRNA-containing granules in dendrites, possibly regulating local protein synthesis in developing dendritic spines.

Disruption of *POGZ* is associated with ID, ASDs (Stessman et al., 2016, 2017) and impaired cortical development (Matsumura et al., 2020). Markenscoff-Papadimitriou et al. (2021) reported that ***Pogz***

(−2.2) promotes chromatin accessibility and expression of clustered synaptic gene; moreover, brain-specific conditional knockout of *Pogz* results in gene expression changes associated with synaptic function as well as an autistic-like behavioral phenotype (Suliman-Lavie et al., 2020). The X-linked gene, ***Ddx3x* (−1.6),** encodes an RNA helicase that functions in corticogenesis and synaptogenesis (Lennox et al., 2020; (Hoye et al., 2022). Mutations in *DDX3X* account for approximately 2% of intellectual disability in females (DDX3X syndrome) (Boitnott et al., 2021; Weil et al., 2020). Knockdown of the RNA binding protein, ***Csde1* (−2.0)**, has been reported to cause abnormal dendritic spine morphology and synapse formation (Guo et al., 2019).

Dendrite development. ***P2rx7* (−2.0)** (ionotrophic P2X7 receptor, which is activated by extracellular ATP) has been described as the "central hub of brain diseases" (Andrejew et al., 2020). Inhibition of P2X7 receptors ameliorated dendritic spine pathology under pathological conditions (in *Mecp2*-deficient mice) (Garré et al., 2020), while P2rx7 deficiency caused dendritic branching deficits on otherwise normal mice (Mut-Arbona et al., 2023). Interestingly, ***Tmem163* (−1.7)** is required for full function of P2X7 receptors (Salm et al., 2020). ***Sema3a* (−2.0)** regulates dendritic complexity via ***Farp1* (−1.7)** in an activity dependent manner (Cheadle and Biederer, 2014). *Farp1* links postsynaptic cytoskeletal dynamics and transsynaptic organization to coordinate synaptic development (Cheadle and Biederer, 2012). In contrast, ***Itpka* (+14.1)**, which has been reported to regulate dendrite morphology (Windhorst et al., 2012), was overexpressed by 14-fold in fetal brain exposed to VPA. Thus, the reduction in expression of *P2rx7*, *Tmem163*, *Sema3a* and *Farp1* and the increase in *Itpka* induced by VPA may work together to alter dendritic branching of developing neurons in the E12.5 brain. ***Icam5* (+16.6)** (intercellular adhesion molecule 5) is overexpressed in the brain of the *Fmr1* knockout mouse (which models fragile X syndrome, an ASD), leading to dendritic spine abnormalities. The massive increase in *Icam5* expression induced by VPA has the potential to severely disrupt the connectivity of the developing brain leading to autism-like behavior. ***Gas7* (−1.7)** encodes a spine initiation factor that responds to neuronal activity; *Gas7* knockdown decreased spine density in hippocampal neurons (Khanal et al., 2023). ***Dlg5* (−3.0)** encodes a membrane-associated guanylate kinase (MAGUK) protein, which is required for dendritic spine formation and synaptogenesis (Wang et al., 2014), raising the possibility that synaptic development and organization may be impacted by the 67% reduction *Dlg5* expression in fetal brain exposed to VPA at E12.5. Normal expression levels of ***Disc1* (−1.9)** (Disrupted in schizophrenia-1) are required for microtubule function and depletion of *Disc1* or dominant negative *Disc1* constructs impairs neurite outgrowth *in vitro* and proper development of the cortex *in vivo* (Kamiya et al., 2005). Increased expression of Twinfillin-2 which is encoded by ***Twf2* (+1.6)**, increases of thin dendritic spine length in hippocampal neurons (Walker et al., 2023).

Postsynaptic cytoskeleton. The organization and assembly of glutamate receptors in the postsynaptic membrane are dependent on scaffold proteins including members of the HOMER and SHANK families (Guang et al., 2018). ***Homer1* (+1.6)** and ***Shank2* (−2.3)** are associated with autism by GWAS (Table S3) and are responsible for rare syndromic cases of autism. ***Homer3* (−1.4)** is also dysregulated in the fetal brain. (Eltokhi et al., 2018; Ronesi et al., 2012; Zaslavsky et al., 2019; Yoon et al., 2021). Disruption of *Homer* and *Shank* gene expression by VPA would be expected to alter synaptic function throughout development.

Excitation-inhibition. A widely discussed hypothesis is that autism is caused by an imbalance between neuronal excitation and inhibition due to increased excitation, reduced inhibition, or both (Rubenstein and Merzenich, 2003; Nelson and Valakh, 2015). Consequently, alterations in the developmental program in the fetal brain that result in too many excitatory (glutamatergic) or too few inhibitory (GABAergic) neurons (or synapses) could contribute to autistic behavior. Two related genes, ***Maf* (−1.6)** and ***Mafb* (−1.6)**, which are both downregulated by about 40% by VPA, have been reported to play redundant roles in the generation of interneurons from the fetal median ganglionic eminence; their deletion results in decreased numbers of cortical somatostatin-releasing, inhibitory interneurons (Pai et al., 2019). ***Gad1* (−2.5)** and ***Gad2* (−3.3)** encode isoforms of the enzyme that synthesizes the inhibitory transmitter, GABA*; Gad1* and *Gad2* were reduced by 60% and 70%, respectively; however, reduced *Gad* expression may be a consequence of a deficit in the number inhibitory GABAergic neurons. ***Insyn1* (−1.4)** encodes a component of the dystroglycan complex at inhibitory synapses; loss of *Insyn1* alters the composition of the GABAergic synapses, excitatory/inhibitory balance, and cognitive behavior (Uezu et al., 2016, 2019) Taken together, these findings are consistent with VPA increasing net brain excitation by decreasing the number of inhibitory interneurons generated during fetal brain development. These reductions in gene expression may contribute to a decrease in inhibition in mice exposed to VPA *in utero* (Markram et al., 2008). ***Nf1* (−1.3)** encodes NF1, which is mutated in neurofibromatosis type 1, an inherited neurocutaneous disorder associated with NDDs including autism. *Nf1* deletion results in the specific loss of parvalbumin-expressing inhibitory cortical interneurons (Angara et al., 2020). In the present study, *Nf1* was downregulated by about 25% in fetal mouse brains exposed to VPA.

***Hapln1* (−3.7)** and ***Hapln4* (+25.7)**, encode extracellular matrix proteins that are components of perineuronal nets (PNNs) (Bosiacki et al., 2019; Nojima et al., 2021). Ramsaran et al. (2023) recently reported that *Hapln1* mediates the functional maturation of hippocampal parvalbumin neurons through assembly of PNNs; this mechanism mediates the development of memory precision during early childhood. *Hapln4* is a selective regulator of the formation of GABAergic synapses between Purkinje and deep cerebellar nuclei neurons (Edamatsu et al., 2018); the cerebellum is one of many brain regions

implicated in the etiology of autism (Mapelli et al., 2022). The 70% decrease in *Hapln1* expression and the massive, 25-fold increase in *Hapln4* induced by VPA would be expected to alter PNN density which could disrupt the connectivity of neuronal circuitry leading to cognitive behavioral deficits.

Dopaminergic system. Several lines of evidence link autistic symptoms with dysfunction in the mesencephalic DA system (Langen et al., 2014; Subramanian et al., 2017; DiCarlo and Wallace, 2022). This can be explained by abnormal circuitry (Golden et al., 2018), notably in the indirect pathway downstream from D2-receptor expressing medium spiny neurons (MSNs). The subthalamic nucleus (STN), an excitatory nucleus in the indirect pathway downstream from the D2-MSNs, is a key factor in the normal functioning of the basal ganglia. ***Foxa1* (−1.6)**, which is reduced by 38% by VPA, has been reported to be essential for the development and functional integrity of the STN (Ferri et al., 2007; Lee et al., 2010; Gasser et al., 2016). *Foxa1* and ***Lmx1a* (−1.7)**, which are both reduced by about 40% in the E12.5 brain exposed to VPA (Table S4), have been shown to promote mesencephalic DA neuron development and diversity (Yan et al., 2011; Doucet-Beaupré et al., 2015; Lin et al., 2009; Deng et al., 2011; Bodea and Blaess, 2015). The basic helix-loop-helix transcription factor ***Srebf1* (+1.4** significant in males only) has been shown to be both necessary and sufficient for midbrain dopaminergic neurogenesis (Toledo et al., 2020). Engeln et al. (2021) reported that transgenic mice with the BDNF receptor, trkB, knocked out specifically in D1 MSNs, displayed autism-like stereotypic behavior that was reversed by chemogenetic inhibition of D1-MSNs. Using RNA-seq of ribosome-associated mRNA, the authors found that 26 genes were dysregulated in the transgenic mouse D1-MSNs. Of these 17 were significantly dysregulated by VPA in the present study [***Slc9a3r1* (+3.0)**, ***Csf1r* (−1.4)**, ***Sparc* (−1.4)**, ***Mcam* (+1.7)**, ***Robo4* (−2.2)**, ***Emp2* (−1.6)**, ***Klf4* (−1.6)**, ***Rtn4rl1* (+2.0)**, ***Bdnf* (+2.8)**, ***C1qtnf1* (−1.4)**, ***Tmem108* (+1.4)**, ***Smyd2* (−1.3)**, ***Wnt7b* (−1.8)**, ***Palm3* (+1.9)**, ***Adora2a* (−1.3)**, ***Drd2* (+2.0)**, ***S1pr1* (−1.3)**]. As discussed above, another gene linked to the development of the DA system is ***Nr2f1* (−1.8)**, which was downregulated by 45% by VPA. It is of interest that all three DA receptor subtypes (*Drd1*, *Drd2*, *Drd3*) are linked to autism by GWAS (SFARI List) and that partial antagonists of the D2 DA receptor (encoded by *Drd2*) have demonstrated utility in reducing stereotypic behaviors in human subjects (Mandic-Maravic et al., 2022); ***Drd2* (+2.2)** expression was doubled by VPA in the present study. Tyrosine hydroxylase encoded by the ***Th* (−1.4)** gene is the rate limiting step in the biosynthesis of catecholamines including DA; *Th* is downregulated by about 35% in fetal brain exposed to VPA.

***Ppp1r1b* (+3.7)**, encodes the dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32). DARPP-32 amplifies and/or mediates many actions of cyclic AMP-dependent protein kinase at the plasma membrane and in the cytoplasm, with a broad spectrum of potential targets and functions within the dopaminergic system and throughout the brain (Yger and Girault, 2011).

Cholinergic system. Cholinergic projection neurons in the nucleus basalis and cholinergic interneurons in the striatum are specified in the embryonic forebrain beginning at about E10 in mouse (Allaway and Machold, 2017). This process is regulated by several transcription factors including ***Lhx8* (−2.7)**, ***Isl1* (−1.5)**, and ***Gbx1* (−2.3)** all of which are downregulated in response to VPA in the fetal mouse brain. In the developing forebrain, *Isl1-Lhx8* hexameric complexes promote cholinergic identity (Cho et al., 2014). Conditional deletion of *Isl1* depletes cholinergic projection- and interneurons resulting in abolition of cholinergic innervation of the cortex (Elshatory and Gan, 2008). ***Nkx2-1* (−2.5**, significant in females only), a homeodomain transcription factor, is required for the development of cholinergic septohippocampal neurons and large subsets of basal forebrain cholinergic neurons (Magno et al., 2017). The 33−67% decrease in the expression of these transcription factors induced in the fetal brain by VPA has the potential to alter the proper development of circuits critical for normal brain function. Indeed, the loss of these neurons due to lack of *Nkx2-1* causes alterations in hippocampal theta rhythms and learning and memory defects (Magno et al., 2017). In addition, *Nkx2-1* acting together with *Lhx6* (not affected by VPA), is necessary to specify pallidal projection neurons and forebrain interneurons in the medial ganglionic eminence (Sandberg et al., 2016).

Endocannabinoid system. ***Cnr1* (−2.0)**, encodes the primary endocannabinoid **(**eCB) receptor in the brain; *Cnr1* has been linked to autism in GWAS. eCBs have been reported to regulate a wide range of embryonic neurodevelopmental processes (Harkany et al., 2007; Gomes et al., 2020) including neural progenitor proliferation (Aguado et al., 2005), long-range axon patterning (Mulder et al., 2008), interneuron migration and morphogenesis (Berghuis et al., 2005), and neuronal fate specification (Harkany et al., 2008). A 50% decrease in expression of *Cnr1* would be predicted to alter the cellular responses to eCB and could disrupt normal fetal brain development (Chakrabarti et al., 2015).

Autism and Down syndrome (DS). While the cause of autism is unknown, DS is caused by triplication of human chromosome 21 (Hsa21), leading to the hypothesis that a 50% increase in gene dosage of one or more Hsa21 genes is the cause of DS symptoms. ASDs and DS share ID as a common feature; moreover, DS individuals are diagnosed with ASD at a higher frequency than the general population (Hepburn et al., 2008; Rachubinski et al., 2017). In fact, several genes triplicated in DS that have been liked to autism or to prenatal brain development are up- or downregulated by VPA in the fetal brain; these include ***Dyrk1a* (−1.5)**, ***Brwd1* (−1.3)**, ***Cbs* (+5.0)** and ***Wdr4* (−1.4)** (Park and Chung, 2013; Duchon and Herault, 2016; Fulton et al., 2022; Marechal et al., 2019). ID in both ASDs and DS has been attributed to overexpression of *Cbs* (cystathionine beta synthase) (Conan et al., 2023; Tisato et al., 2021); dysregulation of *Cbs* and dihydrofolate reductase **(*Dhfr*, +1.5)** contributes to inborn errors of amino acid metabolism and is linked to ASDs (Zigman et al., 2021; Hoxha et al., 2021). *Dyrk1a*

(which encodes dual specificity tyrosine phosphorylation regulated kinase 1A) is associated with autism in all five GWAS (Table 1) and is believed to underlie intellectual disability in DS due to its increased gene dosage in DS (Duchon and Herault, 2016). In contrast, haploinsufficiency of *DYRK1A* results in a (non-DS) syndrome characterized by intellectual disability including impaired speech development, autism spectrum disorder including anxious and/or stereotypic behavior problems, and microcephaly (Levy et al., 2021; Dang et al., 2018).

Circadian rhythms. Eleven genes involved in generating circadian rhythms were significantly affected by VPA in the fetal mouse brain (Table 3). Two of these (*Per1* and *Per1*) are SFARI risk genes. While ***Per1* (+1.9), *Per2* +1.7), *Cry1* (+2.5), *Cry 2* (+2.0), *Arntl* (+2.0) and *Mef2d* (+1.5)** (Geoffray et al., 2016; Mohawk et al., 2019; Lorsung et al., 2021) were upregulated by VPA, *Clock* was not affected. ***Fbxl3* (−1.8)** a negative regulator of *Cry1* and *Cry2*, is downregulated by VPA whereas ***Fbxl21* (+3.2)**, which is up-regulated by VPA, down-regulates *Fbxl3* (Hirano et al., 2013). Thus, these VPA-induced changes lead to increased *Per* and *Cry* gene expression. ***Arntl* (*Bmal1*) (+2.1)**, together with *Clock*, activates rhythmic transcription of *Per* and *Cry* genes; knockout of *Arntl* (*Bmal1*) induces autistic-like behavior and cerebellar dysfunction which was ameliorated by mTORC1 inhibition (Liu et al., 2022).

Daily rhythms in the fetal brain are thought to be entrained by the mother, however, whether there is a functional *Per/Cry/Clock* cycle in the fetal brain has not been explored. It is possible that disrupting the expression of these genes could alter early embryonic brain development through a mechanism unrelated to sleep. For example, (Noda et al., 2019) reported that *in utero* knockdown of *Per3* caused abnormal positioning of cortical excitatory neurons as well as impaired axon extension and dendritic arbor formation.

If VPA-induced changes in circadian rhythm gene expression were to persist postnatally, they could cause disrupted sleep patterns, a common occurrence in autism. Indeed, exposure of fetal rats to VPA on E12.5 caused sleep disturbances that were similar to those reported in autistic children (Cusmano and Mong, 2014); moreover, a VPA-induced reduction in GAD-67 (*Gad1*) was also found in the juvenile rat brains. In the present study, ***Gad1* (−2.5)** was reduced by 60% while RNAs encoding GABA-A and -B receptors subunits, encoded by ***Gabra4* (+2.5)** and ***Gabbr2* (−2.4),** were found to be upregulated 2.5-fold and downregulated 60% by VPA, respectively.

Neuroinflammation. Immune dysregulation is believed to play a key role in the pathogenesis of autism. Neuroinflammatory molecules such as insulin-like growth factors [***Igf1* (−3.2), *Igf2* (−2.7)**], transforming growth factor-β isoforms [***Tgfb2* (−2.4), *Tgfb3* (−2.0)**], and the TGF-β receptor, ***Tgfbr2* (−2.5)**, are decreased in fetal brain in response to VPA exposure. Expression of several heat-shock proteins [***Hsp1a* (+5.9), *Hsp1b* (+5.6), *Hsp2* (+6.2) and *Dnajc12* (+4.5)**] was dramatically increased by VPA

exposure; this may be a response to immune dysregulation following the administration of a high dose of VPA**.** *DNAJC12* encodes a chaperone protein responsible for the proper folding of phenylalanine hydrolase and *DNAJC12* deficiency causes hyperphenylalaninemia leading symptoms ranging from mild autistic features or hyperactivity to severe intellectual disability (Blau et al., 2018; Anikster et al., 2017). ***Cd200* (−2.0)** is a surface glycoprotein expressed by neurons and its receptor, *Cd200r*, is expressed on microglia. Disturbances in this signaling pathway result in microglial activation and can lead to neuroinflammation (Zawadzka et al., 2021) as is observed in MIA, a cause of autism.

Epigenetic regulation of gene expression in the action of VPA. The mechanism(s) by which VPA alters gene expression in the fetal brain is not known; however, VPA is a Class I histone deacetyase (HDAC) inhibitor, suggesting that many of the changes reported here may involve increased histone acetylation (Bourin, 2020; Gurvich et al., 2004). Indeed, Konopko et al. (2017) reported that VPA increased the acetylation of several histones at the promoter regions of *Bdnf* 5' untranslated exons 1, 4, and 6 and that this was positively correlated with increased expression of exon 9, the protein coding region.

It is also likely that many of the genes targeted by VPA are themselves epigenetic writers, erasers, or readers; moreover, there is well-established crosstalk among epigenetic marks (Berger, 2007), including between DNA CpG methylation and covalent histone modifications, which could explain why HDAC inhibition could both increase and decrease gene expression. ***Setd1b* (−2.2)** encodes a lysine-specific methyltransferase which has been linked to autism in GWAS studies (Table S3). De novo variants of *SET1B* are associated with intellectual disability, ASD and epilepsy (Hiraide et al., 2019; Roston et al., 2021). ***Kmt5b* (−6.5)** encodes the epigenetic writer, lysine methyl transferase 5B, which methylates lysine residues on histones, resulting in gene activation or silencing, depending on the histone and lysine residue. Loss of function mutations in *KMT5B* lead to developmental delay and ASD (Eliyahu et al., 2022) (SFARI List). *Kmt5b* expression is reduced by 85% in fetal mouse brains exposed to VPA (Table S3). Pathogenic mutations in ***Ehmt1* (−1.4)** (SFARI List), a lysine methyltransferase, and ***Smarcb2* (−1.7)**, an actin-dependent regulator of chromatin, are causative for Kleefstra Syndrome Spectrum, an ASD; reduced expression of these genes leads to increased neuronal excitability (Kleefstra et al., 2012; Frega et al., 2020).

***Mecp2* (−1.6)** is an X-linked gene that encodes the epigenetic reader, methyl cytosine binding protein 2**.** Mutations in *MECP2* cause Rett syndrome, an ASD that affects mostly girls (Chahrour and Zoghbi, 2007); *MECP2* is on the SFARI autism list. MeCP2 binds to methylated CpGs in DNA mediating gene repression (Ip et al., 2018, Gonzales and LaSalle, 2010; Guy et al., 2011). Either increased or decreased expression of MeCP2 can cause developmental brain disorders. MeCP2 binds to CpGs

throughout the genome; reduced MeCP2 would be expected to alter the expression of many genes. One such gene is ***Bdnf* (+2.8)**. MeCP2 binds to CpGs in the 5'UTR of exon 4 of *Bdnf* leading to suppression of BDNF expression (Martinowich et al., 2003). Reduction of MeCP2 as observed with VPA (Table S3), would be predicted to decrease normal epigenetic suppression of CpG methylated genes. Although reduced *Mecp2* expression induced by VPA would be expected to increase BDNF expression, VPA did not alter CpG methylation in the regulatory regions of *Bdnf*; instead, increased *Bdnf* expression was due to increased histone acetylation and methylation (Konopko et al., 2017).

Paulsen et al. (2022) reported that haploinsufficiency of the chromatin remodelers, *Kmt5b, Arid1b* and *Chd8*, increased the numbers of inhibitory GABAergic neurons in organoid models of human cortex leading to reduced spontaneous circuit activity. In the present study, ***Kmt5b* (−6.5)** and ***Arid1b* (−2.1)** were downregulated in the fetal brain by 85% and 50% respectively, in response to VPA. Although *Chd8* expression was unaffected by VPA, ***Chd2***, ***Chd3*** and ***Chd7***, which have similar chromatin remodeling functions (Marfella and Imbalzano, 2007), were reduced by 23%, 50% and 23% respectively.

Limitations:

1. The results reported here are restricted to VPA-induced changes in RNA levels; the extent to which these changes reflect commensurate changes in the expression levels of the proteins encoded by these genes is not known. Although in many cases, RNA and protein levels are correlated, the extent to which RNA levels of a given gene track its corresponding protein levels may vary.
2. These results represent VPA-induced changes in gene expression at a single point in time (3 hr after VPA administration on E12.5). Whether the changes are transient and reverse as the VPA levels dissipate, as has been reported for *Bdnf* (Almeida et al., 2014), or are persistent, is not known. It is also not known whether the same VPA induced changes would be observed after VPA administration on a different gestational day.
3. There are many genes for which expression is increased or decreased by ≥ 1.25-fold or ≤ −1.25-fold in response to VPA but the FDR-corrected p-value did not reach the $p_{FDR} \leq 0.025$ criterion applied in this study (Supplemental Table S1). Higher statistical power would be needed to determine whether these changes are significant.
4. The ± 1.25 FC cutoff used here is arbitrary; it is possible that smaller changes in the expression of one or more critical genes have profound effects on fetal brain development and these would not be identified in this analysis.
5. Only a fraction of the approximately 7,300 genes whose expression is significantly affected by VPA were curated in this study. It is likely that other genes, in addition to those shown in Tables S3 and S4, could play a role in mediating the effects of VPA on fetal brain development.

Reproducibility:

The stimulation by VPA of *Bdnf* expression (Table S4) has been independently confirmed by quantitative RT-PCR (Almeida et al., 2014; Konopko et al., 2017).

By separately analyzing gene expression in male and female brains, the results of this study were effectively replicated with independent biological samples (7 of each sex ± VPA, each from a different pregnancy). With very few exceptions, male and female gene expression levels were similar (c.f., Figure 1) demonstrating reproducibility across independent samples.

SUMMARY:

The approach for this study can be described as "hypothesis generating" in that we began without any preconceived idea (hypothesis) as to the mechanism by which a brief, transient dose of VPA, administered to the pregnant mouse at E12.5, can cause abnormal, autistic-like behavior in her offspring many weeks later. The analysis identified approximately 7,300 genes, expression of which was significantly affected by VPA. This set of genes was narrowed down in two ways, viz., identifying those genes significantly affected by VPA that were a) also linked to autism by GWAS (SFARI List; Table S3) or b) not on the SFARI list (Table S4), but known to play a role in critical steps of early brain development such as neuroprogenitor proliferation and fate determination, axon and dendrite growth, and synaptogenesis. Interference with one or more of these steps in the fetal brain has the potential to interfere with the "program" directing brain development to create a persistent pathological "signature" that leads to abnormal neuronal circuitry in the adult, long after the increase in VPA in the fetal brain and the initial changes in gene expression have dissipated. Of these 651 genes, about half have known mechanisms of action associated with brain development or function; none showed significant sexually-dimorphic effects of VPA. Several genes affected by VPA in the fetal mouse brain appear in multiple GWAS studies (c.f., Tables 1 and 2) including *Adnp*, *Arid1b*, *Ash1l*, *Cdh2*, *Cdk5l*, *Dyrk1a*, *Kmt5b*, *Mecp2*, *Nr2f1*, *Pogz*, *Pten*, *Tbr1*, *Tcf7l2*, and *Wac*. This "short list" is a potential starting point for future hypothesis-driven studies to determine whether dysregulation of one or more of these genes by VPA is a proximal cause of the behavioral abnormalities identified in the adult animals. Nevertheless, it remains possible that dysregulation of one or more of the other genes altered by VPA contributes to the proximal cause of the autistic-like behavior. This hypothesis could be tested by determining the effect of manipulating gene expression (singly or in combination) on VPA-induced behavioral abnormalities.

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