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 RNAseq data obtained from fetal mouse brains 3 hr after VPA administration revealed that VPA significantly [p(FDR) ≤ 0.025] 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 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 400 autism risk genes was significantly altered by VPA as was expression of 247 genes that have been reported to play fundamental roles in the development of the nervous system, but are not linked to autism by GWAS. 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 neurodevelopmental disorders such as autism.

INTRODUCTION:

Autism is a neurodevelopmental disorder 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 the 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), increase the incidence of autism and other neurodevelopmental disorders 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 syndromic ASD 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 (RNAseq). In the majority of those animal studies, the fetus was exposed to the drug during mid-gestation, but RNA expression was analyzed in adult animals, typically following behavioral testing (e.g., see Qian et al., 2018; Zhao et al., 2019; Matsuo et al., 2022; Zhang et al., 2018). 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 are likely 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 genes 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 about 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 RNAseq three hours after VPA administration to the pregnant dam; male and female fetuses were analyzed separately. Using this protocol, 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 400 are among the 1,115 genes on the SFARI List and at least 247 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 (400mg/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 RNAseq 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:

RNAseq. 19,721 individual genes were analyzed in fetal mouse brain by RNAseq. 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 Supplementary Table I (S1). 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 Supplementary Table 2 (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 tabs 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 400 common genes (Table 1). 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 247 genes 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 (Tables 2 and 5). 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.

Table 1

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
Abat	8490	1.58	2.4E-08	1.68	1.7E-10
Abca7	4344	2.59	3.7E-24	2.58	7.2E-24
Abce1	14484	-1.25	2.2E-03	-1.20	1.3E-02
Abl2	6428	-1.33	1.3E-03	-1.25	1.4E-02
Ache	892	1.73	1.1E-03	1.82	3.1E-04
Actb	107135	-1.36	2.4E-08	-1.48	4.5E-13
Actn4	15736	1.43	3.2E-05	1.37	2.6E-04
Acy1	1770	1.70	2.3E-17	1.68	1.2E-16
Ada	284	1.30	1.9E-02	1.35	7.7E-03
Adcy3	1057	-1.69	1.4E-08	-1.81	1.1E-10
Adk	3332	1.30	1.4E-02	1.30	1.4E-02
Adnp	233	-1.63	4.0E-07	-1.58	3.0E-06
Adora2a	296	-1.25	4.9E-02	-1.36	4.9E-03
Ago3	5603	1.60	3.5E-07	1.90	1.9E-12
Agtr2	720	-6.44	2.7E-04	-4.45	4.1E-03
Ahi1	10423	1.57	1.1E-04	1.60	5.6E-05
Ahnak	9132	-3.07	1.2E-07	-2.64	5.6E-06
Akap9	18776	-2.30	5.3E-09	-1.85	2.4E-05
Aldoa	19582	1.32	1.1E-03	1.25	1.2E-02
Ankrd17	2867	-1.72	7.9E-13	-1.66	2.6E-11
Anxa1	480	-4.00	4.4E-05	-3.16	8.3E-04
Ap1s2	5451	1.35	1.5E-03	1.33	2.7E-03
Apba2	6951	-1.57	2.4E-04	-1.59	1.3E-04
Aph1a	4926	1.43	2.2E-08	1.31	2.6E-05
Arf3	12109	1.27	5.6E-06	1.20	9.6E-04
Arhgef2	9820	-1.95	1.2E-15	-2.08	9.6E-19
Arid1b	5659	-2.13	1.2E-16	-2.17	2.7E-17
Arid2	11217	-1.62	2.8E-15	-1.59	4.7E-14
Arntl2	511	1.71	3.1E-06	2.17	1.2E-11
Ash1l	9264	-1.69	1.4E-05	-1.40	6.9E-03
Asxl3	5208	-1.75	1.5E-10	-1.57	2.7E-07
Atp10a	473	-1.89	3.3E-16	-1.89	4.2E-16
Atp6v0a2	6136	1.42	2.4E-14	1.40	2.6E-13
Atrx	29927	-1.80	2.5E-06	-1.44	5.0E-03
Bace1	6271	1.83	4.3E-22	1.90	2.0E-24
Baiap2l1	386	3.78	2.5E-09	3.63	8.1E-09
Bcas1	279	-1.78	1.5E-03	-1.52	2.4E-02
Bcl11a	10078	-1.59	6.6E-05	-1.70	5.2E-06
Bcorl1	4918	1.89	1.9E-10	1.80	5.5E-09
Brinp2	2925	2.82	2.7E-12	2.61	1.2E-10
Brsk2	7140	-1.74	2.6E-04	-1.86	4.1E-05
Brwd3	6365	1.42	2.2E-03	1.66	4.9E-06
Btaf1	10163	1.41	1.9E-02	1.62	6.9E-04
Cacna1b	6214	1.34	4.7E-02	1.42	1.7E-02
Cacna1c	3446	-1.47	3.1E-03	-1.38	1.5E-02
Cacna2d1	9393	-1.84	2.7E-08	-1.59	3.0E-05
Cacnb2	498	-2.08	9.5E-13	-1.80	1.2E-08
Cadm1	12690	1.25	1.3E-02	1.28	5.3E-03
Camk2a	4142	1.94	1.6E-07	1.84	1.4E-06
Caprin1	3184	1.25	1.4E-03	1.31	1.0E-04
Cask	10174	-1.30	4.7E-03	-1.24	2.3E-02
Casz1	1306	2.43	2.5E-08	3.00	3.2E-12

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
Cc2d1a	2014	-1.38	3.6E-03	-1.44	1.1E-03
Ccdc91	1888	-1.95	2.9E-49	-1.82	4.0E-40
Ccng1	5463	1.49	2.9E-08	1.58	1.4E-10
Cd276	3140	-2.13	5.0E-36	-2.18	2.4E-38
Cd38	232	-1.84	8.7E-05	-1.90	3.5E-05
Cdc42bpb	14607	1.34	2.8E-04	1.31	9.7E-04
Cdh11	12820	-2.40	2.1E-06	-2.37	3.3E-06
Cdk19	6534	1.17	1.4E-02	1.25	3.0E-04
Cdk5rap2	8016	-1.37	6.2E-04	-1.27	1.2E-02
Cdkl5	3056	1.71	5.9E-08	1.95	9.5E-12
Cdon	13736	-1.37	4.4E-04	-1.20	4.7E-02
Celf6	1504	1.51	1.1E-03	1.44	4.8E-03
Cep135	2358	-1.51	7.3E-09	-1.38	9.7E-06
Cep41	1580	-1.91	2.3E-44	-1.94	1.8E-46
Chd2	12282	1.21	2.5E-02	1.35	3.5E-04
Chd3	39510	-1.98	9.7E-21	-2.00	3.8E-21
Chd7	13012	-1.35	1.4E-02	-1.33	2.0E-02
Chrna7	407.96	1.42	1.9E-04	1.28	9.1E-03
Clcn4	10609	-1.57	1.3E-06	-1.48	3.5E-05
Cln8	2506	1.77	2.3E-31	1.68	4.8E-26
Cmpk2	785	1.26	2.5E-02	1.26	2.4E-02
Cnr1	6596	-2.09	6.8E-05	-1.84	1.2E-03
Cntnap2	11429	1.42	7.8E-04	1.26	3.6E-02
Cpeb4	8073	1.60	9.9E-05	1.56	2.5E-04
Cpsf7	11294	-1.50	5.6E-09	-1.61	5.6E-12
Cpt2	1271	1.38	4.1E-04	1.29	5.1E-03
Crebbp	5496	-1.93	1.7E-10	-2.06	2.0E-12
Csde1	31901	-1.98	6.1E-40	-2.01	3.0E-41
Csnk1e	14294	-1.44	1.2E-06	-1.54	6.5E-09
Csnk2a1	16382	-1.27	5.8E-08	-1.26	8.2E-08
Csnk2b	5717	1.39	1.0E-11	1.32	1.4E-08
Ctcf	9148	-2.34	1.8E-38	-2.56	6.6E-47
Ctr9	13165	1.54	1.2E-27	1.54	1.4E-27
Cul7	10139	-1.69	4.5E-14	-1.80	1.2E-17
Cx3cr1	687	-2.05	1.2E-17	-2.28	1.3E-22
Cyfip1	6506	-1.45	1.4E-06	-1.45	1.6E-06
Ddc	823	-1.30	1.8E-02	-1.35	6.1E-03
Ddx3x	52188	-1.58	5.5E-09	-1.63	4.1E-10
Deaf1	3240	-1.47	4.7E-07	-1.57	2.5E-09
Depdc5	6414	1.60	1.5E-25	1.66	5.1E-29
Dhcr7	4286	1.56	6.0E-08	1.38	1.2E-04
Dhx30	9045	-1.55	3.6E-20	-1.65	7.1E-26
Dip2c	3203	-2.00	2.1E-11	-1.94	1.9E-10
Disc1	150	-2.17	8.3E-13	-1.71	1.1E-06
Dixdc1	6724	1.41	1.4E-06	1.50	1.0E-08
Dmpk	2879	1.32	4.3E-03	1.26	2.2E-02
Dnah10	231	2.35	4.2E-06	2.49	8.6E-07
Dnah17	157	5.09	4.6E-23	5.86	7.3E-27
Dnmt3a	15809	-2.22	4.4E-38	-2.11	3.4E-33
Dock1	4066	-1.49	7.5E-04	-1.38	7.5E-03
Dock8	232	-2.26	2.3E-04	-2.08	1.0E-03
Dolk	1049	1.29	1.9E-04	1.22	5.4E-03

Table 1 (cont)

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
Dpp4	163	-1.87	5.5E-03	-1.69	2.3E-02
Dpysl2	11973	-1.40	2.7E-02	-1.70	2.8E-04
Dpysl3	81721	-1.46	4.0E-03	-1.42	8.2E-03
Drd2	191	1.98	7.8E-06	1.92	2.1E-05
Dusp15	477	2.05	1.4E-05	2.24	9.4E-07
Dyrk1a	7472	-1.44	2.6E-08	-1.54	2.1E-11
Ebf3	12814	-1.68	2.6E-04	-1.60	1.1E-03
Ecpas	9645	-1.62	2.0E-10	-1.50	1.6E-07
Efr3a	5605	1.74	1.0E-15	1.91	5.7E-21
Ehmt1	11001	-1.32	3.2E-07	-1.44	5.5E-12
Ep300	10587	-1.39	1.2E-05	-1.31	4.0E-04
Epc2	5022	-3.53	7.0E-57	-3.45	8.2E-55
Ephb2	25546	1.44	1.4E-03	1.29	2.8E-02
Erbin	14418	-2.02	2.6E-26	-1.88	3.6E-21
Erg	1097	-2.37	4.0E-09	-2.44	1.2E-09
Exoc3	8530	-1.26	7.8E-06	-1.23	4.7E-05
Exoc6	1031	-1.67	5.4E-12	-1.60	2.1E-10
Ext1	5543	1.28	3.9E-04	1.29	2.6E-04
Fabp5	12171	-1.45	1.2E-07	-1.46	7.7E-08
Fam98c	976	2.59	4.5E-43	2.47	5.2E-39
Fbxo11	13248	-1.37	3.5E-04	-1.41	1.1E-04
Fbxo33	1584	1.59	1.5E-17	1.51	6.6E-14
Fezf2	1417	-1.73	5.8E-03	-1.99	4.1E-04
Foxp2	6899	-1.49	2.2E-08	-1.58	1.6E-10
Frk	338	-3.44	2.6E-06	-3.42	3.2E-06
Fxn	615	-1.96	1.2E-17	-2.04	1.4E-19
Gabbr2	570	-2.47	7.9E-03	-2.25	1.9E-02
Gabra4	739	2.46	3.0E-08	2.63	2.6E-09
Galnt10	4324	1.96	2.5E-16	1.91	4.3E-15
Galnt14	565	-2.75	7.3E-21	-2.53	1.2E-17
Gas2	1719	-1.55	2.7E-04	-1.45	2.1E-03
Gbe1	851	-1.62	4.7E-07	-1.67	7.3E-08
Gnai1	4191	1.42	4.4E-03	1.43	3.8E-03
Gnb1l	483	1.57	2.1E-15	1.60	1.3E-16
Grb10	45862	-1.51	5.4E-03	-1.49	6.7E-03
Grid2ip	169	2.70	6.3E-24	3.40	1.4E-34
Grik4	520	-1.44	1.2E-03	-1.38	4.7E-03
Grik5	7313	-1.31	2.2E-02	-1.40	3.6E-03
Grin2a	270	-2.28	1.5E-08	-2.27	2.0E-08
Gtf2i	23958	-2.36	1.2E-34	-2.54	8.0E-41
Hcfc1	17725	-2.30	2.7E-29	-2.29	4.5E-29
Hdlbp	25696	-1.29	9.1E-03	-1.25	2.5E-02
Hecw2	1165	-2.41	4.0E-22	-2.08	1.7E-15
*Hivep2**	2898	-1.46	1.3E-02	-1.32	8.0E-02
Hnrnpk	69304	-1.30	9.0E-05	-1.36	3.4E-06
Hnrnpu	3018	-1.59	3.3E-04	-1.59	3.4E-04
Homer1	5300	1.61	9.6E-20	1.63	7.6E-21
Ica1	848	-1.41	2.3E-04	-1.30	5.0E-03
Igf1	2781	-3.30	8.9E-04	-3.18	1.3E-03
Ikzf1	271	-1.58	1.5E-05	-1.59	1.2E-05
Inpp1	1129	-2.01	6.8E-32	-2.01	8.4E-32
Ints6	3398	-1.53	3.8E-20	-1.47	2.7E-16

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
Iqgap3	4143	1.40	1.9E-03	1.39	2.5E-03
Iqsec2	1627	1.47	8.2E-05	1.40	7.0E-04
Irf2bpl	5211	-1.30	2.7E-02	-1.52	3.4E-04
Itpr1	4428	1.77	6.2E-07	2.01	6.7E-10
Kansl1	8932	-1.43	1.0E-14	-1.47	3.6E-17
Kansl1l	2089	-1.80	1.7E-19	-1.61	4.1E-13
Kat2b	1923	-1.71	1.0E-09	-1.73	4.2E-10
Kat6b	7883	-2.68	1.9E-23	-2.43	3.8E-19
Katnal1	2730	-1.97	2.7E-24	-2.04	9.5E-27
Kcnb1	1903	1.44	4.3E-03	1.49	1.6E-03
Kcnc3	5043	5.14	3.4E-42	4.74	3.4E-38
Kcnj10	1277	2.72	9.7E-28	2.66	1.8E-26
*Kcnq2**	10691	-1.57	1.6E-02	-1.42	6.4E-02
Kdm1b	5673	2.26	3.4E-130	2.28	4.1E-133
Kdm5c	12138	-1.40	1.1E-14	-1.53	3.7E-23
Kdm6b	8176	-1.30	4.8E-02	-1.51	1.4E-03
Kif13b	3411	1.29	4.0E-03	1.48	4.3E-06
Kif14	2071	-1.46	4.8E-04	-1.48	2.9E-04
Klf16	1425	2.00	1.5E-16	1.78	8.3E-12
Klf7	6968	-1.26	2.5E-02	-1.27	2.3E-02
Kmt2e	18204	-1.33	3.4E-04	-1.36	8.9E-05
Kmt5b	6543	-6.45	2.2E-62	-6.59	9.4E-64
Kptn	941	-2.00	4.4E-45	-2.16	2.4E-55
Lamb1	8515	-1.45	4.2E-03	-1.39	1.3E-02
Las1l	6968	1.40	4.1E-24	1.37	1.2E-21
Ldb1	15935	-1.30	6.7E-05	-1.40	3.0E-07
Ldlr	2768	-1.36	3.2E-02	-1.57	1.1E-03
Lin7b	298	6.34	4.5E-69	6.45	1.5E-69
Lnpk	5745	1.28	3.6E-03	1.46	3.3E-06
Lrfn2	233	-1.63	3.9E-02	-1.67	3.1E-02
Lrp2	2856	1.43	3.2E-02	1.64	2.8E-03
Lrrc1	2446	1.64	7.0E-12	1.59	9.9E-11
Lrrc4	888	-3.27	1.1E-12	-3.64	8.2E-15
Lztr1	7298	-1.32	3.1E-15	-1.33	6.8E-16
Lzts2	3586	-1.20	3.2E-02	-1.33	5.7E-04
Maoa	3840	1.36	9.8E-05	1.39	2.0E-05
Map1a	10875	1.62	2.0E-04	1.61	2.3E-04
Map4k1	311	-1.27	6.1E-03	-1.37	3.2E-04
Mapk8ip1	12031	1.45	1.9E-07	1.37	1.0E-05
Mark1	5757	-1.39	3.0E-03	-1.42	1.7E-03
Mast3	433	2.89	1.1E-05	2.18	1.5E-03
Mbd3	6414	-2.01	1.9E-13	-2.27	3.7E-18
Mbd4	2576	1.30	1.3E-05	1.33	1.8E-06
Mboat7	5265	1.93	3.0E-26	1.91	2.5E-25
Mecp2	11258	-1.59	2.3E-05	-1.54	7.6E-05
Megf10	3621	-1.96	6.1E-22	-1.80	9.4E-17
Meis2	15231	-1.49	9.6E-04	-1.39	7.0E-03
Mink1	6672	1.33	6.4E-04	1.25	9.3E-03
Mrtfb	3074	-1.45	6.7E-10	-1.39	5.4E-08
Mtf1	1803	-1.16	2.5E-02	-1.25	5.6E-04
Mtor	10534	1.48	7.4E-11	1.56	1.1E-13
Myh9	16822	1.30	3.5E-02	1.29	4.4E-02

Table 1 (cont)

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
Mylk	1785	-1.20	4.4E-02	-1.25	1.5E-02
Myo1e	3165	3.35	1.4E-64	3.28	2.9E-62
*Myo5a**	17678	-1.36	7.3E-03	-1.22	9.8E-02
Myo9b	12403	1.43	3.5E-08	1.52	5.1E-11
Nacc1	8224	-1.15	1.4E-01	-1.29	3.2E-03
Nexmif	3129	-2.12	1.7E-03	-1.76	2.3E-02
Nf1	8492	-1.34	1.2E-04	-1.27	1.9E-03
Nfe2l3	803	4.19	8.5E-61	3.97	1.5E-56
Nfia	6750	-1.71	1.4E-03	-1.83	3.0E-04
Nfib	18590	-2.04	5.5E-04	-2.04	5.8E-04
Nfix	5859	-2.03	4.0E-03	-2.11	2.3E-03
Nipa2	4612	1.32	9.0E-08	1.36	3.8E-09
Nkx2-2†	355	-1.14	6.4E-01	-1.77	1.6E-02
Nr1d1	234	2.42	2.9E-08	2.09	4.4E-06
Nr2f1	15518	-1.63	9.4E-06	-1.93	1.2E-09
Nr3c2	254	2.04	7.0E-04	2.10	4.3E-04
*Nr4a2**	2860	1.35	1.7E-02	1.26	7.4E-02
Nsd1	20486	-1.74	2.9E-11	-1.58	4.4E-08
Nsd2	33101	-1.33	1.0E-04	-1.28	9.4E-04
Nsmce3	1585	1.44	1.8E-18	1.36	2.3E-13
Ntrk2	1174	-1.42	1.6E-02	-1.44	1.2E-02
Nxf1	17752	1.27	4.8E-08	1.26	1.6E-07
Nxph1	911	-2.60	8.0E-07	-2.48	3.3E-06
Ofd1	2004	-1.35	8.8E-09	-1.34	2.4E-08
*Ophn1**	3837	-1.28	1.2E-02	-1.18	1.1E-01
Patj	1326	-2.29	1.3E-31	-2.20	1.1E-28
Pcca	2377	1.50	4.9E-07	1.51	4.8E-07
Pccb	2484	-1.67	1.1E-47	-1.81	4.0E-63
Pcdh10	8761	1.47	4.4E-03	1.54	1.4E-03
Pcdha11	113	-2.30	2.5E-04	-1.94	4.1E-03
Pcdha3	113	-2.00	1.2E-03	-1.97	1.7E-03
Pcdha7	112	-1.90	1.3E-02	-2.01	6.8E-03
Pdk2	1859	2.00	1.8E-19	2.02	3.9E-20
Pdzd8	8176	1.44	5.7E-22	1.50	1.2E-26
Per1	3226	1.95	1.1E-11	1.77	8.2E-09
Per2	1092	1.71	1.0E-05	1.68	2.2E-05
Pex7	836	-2.40	3.4E-59	-2.40	1.2E-58
Phb	6881	1.29	2.3E-05	1.27	1.1E-04
Phf2	6735	-1.85	5.0E-08	-2.00	5.6E-10
Phf14	11218	-1.74	2.7E-18	-1.77	2.0E-19
*Phip**	21455	-1.44	1.2E-03	-1.22	9.0E-02
Pik3cg	194	-2.20	4.0E-09	-2.41	6.5E-11
Pitx1	157	-3.67	5.1E-03	-5.34	2.6E-04
Plxnb1	17385	-1.79	1.2E-08	-1.91	2.6E-10
Pnpla7	498	-1.27	2.0E-03	-1.28	1.4E-03
Pogz	8242	-2.18	2.3E-29	-2.25	8.9E-32
Polr2a	20372	-1.35	4.3E-04	-1.38	1.2E-04
Pola2	4393	1.67	7.1E-08	1.57	3.1E-06
Pomgnt1	3228	-1.63	1.2E-31	-1.69	7.8E-37
Pomt1	2351	-1.30	2.8E-07	-1.29	7.1E-07
Ppm1d	2918	1.33	2.6E-08	1.23	7.7E-05
Ppp1r1b	299	3.35	1.3E-04	4.05	8.5E-06

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
*Ppp1r9b**	13672	1.28	5.3E-03	1.17	9.4E-02
Ppp2r1b	7788	1.54	2.2E-11	1.44	2.0E-08
Ppp2r5d	10594	1.79	5.1E-25	1.68	2.5E-20
Prex1	6987	1.55	5.5E-08	1.62	2.4E-09
Prkd1	1487	1.33	9.2E-03	1.33	8.9E-03
*Prr12**	7437	-1.31	6.4E-02	-1.48	4.9E-03
Ptbp2	26867	-1.53	5.5E-08	-1.56	1.3E-08
*Ptchd1**	234	-1.83	1.6E-02	-1.65	5.1E-02
Ptdss1	5032	1.32	6.1E-07	1.38	8.6E-09
Ptpn4	9452	1.32	1.6E-02	1.60	3.4E-05
Prkd2	1619	1.69	3.1E-11	1.65	3.4E-10
Pten	12976	1.37	3.9E-06	1.37	3.9E-06
Ptk7	9855	1.29	1.8E-02	1.27	2.5E-02
Ptpn11	16665	1.31	9.8E-16	1.27	8.7E-13
Ptprb	1124	-1.77	2.0E-08	-1.83	2.6E-09
Pxdn	15773	-1.48	4.5E-05	-1.38	1.1E-03
Rab11fip5	3422	2.08	6.9E-20	2.09	3.7E-20
Rad21	24544	-1.30	9.2E-06	-1.37	7.5E-08
Rai1	7722	-1.57	2.5E-07	-1.56	3.3E-07
Ranbp17	928	-1.59	3.6E-07	-1.48	1.9E-05
Rassf5	168	1.54	2.2E-02	1.66	6.4E-03
Reep3	6903	-1.26	5.0E-10	-1.26	5.5E-10
Rfx3	5786	-1.47	5.0E-08	-1.45	2.2E-07
Rfx4	2512	-1.73	7.1E-04	-1.78	3.4E-04
Rims2	3497	-1.38	1.1E-02	-1.48	1.7E-03
Rims3	4156	1.95	2.0E-08	1.98	1.2E-08
Rnf135	168	-1.99	4.9E-08	-1.97	8.1E-08
*Rorb**	880	1.72	3.9E-04	1.32	8.8E-02
Rps6ka2	5307	1.80	8.1E-20	1.80	8.6E-20
Rps6ka3	11427	1.21	3.3E-03	1.29	3.3E-05
Runx1t1	7184	-1.62	4.1E-03	-1.61	4.8E-03
Samd11	170	-1.90	1.4E-04	-2.03	2.3E-05
Sbf1	12325	1.57	4.1E-08	1.53	2.8E-07
Scaf4	5878	1.30	4.1E-04	1.26	1.8E-03
*Scn9a**	2003	-2.28	2.2E-02	-1.29	5.3E-01
Sdc2	2896	-1.53	2.6E-02	-1.49	3.8E-02
*Sema5a**	8354	-1.30	1.9E-02	-1.16	2.1E-01
Set	42386	-1.41	4.4E-04	-1.59	1.3E-06
Setbp1	9254	-3.28	1.7E-29	-3.22	1.2E-28
Setd1b	4177	-2.04	1.6E-07	-2.28	1.1E-09
Setdb2	1135	1.56	1.1E-07	1.63	5.2E-09
Sgsm3	2524	1.76	8.5E-29	1.69	8.3E-25
Shank2	2071	-2.21	1.8E-05	-2.32	4.8E-06
Sik1	2688	1.25	1.0E-02	1.26	8.0E-03
Sin3a	12950	1.69	3.1E-27	1.62	8.5E-23
Slc22a15	1103	-2.14	3.7E-39	-1.90	6.7E-28
Slc25a12	5108	1.38	2.3E-10	1.35	6.1E-09
Slc25a39	4509	1.70	1.8E-14	1.57	9.8E-11
Slc29a4	3649	1.55	1.5E-05	1.54	2.4E-05
Slc38a10	11004	1.62	9.3E-17	1.52	6.0E-13
Slc6a8	5359	1.72	5.6E-29	1.62	4.3E-23
Slc7a3	316	2.75	5.8E-38	2.79	4.9E-39

Table 1 (cont)

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
Slc7a5	5758	1.57	2.8E-06	1.36	2.2E-03
Slc7a7	293	-1.48	5.4E-05	-1.62	4.5E-07
Slc9a6	7768	1.31	7.2E-03	1.39	1.1E-03
Slitrk5	2183	-1.56	6.1E-09	-1.59	1.4E-09
Smarca2	10932	1.25	3.1E-02	1.28	2.0E-02
Smarca4	29774	-1.59	5.2E-06	-1.69	2.5E-07
Smarcc2	27817	-1.55	1.9E-08	-1.63	4.3E-10
Smc1a	18407	-1.37	2.0E-06	-1.37	1.7E-06
Snap25	6289	1.64	8.5E-05	1.69	3.2E-05
Snd1	10450	1.27	3.4E-04	1.22	3.4E-03
Snx14	3275	1.73	5.1E-49	1.81	1.9E-56
*Son**	59465	-1.32	3.8E-03	-1.14	2.2E-01
Sox6	5597	-1.29	2.3E-02	-1.32	1.4E-02
Sparcl1	6073	-1.59	2.4E-04	-1.48	2.5E-03
Srcap	371	-3.68	8.6E-20	-3.26	1.9E-16
Ssrp1	22251	-1.31	7.7E-05	-1.43	8.9E-08
St7	2111	-1.57	5.2E-08	-1.50	1.5E-06
St8sia2	9960	-1.84	8.8E-05	-1.91	3.1E-05
Stag1	8175	-2.42	1.3E-51	-2.34	7.8E-48
Stx1a	2912	1.38	5.2E-03	1.36	7.0E-03
Stxbp5	4749	1.46	3.0E-06	1.50	4.8E-07
Syap1	4114	1.57	3.4E-09	1.54	1.7E-08
Sybu	2165	-1.37	4.5E-02	-1.44	1.7E-02
Syn2	1795	1.63	3.8E-02	1.78	1.3E-02
Syncrip	22048	-1.48	7.5E-10	-1.51	7.8E-11
Synj1	9528	1.46	1.1E-03	1.54	1.8E-04
Taf1	11862	1.26	3.9E-04	1.36	1.7E-06
Taf4	3798	1.37	4.0E-04	1.28	7.5E-03
Taf6	5567	1.28	8.3E-05	1.18	1.1E-02
Taok1	12512	-1.49	4.8E-06	-1.36	4.8E-04
Tbc1d31	3810	2.35	1.3E-34	2.40	3.0E-36
Tbc1d5	1509	-1.60	6.8E-26	-1.59	4.1E-25
Tbl1x	11400	-1.64	2.6E-32	-1.68	4.3E-35
Tbl1xr1	4323	-1.42	2.5E-07	-1.57	2.4E-11
Tbr1	3672	-2.68	2.6E-05	-2.85	7.8E-06
Tbx22	155	-4.84	3.4E-02	-8.10	4.5E-03
Tceal1	996	-1.82	6.2E-13	-1.66	1.7E-09
Tcf4	24203	-1.55	9.7E-07	-1.48	1.4E-05
Tcf7l2	10030	-1.73	1.7E-02	-2.47	5.4E-05
Tet2	6298	-1.41	3.8E-05	-1.30	2.0E-03
Tet3	11236	-1.39	1.7E-03	-1.50	8.5E-05
Thbs1	7121	-1.91	1.8E-02	-1.90	1.9E-02
Tlk2	6144	-1.42	1.2E-14	-1.44	8.3E-16
Tm9sf4	4866	-1.34	2.6E-21	-1.36	1.1E-23

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
Tmem39b	1217	-2.03	9.6E-28	-2.26	2.6E-36
Tnrc6b	8717	-1.41	1.8E-06	-1.32	1.3E-04
Tns2	1379	-3.34	4.5E-19	-3.35	3.7E-19
Top3b	3624	-1.49	1.6E-43	-1.49	2.3E-43
Traf7	9194	1.42	3.8E-11	1.32	1.8E-07
Trappc6b	2765	-1.19	1.6E-02	-1.27	6.3E-04
Trim33	11213	-1.29	6.9E-05	-1.24	9.5E-04
*Trio**	14535	-1.30	1.8E-02	-1.19	1.4E-01
Trrap	17379	-1.49	2.6E-06	-1.36	3.2E-04
Tsc2	11894	1.25	9.6E-04	1.25	8.7E-04
Tshz3	1699	1.28	4.1E-02	1.43	2.1E-03
Tspan17	1100	2.74	6.1E-25	2.80	7.2E-26
Tspan4	996	1.40	1.0E-03	1.36	2.8E-03
Ttn	914	-15.26	1.8E-03	-8.61	1.6E-02
Tubgcp5	3696	-1.29	2.7E-04	-1.21	8.0E-03
Ube2h	11325	1.37	7.8E-08	1.29	1.7E-05
Ubr1	5748	-1.47	1.3E-06	-1.37	9.7E-05
*Ubr5**	28114	-1.28	3.3E-03	-1.18	5.5E-02
Unc13a	10790	2.41	2.4E-07	2.38	4.1E-07
Unc79	2448	-2.25	1.4E-06	-2.15	5.9E-06
Upf2	7646	1.31	1.6E-13	1.39	5.9E-19
Usp15	8350	1.26	6.5E-04	1.37	1.2E-06
Usp2	685	3.08	1.5E-34	2.78	1.2E-28
Usp30	2549	1.25	4.6E-09	1.15	3.5E-04
Usp45	4065	1.41	1.6E-14	1.43	1.5E-15
Vdr	170	6.19	1.5E-10	6.71	2.3E-11
*Vezf1**	398	-1.31	4.8E-02	-1.11	4.8E-01
Vwa7	164	2.50	6.6E-09	2.60	1.4E-09
Wac	11365	-1.34	1.4E-10	-1.38	6.3E-13
Wdfy3	12067	-2.06	5.2E-11	-1.89	8.4E-09
Wnt1	77	-2.22	1.4E-02	-2.23	1.4E-02
Xpc	3233	1.40	2.2E-17	1.41	1.2E-17
Xrcc6	3705	1.36	7.6E-09	1.32	2.1E-07
Yeats2	8743	-1.43	3.0E-18	-1.38	3.4E-15
Ywhae	73412	-1.26	1.0E-04	-1.36	1.2E-07
Ywhaz	43502	-1.35	1.8E-10	-1.44	9.7E-15
Zbtb16	881	1.86	2.2E-04	1.70	1.7E-03
Zbtb20	6592	-2.52	2.0E-14	-2.13	5.5E-10
Zc3h4	6993	-2.62	3.8E-24	-2.79	3.1E-27
Zfyve26	2417	-1.46	1.9E-09	-1.33	1.0E-05
Zmiz1	19376	-1.45	1.1E-03	-1.53	1.8E-04
Zmym2	16595	-1.41	2.1E-06	-1.28	6.7E-04
Zmynd11	13113	-1.87	7.8E-23	-1.83	1.8E-21
Zmynd8	7708	-1.94	1.1E-19	-1.96	3.1E-20

* Significant effect of VPA in one sex only

Table 1. 400 genes that have been reported to be linked to autism by GWAS (SFARI list) that are also significantly up- or downregulated by VPA (from Table S2) in the fetal mouse brain.

Table 2

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
Adra2a	747	-1.66	2.3E-09	-2.01	1.1E-16
*Aif1**	113	-1.49	2.7E-04	-1.15	2.4E-01
Ak2	7116	1.41	2.2E-04	1.27	1.2E-02
Akap8	17422	-1.26	3.2E-05	-1.29	6.1E-06
Akap8l	8525	1.60	5.9E-12	1.61	2.6E-12
Akt1	15951	-1.40	5.5E-09	-1.45	4.7E-11
Akt2	4906	-2.03	2.9E-50	-2.08	5.6E-54
Amotl2	6480	-1.60	8.2E-11	-1.72	4.1E-14
Apbb1ip	142	-1.73	1.1E-04	-1.57	1.7E-03
Apc2	25582	-1.41	3.9E-02	-1.48	1.7E-02
Arc	170	1.64	1.7E-04	1.66	1.4E-04
Arntl	1203	2.17	8.8E-45	2.07	1.6E-39
Asxl1	9564	-1.39	7.4E-06	-1.42	1.8E-06
Bcl10	2338	1.68	2.6E-19	1.59	1.4E-15
Bcl11b	7112	-1.58	1.7E-02	-1.67	8.1E-03
Bdnf	423	2.57	9.7E-12	3.10	3.3E-16
Bmp2	291	-1.49	3.6E-02	-1.68	6.3E-03
Bola2	2477	1.59	2.6E-05	1.48	5.2E-04
Brca1	5043	1.43	4.0E-03	1.48	1.7E-03
Brd1	7073	-1.39	1.9E-04	-1.47	1.5E-05
Brd2	19021	-1.26	1.3E-04	-1.35	6.2E-07
Brwd1	13743	-1.37	5.2E-06	-1.29	2.3E-04
C1qtnf1	444	-1.45	2.7E-04	-1.43	4.5E-04
C2cd5	6100	-1.54	1.9E-20	-1.50	3.3E-18
*Cacnb4**	2101	-1.68	3.9E-03	-1.37	9.8E-02
Calm1	52234	1.44	1.0E-23	1.44	2.4E-24
Camk1	1971	-1.61	5.9E-24	-1.60	5.7E-23
Camkk1	2077	2.66	7.2E-41	2.53	6.9E-37
Camkv	1980	2.50	3.4E-28	2.30	1.1E-23
Carm1	6783	-1.40	1.9E-06	-1.52	1.4E-09
Casq2	132	-14.04	5.9E-04	-7.48	1.1E-02
Cbln2	2008	-1.82	1.5E-05	-1.71	1.2E-04
Cbs	736	5.02	2.1E-38	5.08	7.7E-39
*Cdh7**	13012	-1.35	8.4E-03	-1.24	6.8E-02
*Cdh15**	136	-4.45	6.9E-03	-2.61	9.7E-02
Clic6	1509	-1.80	3.4E-04	-1.58	6.3E-03
Col6a6	212	-4.15	3.8E-04	-4.13	4.2E-04
Creb3	2520	1.42	4.5E-15	1.43	1.2E-15
Cry1	3590	2.57	1.2E-98	2.41	5.4E-86
Cry2	3881	2.01	2.8E-27	1.94	1.1E-24
Csf1r	1501	-1.39	8.8E-04	-1.46	1.2E-04
Csgalnact1	179	-4.40	2.5E-08	-3.40	5.4E-06
*Cux1**	12750	-1.17	5.2E-02	-1.27	2.6E-03
Cxcl14	1869	-3.84	2.6E-04	-3.31	1.3E-03
Dab2	6440	-1.69	1.0E-02	-1.63	1.8E-02
Dcbld2	9176	1.74	2.6E-25	1.68	3.3E-22
Dcps	2121	-1.27	3.8E-03	-1.34	3.2E-04
Dhfr	4614	1.56	3.7E-04	1.38	1.1E-02
Dlg5	6522	-2.95	3.9E-61	-2.96	1.3E-61
*Dlx1**	5903	-1.77	1.2E-01	-2.29	2.0E-02
Dnajc12	1458	4.40	5.0E-179	4.66	1.6E-192
Dop1b	4122	1.41	4.7E-03	1.47	1.5E-03

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
Draxin	7093	-1.46	1.0E-02	-1.48	8.0E-03
Dyrk1b	1249	-2.21	3.1E-12	-2.12	3.7E-11
Ebf1	6893	-1.54	6.9E-05	-1.34	9.0E-03
Efna3	1500	2.61	1.2E-19	2.86	3.1E-23
Egfr	1567	-1.89	1.1E-02	-1.69	3.9E-02
Egr1	400	1.74	2.8E-04	1.71	4.8E-04
Elavl4	13824	-1.58	1.1E-05	-1.45	4.3E-04
Emp2	457	-1.49	4.9E-02	-1.62	1.6E-02
Eomes	3938	-1.81	1.6E-02	-1.86	1.1E-02
Ets2	2942	-1.45	8.6E-03	-1.42	1.5E-02
*Evx1**	102	-3.25	1.3E-02	-1.42	5.1E-01
Farp1	6684	-1.72	5.0E-11	-1.72	7.1E-11
Fbxl21	373	3.18	2.4E-56	3.23	2.4E-57
Fbxl3	4323	-1.77	5.5E-43	-1.70	2.2E-37
*Fezf1**	950	-1.20	4.6E-01	-1.68	2.3E-02
Fgf2	295	-2.94	3.9E-15	-2.79	1.1E-13
*Fgf17**	317	-1.64	1.2E-02	-1.45	6.85E-02
Fgfr1op2	6435	1.26	5.9E-05	1.31	3.4E-06
Flrt2	7693	-1.73	2.6E-08	-1.52	3.0E-05
Fos	108	1.92	1.0E-03	2.49	3.2E-06
Foxa1	549	-1.47	2.2E-02	-1.75	5.7E-04
Foxc1	1824	-1.79	6.9E-04	-2.13	7.9E-06
Foxo1	2595	1.84	2.8E-13	1.80	1.8E-12
Foxo3	4755	1.66	1.9E-11	1.70	2.6E-12
Foxo4	3776	1.37	1.8E-05	1.36	3.1E-05
Foxo6	990	1.56	1.2E-02	1.50	2.3E-02
Fzd5	608	2.35	1.0E-10	1.80	1.3E-05
Gabra1	591	-1.75	2.6E-03	-1.68	5.9E-03
Gad1	3462	-2.50	1.0E-04	-2.54	7.7E-05
Gad2	5532	-3.18	2.9E-08	-3.38	4.9E-09
Gas7	1662	-1.64	6.9E-03	-1.67	4.8E-03
Gbx1	311	-2.29	3.5E-03	-2.39	2.2E-03
Gch1	460	5.58	7.6E-65	5.25	1.8E-60
Gfpt2	1177	1.67	1.2E-07	1.78	2.5E-09
Gls2	319	4.06	2.3E-62	4.84	4.0E-75
Grb2	9573	1.44	5.9E-08	1.37	3.4E-06
Grm2	*321*	-1.39	2.8E-02	-1.49	7.1E-03
Grm3	*394*	-1.84	2.7E-03	-1.59	2.7E-02
Hapln4	275	27.66	3.9E-126	23.71	2.8E-119
Hes1	1561	-1.52	3.5E-06	-1.70	2.6E-09
Hnrnpab	47049	-1.53	2.7E-05	-1.75	1.5E-08
Hnrnph1	93936	-1.60	9.0E-14	-1.61	5.2E-14
Homer3	3874	-1.27	1.0E-02	-1.43	9.4E-05
Hrh3	823	2.58	5.1E-15	2.34	2.6E-12
Hsp1a	235	6.27	1.4E-31	5.59	6.7E-28
Hsp1b	412	5.93	3.1E-35	5.20	1.9E-30
Hsp2	996	6.34	1.0E-96	6.09	1.8E-92
Icam5	104	16.47	6.4E-39	16.73	3.0E-37
Isl1	3389	-1.54	1.4E-03	-1.45	6.9E-03
Itpka	175	17.28	1.1E-58	10.83	3.3E-44
JunB	206	2.19	5.8E-10	2.20	5.4E-10
Kbtbd2	4187	-1.24	1.2E-05	-1.31	2.0E-08

Table 2 (cont)

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
*Kcnmb2**	329	-2.11	5.5E-04	-1.45	1.1E-01
Kif17	387	4.17	2.5E-27	4.83	1.1E-32
Klf4	882	-1.56	1.3E-02	-1.59	9.9E-03
Kras	16185	1.38	2.1E-07	1.32	6.2E-06
Lamp5	587	-6.05	9.0E-04	-4.36	7.7E-03
Lhx8	610	-2.58	7.4E-03	-2.81	3.5E-03
Lmx1a	1011	-1.57	1.5E-03	-1.81	1.9E-05
*Lrp6**	10654	-1.26	1.0E-04	-1.14	3.4E-02
Lrrk1	709	-2.99	2.8E-13	-2.85	3.1E-12
Lrrk2	647	-2.40	1.1E-08	-2.20	2.9E-07
Maf	3212	-1.63	3.8E-06	-1.64	2.8E-06
Mafb	999	-1.57	8.5E-05	-1.71	2.2E-06
Map2	68999	-1.59	2.1E-04	-1.41	7.8E-03
Map2k1	4241	1.70	2.3E-18	1.67	3.6E-17
Mapk1	20963	1.33	3.2E-15	1.29	7.0E-13
Mapk14	7022	1.55	4.6E-40	1.52	9.9E-36
Marcks	64056	-2.10	3.1E-25	-2.26	2.7E-30
Mcam	4295	1.79	4.6E-17	1.65	5.1E-13
Mef2d	6108	1.54	1.3E-05	1.47	1.1E-04
Metrn	4092	2.39	5.0E-10	2.07	2.6E-07
Mllt11	16031	-1.56	2.5E-03	-1.49	8.0E-03
Ncoa3	5325	1.35	1.9E-06	1.38	3.7E-07
Ndst3	2340	-1.64	9.5E-05	-1.47	3.0E-03
*Nell2**	9177	-1.22	5.5E-02	-1.29	1.1E-02
Neurod1	2403	-1.43	1.4E-03	-1.58	3.5E-05
Neurog1	475	-2.00	4.6E-03	-2.49	1.5E-04
*Neurog2**	6066	-1.37	1.4E-01	-1.66	1.3E-02
Nfatc1	283	-1.52	3.3E-04	-1.63	2.2E-05
Nfatc2	689	-1.65	4.9E-03	-1.60	8.5E-03
Nfatc4	3812	-2.04	9.2E-09	-2.10	2.2E-09
Nhej1	194	-2.15	1.1E-16	-2.40	3.4E-21
Nhlh1	2918	-1.63	8.1E-06	-1.62	1.1E-05
Nhlh2	5362	-2.89	1.7E-16	-2.74	4.9E-15
*Nkx2-1**	2086	-1.74	1.2E-01	-2.46	8.6E-03
Nkx2-4†*	120	-1.30	2.9E-01	-2.42	8.7E-05
Nprl3	1675	-1.37	3.3E-11	-1.29	1.2E-07
Nras	15777	-1.36	4.5E-09	-1.42	1.2E-11
Ntn4	1222	-1.88	2.1E-07	-2.09	1.1E-09
Odc1	24222	1.49	5.0E-06	1.43	5.3E-05
Ogt	39637	-1.51	1.5E-06	-1.30	2.8E-03
*Olig1**	341	2.11	7.4E-05	1.49	4.5E-02
Otp	1318	-2.64	2.1E-08	-2.31	1.8E-06
P2rx7	210	-2.26	6.8E-06	-1.67	6.3E-03
Palm3	720	1.99	7.7E-06	1.89	4.2E-05
Pax7	1240	-1.74	3.4E-03	-1.68	7.0E-03
Pdk1	4064	1.24	1.1E-02	1.33	6.5E-04
Per3	1173	1.85	8.7E-21	1.92	5.5E-23
Pigh	1044	-1.39	5.5E-07	-1.38	8.7E-07
Pigp	2067	-1.62	3.5E-20	-1.60	1.9E-19
Pik3cb	2150	1.73	3.6E-22	1.77	1.2E-23
Pik3r4	3800	1.26	3.3E-05	1.24	1.7E-04
Plcg2	339	1.95	1.2E-07	1.74	1.6E-05

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
Plk1	3447	-1.54	5.4E-03	-1.83	7.0E-05
Pnisr	26071	-1.95	1.5E-24	-1.81	1.0E-19
Ppm1j	282	7.24	1.4E-36	7.60	4.4E-38
Ppp3cb	14492	-1.25	1.0E-02	-1.29	3.0E-03
Ppp3cc	516	-1.27	6.9E-03	-1.25	1.2E-02
Ptk2b	117	1.75	1.6E-03	1.68	3.7E-03
Ptpn1	7485	1.59	8.2E-10	1.45	1.3E-06
Ptpn5	4140	-1.59	1.2E-02	-1.49	3.7E-02
Ptpro	3982	-1.70	1.0E-02	-1.67	1.4E-02
Pttg1ip	4768	1.30	1.4E-04	1.28	3.9E-04
Pura	2394	1.32	2.7E-05	1.35	5.1E-06
Raf1	11657	-1.62	2.3E-24	-1.60	1.8E-23
Rarb	1030	-2.09	2.6E-05	-2.27	3.0E-06
Rcan1	3802	1.67	4.1E-27	1.59	4.6E-22
Rcan1	3802	1.67	4.1E-27	1.59	4.6E-22
Rmnd5b	2194	1.35	3.1E-10	1.32	7.3E-09
Rnd3	17191	-1.47	1.3E-07	-1.44	6.9E-07
Rnf19b	2770	1.38	7.2E-05	1.32	7.2E-04
*Robo3**	6624	-1.73	5.8E-03	-1.23	3.4E-01
Robo4	695	-2.11	1.5E-17	-2.21	1.1E-19
Rtn4rl1	469	2.04	4.0E-09	2.01	7.6E-09
Runx1	381	-3.19	1.6E-04	-2.98	4.3E-04
Runx2	782	-3.89	1.5E-05	-3.99	1.1E-05
Rxrb	4358	-1.27	5.6E-06	-1.34	3.0E-08
S1pr1	1963	-1.25	2.4E-06	-1.38	4.0E-12
Sema3a	2749	-2.24	2.5E-07	-1.77	3.5E-04
Setd4	686	-1.85	1.3E-19	-1.92	5.7E-22
Sh3bgr	135	1.74	1.1E-02	1.97	1.9E-03
Shc2	2467	-1.42	1.9E-05	-1.41	3.2E-05
Shc4	325	-1.69	1.4E-05	-1.50	1.0E-03
Shox2	3795	-1.95	6.0E-05	-2.26	8.6E-07
Sim2	224	-2.06	9.2E-03	-2.27	2.9E-03
Sirt1	3366	-1.49	5.2E-15	-1.48	1.1E-14
Slc17a6	5910	-2.41	2.9E-09	-2.42	2.4E-09
Slc17a7	847	1.63	1.3E-02	1.63	1.4E-02
Slc17a8	404	-1.72	1.1E-03	-1.43	3.8E-02
Slc17a9	221	5.87	1.2E-33	6.74	2.1E-38
Slc35e1	5103	1.48	2.7E-16	1.42	3.2E-13
Slc8a1	4042	-2.30	2.5E-05	-1.79	4.2E-03
Slc8a2	3842	1.99	4.1E-15	2.04	4.2E-16
Slc9a3r1	2902	3.04	2.4E-31	2.94	1.9E-29
Slit2	10361	1.31	2.6E-04	1.46	2.2E-07
Slitrk1	1872	-1.44	8.6E-03	-1.46	6.4E-03
Slitrk4	1131	1.62	2.8E-05	1.72	2.3E-06
Smyd2	1864	-1.24	3.7E-04	-1.28	4.3E-05
Sos1	9101	1.25	6.5E-03	1.29	2.0E-03
*Sox2**	10110	-1.21	3.3E-01	-1.50	2.4E-02
Sp1	8616	-1.26	7.3E-07	-1.20	7.9E-05
Sp2	1254	-1.49	2.2E-03	-1.63	1.5E-04
Sp3	9744	-1.51	3.1E-11	-1.49	7.0E-11
Sparc	25277	-1.37	2.6E-02	-1.40	1.8E-02
Src	5937	-2.49	4.8E-21	-2.70	7.6E-25

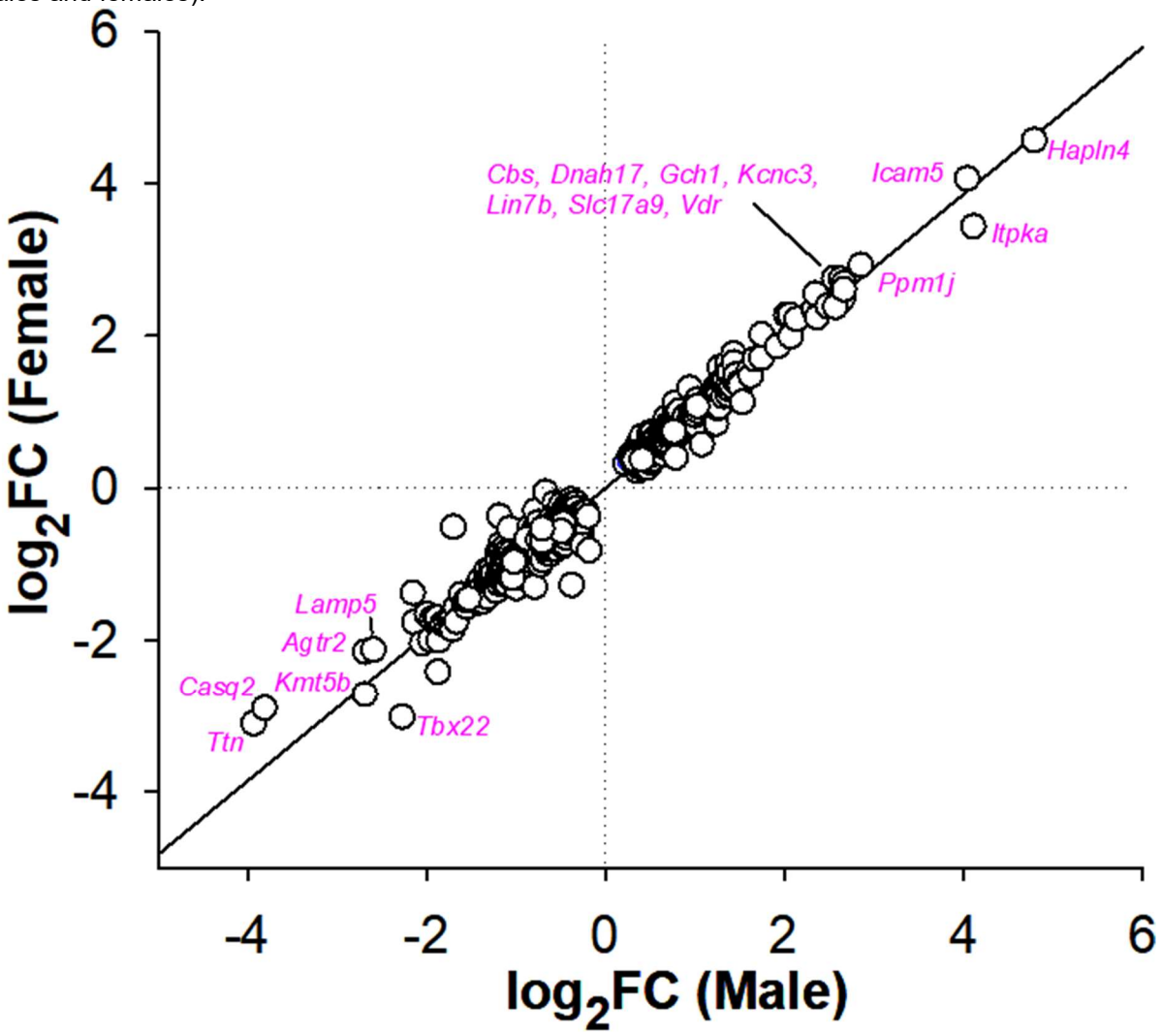
Table 2 (cont)

		Male		Female	
Gene	Counts	FC	p_{FDR}	FC	p_{FDR}
*Srebf1**	4265	1.38	5.8E-04	1.21	5.7E-02
Sst	803	-1.79	1.0E-02	-1.86	6.2E-03
Stat3	5850	2.72	1.2E-44	2.69	1.7E-43
Stc1	534	1.35	8.9E-03	1.35	9.0E-03
Stc2	494	2.83	2.1E-23	2.55	4.8E-19
Stx18	1175	-1.59	1.1E-09	-1.68	9.8E-12
Sumo3	6505	-1.39	4.8E-09	-1.51	3.1E-13
Tacc2	7124	-1.50	5.9E-06	-1.52	2.5E-06
Tctn1	4106	1.47	1.8E-08	1.41	6.7E-07
Tctn3	1997	1.94	6.3E-22	1.99	1.9E-23
Tfap2a	954	-1.85	2.8E-03	-1.71	1.1E-02
Tgfb2	2234	-2.52	4.1E-13	-2.34	2.9E-11
Tgfb3	1115	-2.03	1.7E-04	-1.96	4.0E-04
Th	476	-1.42	5.1E-03	-1.52	6.6E-04
Tlr3	167	-1.75	1.2E-10	-1.95	2.6E-14
Tmem108	1225	1.53	7.9E-06	1.37	1.4E-03
Tmem163	1496	-1.62	1.4E-05	-1.68	2.4E-06
Top3a	2357	1.71	1.3E-20	1.66	1.2E-18
Trib2	8489	-1.73	3.2E-07	-1.73	3.4E-07
Trnp1	298	1.45	3.8E-02	1.55	1.4E-02
Trpm4	1776	2.04	1.2E-15	2.11	5.0E-17
Tubb3	58234	-1.25	1.8E-02	-1.27	1.4E-02
Tug1	14599	-1.19	1.6E-04	-1.26	2.8E-07
Unc5b	3707	1.69	4.9E-08	1.41	4.4E-04
Unc5c	4631	-2.20	1.5E-06	-1.86	2.1E-04
*Usp34**	19191	-1.41	6.4E-03	-1.17	2.7E-01
Vegfc	373	-1.52	4.0E-06	-1.73	1.1E-09
Vegfd	111	-2.21	2.6E-04	-1.91	3.5E-03
Wdr4	3027	-1.43	2.4E-11	-1.47	9.8E-13
Wdr41	1513	-2.70	1.1E-116	-2.73	2.1E-118
Wnt10b	116	-3.73	2.4E-03	-3.29	6.8E-03
Wnt16	127	2.71	1.2E-03	3.13	2.0E-04
*Wnt3**	639	-1.19	1.3E-01	-1.37	5.3E-03
Wnt3a	207	-3.64	1.6E-12	-4.01	2.7E-14
*Wnt4**	470	-1.70	2.3E-02	-1.39	1.8E-01
Wnt7b	4194	-1.68	1.4E-06	-1.97	1.5E-10
Wsb1	31033	-1.42	6.3E-08	-1.35	2.8E-06
Zfp804a	553	-1.88	3.6E-03	-1.68	2.0E-02
Zmym4	12662	-1.53	7.8E-14	-1.45	5.7E-11

* Significant effect of VPA in one sex only

Table 2. 247 genes involved in brain development and neurodevelopmental disorders that are dysregulated in the fetal brain by VPA (from Table S2) that have ***not*** been reported to be as sociated with autism in GWAS.

No significant sexually-dimorphic effects of VPA. Fig. 1 is a plot of the $\log_2$ fold-change (FC) of males versus females for the 647 genes in Tables 1 and 2. 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.17 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 CaCh β-subunit
Calm1, calmodulin
Chrna7, α7 nAChR
Cnr1, CB1 eCB receptor
Dop1b, Dopey2
Eomes, Tbr2
Gad1, GAD67
Kcnc3, Kv3.3 K channel
Kcnmb2, BK Ch β-subunit
Map2k1, Mek
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
Slc7a5, large amino acid transporter
Slc8a1/2, NCX1/2
Syn2, synapsin II

DISCUSSION:

The overarching hypothesis for this research is that neurodevelopmental disorders involving cognitive function such as ASDs and intellectual disability (ID) are the result of abnormalities in the 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 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 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.

More than 7,300 genes were significantly up- or downregulated in the E12.5 fetal brain 3 hr after exposure to VPA (Table S2). 400 of those genes have been linked to autism in GWAS (Table 1), although in many cases the mechanism of action is unknown. In addition, at least 247 of the genes dysregulated by VPA but not on the SFARI Autism Risk list (Table 2), 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 AND

b. known to be linked to autism in GWAS 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 would provide potential targets for future hypothesis-driven approaches to understanding the underlying proximal causes of defective brain connectivity in neurodevelopmental disorders such as autism.

It was not practical to vet each of the more than 7,300 VPA-dysregulated genes for a potential role in abnormal brain development. However, as described below, the 247 genes in Table 2 and some of the genes in Table 1 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 neurodevelopmental disorders. 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 3, many of the genes identified in these reports are also dysregulated by VPA in the fetal mouse brain (Tables 1 and 2). Genes discussed in the respective papers are listed in each column in Table 3; genes that are also dysregulated by VPA in the present study are shown in **bold**. Five VPA-regulated genes in Tables 1 and 2 that also appear in all five of the published reports are in **red**. (*Arid1b*, *Dyrk1a*, *Pogz*, *Pten*, *Tbr1.*) Six genes in Tables 1 and 2 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 3, three (*Adnp*, *Dyrk1a*, *Pten*) appear in Table 4 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 1). 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 2). All of these genes are on the SFARI autism risk gene list (Table 1) and the three seed genes were identified in all 5 reports analyzed in this study (Table 3). 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 3

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 3 (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 1 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 4). Shown in red ***bold*** are 11 of these genes (38%) that were significantly up- or downregulated by VPA (i.e., in Table 1). 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 3 and 4 revealed that *Pten* and *Dyrk1a* are common to the Lin et al., (2016) study and to all five of the GWAS. It is suggested that these two genes may be good candidates for investigating the molecular basis for the proximal causes of autism.

Table 4

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 4. 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 1). 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 1).

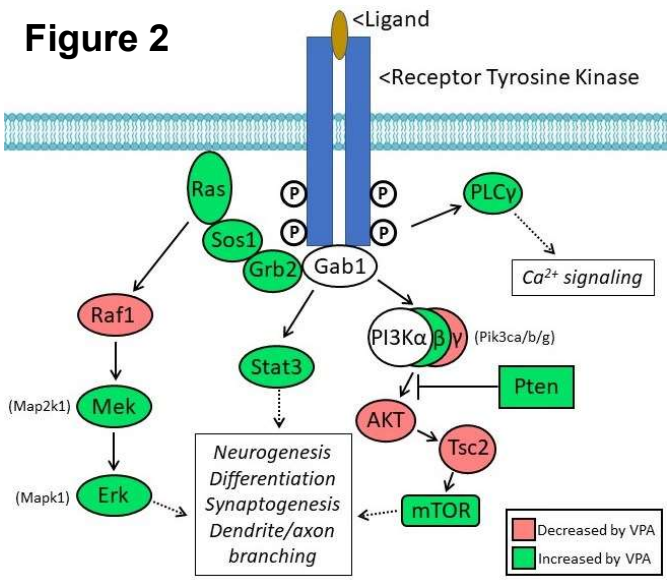
Biological roles of genes dysregulated by VPA. Table 5 (next page) shows the biological function of genes that were significantly dysregulated by VPA in the E12.5 fetal brain. One or more developmental errors caused by these changes could induce a "signature" of abnormal brain circuitry resulting in 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 5, the rationale for inclusion of the genes in the 18 categories is provided together with references to the literature.

Table 5

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)**, ***Hnrnpab*** **(−1.6)**, ***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	***Cdkrrap2*** **(−1.3)**, ***Ebf1*** **(−1.4)**, ***Eomes*** **(*Tbr2*) (−1.8)**, ***Fgf17*** **(−1.6)**, ***Foxc1*** **(−1.9)**, ***Lrrk1*** **(−2.9)**, ***Neurog1*** **(−2.2)**, ***Plk1*** **(−1.7)**, ***Trnp1*** **(+1.5)**
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)**, ***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	***Cbln2*** **(−1.8)**, ***Csde1*** **(−2.0)**, ***Ddx3x*** **(−1.6)**, ***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)**,
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)**, ***Hapln4*** **(+25.7)**, ***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)**, ***Dyrk1a*** **(−1.5)**, ***Cbs*** **(+5.0)**, ***Dop1b*** **(+1.4)**, ***Erg*** **(−2.4)**, ***Ets2*** **(−1.4)**, ***Pigp*** **(−1.6)**, ***Setd4*** **(−1.9)**, ***Shebgr*** **(+1.8)**, ***Sim2*** **(−2.2)**
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	***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)**

Table 5. The biological roles of genes that are dysregulated by VPA in the fetal brain and are associated with autism or prenatal neurodevelopmental processes (Tables 1 and 2). 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.

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 4) (Lin et al., 2016).



Twelve additional genes associated with intracellular signaling are included in Table 5. 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 variation 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).

Ca^{2+} 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 1) were dysregulated 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 (Table 1). *Cacna1c* is associated

with Timothy syndrome, an ASD characterized by ID as well as long QT syndrome, a frequently lethal defect in heart rhythm (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. The RA receptor ***Rarb*** **(−2.2)**, which encodes 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 include loss of GABAergic striatal projection neurons and interneurons (Chatzi et al., 2013), reduced expression of D2 dopamine receptors in the ventral striatum (Krezel et al., 1998) and reduced 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, RXRG and RARB, and genetic deletions of ***Rxrb*** **(−1.3)** and *Rarb* demonstrated that these enzymes 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 doubled the expression the D2 DA receptor, ***Drd2*** **(−1.9)**. 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 regulate 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 extracellular 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 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. ***Neurog1* (−2.2)** has been reported to play a role as a negative regulator of cortical neurogenesis (Han et al., 2018). ***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). The conversion from symmetrical to asymmetrical mitoses associated with the generation of post-mitotic neurons is regulated by the ***Plk1* (−1.7)** – ***Lrrk1* (−2.9)** – ***Cdk5rap2* (−1.3)** cascade (Hanafusa et al., 2015).

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 2). 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 in the fetal brain at E12.5 contribute to VPA-induced, 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.

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 3). VPA reduced *Tbr1* expression by 63% in the fetal mouse brain (Table 1). *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 wiring 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* also has 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).

<u>Axon growth/guidance</u>. *Marcks* (−2.2). Myristoylated Alanine-Rich C-kinase Substrate (MARCKS) 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).

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. ***Slitrk*s** 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, viz., *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 neurodevelopmental disorders (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 2). ***Cbln2* (−1.8)**. The cerebellins (CBLNs) are secreted glycoproteins that link presynaptic neurexins with postsynaptic δ1 glutamate receptors

(GluDs) 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* 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 1). *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 genes. 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 abnormal dendritic spine morphology and synapse formation (Guo et al., 2019).

***Hapln4* (+25.7)**, which encodes an extracellular matrix protein that is a component of perineuronal nets (PNNs), 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 massive, 25-fold increase in *Hapln4* induced by VPA and the expected alterations in PNN density could alter the connectivity of cerebellar circuitry leading to cognitive behavioral deficits.

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, was overexpressed by 14-fold in fetal brain exposed to VPA (Windhorst et al., 2012). Thus, the reduction in expression of *P2rx7*, *Tmem163*, *Sema3a* and *Farp1* and the increase in *Itpka* induced by VPA may work synergistically 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. ***Disc1* (−1.9)** (Disrupted in schizophrenia-1) has been implicated in schizophrenia. Normal expression levels of *Disc1* 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).

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 1) 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).

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 resulting 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. Taken together, these findings are consistent with VPA increasing net brain excitation by decreasing the number of

inhibitory interneurons generated during fetal brain development. ***Nf1* (−1.3)** encodes NF1, which is mutated in neurofibromatosis type 1, an inherited neurocutaneous disorder associated with neurodevelopmental disorders 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. These reductions in gene expression may contribute to a decrease in inhibition in mice exposed to VPA *in utero* (Markram et al., 2008).

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). The former 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 2), 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 RNAseq 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 liked 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 are up- or downregulated by VPA in the fetal brain; these include ***Dyrk1a* (−1.5)**, ***Brwd1* (−1.3)** and ***Cbs* (+5.0)** (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 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 5). 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. *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).

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 1). 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 1). ***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 1), 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 in 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 organoids. In the present study, ***Kmt5b* (−6.5)** and ***Arid1b* (−2.1)** were down regulated 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.

Caveats:

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 (Supplementary 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 1 and 2, could play a role in mediating the effects of VPA on fetal brain development.

Reproducibility:

The stimulation by VPA of *Bdnf* expression (Table 2) 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 1) and b) not on the SFARI list (Table 2), 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 change in gene expression have dissipated. Of these 647 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 3 and 4) 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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