Published in final edited form as:
*Int J Dev Neurosci*. 2013 December ; 31(8): . doi:10.1016/j.ijdevneu.2013.09.002.

# ACUTE PRENATAL EXPOSURE TO A MODERATE DOSE OF VALPROIC ACID INCREASES SOCIAL BEHAVIOR AND ALTERS GENE EXPRESSION IN RATS

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## Abstract

Prenatal exposure to moderate doses of valproic acid (VPA) produces brainstem abnormalities, while higher doses of this teratogen elicit social deficits in the rat. In this pilot study, we examined effects of prenatal exposure to a moderate-dose of VPA on behavior and on transcriptomic expression in three brain regions that mediate social behavior. Pregnant Long-Evans rats were injected with 350 mg/kg VPA or saline on gestational day 13. A modified social-interaction test was used to assess social behavior and social preference/avoidance during early and late adolescence and in adulthood. VPA-exposed animals demonstrated more social investigation and play fighting than control animals. Social investigation, play fighting, and contact behavior also differed as a function of age; the frequency of these behaviors increased in late adolescence. Social preference and locomotor activity under social circumstances were unaffected by treatment or age. Thus, a moderate prenatal dose of VPA produces behavioral alterations that are substantially different from the outcomes that occur following exposure to a higher dose. At adulthood, VPA-exposed subjects exhibited transcriptomic abnormalities in three brain regions: anterior amygdala, cerebellar vermis, and orbitofrontal cortex. A common feature among the proteins encoded by the dysregulated genes was their ability to be modulated by acetylation. Analysis of the expression of individual exons also revealed that genes involved in post-translational modification and epigenetic regulation had particular isoforms that were ubiquitously dysregulated across brain regions. The vulnerability of these genes to the epigenetic effects of VPA may highlight potential mechanisms by which prenatal VPA exposure alters the development of social behavior.



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**Keywords**

adolescence; autism; gene expression; sex differences; social interaction; teratogen

## 1. INTRODUCTION

During nervous system development, neurons exhibit periods of vulnerability to teratogens. One period of vulnerability is neuronal birthdate; *i.e.*, the day on which neurons undergo their final mitosis. In the rat, neurons in some of the cranial nerve nuclei are born on gestational day (G) 12 or G13. These include the principal sensory nucleus of the trigeminal nerve and the motor nuclei of the trigeminal, facial, and hypoglossal nerves (Altman & Bayer, 1980a; b; c). Exposure to ethanol on G12 or G13 results in a permanent reduction in the number of neurons in some cranial nerve nuclei (Mooney & Miller, 2007), as well as alterations in social behavior and gene expression (Mooney & Varlinskaya, 2011; Middleton *et al.*, 2012). The pronounced and permanent social deficits seen throughout adolescence and adulthood were most apparent in male offspring (Mooney & Varlinskaya, 2011; Middleton *et al.*, 2012). Specifically, males prenatally exposed to ethanol and tested as early adolescents, late adolescents or young adults exhibited a significant reduction of social investigation, contact behavior, and play fighting regardless of age. Older adolescent and adult males and females demonstrated social anxiety indexed by transformation of social preference into social avoidance.

Administration of another teratogen, valproic acid (VPA) at a dose of 350 mg/kg, during the same critical period also decreases neuronal number in cranial nerve nuclei (Rodier *et al.*, 1996) but the effect on social behavior, anxiety-like responses under social circumstances, or gene expression is unknown. For this reason we decided to use this dose to explore the effects of prenatal VPA. Many studies examining behavioral outcome after exposure to VPA use a high dose of the drug (500 – 800 mg/kg). In these models, animals show altered nociception (Schneider *et al.*, 2001; Schneider & Przewlocki, 2005; Schneider *et al.*, 2008), abnormal fear conditioning and increased anxiety (Markram *et al.*, 2008), repetitive, stereotypic-like behaviors (Schneider & Przewlocki, 2005; Markram *et al.*, 2008; Schneider *et al.*, 2008), decreases in social interactions (Schneider & Przewlocki, 2005; Markram *et al.*, 2008; Schneider *et al.*, 2008; Dufour-Rainfray *et al.*, 2010), and alterations in eye-blink conditioning that are similar to the changes seen in humans with ASD (Stanton *et al.*, 2007). Acute prenatal exposure to a high dose of VPA also has anatomical effects; for example, it reduces the number of Purkinje cells in the cerebellum (Ingram *et al.*, 2000), alters the location of serotonergic cells (Kuwagata *et al.*, 2009), decreases serotonin expression in the hippocampus (Stanton *et al.*, 2007), and alters cortical neuronal connectivity (Rinaldi *et al.*, 2008). Chronic prenatal exposure to a moderate dose (300 or 350 mg/kg) alters hippocampal synaptic plasticity (Zhang *et al.*, 2003), increases complexity of apical dendrite branching in motor cortex (Snow *et al.*, 2008), and decreases complexity of dendrite branching in hippocampal neurons (Raymond *et al.*, 1996). But high doses of this drug can also be toxic to the dam and/or cause fetal death (Vorhees, 1987).

VPA has several mechanisms. Its acute effects may be driven by increases of gamma-aminobutyric acid (GABA) concentrations in the brain (Dufour-Rainfray *et al.*, 2010) *via* inhibition of GABA transaminase (Rosenberg, 2007b). VPA can also have more sustained effects that are mediated by manipulation of DNA-processing and changes in gene transcription that result from its ability to act as a histone deacetylase inhibitor (HDACi) (Phiel *et al.*, 2001; Rosenberg, 2007a). Histone acetylation is a global mark and facilitator of gene activity (Brownell & Allis, 1996). Acetylation yields a negative charge, acting to neutralize the positive charge on histones and decrease the interaction of the N-termini of

histones with the negatively charged phosphate groups of DNA. As a consequence, the condensed chromatin is transformed into a more relaxed structure, which facilitates transcription. An even more prolonged influence proposed for VPA is through the induction of replication-independent DNA demethylation (Detich *et al.*, 2003b; a). VPA can reset stable DNA methylation patterns in established non-dividing cells and, therefore, can have wide-ranging and long-term effects on all cell types found in the brain and at any period of life (Detich *et al.*, 2003b; Rosenberg, 2007a). Equally interesting is the ability of VPA to also affect the level of acetylation of non-histone proteins (Mannaerts *et al.*, 2010).

Brain regions important for social behavior include the orbitofrontal cortex (OFC), the amygdala, and the cerebellar vermis, among others. A lesion made to the OFC or the amygdala can cause deficits in social behavior (Daenen *et al.*, 2002; Diergaarde *et al.*, 2004; Mah *et al.*, 2004; Rudebeck *et al.*, 2007). Cerebellar vermis forms connections with the limbic system (Strick *et al.*, 2009), and abnormalities of the vermis are associated with a number of disorders, including attention-deficit/hyperactivity disorder, schizophrenia, bipolar disorder, depression, anxiety, and autism (DelBello *et al.*, 1999; Ichimiya *et al.*, 2001; Loeber *et al.*, 2001; Kaufmann *et al.*, 2003; Mackie *et al.*, 2007; Picard *et al.*, 2008; Strick *et al.*, 2009).

In the present study, we tested whether acute exposure to a moderate dose of VPA during a critical period of gestation: (1) produces social deficits and increases social anxiety during adolescence and/or adulthood; and (2) alters gene expression in three areas of the brain that are important for normal social behavior. Different forms of social behavior and social preference/avoidance (an index of social anxiety-like behavior) (Varlinskaya & Spear, 2010) were assessed using a modified social interaction test (Varlinskaya *et al.*, 1999; 2001), which allows assessments of different components of social behavior, and of social motivation indexed *via* a coefficient of social preference/avoidance. Transcriptome expression was also evaluated at two levels: as an aggregate measure of each gene's overall expression which encompassed all expressed isoforms, and at the level of individual exons to identify particular differentially spliced isoforms of each gene.

## 2. METHODS

### 2.1. Animals

Timed-pregnant Long Evans rats (Taconic, Germantown NY) were injected intraperitoneally (i.p.) with 350 mg/kg VPA (Sigma; St Louis MO) or an equivalent volume of saline on gestational day (G) 13. G1 was designated as the first day on which a sperm-positive plug was identified. All procedures were approved by the Committee for Humane Use of Animals at Upstate Medical University and the Institutional Animal Care and Use Committee at the Syracuse Veterans Affairs Medical Center.

Within 24 hours of birth (on postnatal day (P) 0), litters were culled to ten, maintaining the ratio of male and female pups at 1:1 as well as possible. Pups were weaned on P21 and subsequently housed in same-sex groups of four littermates on a reverse light-dark cycle (*i.e.*, lights off from 6am–6pm).

### 2.2. Social Behavior Study

**2.2.1. Modified Social Interaction Test—**Animals were tested on P28, P42, or P75 (early and late adolescence and young adulthood, respectively) as described previously (Mooney & Varlinskaya, 2011). One male and one female from each litter were tested at each age, and each animal was only tested once ($n$=10 per sex/age for saline-exposed

animals, *n*=8 per age for VPA-exposed males, *n*=9 for VPA-exposed females tested at P28 or P42, *n*=7 for VPA-exposed females tested at P75).

Testing was conducted under dim light using Plexiglas (Binghamton Plate Glass, Binghamton, NY) test apparatuses. The test apparatus was divided into two compartments of the same size by a clear Plexiglas partition. The partition contained a semi-circular aperture that allowed animals to move between compartments such that only one animal was able to move through the aperture at a time (Varlinskaya *et al.*, 1999; 2001). The box used for adolescent animals was smaller than that used for adult animals (30 × 20 × 20 cm for adolescents, 45 × 30 × 20 cm for adults).

On the day prior to testing, each experimental animal spent 30 min alone in the testing apparatus. Allowing the animal to familiarize itself with the apparatus increases the frequency of social interactions in the later test situation (File & Hyde, 1978; File & Seth, 2003). On the test day, experimental animals were marked with indelible ink for later identification, and placed alone into a holding cage for 30 min, another way to increase social interactions. Animals were then placed into the testing apparatus. Five min later, a non-manipulated same-sex, same-age novel rat was also placed into the testing apparatus (Varlinskaya & Spear, 2002; 2008). Animals were video-taped for 10 min with the investigator outside the room. Between tests, the testing apparatus was cleaned with 3% hydrogen peroxide.

**2.2.2. Behavioral Measures—**Four separate behavioral measures were scored and analyzed: frequency of social investigation (sniffing of the partner), contact behavior (crawling under or over the partner, social grooming), and play fighting (following, chasing, playful nape attacks and pinning), and also social preference/avoidance (Mooney & Varlinskaya, 2011). Social preference/avoidance was assessed by scoring the number of crossovers (movements between compartments) demonstrated by the experimental animal toward the partner and away from the partner (for details see 5). In addition, the total number of crossovers from one compartment to another was used as an index of locomotor activity in the social context. The frequency of each behavior for each test subject within the 10 min period was noted (Meaney & Stewart, 1981; Thor & Holloway, 1984; Vanderschuren *et al.*, 1997; Varlinskaya *et al.*, 1999; 2001; Mooney & Varlinskaya, 2011). Behavioral data were scored by a trained observer without knowledge of experimental condition of any animal.

**2.2.3. Statistical Analysis—**Statistical analysis of each behavioral measure was performed using separate 2 (prenatal exposure) × 3 (test age) × 2 (sex) between-group analyses of variance (ANOVAs). Changes in social behavior induced by prenatal exposure to VPA were assessed by *post-hoc* comparisons (Fisher's planned least significant difference tests) between VPA-exposed animals and their age-matched saline-exposed controls. Statistical analyses were performed using Statistica software.

### 2.3. Microarray Study

**2.3.1. Tissue Samples—**Sixty to 90 minutes after behavioral testing, 75-day-old rats were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine, i.p.) and decapitated. Brains were removed and the forebrain was separated from the brainstem and cerebellum by a coronal cut between the colliculi. Forebrains were separated by a cut in the mid-sagittal plane. The cerebellum was removed from the brainstem by cutting through the peduncles. Brain pieces were frozen rapidly on dry ice, then stored at −80°C. Three male and three female rats, each from a different litter, were taken from each of the two treatment groups.

**2.3.2. RNA Extraction—**Segments enriched in three brain regions were obtained from each of the six saline-exposed animals and six VPA-exposed animals. Target brain regions included those that had been previously associated with social behavior: orbitofrontal cortex, amygdala, and cerebellar vermis. All tissue was taken from the left hemisphere, and dissections were performed using a rat atlas as a guide (Paxinos G., 2009). To isolate orbitofrontal cortex, the anterior 1 mm was sectioned from the hemisphere, the olfactory tubercle was removed, then a triangle of tissue was cut from the central part of the ventral half of the remaining tissue. The amygdala tissue was dissected from a 2 mm-thick slab taken from the middle of the forebrain. The slab was laid flat and a cut was made parallel and slightly lateral to the internal capsule. A second cut was made approximately 2 mm lateral to the first to separate much of the piriform cortex. A final cut was made in the horizontal plane 1 mm below the rhinal fissure. This resulted in a block of tissue enriched in amygdala. The cerebellar vermis was defined as the middle one third of the cerebellum; *i.e.*, the tissue separating the cerebellar hemispheres. The cerebellum and brainstem were isolated from the rest of the brain by a coronal cut made between the superior and inferior colliculi, then the cerebellum was removed by severing the peduncles. The hemispheres were cut away to reveal the vermis.

Samples were prepared for RNA extraction using a mortar and pestle to disrupt the tissue, then a QIAshredder (Qiagen, Valencia, CA) to homogenize it. RNA was extracted from each sample using an AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA). The purity, yield, and quality of RNA were evaluated *via* ultraviolet spectrophotometry and comparison of 28S:18S ratios using a Bioanalyzer RNA Nanochip (Agilent, Palo Alto CA). All RNA samples were of high quality and showed no signs of DNA contamination. RNA amplification was performed using the WT Expression Kit (Ambion, Austin, TX) in preparation for fragmentation and labeling.

**2.3.3. Gene Expression Quantification—**Genome-wide quantification of mRNA expression was accomplished using Affymetrix GeneChip Rat Exon 1.0 ST Arrays (Affymetrix, Santa Clara, CA). This chip contained approximately 40 probes per gene and four probes per exon, thus providing information sufficient for determining differential gene expression as well as alternative splicing between the control- and VPA-exposed rat groups. Hybridization and scanning of microarrays were performed as previously described (Glatt *et al.*, 2009).

**2.3.4. Statistical Analysis—**CEL files containing microarray probe intensities were imported into Partek Genomics Suite Software (Partek Inc., St. Louis, MO) for all subsequent analyses. Data for 80,908 probes on the microarray were summarized in two steps: 1) probes for the same exon were summarized by Tukey's biweight to get one measurement per exon; and 2) exons of the same gene were also summarized by Tukey's biweight to get one measurement per gene. Chips were quantile-normalized by robust multi-array average and probe intensities were *log*-2 transformed to yield data that more closely approximated normality. Probes with a signal:noise ratio less than 3.0 were not used in the analysis.

For analyses of gene (*i.e.*, full-length transcript) expression, data in each brain region (within-subjects) were analyzed jointly in a mixed analysis of variance (ANOVA) to isolate regionally specific and ubiquitous effects of treatment group (between-subjects). In addition to the main effects of prenatal exposure and brain region, we also modeled the interaction of these two terms, and compared the results of *post-hoc* analyses (one-way ANOVAs with prenatal treatment as the lone factor) in each brain region *via* intersection/union tests (IUTs). Due to the large number of tests performed and the small number of subjects, we would not anticipate observing results that surpassed a Bonferroni-corrected threshold for statistical

significance, nor would we ascribe much weight to nominally significant results for individual genes found to be dysregulated in only one brain region; therefore, the main outcome of these analyses involved the identification of gene ontologies, pathways, and protein domains that were over-represented among sets of genes dysregulated in each brain region and across brain regions. These pathway/ontology/domain analyses were conducted using DAVID (Dennis *et al.*, 2003).

For analyses of alternatively spliced transcript isoforms and their individual exons, separate models were employed. Prenatal VPA- and saline-exposed groups were compared on mean expression levels of all exons in each gene on a gene-by-gene basis through ANCOVAs and inspection of interaction terms, as described previously (Partek Incorporated, 2008; Glatt *et al.*, 2009). Briefly, treatment was added as a between-subjects factor, and since not all exons in a gene express at the same level, exon identity (ID) was added to the model to account for exon-to-exon differences. Also, since multiple measurements (on the multiple exons) come from the same subject, subject ID was added to the model to accommodate the assumption of independence that is fundamental to ANCOVA. The final term included was the interaction of exon ID with treatment group, which allowed for the detection of differences in the expression of one or more (but not all) exons in a gene between the two treatment groups (Partek Incorporated, 2008). The significance of these interaction terms (one per gene) was judged against a stringent Bonferroni-corrected threshold, and post-hoc *F*-tests were used to identify the specific dysregulated exon(s) in the genes showing significant interactions. The analysis was performed separately in each of the three brain regions and ubiquitously dysregulated isoforms were identified by IUTs of the results from each region. Ontological analysis of genes that show dysregulated alternative splicing was also conducted using DAVID for each brain region in order to discover enriched biological themes.

## 3. RESULTS

### 3.1. Litter outcomes

One-way ANOVA identified a treatment-induced difference in average pup weight (untreated animals 7.85 g, saline-exposed animals 8.07 g, VPA-exposed animals 7.20 g; $F_{2,25}$=3.627; $p$=0.043). A *post-hoc* Tukey B-test found that the difference in body weight of animals exposed to VPA compared with saline-exposed animals was just shy of significance ($p$=0.051). The number of pups and the proportion of males was unaffected by treatment. For all treatment groups, there was an average of 11.3 pups per litter ($F_{2,25}$=0.0402; $p$=0.961), and 50% of untreated or saline-exposed pups and 55% of VPA-exposed pups were male ($F_{2,25}$=0.339; $p$=0.716). We observed similar maternal culling between the different treatments, and dams seemed to normally retrieve pups into nest regardless of maternal exposure. This is in agreement with others who report that exposure to VPA during pregnancy does not alter maternal behavior (Mychasiuk *et al.*, 2012)

### 3.2. Social behavior study

A modified social interaction test was performed and four indices of social behavior as well as locomotor activity were scored and analyzed (Fig. 1). Play fighting differed as a function of age ($F_{2,97}$=13.54, $p$<0.001), with 42-day-old animals showing more play than their older and younger counterparts. Prenatal exposure to VPA increased this form of social interaction regardless of age (main effect of treatment, $F_{1,97}$=4.09, $p$<0.05).

For social investigation, there was also a significant main effect of test age, ($F_{2,97}$=5.55, $p$<0.01) with 42-day-old animals demonstrating more social investigation than rats tested on P28 or P75. Social investigation also differed as a function of prenatal treatment

($F_{1,97}$=7.05, $p$<0.01), with animals exposed to VPA demonstrating more social investigation than controls.

Contact behavior was not affected by prenatal treatment and differed as a function of age only ($F_{2,97}$=7.52, $p$<0.001), with 42-day-old adolescents demonstrating more contact behavior than younger and older animals.

The coefficient of social preference did not differ as a function of age ($F_{2,97}$=1.82, $p$=0.1673) or treatment ($F_{1,97}$=1.40, $p$=0.2398), with all animals demonstrating equivalent levels of social preference. Locomotor activity within the social context, as determined by the number of crossings from one part of the test box to the other, was not altered by treatment ($F_{1,97}$=1.51, $p$=0.2215) or by age ($F_{2,97}$=1.79, $p$=0.1716).

### 3.3. Microarray Study

Gene-expression analysis was done in the 75-day-old animals as this age targets those genes that most likely are permanently altered. No sex differences were seen in the behavioral analyses; thus, gene expression data from male and female rats were combined to maximize statistical power to detect the main effect of interest (VPA exposure).

Quality-control analyses on normalized transformed data involved inspection of sample histograms of *log2* expression intensities of each probe and box-plots of the mean and variance of *log*2 expression intensities, neither of which identified any outliers. The high degree of overlap in these parameters across all samples demonstrated that, after transformation and normalization, all samples had highly comparable distributions, thus allowing for their comparison *via* parametric inferential statistics. The first three principal components of transcriptome-wide gene expression were also visualized, identifying three distinct clusters of data segregated by brain region (Fig. 2a). Two samples (from two brain regions of the same subject) did not map to the appropriate cluster, which most likely reflected an experimental error of labeling or processing, therefore these two data points (as well as the third brain-region data point from this same subject) were removed from all further analyses. Data from each of the three brain regions clustered tightly together (Fig. 2a), but there was no clustering by prenatal treatment, suggesting that the exposure to VPA in pregnancy does not alter the fundamental and biologically critical correlations between expression levels of most genes. In other words, when certain genes are highly expressed, we would expect, biologically, that others would be highly expressed while some others would be expressed at very low levels. VPA exposure during pregnancy does not alter this fundamental biological co-expression and joint regulation; however, clearly the co-expression and joint regulation of sets of genes is different in discrete brain regions.

702 (8.0%) of the 8,762 genes represented by the nearly 81,000 probes on the utilized microarray showed a nominally significant main effect of prenatal treatment ($p$<0.05). Although none surpassed a strict Bonferroni-corrected significance level, this is not surprising given the small sample size of this pilot study. Critically, however, the top 163 results had a false-discovery rate $q$-value of just 0.40, meaning that while 40% of these results could be false-positives, 60% could be true discoveries. Yet, beyond simply identifying individual genes that were dysregulated by prenatal VPA exposure, we primarily hypothesized that VPA exposure would affect biologically related groups of genes, which was assessed using annotation-enrichment analyses in DAVID. Among the 702 nominally significantly dysregulated genes, there was a 1.5-fold enrichment of genes (121) that encode acetylation-sensitive proteins, relative to the ratio of acetylation-sensitive genes expected based on their number in the rat genome. This enrichment was highly significant ($p$=2.3$e^{-6}$), and remained significant even after applying the Bonferroni correction for the number of pathways, ontologies, and protein domains evaluated in DAVID (corrected $p$=1.0$e^{-3}$). This

finding lends support to our principal hypothesis that prenatal VPA exposure affects gene expression *via* its actions as a deacetylation inhibitor. Two other annotation categories survived Bonferroni correction, including "FAD-dependent pyridine nucleotide-disulphide oxidoreductase" (corrected $p=5.6e^{-3}$) which showed a 9.9-fold enrichment, and "negative regulation of catalytic activity" (corrected $p=2.8e^{-2}$) which showed a 2.6-fold enrichment.

Beyond the main effect of prenatal VPA treatment across brain regions, we also found that 247 (2.8%) of the 8,762 genes assayed showed a nominally significant interaction between prenatal treatment and brain region ($p<0.05$). Again, because of the small sample size and the relatively lower power to detect interactions than main effects in general, none of the detected interactions surpassed a Bonferroni-corrected significance level. Closer inspection of the regional distribution of those genes that did evince a significant interaction of brain region with prenatal treatment revealed some quite strong and consistent regional differences between the two prenatal treatment groups. As shown in figure 2b, 394 genes were significantly dysregulated in AA, 392 in CV, and 564 in OFC (Supplementary Tables 1, 2, and 3 respectively). There are also instances ($k=149$) where prenatal VPA exposure had effects on gene expression in two of the three regions (with greatest similarity between anterior amygdala and orbitofrontal cortex), as well as some instances ($k=5$) where the effects of VPA were persistent and ubiquitous across all three brain regions examined (Fig. 2b). Each of the five ubiquitously dysregulated genes exhibited a consistent effect (*i.e.*, changed in the same direction) across all brain regions (Table 1).

Brain region-specific ontological analysis of all nominally significant whole genes did not uncover enrichments that surpassed Bonferroni-corrected significance at any brain region. However, independent analysis of genes that displayed increased expression and genes that displayed decreased expression did identify enrichments that surpassed this correction (Table 3).

Alternative splicing was also evaluated by identifying genes that have differential expression of one or more exons in response to prenatal VPA exposure (Fig. 3). Unlike the analysis of full-length gene expression aggregating all isoforms, the exon-level analysis was able to detect several effects that surpassed Bonferroni-corrected significance thresholds, perhaps due in part to the fact that exon expression is better controlled via comparison to other exons in the same gene, and perhaps because exons (which index particular alternatively spliced isoforms) are more biologically meaningful elements than genes. Nominally significant interaction for alternative splicing and treatment was seen in 821, 821, and 820 genes in AA, CV, and OFC; of these 22, 11, and 11, respectively, survived Bonferroni correction (Supplementary Tables 4, 5, and 6 respectively). The differential exon expression of prolyl endopeptidase *(Prep)* in VPA-exposed subjects surpassed a Bonferroni-corrected level of significance in all three brain regions (AA, CV, and OFC). Further analysis of ubiquitous alternative-splicing anomalies revealed 51 genes that were nominally significant in all three brain regions (Table 2), many of which surpassed Bonferroni-corrected significance levels in one or two of the three regions.

Brain region-specific ontological analysis of genes that showed dysregulated alternative splicing revealed Bonferroni-corrected significant enrichments that were unique to the specialized brain region; however, most seem to span at least two, if not all three, brain regions (Table 3). While, "calcium ion binding" and "calcium ion transport" enrichments were found exclusively in the CV, "cell projection" and "vesicle" enrichments were found in three and two brain regions, respectively.

# 4. DISCUSSION

## 4.1 Behavior

In our study, acute prenatal exposure to a moderate dose of VPA appeared to enhance social behavior. Social investigation and play fighting occurred at a significantly ($p<0.05$) higher frequency in animals exposed to VPA than in saline-exposed animals at all ages of testing. In addition, social investigation, play fighting, and contact behavior differed as a function of age, with higher frequencies of these behaviors apparent in 42-day-old animals. All animals, regardless of prenatal treatment, showed social preference suggesting that prenatal exposure to VPA had no anxiogenic effects. Locomotor activity under social circumstances was also not affected by treatment or age.

The fact that exposure to a moderate dose of VPA (350 mg/kg) enhances social behavior is intriguing as it is in direct contrast to reports that social behavior is negatively affected by exposure to higher doses of VPA given at the same gestational age (Schneider *et al.*, 2001; Dennis *et al.*, 2003; Schneider & Przewlocki, 2005; Stanton *et al.*, 2007; Markram *et al.*, 2008; Schneider *et al.*, 2008; Dufour-Rainfray *et al.*, 2010). The different outcomes may be partly attributable to differences in the social behavior tests, but are also likely to be a function of the different doses used. It should also be noted that increased social behavior is as aberrant as decreased and thus, should not be considered an auspicious outcome. Combining the current data with that reported previously by others, we show that the dose of this teratogen defines the outcome.

**4.2.1 Gene Expression: Whole gene—**The microarray analysis identified a nominally significant main effect of prenatal treatment in 8.0% (702) of the 8,762 genes probed, and 2.8% (247) of genes showed a nominally significant interaction between prenatal treatment and brain region. Among the 702 genes that show main effect of treatment, there was a 1.5-fold enrichment of genes encoding acetylation-sensitive proteins, consistent with the role of VPA as a histone and non-histone deacetylase inhibitor (Phiel *et al.*, 2001; Mannaerts *et al.*, 2010). Our experimental outcomes represent the long-term effects of prenatal manipulations, thus, it is reasonable to consider these as epigenetic effects of VPA; however, manipulation of protein activity during critical periods can also lead to lifelong changes in gene expression (Detich *et al.*, 2003b; a). Brains for mRNA analysis were taken from animals after completion of a social behavior test; thus, the described gene effects may be an interaction of prenatal exposure to VPA and the sum of all postnatal experiences, including the social interaction test.

Brain region-specific whole-gene dysregulations are particularly interesting when considering the specialized behavioral-related function of each of the brain regions analyzed, however, none of our transcripts surpassed a Bonferroni-corrected level of significance when both up-regulated and down-regulated genes were used. VPA acts as a histone deacetylase inhibitor, thereby promoting gene expression, thus, we divided each brain-region analysis into separate groups of up- and down-regulated genes. Interestingly, acetylation-sensitive proteins showed enrichment in the down-regulated genes. This enrichment was significant in the AA and marginally significant in the CV. The AA and CV also showed similarities in enrichments from the up-regulated genes, perhaps signifying similar pathologies (Table 3). The enrichments found from whole-genes dysregulated in the OFC seemed to be the most localized, and were more obviously relevant to social interaction behaviors. This is likely due to the specialized cognitive function of the OFC. A closer look at the individual genes that make up these enrichments and their interaction with each other will be an interesting avenue for future work.

Five genes were ubiquitously dysregulated by prenatal exposure to VPA ; that is, they changed in the same direction across all brain regions examined. Of these, one was up-regulated and four were down-regulated in all three regions (Table 1). The gene that was up-regulated, *Kcnmb1 (Calcium-activated potassium channel subunit beta-1)*, is a regulatory subunit of the calcium activated potassium KCNMA1 channel, controlling both the sensitivity and gating kinetics of this channel (Meera *et al.*, 1996). Genes that were down-regulated did not fall into any defined categories: *Cttn* (Src substrate cortactin) plays a role in cell migration and adhesion (von Holleben *et al.*, 2011),, *Pcmt1* (Protein-L-isoaspartate (D-aspartate) O-methyltransferase) is involved in protein repair and degradation, *Plin2 (Perlipin-2)* is involved with maintenance of adipose tissue, and *Hsp17b8 (Estradiol 17-beta-dehydrogenase 8)* is important for biosysthesis of fatty acids (Chen *et al.*, 2009; McIntosh *et al.*, 2012). In mice, Hsp17b8 has been found to regulate estradiol and testosterone activity (Ohno *et al.*, 2008).

**4.2.2 Gene Expression: Exon-level analysis—**Results from the individual exon-level analysis showed that each brain region had a distinct pattern of significantly dysregulated exons. Most gene-annotation enrichments that surpassed a Bonferroni-corrected level of significance, including “neuronal projection”, “vesicle”, and “plasma membrane”, were not localized in one particular brain region (Table 3). However, calcium-related enrichments were found exclusively in the CV and could suggest a localized disturbance in that pathway.

Genes that showed differential exon expression across all brain regions should perhaps take priority for follow-up studies since they signify a more global, and thus, a more traceable effect of exposure (Table 2). CREB binding protein, encoded by the *Crebbp* gene, showed an interaction of treatment and exon ID that surpassed Bonferroni-corrected significance in AA and had nominal significance in the CV and OFC. This gene plays a key role in embryonic development. Deletions of chromosome region 16p13.3 which harbors this gene causes Rubinstein-Taybi syndrome (RTS) in humans (Hennekam *et al.*, 1993), and duplication 16p13.3 syndrome is thought to be directly caused by over-activity of the *CREBBP* gene (Demeer *et al.*, 2013). This highlights phenotypes that are highly dosage dependent. CREBBP binds to phosphorylated cAMP response element binding protein (CREB) further synergizing its activity and increasing the expression of cAMP-responsive genes. CREB is a central player in dendritic spine organization of pyramidal neurons in the hippocampus and, consequently, of memory formation (Middei *et al.*, 2012). CREBBP also plays an important role in controlling transcription by acetylation of histones and non-histone proteins (Chen *et al.*, 1999; van der Horst *et al.*, 2004). Synergy between the acetylating role of CREBBP and the deacetylation-inhibiting role of VPA remains to be explored.

The only gene with dysregulated alternative splicing that surpassed Bonferroni-corrected significance in all three brain regions was prolyl endopeptidase (*Prep*). Prep is a serine protease that cleaves prolyl bonds of small (less than 3 kDa) hormones and neuropeptides, including oxytocin, arginine vasopressin, neurotensin, and substance P (Welches *et al.*, 1993). These peptides are key regulators of social behavior and therefore, impaired PREP activity has been linked to several psychiatric disorders (Peltonen & Mannisto, 2011). While activity of PREP has been negatively correlated with the severity of depression, an increased activity is seen in serum collected from individuals with schizophrenia and mania (Maes *et al.*, 1995; Peltonen & Mannisto, 2011). The discovery that VPA inhibits PREP activity has allowed some speculation about the therapeutic mechanisms of VPA (Maes *et al.*, 1995; Cheng *et al.*, 2005). However, how this activity is regulated *in vivo*, and the long-term effects of VPA exposure during developmental stages are still unclear.

Exon-level analysis of *Prep* revealed several differentially expressed exons for this gene (Fig. 3c). The two exons that showed differential expression across all three brain regions were exon 1 and 8. In the VPA group exon 1 had a 1.5-fold increase in expression, while exon 8 had a 3-fold decrease. Although these don't seem to affect any of the amino acids in the catalytic triad (Ser554, Asp641, and His680), other properties such as ligand specificity may be affected (Fulop *et al.*, 1998). Dysregulation of an exon that could disturb the catalytic triad (exon 13) was only seen in the anterior amygdala. Exon 13 harbors Ser554 and therefore is crucial for *Prep*'s protease activity.

**4.2.3 Gene Expression: Comparison of whole-gene and exon-level changes—** An interesting interaction is seen when comparing genes that showed differential exonic expression with genes that showed differential whole-gene expression. We observed ubiquitous dysregulation of an exon in the *Pkn1* gene coding for the PKC-related serine/threonine-protein kinase and ubiquitous dysregulation of the aggregated transcripts of cortactin (*Cttn*). In humans, *PKN1* is involved in a wide range of cellular processes, including actin polymerization, cell migration, and dendritic spine morphogenesis. *PKN1* also has epigenetic influence and can directly phosphorylate histones and co-activate androgen receptor-dependent transcription (Metzger *et al.*, 2003); it also phosphorylates HDACs, which restricts their import to the nucleus (Harrison *et al.*, 2010). Meanwhile, *CTTN* is involved in actin polymerization and activation of the Arp2/3 complex, and thus is also involved in cell migration (Ammer & Weed, 2008). Combining our data with evidence that *PKN1* directly phosphorylates *CTTN* (Grassart *et al.*, 2010), further strengthens the association of these two genes and suggests that their expression patterns may be interconnected.

Our results specify an exclusion of exon 19 of *Pkn1*, which in the human, corresponds to amino acids (aas) 766–812 (Fig. 3b). Dysregulation of this exon surpassed a Bonferroni-corrected threshold for statistical significance in the AA and the CV and had nominal significance in the OFC. This region does not contain the whole catalytic domain (aas 615–874); however, it does contain two known phosphorylation sites (phosphothreonine at aa 774 and aa 778). Threonine 774 is phosphorylated by phosphoinositide-dependent protein kinase-1 (PDK1) in response to insulin, and facilitates cytoskeleton reorganization (Dong *et al.*, 2000). Other studies report that complete inhibition of the catalytic activity of *Pkn1* ameliorates the cellular and behavioral deficits commonly seen in fragile × mental retardation 1 (*Fmr1*) knockout mice, an animal model of fragile × syndrome. (Hayashi *et al.*, 2007). Thus, the dendritic spine abnormalities and behavioral deficits in this animal model are thought to be, at least partly, due to the catalytic activity of Pkn1. Indeed, *Fmr1* knockout mice show reduced interaction of *Cttn* with actin (Seese *et al.*, 2012), consistent with increased phosophorylation of this protein (Webb *et al.*, 2006). This may suggest that VPA alters developmental alternative splicing patterns or perhaps causes a compensatory mechanism that involves exclusion of exons with catalytic domains.

**4.2.4 VPA-associated Differential Expression Considerations—**It is important to remember that changes in whole-gene or exon expression may be a compensatory response to VPA and not a direct effect. Alternative splicing can manipulate transcripts in response to environmental and other cellular signals in order to allow an organism to adapt. Therefore, exons that show consistent dysregulation in response to exposure may be important for cellular "coping" mechanisms and should be considered for replication and follow up studies.

Certain cellular factors, such as differential microRNA expression and protein degradation, could cause mRNA expression that is inconsistent with protein expression. However, a dysregulated exon indicates that the amino acids that are coded in that exon are missing in

the expressed protein. Alternative splicing regulators are activated and directed to exclude or include a particular exon or exons from the mature mRNA transcript in order to control the type of isoform that is translated. Therefore, this cellular behavior is a functional effort, regardless of the amount of active protein found in the cell. Furthermore, it is important to note that genes that showed dysregulated exon expression (alternative splicing) did not show whole-gene expression differences. That is, the whole gene cluster averages were not different between VPA- and Saline-exposed animals. The only difference in expression that is expected is for the amino acids that are coded by the particular exon that is dysregulated. These results underscore the importance of exon-level analysis, as without it biologically important changes in expression will be missed in the analysis of whole-gene expression data.

## 5. Conclusion

In contrast to prior studies with higher doses of prenatal VPA exposure, none of the behavioral changes observed in this study appeared homologous to autistic behavior in humans; however, this is perhaps not unexpected due to the lower dose and acute exposure we employed. It is quite remarkable, however, that this relatively mild exposure to VPA had long-lasting behavioral and neuromolecular consequences. The opposite behavioral outcomes seen between high- and moderate-dose prenatal VPA exposures clearly illustrate the need for a more comprehensive dose-response curve. Aside from what we can learn about the teratogenicity of VPA, region-specific and ubiquitous changes in gene- and exon-expression may give insight into the mechanism(s) of VPA's influence on social behavior.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Karen Gentile, Renee Mezza, Wendi Burnette, and Terri Novak for their technical assistance. This research was supported by Autism Speaks (SMM), the National Institute of Alcohol Abuse and Alcoholism (AA018693 and AA178231 to SMM; AA012453 and AA178231 to EIV), the National Institute of Mental Health, the National Institute of Neurological Disorders and Stroke, and the National Institute of Child Health and Human Development (1P50MH 081755-020003 to SJG), and the Gerber Foundation (SJG).

## ABBREVIATIONS

| | |
|---|---|
| **aa** | amino acid |
| **AA** | anterior amygdala |
| **ANCOVA** | analysis of covariance |
| **ANOVA** | analysis of variance |
| **CREB** | cAMP response element binding protein |
| **CREBBP** | CREB binding protein |
| **CV** | cerebellar vermis |
| **DNA** | deoxyribose nucleic acid |
| **G** | gestational day |
| **GABA** | gamma-aminobutyric acid |
| **HDAC** | histone deacetylase |

| | |
|---|---|
| **HDACi** | histone deacetylase inhibitor |
| **ID** | identity |
| **i.p.** | intraperitoneal |
| **IUTs** | intersection/union tests |
| **mg/kg** | milligrams per kilogram body weight |
| **mRNA** | messenger RNA |
| **OFC** | orbitofrontal cortex |
| **P** | postnatal day |
| **P. C.** | principal component |
| **RNA** | ribonucleic acid |
| **RTS** | Rubinstein-Taybi syndrome |
| **VPA** | valproic acid |

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- A moderate dose of the VPA has long-term effects on social interaction behaviors.
- Behavioral effects are opposite of those described after a high dose of VPA.
- Transcriptomic analysis identified some intriguing differences.
- Differential whole-genes and individual exons expression were identified

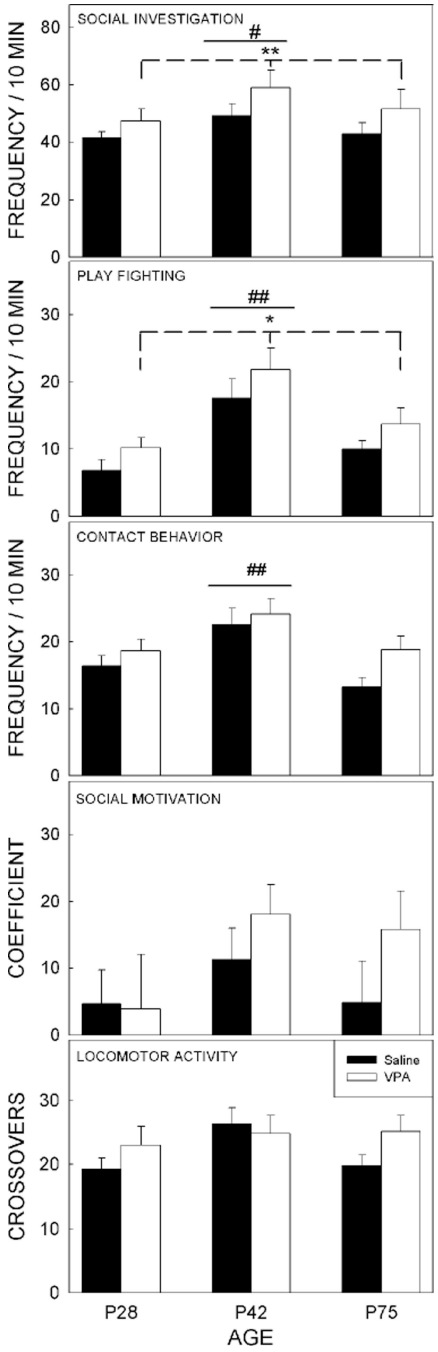


**Figure 1.**

Effects of Exposure to Valproic Acid on Social Behavior.

Four features of social behavior as well as locomotor activity were analyzed from a modified social interaction test. Both social investigation and play fighting, were significantly increased in animals exposed to valproic acid when the data were collapsed across age. Three features, social investigation, play fighting, and contact behavior, were significantly increased in 42-day-old animals compared with those tested on P28 or P75 (data collapsed across treatment). Neither social motivation nor locomotor activity showed a significant effect of age or prenatal treatment.

Significant differences between saline- and valproic acid-exposed animals: * $p < 0.05$, ** $p<0.01$. Significant differences between age groups: # $p < 0.01$, ## $p<0.001$. Bars show the mean for each group, t-bars depict the standard error of the mean. No differences between the sexes were identified for any of the measures, thus data were collapsed across sex.

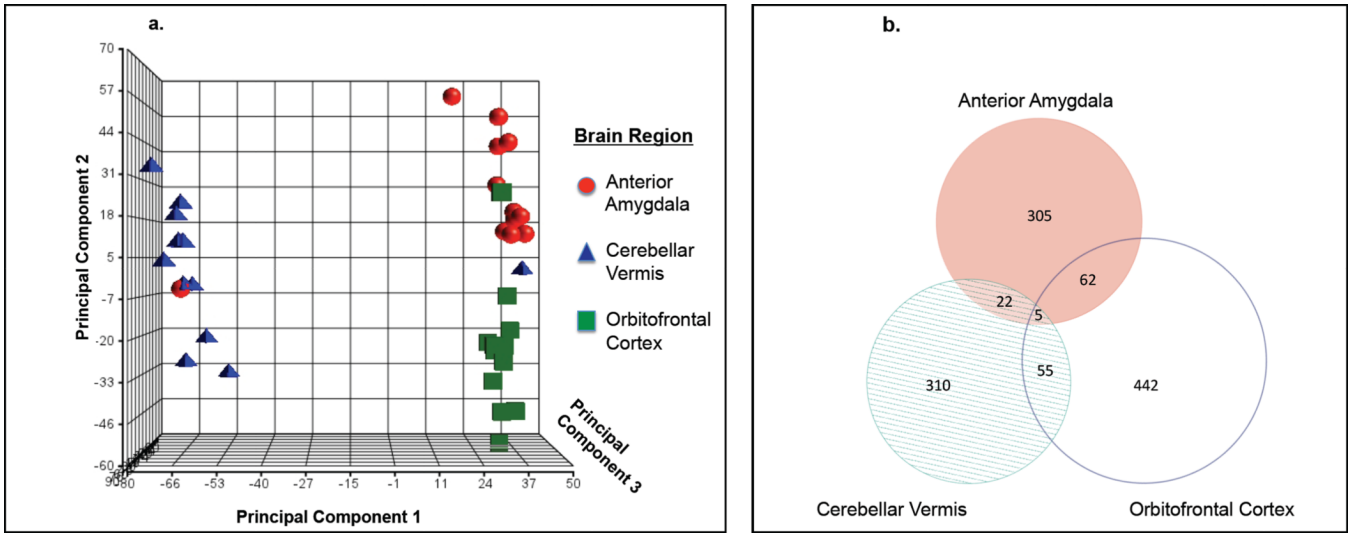


**Figure 2.**

a. Principal Components Analysis of Rat Brain Transcriptome.

Individual data points (three per subject, one per brain region) were mapped to the first three principal components (P. C.) of gene expression variance derived from the entire rat transcriptome. Data points from each brain region showed very strong correspondence with each other but no relation to prenatal treatment, while data from each individual subject was clearly distributed across the three clusters defined by brain region, with the exception of a single subject that was removed from all subsequent analyses. This demonstrates the brain-regional specificity of the co-expression of genes in the rat transcriptome and the relative integrity of its global characteristics and gene-wise correlations despite prenatal VPA exposure.

b. Venn Diagram of Whole-Gene Clusters Significantly Dysregulated by VPA Treatment in Each Brain Region

Most genes dysregulated by prenatal VPA treatment were only affected in one of the three evaluated brain regions. In contrast to these regionally specific effects of prenatal VPA exposure on gene expression, there were also 139 instances where prenatal VPA exposure had long-lasting effects on gene expression in two of the three regions, with the anterior amygdala and orbitofrontal cortex highest in similarity. There were also 5 instances where the effects of VPA were persistent and ubiquitous across all three brain regions

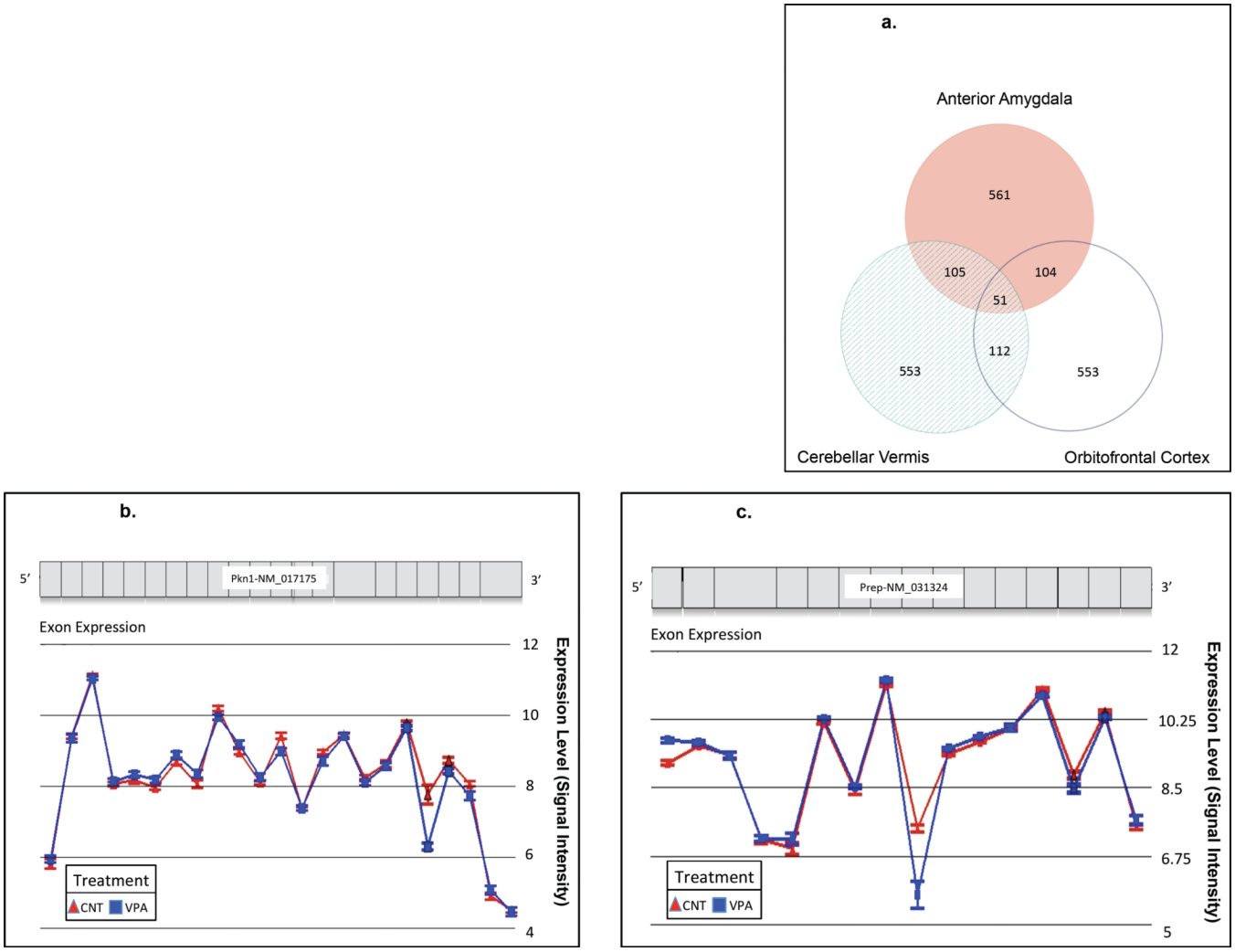


**Figure 3.**

**a.** Venn Diagram of Genes with Significantly Dysregulated Alternative Splicing by VPA Treatment in Each Brain Region

Most genes with dysregulated alternative splicing by prenatal VPA treatment were only affected in one of the three evaluated brain regions. In contrast to these regionally specific effects of prenatal VPA exposure on gene expression, there were also 321 instances where prenatal VPA exposure had long-lasting effects on gene expression in two of the three regions, with the cerebellar vermis and orbitofrontal cortex having highest in similarity. There were also 51 instances where the effects of VPA were persistent and ubiquitous across all three brain regions.

**b.** Exonic expression of Serine/threonine-protein kinase N1 (*Pkn1*).

Exonic expression of *Pkn1* across all three brain regions: anterior amygdala (AA), cerebellar vermis (CV), and oribitofrontal cortex (OFC) from 5 prenatally valproic acid (VPA) exposed rats and 6 saline control (CNT) rats. Differential expression of exon 19 is seen between exposure groups.

**c.** Exonic expression of Prolyl endopeptidase (*Prep*)

Exonic expression of *Prep* across all three brain regions: anterior amygdala (AA), cerebellar vermis (CV), and oribitofrontal cortex (OFC) from 5 prenatally valproic acid (VPA)

exposed rats and 6 saline control (CNT) rats. Differential expressions of exons 1 and 8 are seen between exposure groups for all three brain regions.

**Table 1**

Genes Significantly Dysregulated in All Three Evaluated Brain Regions after Prenatal Valproic Acid Treatment

| | | | | Anterior Amygdala | | Cerebellar Vermis | | Orbitofrontal Cortex | |
|---|---|---|---|---|---|---|---|---|---|
| **Transcript Cluster ID** | **Reference Sequence** | **Gene Symbol** | **Gene Product** | ***p*** | ***Log2* Fold-Change** | ***p*** | ***Log2* Fold-Change** | ***p*** | ***Log2* Fold-Change** |
| 7043913 | NM_013073 | Pcmt1 | Protein-L-isoaspartate(D-aspartate) O-methyltransferase | 0.0243 | −1.19 | 0.0127 | −1.25 | 0.0419 | −1.19 |
| 7059188 | NM_021868 | Cttn | Src substrate cortactin | 0.0059 | −1.17 | 0.0324 | −1.09 | 0.0379 | −1.16 |
| 7066168 | NM_019273 | Kcnmb1 | Calcium-activated potassium channel subunit beta-1 | 0.0288 | 1.25 | 0.0448 | 1.31 | 0.0423 | 1.44 |
| 7216980 | NM_212529 | Hsd17b8 | Estradiol 17-beta-dehydrogenase 8 | 0.0297 | −1.37 | 0.0093 | −1.33 | 0.0263 | −1.34 |
| 7288291 | NM_001007144 | Plin2 | Perilipin-2 | 0.0002 | −1.36 | 0.0222 | −1.47 | 0.0207 | −1.33 |

**Table 2**

Alternative Splicing Significantly Dysregulated in All Three Evaluated Brain Regions after Prenatal Valproic Acid Treatment

| | | | | Anterior Amygdala | | Cerebellar Vermis | | Orbitofrontal Cortex | |
|---|---|---|---|---|---|---|---|---|---|
| **Transcript Cluster ID** | **Reference Sequence** | **Gene Symbol** | **Gene Product** | ***F*** | ***p*** | ***F*** | ***p*** | ***F*** | ***p*** |
| 7213582 | NM_012804 | *Abcd3* | ATP-binding cassette sub-family D member 3 | 3.481 | 6.93E-08* | 1.532 | 4.73E-02 | 2.574 | 5.54E-05 |
| 7082684 | NM_016987 | *Acly* | ATP-citrate synthase | 1.914 | 4.95E-03 | 1.721 | 1.61E-02 | 1.657 | 2.35E-02 |
| **7123967** | **NM_012493** | ***Afp*** | **alpha-fetoprotein** | **1.973** | **1.61E-02** | **3.672** | **7.32E-06** | **1.983** | **1.54E-02** |
| 7337058 | NM_012903 | *Anp32a* | acidic (leucine-rich) nuclear phosphoprotein 32 family, member A | 4.110 | 7.62E-03 | 5.783 | 1.06E-03 | 4.233 | 6.55E-03 |
| 7377029 | NM_001024367 | *Armcx1* | Armadillo repeat-containing X-linked protein 1 | 3.937 | 2.46E-03 | 2.45 | 3.62E-02 | 4.330 | 1.23E-03 |
| 7349069 | NM_012913 | *Atp1b3* | ATPase, Na+ | 5.745 | 4.01E-02 | 10.564 | 9.99E-03 | 5.188 | 4.88E-02 |
| 7268482 | NM_012508 | *Atp2b2* | Plasma membrane calcium-transporting ATPase 2 | 4.219 | 1.75E-08* | 2.605 | 2.42E-04 | 2.217 | 2.14E-03 |
| 7378504 | NM_133288 | *Atp2b3* | Plasma membrane calcium-transporting ATPase 3 | 2.751 | 4.02E-05 | 2.628 | 8.95E-05 | 3.634 | 1.12E-07* |
| 7385140 | NM_031785 | *Atp6ap1* | ATPase, H+ transporting, lysosomal accessory protein 1 | 2.437 | 9.86E-03 | 3.778 | 1.56E-04 | 3.458 | 4.21E-04 |
| 7384924 | NM_001004224 | *Bcap31* | B-cell receptor-associated protein 31 | 2.272 | 3.15E-02 | 2.597 | 1.49E-02 | 2.138 | 4.29E-02 |
| 7137201 | NM_012827 | *Bmp4* | bone morphogenetic protein 4 | 2.235 | 4.28E-02 | 2.307 | 3.69E-02 | 2.722 | 1.57E-02 |
| **7335781** | **NM_001012201** | ***Cadm1*** | **cell adhesion molecule 1** | **3.122** | **7.81E-04** | **3.65** | **1.32E-04** | **5.360** | **4.69E-07*** |
| 7092876 | NM_019195 | *Cd47* | Cd47 molecule | 2.314 | 2.27E-02 | 3.021 | 3.69E-03 | 2.480 | 1.49E-02 |
| 7215277 | NM_201419 | *Clca4* | chloride channel accessory 4 | 8.108 | 9.65E-16* | 2.093 | 6.89E-03 | 2.371 | 1.82E-03 |
| **7065253** | **NM_133381** | ***Crebbp*** | **CREB binding protein** | **2.778** | **5.13E-07*** | **1.909** | **1.48E-03** | **1.991** | **7.44E-04** |
| 7083616 | NM_001007613 | *Ddx5* | DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 | 2.503 | 3.28E-02 | 2.000 | 3.09E-02 | 1.892 | 4.30E-02 |
| **7089864** | **NM_001012472** | ***Dgcr14*** | **DiGeorge syndrome critical region protein 14** | **2.849** | **4.00E-03** | **2.047** | **3.73E-02** | **2.067** | **3.53E-02** |
| 7198615 | NM_013057 | *F3* | Tissue factor Precursor | 5.375 | 3.39E-06* | 3.932 | 1.84E-04 | 3.595 | 4.78E-04 |
| 7340407 | NM_021660 | *Ip6k2* | Inositol hexakisphosphate kinase 2 | 9.178 | 9.05E-08* | 3.964 | 1.20E-03 | 4.646 | 3.05E-04 |
| 7231634 | NM_031045 | *Itpka* | inositol 1,4,5-trisphosphate 3-kinase A | 2.185 | 2.56E-02 | 2.495 | 1.09E-02 | 2.672 | 6.61E-03 |
| 7326239 | NM_212523 | *Kif5a* | kinesin family member 5A | 1.850 | 6.62E-03 | 1.634 | 2.49E-02 | 1.639 | 2.42E-02 |
| 7070380 | NM_001012011 | *Lig3* | DNA ligase | 2.548 | 4.40E-04 | 1.891 | 1.35E-02 | 2.154 | 3.59E-03 |
| 7333434 | NM_001025054 | *LOC500956* | hypothetical protein LOC500956 | 2.512 | 1.04E-02 | 4.395 | 5.00E-05 | 5.167 | 5.96E-06* |
| 7322009 | NM_021859 | *Matk* | Megakaryocyte-associated tyrosine-protein kinase | 4.746 | 1.47E-06* | 2.455 | 5.55E-03 | 2.407 | 6.57E-03 |
| 7059820 | NM_001015013 | *Mtvr2* | mammary tumor virus receptor 2 | 3.766 | 4.30E-02 | 5.004 | 1.87E-02 | 4.918 | 1.98E-02 |

| Transcript Cluster ID | Reference Sequence | Gene Symbol | Gene Product | Anterior Amygdala | | Cerebellar Vermis | | Orbitofrontal Cortex | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *F* | *p* | *F* | *p* | *F* | *p* |
| **7322533** | **NM_020087** | ***Notch3*** | **Notch homolog 3 (Drosophila)** | **1.647** | **1.02E-02** | **1.474** | **3.65E-02** | **1.433** | **4.83E-02** |
| 7233312 | NM_012746 | *Pcsk2* | Neuroendocrine convertase 2 Precursor | 2.616 | 4.23E-03 | 2.02 | 2.90E-02 | 2.379 | 9.22E-03 |
| 7289582 | NM_199253 | *Pcsk9* | proprotein convertase subtilisin | 1.742 | 4.50E-02 | 2.085 | 1.19E-02 | 1.756 | 4.28E-02 |
| 7320471 | NM_031715 | *Pfkm* | 6-phosphofructokinase | 2.652 | 1.85E-04 | 2.008 | 6.53E-03 | 2.029 | 5.85E-03 |
| **7179966** | **NM_017175** | ***Pkn1*** | **Serine/threonine-protein kinase N1** | **4.128** | **3.01E-08*** | **3.43** | **1.92E-06*** | **3.129** | **1.14E-05** |
| 7329136 | NM_001164298 | *Plec* | plectin | 1.454 | 1.61E-02 | 1.504 | 9.55E-03 | 1.565 | 4.99E-03 |
| 7339147 | NM_057194 | *Plscr1* | phospholipid scramblase 1 | 2.510 | 1.82E-02 | 2.148 | 4.19E-02 | 2.956 | 6.46E-03 |
| 7139367 | NM_053999 | *Ppp2r2a* | protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha | 2.092 | 9.72E-03 | 3.057 | 1.28E-04 | 2.888 | 2.79E-04 |
| **7219948** | **NM_031324** | ***Prep*** | **Prolyl endopeptidase** | **5.697** | **6.12E-09*** | **4.335** | **1.46E-06*** | **5.013** | **9.27E-08*** |
| **7300035** | **NM_019163** | ***Psen1*** | **Presenilin-1** | **5.727** | **4.40E-07*** | **7.000** | **1.20E-08*** | **4.297** | **3.15E-05** |
| 7302329 | NM_031600 | *Ptprn2* | protein tyrosine phosphatase, receptor type, N polypeptide 2 | 1.845 | 9.58E-03 | 1.782 | 1.38E-02 | 2.220 | 9.66E-04 |
| 7042063 | NM_001004261 | *Pyroxd2* | Pyridine nucleotide-disulfide oxidoreductase domain 2 | 3.345 | 5.41E-05 | 2.558 | 1.66E-03 | 4.071 | 2.22E-06* |
| 7119529 | NM_022390 | *Qdpr* | quinoid dihydropteridine reductase | 3.468 | 5.68E-03 | 4.999 | 3.89E-04 | 5.954 | 7.97E-05 |
| 7174465 | NM_017315 | *Slc23a1* | solute carrier family 23 (nucleobase transporters), member 1 | 2.071 | 1.49E-02 | 2.049 | 1.61E-02 | 2.348 | 5.07E-03 |
| 7138066 | NM_053442 | *Slc7a8* | solute carrier family 7 (cationic amino acid transporter, y+ sys | 2.471 | 6.82E-03 | 1.927 | 3.87E-02 | 2.626 | 4.09E-03 |
| 7365728 | NM_173338 | *Slco6c1* | solute carrier organic anion transporter family, member 6c1 | 2.108 | 1.84E-02 | 1.828 | 4.64E-02 | 2.984 | 8.41E-04 |
| 7348411 | NM_031728 | *Snap91* | Clathrin coat assembly protein AP180 | 1.983 | 3.18E-03 | 2.041 | 2.18E-03 | 2.249 | 5.42E-04 |
| 7045017 | NM_175583 | *Taar4* | Trace amine-associated receptor 4 | 3.576 | 4.68E-03 | 3.161 | 9.91E-03 | 5.199 | 2.77E-04 |
| 7320452 | NM_001008358 | *Tmem106c* | transmembrane protein 106C | 2.468 | 2.65E-02 | 2.719 | 1.57E-02 | 2.952 | 9.69E-03 |
| 7303979 | NM_001014195 | *Tmem214* | transmembrane protein 214 | 2.277 | 8.30E-03 | 2.045 | 1.93E-02 | 2.778 | 1.26E-03 |
| 7219299 | NM_001013033 | *Tspyl1* | TSPY-like 1 | 21.453 | 1.23E-03 | 5.538 | 4.31E-02 | 16.525 | 2.82E-03 |
| 7378941 | NM_001013933 | *Ube2a* | ubiquitin-conjugating enzyme E2A | 3.788 | 3.21E-03 | 3.404 | 6.38E-03 | 5.201 | 2.76E-04 |
| 7084520 | NM_138844 | *Unc13d* | Protein unc-13 homolog D | 1.841 | 4.03E-03 | 1.69 | 1.18E-02 | 1.884 | 2.91E-03 |
| 7154203 | NM_001001516 | *Usp19* | ubiquitin specific peptidase 19 | 1.737 | 1.34E-02 | 1.985 | 2.73E-03 | 1.858 | 6.25E-03 |
| 7331440 | NM_017058 | *Vdr* | Vitamin D3 receptor | 3.091 | 4.71E-03 | 3.426 | 2.16E-03 | 3.630 | 1.34E-03 |
| 7295338 | NM_017154 | Xdh | xanthine dehydrogenase | 1.495 | 4.03E-02 | 2.690 | 2.96E-06* | 2.267 | 1.17E-04 |

*Genes that surpassed Bonferroni corrected significance for that brain region.

Genes that may be relevant to social interaction behavior are indicated in bold.

*Int J Dev Neurosci*. Author manuscript; available in PMC 2014 December 01.

**Table 3**

Significant Annotation Clustering Enrichments In Response to VPA by Brain-Region-Specific Ontological Analysis

| | **Anterior Amygdala** | | **Cerebellar Vermis** | | **Orbitofrontal Cortex** | |
|---|---|---|---|---|---|---|
| **Alternative Splicing Dysregulated** | Neuron projection<br>Cell-cell signaling<br>Phosphoprotein<br>Protein domain specific binding<br>Synaptic transmission<br>Vesicle (membrane-bound/cytoplasmic) | | Neuron projection<br>Cellular homeostasis (ion/channel)<br>Phosphoprotein<br>Calcium ion binding and transport<br>Plasma membrane<br>Vesicle (membrane-bound/cytoplasmic) | | Cell projection<br>Homeostatic process<br>Phosphoprotein<br>Purine nucleotide binding<br>Plasma membrane | |
| **Whole Gene Transcript Dysregulated** | **Up-regulated** | **Down-regulated** | **Up-regulated** | **Down-regulated** | **Up-regulated** | **Down-regulated** |
| | Glycoprotein<br><br>Galactose metabolism | Acetylation<br>Nucleoside binding | Glycoprotein | Acetylation (p=0.09)<br><br>Cognition (p=0.09) | Neurological system<br>Sensory perception | Nucleotide binding (p=0.06) |

Gene-annotation enrichments that surpassed Bonferroni-corrected level of significance (p-value indicated for marginally significant enrichments)