

University of Northern Colorado Scholarship & Creative Works @ Digital UNC

School of Psychological Sciences Faculty
Publications

School of Psychological Sciences

2011

Genetic and Maternal Effects on Valproic Acid Teratogenesis in C57BL/6J and DBA/2J Mice

Chris Downing
University of Colorado, Boulder

Jami Biers
University of Colorado, Boulder

Colin Larson
University of Colorado, Boulder

Alexi Kimball
University of Colorado, Boulder

Hali Wright
University of Colorado, Boulder

See next page for additional authors

Follow this and additional works at: <http://digscholarship.unco.edu/spsfacpub>

 Part of the [Cellular and Molecular Physiology Commons](#)

Recommended Citation

Downing, Chris; Biers, Jami; Larson, Colin; Kimball, Alexi; Wright, Hali; Ishii, Takamasa; Gilliam, David; and Johnson, Thomas, "Genetic and Maternal Effects on Valproic Acid Teratogenesis in C57BL/6J and DBA/2J Mice" (2011). *School of Psychological Sciences Faculty Publications*. 2.

<http://digscholarship.unco.edu/spsfacpub/2>

This Article is brought to you for free and open access by the School of Psychological Sciences at Scholarship & Creative Works @ Digital UNC. It has been accepted for inclusion in School of Psychological Sciences Faculty Publications by an authorized administrator of Scholarship & Creative Works @ Digital UNC. For more information, please contact Jane.Monson@unco.edu.

Creator

Chris Downing, Jami Biers, Colin Larson, Alexi Kimball, Hali Wright, Takamasa Ishii, David Gilliam, and Thomas Johnson

Genetic and Maternal Effects on Valproic Acid Teratogenesis in C57BL/6J and DBA/2J Mice

Chris Downing^{*, ‡, §}, Jami Biers^{*}, Colin Larson^{*}, Alexi Kimball^{*}, Hali Wright^{*}, Takamasa Ishii^{*},

David Gilliam[†], Thomas Johnson^{*}

^{*} University of Colorado, Institute for Behavioral Genetics, Campus Box 447, Boulder CO,

80309-0447

[†] University of Northern Colorado, School of Psychological Sciences

[‡] To whom correspondence should be addressed:

Email: cdowning@pharmacy.isu.edu

Telephone: 208-282-3837

[§] Present address:

Idaho State University

College of Pharmacy

Department of Biomedical and Pharmaceutical Sciences

970 S. 5th Avenue, Stop 8334

Pocatello, ID 83209-8334

Running Title: Genetic Variation in VPA Teratogenesis

Suggested Section: Reproductive and Developmental Toxicology

Abstract

Valproic acid (VPA) is used worldwide to treat epilepsy, migraine headaches and bipolar disorder. However, VPA is teratogenic and *in utero* exposure can lead to congenital malformations. Using inbred C57BL/6J (B6) and DBA/2J (D2) mice, we asked whether genetic variation could play a role in susceptibility to VPA teratogenesis. While B6 fetuses were more susceptible than D2 fetuses to digit and vertebral malformations, D2 fetuses were more susceptible to rib malformations. In a reciprocal cross between B6 and D2, genetically identical F₁ mice carried in a B6 mother had a greater percentage of vertebral malformations following prenatal VPA exposure than F₁ mice carried in a D2 mother. This reciprocal F₁ difference is known as a maternal effect and shows that maternal genotype/uterine environment is an important mediator of VPA teratogenicity. VPA is a histone deacetylase inhibitor and it is possible that the differential teratogenesis in B6 and D2 is due to strain differences in histone acetylation. We observed strain differences in acetylation of histones H3 and H4 in both embryo and placenta following *in utero* VPA exposure, but additional studies are needed to determine the significance of these changes in mediating teratogenesis. Our results provide additional support that genetic factors, both maternal and fetal, play a role in VPA teratogenesis. Lines of mice derived from B6 and D2 will be a useful model for elucidating the genetic architecture underlying susceptibility to VPA teratogenesis.

Valproic acid (VPA) is one of the most frequently used anti-epileptic drugs (AEDs) worldwide and has developed into a first-line treatment for migraine headaches and mania associated with bipolar disorder. While VPA has a good side-effect profile with little sedation and few behavioral effects, it is teratogenic. *In utero* VPA exposure can produce growth retardation and congenital malformations, including facial dysmorphism, spina bifida and other neural tube defects (NTDs), urogenital abnormalities, heart anomalies and skeletal malformations (Duncan, 2007; Meador *et al.*, 2008b). Recently revised estimates indicate that in the United States, there are one-half million women of childbearing age with epilepsy, most requiring AEDs; they give birth to 25,000 offspring each year (Harden *et al.*, 2009; Meador *et al.*, 2008a). The number of children exposed to AEDs *in utero* is likely double that because of AED use in treating mood disorders and migraines (Pennell, 2004). VPA warrants particular attention because it is more teratogenic than other AEDs (Harden *et al.*, 2009; Meador *et al.*, 2008a, 2008b), and an increasing number of women of reproductive age are taking VPA for various therapeutic reasons (Koren *et al.*, 2006).

The vast majority of women who take VPA during pregnancy give birth to normal children. It is likely that many factors play a role in susceptibility to the teratogenic effects of VPA. Studies, while few in number, have shown that genetic variation can play a role in the development of morphological and cognitive/behavioral abnormalities in children exposed to VPA *in utero* (Hockey *et al.*, 1996; Kini *et al.*, 2007; Kozma, 2001; Malm *et al.*, 2002). Animal models have shown conclusively that genotype can affect susceptibility to VPA teratogenesis. Inbred strains of mice differ on several teratogenic outcomes, including skeletal malformations and NTDs (Faiella *et al.* 2000; Finnell *et al.*, 1988). Significant changes in expression of many genes have also been reported following prenatal VPA exposure (Kultima *et al.*, 2004; Massa *et al.*, 2005).

While genetic variation in VPA teratogenesis has been observed, the range of variation has not been well characterized, in humans or mice, and susceptibility genes remain unknown.

We examined skeletal teratogenesis in inbred C57BL/6J (B6) and DBA/2J (D2) mice following *in utero* exposure to one of several doses of VPA. We chose B6 and D2 because they have been among the most widely used inbred strains of mice in biomedical research. Their genomes have been well-characterized, which makes lines of mice derived from them valuable for identifying genetic variation mediating many traits. In order to investigate the effects of maternal genotype and uterine environment on VPA teratogenesis, we reciprocally mated B6 and D2 and examined F₁ litters. Valproic acid is a histone deacetylase inhibitor and increased histone acetylation/deacetylation inhibition has been linked to VPA teratogenesis (Eikel *et al.*, 2006; Menegola *et al.*, 2005). Therefore, we used Western blotting to quantify changes in acetylation of histone proteins H3 and H4 following prenatal VPA in both embryo and placenta. Finally, we looked at mRNA levels of several histone deacetylase genes following prenatal VPA.

Materials and Methods

Dose-Response Study. Male and female B6 and D2 mice were obtained from the Jackson Laboratory and housed in the animal facility at the Institute for Behavioral Genetics, Boulder, CO. Males were individually housed, while females were housed 3-5 per cage. Mice were maintained on a 12-hour light/dark cycle, with lights on at 7:00 am. The temperature was kept at a constant $22 \pm 2^\circ$ C. All procedures were approved by the University of Colorado Institutional Animal Care and Use Committee, in accordance with the National Institute of Health guidelines.

From 7:00 – 9:00 am, two females were placed with a male and then examined for a seminal plug as evidence of mating. The morning of plug detection was designated gestational day 0 (GD 0). Mated females were weighed and single-housed. At noon on GD 9 females were weighed to

ascertain a 2 g minimum weight gain as evidence of pregnancy. Dams were then given an intraperitoneal (ip) injection of either saline or 200 mg/kg (0.4 ml/kg), 400 mg/kg (0.8 ml/kg) or 800 mg/kg (1.6 ml/kg) VPA (sodium salt, Sigma). On GD 18, females were sacrificed and caesarean-sectioned (c-sectioned) between 1:30 and 2:30 pm; uterine horns were exposed and a count was made of live, dead and resorbed fetuses. Live fetuses were weighed, sexed and examined for gross morphological malformations. Fetuses were eviscerated and placed in 95% alcohol for two weeks. They were subsequently macerated in a 1% KOH solution for 72 hours and then placed in a 1% KOH solution containing alizarin red for 6-8 hours. Stained fetuses were placed first in a 25% and then a 75% glycerin solution for subsequent skeletal examination.

Maternal variables included maternal weight gain and prenatal mortality. Data were analyzed using analysis of variance (ANOVA) with strain (B6 or D2) and treatment (saline, 200, 400, 800 mg/kg VPA) as between group factors. Offspring variables included fetal weight at c-section and digit, rib and vertebral malformations. Digit malformations included fused or missing digits. Rib malformations included fused, missing, bifurcated or wavy/bulbous ribs. Vertebral malformations included fused, missing or asymmetrical arches and centra. Between groups variables for offspring data included strain, treatment and sex. Litter means (percent litter malformed) were the unit of analysis and litters with only one live fetus were not used in analyses of offspring variables. With the exception of weight at c-section, we saw no effect of sex on any measures of teratogenesis, so we collapsed across sex for means in Table 2. *Post-hoc* analyses consisted of Bonferoni corrected t-tests (treatment) within strain.

Reciprocal Cross. When it became apparent there was a strain difference in response to VPA, we reciprocally bred B6 and D2 mice. On GD 9, pregnant dams were ip injected with either saline or 600 mg/kg (1.2 ml/kg) VPA. We chose the 600 mg/kg dose because of the high rate of

prenatal mortality at the 800 mg/kg dose (B6 = 41%, D2 = 31%). Dams were sacrificed on GD 18 and fetuses prepared and examined as described above.

Western Blots for Acetylated Histones. In order to investigate the effects of *in utero* VPA exposure on histone acetylation in B6 and D2, mice were mated as described above. At noon on GD 9, pregnant dams were intraperitoneally (ip) injected with 600 mg/kg VPA. We included two additional groups in this study, one group of pregnant dams that was intragastrically intubated with 5.8 g/kg ethanol (2.9 ml/kg) and one that received an isocaloric amount of maltose-dextrin (3.5 ml/kg), . We included these additional groups because we have previously shown that, similar to our findings with VPA, B6 are susceptible to digit and vertebral malformations following prenatal alcohol exposure, while D2 are resistant (Boehm *et al.*, 1997; Downing and Gilliam, 1999). Strain differences in histone acetylation following prenatal VPA exposure and prenatal ethanol exposure, while far from conclusive, would suggest a possible common teratogenic mechanism.

Four hours after intubation or injection, dams were sacrificed and embryos and placentae were excised and frozen. Three litters per strain and treatment were produced and within a litter, all embryos were pooled for protein extraction and all placentae were pooled for protein extraction. Tissues were minced with a scissors and then homogenized in a sucrose lysis buffer using a Teflon homogenizer. The homogenate was washed and nuclear and cytoplasmic fractions were separated using low-speed centrifugation. Nuclear pellets were re-suspended in an H₂SO₄ buffer and gently stirred on a shaker overnight at 4° C. Pelleted, non acid soluble proteins were removed by centrifugation and the supernatants containing the acid soluble proteins were precipitated with TCA. Precipitated proteins were then centrifuged and the subsequent protein pellet was washed by centrifugation in ice-cold acetone; this step was repeated twice. Pellets

were then resuspended in 10mM tris HCL, stirred gently overnight at 4° C and stored at -80° C for subsequent analyses. Protein concentrations were determined using a BCA assay with bovine serum albumin (BSA) as the standard.

Nuclear protein extracts (5 µg) were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Invitrogen, LC2000). Membranes were blocked in TBS containing 5% powdered milk and 0.1% TWEEN 20. Membranes were then incubated with antibodies against histone H3 (Abcam, ab1791; 1:2500 dilution), histone H4 (Upstate Biotechnology, 05-858; 1:2500 dilution), acetylated histone H3K9 (Abcam, ab4441; 1:2500 dilution) and acetylated H4 (Upstate Biotechnology, 06-866; 1:2500 dilution). The acetylated H4 antibody recognizes 4 different acetylated lysines in the first 19 amino acids. After incubation with a primary antibody, membranes were incubated with a secondary antibody (anti-rabbit IgG peroxidase, Sigma Aldrich #A0545) and visualized using an ECL Western Blotting Detection System (Amersham, RPN2132). Immunoreactive bands were visualized by exposure to autoradiographic film. Films were scanned and band densities were quantified using ImageJ software (<http://rsb.info.nih.gov/ij>).

Quantitative PCR. We examined expression of several histone deacetylase genes following prenatal VPA or alcohol exposure. B6 and D2 mice were mated as described above. At noon on GD 9, pregnant dams were ip injected with 600 mg/kg VPA, or intragastrically intubated with either 5.8 g/kg ethanol or maltose-dextrin. Four hours later, dams were sacrificed and embryos and placentae were excised. Three litters per strain and treatment were produced and tissues were pooled for extraction. RNA was extracted using a Qiagen RNeasy Midi Kit. Total RNA was then reverse transcribed to generate single-stranded cDNA using a Promega ImProm II Reverse Transcription Kit. Primers for *Hdac1*, *Hdac2*, *Hdac3*, *Hdac4* and *Hdac8* were designed using

Primer Express software (Applied Biosystems; ABI). Quantitative PCR was performed on cDNAs using SYBR green chemistry and an ABI Prism 7000 system. Relative quantification of mRNA levels was determined by normalizing against a control gene, *Gapdh*, using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). All determinations were replicated 3 times.

We chose the four hour time point to examine changes in histone acetylation and gene expression for several reasons. While many studies have shown increased acetylation or changes in gene expression by exposing cell lines or embryos to VPA in culture, to the best of our knowledge, only one study has looked at acetylation *in vivo* in mouse embryos following an acute dose of VPA prenatally (Menegola et al. 2005); they showed increased acetylation one hour after VPA exposure. The Menegola laboratory has also observed increased acetylation in embryos exposed to other HDAC inhibitors *in utero* at several different timepoints, including 4 hours later (DiRenzo et al. 2007a, b, 2008). Two studies have examined changes in gene expression in mouse embryos following an acute dose of VPA *in utero* (Massa et al. 2005; Stodgell et al. 2006) and have shown changes in expression at the four hour time point. In our own work with C57BL/6J (B6) mice, we have shown changes in DNA methylation and gene expression in embryos four hours after intrauterine exposure to ethanol (Downing et al. in revision).

Results

Dose Response

During pregnancy, B6 dams gained more weight than D2 dams ($p < .01$; Table 1). Compared to saline controls, only the 800 mg/kg dose of VPA significantly decreased weight gain ($p < .01$; Table 1) and increased embryolethality ($p < .01$; Table 1) in B6 and D2 litters. B6 fetuses

exposed to either 400 or 800 mg/kg VPA weighed less than saline controls (p 's < .01; Table 1), while for D2, only the 800 mg/kg dose decreased fetal weight (p < .01; Table 1).

D2 fetuses had a very low incidence of fused or missing digits after VPA treatment, while an increase was observed in B6 fetuses at 800 mg/kg (p < .01; Table 2). *In utero* VPA increased rib malformations only at the highest dose in B6 fetuses (p < .01), but both 400 and 800 mg/kg produced a significant increase in D2 (p 's < .01; Table 2). While VPA affected vertebral development in both B6 and D2, the effect was greater in B6. B6 fetuses had 39% and 94% vertebral malformation rates at the 400 and 800 mg/kg doses (p 's < .01) while D2 had 28% and 53% vertebral malformation rates at these doses (p 's < .02 and .01; Table 2). Indeed, following intrauterine exposure to 800 mg/kg VPA, most B6 fetuses had at least one vertebral defect.

In addition to the malformations described above, we observed several other anomalies (data not shown). We found several supernumerary ribs; there were no differences between strains or among treatments. There are six components to the fetal sternum: the manubrium or first segment, segments two through five, and the xiphoid or sixth segment. Sternebral anomalies are not uncommon, even in unexposed mice, and are generally considered as variations rather than malformations; we did not include sternebral anomalies in vertebral or rib malformations described above. However, sternebral anomalies are frequently reported following *in utero* VPA exposure. We observed fused, bipartite, incompletely ossified and asymmetrical sternebrae in both strains. These sternebral anomalies occurred at a higher frequency in B6 fetuses and were observed at all doses, including saline. In addition, at the 800 mg/kg dose, two D2 and three B6 fetuses had exencephaly.

Reciprocal Cross

We reciprocally mated B6 and D2, which produced B6D2 and D2B6 fetal genotypes (maternal genotype is first). On GD 9, dams were ip injected with saline or 600 mg/kg VPA. Dams were sacrificed on GD 18 and fetuses were processed and examined as described above. B6D2 dams gained more weight than D2B6 dams ($p < .01$) and saline-treated dams put on more weight than VPA-treated dams ($p < .02$), but there was no significant genotype x treatment interaction (Table 1). Both genotypes had greater embryolethality when treated with VPA compared to saline controls ($p < .02$; Table 1). Saline-treated fetuses weighed more than VPA-treated fetuses ($p < .01$).

Prenatal VPA exposure significantly increased digit malformations in B6D2 fetuses but not in D2B6 fetuses (p 's $< .01$ and $.07$, respectively; Table 2). While *in utero* VPA increased vertebral malformations in both B6D2 and D2B6 fetuses, the increase was greater in B6D2 fetuses ($p < .01$; Table 2). These results demonstrate that B6 maternal genotype and/or uterine environment increase susceptibility to digit and vertebral malformations, while the D2 genotype/uterine environment is less susceptible. There appears to be a threshold dose of VPA, somewhere between 400 and 600 mg/kg, above which fetuses carried in a B6 mother are particularly susceptible to vertebral malformations. B6 fetuses exposed to 400 mg/kg VPA had a 39% malformation rate, while the malformation rate at 800 mg/kg was 94%; B6D2 fetuses exposed to 600 mg/kg VPA had a 91% malformation rate. Interestingly, for rib malformations the difference between saline- and VPA-exposed fetuses was greater in the D2B6 genotype compared to the B6D2 genotype ($p < .03$; Table 2). In this case, the D2 genotype and/or uterine environment increased susceptibility to rib malformations.

Western Blots and Quantitative PCR

Valproic acid is a histone deacetylase inhibitor and increased histone acetylation/deacetylation inhibition has been linked to VPA teratogenesis (Eikel *et al.*, 2006; Menegola *et al.*, 2005). It is possible that the differences in VPA teratogenesis observed in B6 and D2 are due, at least in part, to strain differences in VPA-induced acetylation/deacetylation inhibition. Therefore, we examined acetylation of histone proteins H3 and H4 in both placenta and embryo following intrauterine VPA or ethanol exposure. We chose H3 and H4 because most previous studies that have examined acetylation/deacetylation with VPA have examined these two histone proteins and not H1, H2A or H2B. There were no differences in band intensity in histones H3 or H4, either among treatments or between strains, so band densities for acetylated H3 (H3ac) or acetylated H4 (H4ac) were normalized to H3 or H4.

Compared to maltose-treated controls, embryos from both strains exhibited a similar increase in acetylated H3 (H3ac) following prenatal VPA (98-101%) and prenatal ethanol (57-64%; Figure 1). D2 embryos showed a greater increase in acetylated H4 (H4ac) compared to B6 following VPA (97% vs. 40%; $p < .01$; Figure 2). Following prenatal alcohol exposure, D2 embryos had a 44% increase in H4ac ($p < .01$; Figure 2), while B6 embryos actually showed a slight decrease. Increases in acetylation were smaller in placenta, compared to embryo, following *in utero* VPA or ethanol exposure (Figures 1-4). B6 placentae had a 93% increase in H3ac following prenatal VPA, compared to 51% for D2 ($p < .02$); the increases in H3ac following prenatal alcohol were 37% and 18%, respectively (Figure 3). Following *in utero* VPA, B6 and D2 had similar increases in H4ac in placentae, but D2 had a larger increase than B6 following prenatal alcohol (63% vs. 32%, $p < .05$; Figure 4).

Valproic acid preferentially inhibits class I (1, 2, 3 and 8) histone deacetylases (Gottlicher et al. 2001). It is possible that if VPA inhibits histone deacetylation and effectively increases acetylation, and HDAC inhibition/increased acetylation is correlated with teratogenesis, there would be a compensatory increase in HDAC mRNA and protein. In culture, VPA has been shown to increase expression of several HDAC mRNAs (Kim, *et al.*, 2008). Therefore, we examined expression of *Hdac1*, *Hdac2*, *Hdac3* and *Hdac8* in embryo and placenta following *in utero* exposure to maltose, ethanol or VPA. We also examined expression of *Hdac4* because it plays a role in skeletal development (Vega *et al.*, 2004). Cycle threshold values were normalized to *Gapdh* and relative fold changes were determined in comparison to maltose-treated controls. Fold changes were small, ranging from a 0.31-fold increase to a 0.22-fold decrease. There were no significant fold changes in any of the *Hdac* genes in either strain (placenta or embryo) following prenatal VPA or alcohol.

Discussion

Valproic acid (VPA) is one of the most frequently used drugs to treat epilepsy, bipolar disorder and migraine headaches, but VPA is teratogenic. While few studies have looked for genetic variation in susceptibility to VPA teratogenesis in humans, several studies have demonstrated genetic variation in mice. We found that following intrauterine VPA exposure, fetuses from C57BL/6J (B6) and DBA/2J (D2) inbred strains of mice showed altered skeletal development, but differed in degree of susceptibility to specific malformations. While B6 fetuses showed an increase in fused and missing digits at the highest dose of VPA, D2 fetuses were resistant to digit malformations. Rib development was altered in both strains, but the effect was greater in D2 fetuses. In contrast, while vertebral development was also altered in both strains, the effect was

greater in B6 fetuses. Results from our study agree with Faiella et al. (2000), who reported that B6 fetuses were more susceptible to digit and vertebral malformations than D2 following *in utero* VPA, while D2 fetuses was more susceptible to rib malformations. These studies have identified genetic variation in response to prenatal VPA but do not reveal to what extent maternal and embryonic genes play a role in teratogenesis.

One simple strategy for determining maternal and fetal genetic contribution is to reciprocally cross two inbred strains. If the reciprocally bred, genetically identical F₁ offspring differ on the trait, this is known as a maternal effect and suggests that maternal genotype can play an important role in the trait. We found that F₁ offspring carried in a B6 mother were more susceptible to vertebral malformations than F₁ offspring carried in a D2 mother, while F₁ offspring carried in a D2 mother were more susceptible to rib malformations. Beck (1999, 2001) reported a maternal effect following *in utero* VPA, with fetuses carried in a B6 mother being comparatively resistant to exencephaly, compared to fetuses carried in susceptible SWV mothers. In a reciprocal cross between D2 and P/J inbred mice, fetuses carried in a D2 mother were relatively resistant to vertebral malformations (Faiella *et al.*, 2000). These findings indicate that maternal effects can be an important determinant of VPA teratogenesis and the same maternal genotype/uterine environment can increase susceptibility to some teratogenic effects but not others. Embryo transfers can be used to evaluate the effects of uterine environment.

One potential mechanism that can account for a maternal effect is genomic imprinting, an epigenetic phenomenon in which only one of two parental alleles at a locus is expressed. At the molecular level, genomic imprinting is mediated in large part by differential DNA methylation and covalent modification of histone proteins. One of the best characterized histone modifications is acetylation, where acetyl groups are attached to histones by histone

acetyltransferases (HATs) and removed by histone deacetylases (HDACs). HDACs have been shown to play a role in somitogenesis, skeletogenesis and limb/digit development (Morrison and D'Mello, 2008; Westendorf, 2007). Valproic acid is a HDAC inhibitor (HDACi) and increases acetylation, an effect that is correlated with its' teratogenic properties (Menegola *et al.* 2005). It is possible that the differential teratogenesis in B6 and D2 fetuses is due, at least in part, to differential HDACi activity of VPA in these two strains. As a first step in testing this hypothesis, we examined acetylation of lysine residues on histones H3 and H4 following *in utero* exposure to VPA. Embryos from both strains showed a nearly two-fold increase in H3ac following intrauterine exposure to VPA. While D2 embryos also showed a nearly two-fold increase in H4ac, the increase in B6 was much smaller. In placenta, where overall acetylation levels were higher, the increases in acetylation following VPA exposure were smaller and the strain differences were reversed. Both strains showed similar increases in H4ac, while B6 had a nearly two-fold increase in H3ac compared to D2. These strain differences in acetylation following *in utero* VPA exposure need to be examined in greater depth before the relationship between differential acetylation/deacetylation and teratogenesis can be determined.

Analogs of VPA that have HDACi activity are teratogenic, while analogs of VPA that do not have HDACi activity are not teratogenic (Eikel *et al.*, 2006; Gurvich *et al.*, 2005). Furthermore, other teratogens such as sodium salicylate, sodium butyrate, apicidin, the synthetic benzamide derivative MS-275, and boric acid all have HDACi activity and produce similar vertebral malformations (Di Renzo *et al.*, 2007a, 2007b, 2008). This provides convincing evidence that histone acetylation/deacetylation can play an important role in vertebral teratogenesis. Given that alcohol produces similar vertebral malformations when administered *in utero*, we examined histone acetylation in B6 and D2 following prenatal alcohol exposure. There were increases in

H3ac and H4ac in both embryo and placenta following prenatal alcohol exposure but the increases were clearly not as large as those observed with VPA. This suggests that, prenatally, alcohol does not have as great of HDACi activity as VPA, although other mechanisms for increased acetylation may be responsible, as discussed below. The only strain difference in acetylation that was consistent between VPA and ethanol was a greater increase in H4ac in D2 embryos compared to B6 embryos.

Our study is a first step towards characterizing genetic variation in teratogenesis and histone acetylation/deacetylation following prenatal VPA exposure. One shortcoming of our study is that we did not examine VPA metabolism in pregnant dams or embryos. It is possible that strain differences in VPA metabolism could contribute, at least in part, to strain differences in VPA teratogenesis. To the best of our knowledge, no studies have looked at VPA metabolism in pregnant B6 or D2 dams or embryos. Additional studies are also needed to clarify the role that increased acetylation and deacetylase inhibition play in VPA teratogenesis. We observed changes in global acetylation but it is likely that there are specific regions of the genome, perhaps imprinted loci, where histone acetylation/deacetylation plays a crucial role in VPA teratogenesis. Chromatin immunoprecipitation assays can be used to interrogate acetylation in specific genomic regions. Acetylation levels will also need to be measured at different time-points following VPA exposure and in more specific tissues (i.e., developing vertebra). While studies often infer HDACi activity by observing increased acetylation, assays that measure the rate of deacetylation are available and can be used to assess the extent to which increased acetylation is due to HDACi activity. Levels of HATs and HAT activity will also need to be examined.

Acetylation and HDAC inhibition may play a role in differential susceptibility to VPA teratogenesis, but additional mechanisms are almost certainly involved. Observing genetic variation will identify strains for further investigation but does not provide information on causal genes, polymorphisms or genetic pathways. Quantitative trait locus (QTL) mapping can be used to identify regions on chromosomes that harbor genes or DNA polymorphisms mediating variation in VPA teratogenesis. B6 and D2 have been two of the most frequently used inbred strains in biomedical research and their genomes have been extensively sequenced. This makes lines of mice derived from B6 and D2, such as the BXD recombinant inbred (RI) strains, a valuable resource for QTL mapping. There is a high degree of synteny and homology between mouse and human genomes, so putative causal polymorphisms and epigenetic modifications identified in mice can be examined in humans. Given the paucity of research investigating genetic variation in humans, mice will be a valuable tool for elucidating genetic mechanisms mediating susceptibility to VPA teratogenesis.

References

- Beck, S.L. (1999). Contributions of dam and conceptus to differences in sensitivity to valproic acid among C57 black and SWV mice. *Reprod. Toxicol.* **13**, 353-360.
- Beck, S.L. (2001). Does genomic imprinting contribute to valproic acid teratogenicity? *Reprod. Toxicol.* **15**, 43-48.
- Boehm, S.L., Lundahl, K.R., Caldwell, J., Gilliam, D.M. (1997). Ethanol teratogenesis in the C57BL/6J, DBA/2J and A/J inbred mouse strains. *Alcohol* **14**, 389-395.
- Di Renzo, F., Broccia, M.L., Giavini, E., Menegola, E. (2007a). Relationship between embryonic histone hyperacetylation and axial skeletal defects in mouse exposed to the three HDAC inhibitors apicidin, MS-275, and sodium butyrate. *Toxicol. Sci.* **98**, 582-588.
- Di Renzo, F., Cappelletti, G., Broccia, M.L., Giavini, E., Menegola, E. (2007b). Boric acid inhibits embryonic histone deacetylases: A suggested mechanism to explain boric acid-related teratogenicity. *Toxicol. Appl. Pharm.* **220**, 178-185.
- Di Renzo, F., Cappelletti, G., Broccia, M.L., Giavini, E., Menegola, E. (2008). The inhibition of embryonic histone deacetylases as the possible mechanism accounting for axial skeletal malformations induced by sodium salicylate. *Toxicol. Sci.* **104**, 387-404.
- Downing, C., Gilliam, D.M. (1999). Cytoplasmic factors do not contribute to a maternal effect on ethanol teratogenesis. *Behav. Genet.* **29**, 31-39.
- Duncan, S. (2007). Teratogenesis of sodium valproate. *Curr. Opin. Neurol.* **20**, 175-180.
- Eikel, D., Lampen, A., Nau, H. (2006). Teratogenic effects mediated by inhibition of histone deacetylases: Evidence from quantitative structure activity relationships of 20 valproic acid derivatives. *Chem. Res. Toxicol.* **19**, 272-278.

- Faiella, A., Wernig, M., Consalez, G.C., Hostick, U., Hofmann, C., Hustert, E., Boncinelli E, Balling, R., Nadeau, J.N. (2000). A mouse model for valproate teratogenicity: parental effects, homeotic transformations, and altered HOX expression. *Hum. Mol. Genet.* **9**, 227-236.
- Finnell, R.H., Bennett, G.D., Karras, S.B., Mohl, V.K. (1988). Common hierarchies of susceptibility to the induction of neural tube defects in mouse embryos by valproic acid and its 4-propyl-4-pentenoic acid metabolite. *Teratol.* **38**, 313-320.
- Gottlicher, M., Minucci S., Zhu, P., Kramer, O.H., Schimpf, A., Giavara, S., Sleeman, J.P., Lo Coco, F., Nervi, C., Pelicci, P.G., Heinzl, T. (2001). Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J.* **20**, 6969-6978.
- Gurvich, N., Berman, M.G., Wittner B.S., Gentleman, R.C., Klein, P.S., Green, J.B.A. (2005). Association of valproate-induced teratogenesis with histone deacetylase inhibition in vivo. *FASEB J* **19**, 1166-1188.
- Harden, C.L., Meador, K.J., Pennell, P.B., Hauser, W.A., Gronseth, G.S., French, J.A., Wiebe, S., Thurman, D., Koppel, B.S., Kaplan, P.W., Robinson, J.N., Hopp, J., Ting, T.Y., Gidal, B., Hovinga, C.A., Wilner, A.N., Vazquez, B., Holmes, L., Krumholz, A., Finnell, R., Hirtz, D., Le Guen, C. (2009). Management issues for women with epilepsy – Focus on pregnancy (an evidence-based review): II. Teratogenesis and perinatal outcomes. *Epilepsia* **50**, 1237-1246.
- Hockey, A., Bower, C., Goldblatt, J., Knowles, S. (1996). Fetal valproate embryopathy in twins: Genetic modification of the response to a teratogen. *Birth Def. Orig. Art. Series* **30**, 401-405.
- Kim, B., Rincon Castro, L.M., Jawed, S., Niles, L.P. (2008). Clinically relevant concentrations of valproic acid modulate melatonin MT₁ receptor, HDAC and MeCP2 mRNA expression in C6 glioma cells. *Eur. J. Pharmacol.* **589**, 45-48.

- Kini, U., Lee, R., Jones, A., Smith, S., Ramsden, S., Fryer, A., Clayton-Smith, J. (2007). Influence of the *MTHFR* genotype on the rate of malformations following exposure to antiepileptic drugs in utero. *Eur. J. Med. Genet* **50**, 411-420.
- Koren, G., Nava-Ocampo, A.A., Moretti, M.E., Sussman, R., Nulman, I. (2006). Motherisk update: Major malformations with valproic acid. *Can. Family Phys.* **52**, 441-447.
- Kozma, C. (2001). Valproic acid embryopathy: Report of two siblings with further expansion of the phenotypic abnormalities and a review of the literature. *Am. J. Med. Genet.* **98**, 168-175.
- Kultima, K., Nystrom, A-M., Scholz, B., Gustafson, A-L., Dencker, L., Stigson, M. (2004). Valproic acid teratogenicity: A toxicogenomics approach. *Environ. Health Persp.* **112**, 1225-1235.
- Livak, K.J., Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402-408.
- Malm, H., Kajantie, E., Kivirikko, S., Kaariainen, H., Peippo, M., Somer, M. (2002). Valproate embryopathy in three sets of siblings: Further proof of hereditary susceptibility. *Neurology* **59**, 630-633.
- Massa, V., Cabrera, R.M., Menegola, E., Giavini, E., Finnell, R.H. (2005). Valproic acid-induced skeletal malformations: associated gene expression cascades. *Pharmacogenetics Genom.*, **15**, 787-800.
- Meador, K.J., Pennell, P.B., Harden, C.L., Gordon, J.C., Tomson, T., Kaplan, P.W., Holmes, G.L., French, J.A., Hauser, W.A., Wells, P.G., Cramer, J.A. (2008a). Pregnancy registries in epilepsy. *Neurology* **71**, 1109-1117.

- Meador, K., Reynolds, M.W., Ceran, S., Fahrbach, K., Probst, C. (2008b). Pregnancy outcomes in women with epilepsy: A systematic review and meta-analysis of published pregnancy registries and cohorts. *Epilepsy Res.* **81**, 1-13.
- Menegola, E., Di Renzo, F., Broccia, M.L., Prudenziati, M., Minucci S., Massa V., Giavini E. (2005). Inhibition of histone deacetylase activity on specific embryonic tissues as a new mechanism for teratogenicity. *Birth Def. Res.* **74**, 392-398.
- Morrison, B.E., D'Mello, S.R. (2008). Polydactyly in mice lacking HDAC9/HDRP. *Exp. Biol. Med.* **233**, 980-988.
- Pennell, P.B. (2004). Pregnancy in women who have epilepsy. *Neurol. Clin.* **22**, 799-820.
- Stodgell, C.J., Ingram, J.L., O'Bara, M., Tisdale, B.K., Nau, H., Rodier, P.M. (2006). Induction of the homeotic gene *Hoxa1* through valproic acid's teratogenic mechanism of action. *Neurotoxicol. Teratol.* **28**, 617-624.
- Vega, R.B., Matsuda, K., Oh, J., Barbosa, A.C., Yang, X., Meadows, E., McAnally, J., Pomajzl, C., Shelton, J.M., Richardson, J.A., Karsenty, G., Olson, E.N. (2004). Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell* **119**, 555-566.
- Westendorf, J. (2007). Histone deacetylases in control of skeletogenesis. *J. Cell. Biochem.* **102**, 332-340.

Figure Legends

Figure 1. Western blot for acetylated histone H3 (H3ac) in embryo following *in utero* exposure to valproic acid (VPA) or ethanol. There were no differences in H3 among treatments or between strains, so band densities for each sample were normalized to H3. Results are presented as the percent change from maltose-treated controls. * indicates a significant increase compared to maltose control, $p < .01$.

Figure 2. Western blot for acetylated histone H4 (H4ac) in embryo following *in utero* exposure to valproic acid (VPA) or ethanol. There were no differences in H4 among treatments or between strains, so band densities for each sample were normalized to H4. Results are presented as the percent change from maltose-treated controls. * indicates a significant increase compared to maltose controls, $p < .01$; **, $p < .05$.

Figure 3. Western blot for H3ac in placenta. Band densities for acetylated H3ac were normalized to H3 for each sample and results are presented as the percent change from maltose-treated controls. * indicates a significant increase, $p < .01$; **, $p < .05$.

Figure 4. Western blot for H4ac in placenta. Band densities for acetylated H4ac were normalized to H4 for each sample and results are presented as the percent change from maltose-treated controls. * indicates a significant increase, $p < .01$.