



Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Jazayeri, D;Braine, E;McDonald, S;Dworkin, S;Powell, KL;Griggs, K;Vajda, FJE;O'Brien, TJ;Jones, NC

Title:

A rat model of valproate teratogenicity from chronic oral treatment during pregnancy

Date:

2020-06-01

Citation:

Jazayeri, D., Braine, E., McDonald, S., Dworkin, S., Powell, K. L., Griggs, K., Vajda, F. J. E., O'Brien, T. J. & Jones, N. C. (2020). A rat model of valproate teratogenicity from chronic oral treatment during pregnancy. *Epilepsia*, 61 (6), pp.1291-1300. <https://doi.org/10.1111/epi.16536>.

Persistent Link:

<https://hdl.handle.net/11343/275757>

DR. FRANK J. VAJDA (Orcid ID : 0000-0001-5570-7538)

DR. NIGEL C JONES (Orcid ID : 0000-0002-1080-8439)

Article type : Full length original research paper

### **A rat model of valproate teratogenicity from chronic oral treatment during pregnancy**

Dana Jazayeri<sup>a,d</sup>, Emma Braine<sup>a,c</sup>, Stuart McDonald<sup>b,c</sup>, Sebastian Dworkin<sup>b</sup>, Kim L. Powell<sup>a,c</sup>, Karen Griggs<sup>b</sup>, Frank J.E Vajda<sup>a,c</sup>, Terence J. O'Brien<sup>a,c</sup>, Nigel C. Jones<sup>\*a,c</sup>

<sup>a</sup>Department of Medicine (Royal Melbourne Hospital), University of Melbourne, Melbourne Brain Centre, Parkville, Victoria, 3050, Australia

<sup>b</sup>Department of Physiology, Anatomy and Microbiology, School of Life Sciences, La Trobe University, Melbourne, Victoria, 3086, Australia

<sup>c</sup>Department of Neuroscience, Central Clinical School, Monash University and Department of Neurology, The Alfred Hospital, Melbourne, Victoria, 3004, Australia

<sup>d</sup>La Trobe Centre for Sport and Exercise Medicine Research, School of Allied Health, Human Services and Sport, La Trobe University, Bundoora, VIC, Australia

#### **\*Author for correspondence:**

Associate Professor Nigel C. Jones.

Department of Neuroscience, Central Clinical School, Monash University, Melbourne, Victoria, 3004, Australia. Email: [nigel.jones@monash.edu](mailto:nigel.jones@monash.edu)

Number of words in abstract: 249

Number of words in article body: 4553

Number of figures: 4

Number of tables: 1

**This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as [doi: 10.1111/EPL.16536](https://doi.org/10.1111/EPL.16536)**

This article is protected by copyright. All rights reserved

## **Abstract**

**Objective:** Sodium Valproate (VPA), the most effective antiepileptic drug for patients with genetic generalised epilepsy (GGE), is a potent human teratogen which increases the risk of a range of congenital malformations, including spina bifida. The mechanisms underlying this teratogenicity are not known, but may involve genetic risk factors. This study aimed to develop an animal model of VPA-induced birth defects.

**Methods:** We used three different rat strains: inbred Genetic Absence Epilepsy Rats from Strasbourg (GAERS) – a model of GGE with absence seizures; inbred non-epileptic controls (NEC); outbred non-epileptic Wistars. Female rats were fed standard chow or VPA (20g/kg food) mixed in standard chow for two weeks prior to conception, and then mated with same-strain males. Treatment continued throughout pregnancy. Fetuses were extracted via C-section on gestational Day 21 and examined for birth defects, including external assessment and spinal measurements.

**Results:** VPA-exposed pups showed significant reductions in weight, length and whole-body development compared with controls of all three strains ( $p < 0.0001$ ). Gestational VPA treatment altered intra-vertebral distances, and resulted in underdeveloped vertebral arches between thoracic region T11 and caudal region C2 in most pups (GAERS 100%; NEC 95%; Wistar 80%), more frequent than in controls (9%; 13%; 19%).

**Significance:** Gestational VPA treatment results in similar developmental and morphological abnormalities in three rat strains, including one with GGE, indicating that the genetic underpinnings of epilepsy do not contribute markedly to VPA-induced birth defects. This model may be used in future studies to investigate mechanisms involved in the pathogenesis of AED-induced birth defects.

**Key words:** valproate; pregnancy; teratogenicity; epilepsy; GAERS; rodent model

## **Highlights**

- Pregnant women taking the anti-seizure drug sodium valproate for epilepsy have a high risk of birth defects in offspring
- Here, we developed a clinically relevant rat model of Valproate-induced birth defects
- Rat fetuses exposed to Valproate throughout gestation showed reductions in whole-body development, and developmental delay of vertebrae.

- Pregnant rats with GGE treated with VPA do not have different outcomes than non-epileptic dams.
- This suggests that the genetic background resulting in epilepsy does not influence the teratogenic effect of VPA.

## **Introduction**

Sodium valproate (VPA) is the most effective anti-epileptic drug (AED) in patients with genetic generalized epilepsies (GGE). However, VPA is also a well-established human teratogen, increasing the risk of birth defects by up to 17-fold in a dose-dependent manner<sup>1,2</sup>, which is a major cause for concern for women of child-bearing age who are required to take this drug to control their seizures<sup>3</sup>. The pathophysiological mechanisms associated with this teratogenicity have not yet been elucidated, but are essential in order to develop strategies to reduce this risk<sup>4,5</sup>. Previous research has utilized animal models of VPA-induced birth defects to study teratogenic mechanisms, including using rat, mouse, rabbit, monkey, *xenopus* and zebrafish models, both *in vivo* and *in vitro* (eg:<sup>6-8</sup>). From such animal models, it is evident that birth defect presentation varies depending on drug dose, gestational time point of administration, and method of delivery<sup>9</sup>. In humans, VPA monotherapy is associated with a significantly increased risk of six main malformation types; spina bifida, atrial septal defect, cleft palate, genital abnormalities such as hypospadias, polydactyly and craniosynostosis<sup>10</sup>. These defects are reflected to some extent in animal models, with the most common malformations in rodent studies being defects of the ribs, vertebrae, craniofacial bones and digits<sup>9</sup>.

Previous rodent studies have assessed VPA teratogenicity using a broad range of drug doses and animal strains resulting in malformations of varying degrees of severity and specificity. For example, Sprague-Dawley rats administered VPA at 400mg/kg/day via oral gavage from gestational day 7-18 exhibited cardiovascular, vertebral, and rib defects, as well as hypoplastic bladder, ectrodactyly and hydronephrosis, while higher doses resulted in embryo resorption and lower doses resulted in less severe malformations<sup>11</sup>. Approximately 20% of the offspring of Wistar rats administered VPA at 300mg/kg ip on the 8-9<sup>th</sup> day of pregnancy were found to have birth defects, such as spina bifida, cleft palate and exencephaly<sup>12</sup>, while rats receiving 600mg/kg VPA sc twice on gestational day 9 resulted in spina bifida occulta in 100% of exposed fetuses<sup>13</sup>. Mouse studies have found strain-dependent effects, with some strains

exhibiting higher rates of rib fusion following 200mg/kg ip VPA than others <sup>14</sup>, suggesting an underlying genetic susceptibility.

While these models recapitulate the clinical observation that VPA exerts teratogenic effects, they do not use doses that would necessarily be therapeutically effective against seizures, and most often do not expose the subject to the drug chronically to mimic a clinical scenario where women with epilepsy are taking the drug 2-3 times per day from before conception throughout pregnancy. Instead, they frequently target specific gestational timepoints, most prominently gestational days 7-9 in rodent studies. The importance of having a clinically relevant treatment regime and method of administration was highlighted in a study comparing continuous infusion of VPA with repeated daily injections in mice <sup>15</sup>. Acute daily injections of 400mg/kg sc VPA on gestational days 7-15 led to extreme peaks in drug concentration, but extended periods without any drug detectable in blood given the short half-life of VPA in rats of 1-2 hours <sup>16</sup>, whereas delivery via osmotic pump resulted in consistent blood levels across the experimental timeline without the peaks and troughs seen in clinical use in humans. The differences in birth defects generated by these two regimes was extreme: repeated acute doses resulted in many malformations with the most severe being exencephaly, whereas constant infusion via mini-pumps resulted in less severe malformations and no exencephaly <sup>15</sup>.

In this current study, we aimed to advance on previous reports of animal models of VPA-induced birth defects, focusing on spina bifida occulta, incorporating a series of strategies. First, we chose to deliver the VPA chronically, in a clinically relevant manner, administering the drug via the animal feed. We also used a variety of rat strains, including a strain with GGE – Genetic Absence Epilepsy Rats from Strasbourg (GAERS) <sup>17</sup>. We also studied the genetically related Non-Epileptic Control (NEC) rat strain<sup>18</sup>, originating from the same Wistar colony but separated many decades ago, which do not manifest seizures. Outbred Wistar rats were also included to assess an unrelated non-epileptic strain. The comparison of three strains allows us to assess whether there are genetic drivers of VPA-induced birth defects, in particular whether the same genes that cause GGE may also promote risk for VPA teratogenicity.

## **Materials and Methods**

The study was performed in two parts. First, we developed methodology to deliver VPA in the chow of epileptic rats, tailoring the concentration to achieve clinically relevant blood levels which effectively suppress absence seizures in GAERS without major adverse effects. Second,

we investigated the effects of gestational VPA on the anatomy of rodent offspring using three different rat strains, including one with GGE with absence seizures (GAERS).

### **Subjects**

Female and male GAERS<sup>17</sup> (n = 42) and inbred non-epileptic control rats (NEC) (n = 28) were obtained from our breeding colonies at the Department of Medicine, Royal Melbourne Hospital, University of Melbourne. Outbred Wistar rats (n = 20) were sourced from the Animal Resource Centre (ARC), Western Australia. Animals were kept under a 12-hour light-dark cycle (lights on at 7am) maintained at 19 - 22°C. Over the course of the study, we also used n = 370 rat fetuses. Ethics approval was obtained from Florey Animal Ethics committee.

### **Drug delivery paradigm**

For this aspect of the study, we used 16-19 week old GAERS, an age when the seizure phenotype is well-developed<sup>19, 20</sup>. Animals were fed a standard diet (Specialty Feeds, WA<sup>21</sup>) premixed with either 0, 5, 10 or 20g/kg of VPA (Sigma Aldrich, St Louis, MO). This diet persisted for the duration of the study, and the amount consumed measured daily. After 2 weeks treatment, VPA levels in blood serum were assessed between 9-11am. VPA levels in serum were measured by Melbourne Pathology (Royal Melbourne Hospital, Victoria, Australia), using an AxSYM Valproic acid assay.

### **Electrode implantation**

Another cohort of GAERS underwent surgical implantation of EEG electrodes to facilitate measurement of seizures, as previously described<sup>22</sup>. Briefly, rats were anaesthetized with isoflurane, and the skull surface was exposed. Six small burr holes drilled into the surface, and six electrodes (E363/20/SPC Plastics One, Roanoke, VA, USA) were screwed into the holes. Electrodes were inserted into a pedestal, and the entire assembly stabilized with cyanoacrylate glue.

### **EEG recording and seizure analysis**

After recovery from surgery, GAERS underwent EEG recordings, as previously described<sup>23</sup>. This entailed connecting the animal's headpiece to an amplifier via a cable (Plastics One), and then to a computer. The EEG was visualized using Profusion software (Compumedics, Australia). Two recording sessions of 24 hours each were conducted one week prior to treatment initiation to confirm all animals were seizing and to obtain the pre-treatment seizure

frequency. The following week, treatment was initiated with rats exposed to 20g VPA/kg chow. After one week of drug treatment, another two EEG recording sessions were conducted. Six hours of each recording session were analysed for spike-wave discharges (3 hours during the day, 3 during night). EEG recordings were analysed using SpikeWave Complex Finder (Spikewave Finder, Netherlands). No differences were found between day and night, or between the first and second recording sessions, so data was pooled.

### **Gestational drug delivery study**

Once we established that a concentration of 20g/kg resulted in appropriate blood drug levels, and reduced seizures in GAERS, we embarked upon our primary study – assessment of teratogenic effects of VPA. Nine week old female rats of the three strains were fed a standard rodent diet pre-mixed with either 20g/kg of VPA (GAERS n=5 rats, NEC n=6, Wistar n=6) or control diet (GAERS n= 5, NEC n=8, Wistar n= 4). After 2 weeks, rats were mated with males of the same strain and age in cages with mesh floors overnight, which were provided to allow identification of the copulatory plug. The presence of a plug in the morning indicated day 0 of pregnancy, at which time the males were removed. The respective diets were continued for the duration of the pregnancy. Within 24 hours of day 21 of pregnancy, females underwent C-section.

### **Teratology studies**

Dams were sacrificed via carbon dioxide euthanasia, and an incision made in the abdominal wall, the uterus was cut open and fetuses removed and separated. Fetuses were immediately sacrificed by freezing, and then photographed, weighed and crown-rump length recorded. Each animal was assessed for external malformations such as external spina bifida, and any other signs of abnormal development. They were then fixed in 10% formalin for vertebral integrity studies.

### **Morphological scoring system**

A 4-point visual scoring system was compiled to score the development of the pups based on their morphological appearance. This was modified from a previous system<sup>24</sup>. Pups with a score of four required a ‘structure entirely normal and pup fully developed and healthy’; score of 3 - ‘structure is largely normal but pup looks slightly underdeveloped’; score of 2 – ‘some structure is evident but pup is severely underdeveloped’; score of 1 – ‘no structure is evident, pup represents a small attachment to the placenta or a resorption’.

### **Vertebral integrity studies**

To assess the integrity of the spine, cartilage and bone of fetuses were stained using a previous method<sup>25</sup>. This was done in a subset of litters. Fetuses were put in 10% formalin solution with a buffer of magnesium carbonate (0.1g/200ml) for 2 days and then skinned and eviscerated. The pups were then stained for 72h with 0.01% alcian blue (Sigma-Aldrich, Australia), followed by immersion in reducing concentrations of ethanol over 6h, then in 2% KOH (Sigma) for 48h. They were then put in PBS for 24 hrs, and immersed in 0.001% alizarin red (Sigma) in 1% KOH for 24h. They were then washed in 1% KOH for 2h, and put into increasing concentrations of glycerol in dH<sub>2</sub>O over 24h. The fetuses were removed from the glycerol and imaged under stereomicroscope using Leica IM50, v.5 Leica Microsystems (Switzerland). The distance between the cartilaginous ends of each vertebral arch measured using LeicaQWin Standard v3.5.1, Leica Microsystems by an investigator blinded to strain and treatment. When doing this, we noted several instances where vertebrae were unstained. Alcian blue stains glycosaminoglycans (GAGs) present in mature cartilage, and so the development of these unstained vertebrae were considered to be delayed and not yet representing mature cartilage. The frequency of unstained vertebrae in each foetus was noted between T11 – C2.

### **Statistical Analysis**

First, all data was tested for normality. In all cases, data followed a normal distribution, and so parametric tests were conducted to assess effects of diet. A paired t-test was performed to compare effect of diet on seizure frequency before and after initiation of diet. One-way ANOVA was used to compare consumption rates of rats exposed to different doses of VPA in food. Two-way ANOVA were used to compare the effects of treatment and strain on litter size, pup length, and morphological scores. For spinal measurements, we included all vertebral arches where information was available. If information was not available (in some circumstances, poor image quality prevented definitive measurement being made), all other measurements from that spine were still included. Therefore, we used mixed models ANOVA to compare the intra-vertebral distances in each strain, with Sidak's multiple comparison post-hoc test conducted as appropriate. Chi square analysis assessed the proportion of treated and untreated offspring with underdeveloped vertebral arches in each strain, and Pearson's correlation to determine the relationship between VPA concentration provided food and observed in blood. Because the laced food was a different color than control food, food intake, litter sizes, pup morphology and pup lengths were not calculated in a blinded fashion. All other

outcomes were analysed blindly following coding of the respective stored files/samples.

## **Results**

### **Adding VPA to rodent diet does not impact food intake, and linearly increases blood VPA levels.**

First, we established the palatability and bioavailability of VPA delivered via the chow. Rats fed a diet containing various concentrations of VPA up to 20g/kg consumed a similar amount of food as standard chow-fed rats ( $F_{(3,16)}=1.64$ ;  $p=0.22$ ; Figure 1A). Blood taken from these animals demonstrated a significant linear relationship between concentration of drug in food and concentration in blood ( $r=0.90$ ;  $p<0.0001$ ; Figure 1B), with the highest palatable dose of 20g/kg leading to blood levels of  $\sim 220\mu\text{mol/L}$ . Rats treated with 20g VPA/kg chow equates to  $\sim 1000\text{mg/kg/day}$  VPA. We also exposed some animals to 40g/kg VPA, but this concentration was not consumed, and so these subjects were discontinued prematurely.

### **Dietary VPA reduces seizures in GAERS**

Next, we probed whether treating epileptic rats with VPA via the food was therapeutically effective. We measured the number of seizures based on the spike-wave discharge frequency recorded on the EEG (Figure 1C for example), both before and after GAERS were treated with 20g/kg VPA in food. The addition of VPA to the diet resulted in a significant suppression in the number of absence seizures recorded per hour on the EEG ( $t_{(4)}=3.74$ ,  $p=0.020$ ; Figure 1D).

### **Gestational VPA results in reduced offspring length, but does not impact litter size**

After having established the appropriate concentration of VPA in the food which resulted in adequate blood levels and reduction in seizures, we embarked upon our major study: to assess the impact of gestational VPA on rodent offspring anatomy in three rat strains. In all strains, VPA was detected in the blood of the pregnant females exposed to dietary VPA, and this did not differ between strains ( $F_{(2,25)}=1.06$ ;  $p=0.36$ ; Figure 2A). VPA was undetectable in rats not treated with VPA.

The length of each pup was measured on the day of the C-section. Firstly, the average size of each litter did not differ between treatment groups ( $F_{(1,30)}=2.24$ ;  $p=0.14$ ), but did vary by strain ( $F_{(1,30)}=5.29$ ;  $p=0.011$ ; Figure 2B), with Wistar rats having the greatest litter sizes. Pups treated with VPA were significantly shorter ( $F_{(1,96)}=160.2$ ;  $p<0.0001$ ; Figure 2C). In addition, there were significant differences in pup length in the different rat strains ( $F_{(2,96)}=88.3$ ;  $p<0.0001$ ), and a significant treatment x strain interaction ( $F_{(2,96)}=19.5$ ;  $p<0.0001$ ), with NEC

rat pups only relatively modestly affected by VPA exposure. In terms of the dams, these gained weight at different rates throughout pregnancy and were as follows: GAERS control -  $58.6 \pm 22.1$ g; GAERS VPA -  $39.4 \pm 8.8$ g; NEC control -  $90.35 \pm 6.9$ g; NEC VPA -  $58.1 \pm 7.9$ g; Wistar control -  $245.9 \pm 9.4$ g; Wistar VPA -  $182.5 \pm 84.8$ g. Statistically, there was a main effect of strain ( $F_{(2,9)}=19.5$ ;  $p=0.001$ ), but not of treatment ( $F_{(1,9)}=19.5$ ;  $p=0.15$ ), and no interaction ( $F_{(2,9)}=19.5$ ;  $p=0.77$ ).

### **Gestational VPA causes morphological abnormalities in offspring**

Using our modified morphological scoring system (examples given in Figure 3A), we found striking changes in embryonic development following gestational exposure to VPA. Rat offspring treated with gestational VPA exhibited significantly lower morphological scores, compared to control treated offspring ( $F_{(1,348)}=143$ ;  $p<0.0001$ ; Figure 3B). This also varied significantly with strain ( $F_{(2,348)}=11.3$ ;  $p<0.0001$ ) and there was a significant treatment x strain interaction ( $F_{(2,348)}=5.5$ ;  $p=0.0044$ ), such that Wistar rats appeared relatively protected from VPA. We designed these studies to examine the individual offspring, and so treated each pup as a biological replicate. However, we also conducted analysis examining the litter as the replicate. In this case, we averaged the morphological score from each pup in a given litter, then ascribing that score to the litter, and performed the same statistical analyses. In this case, effect of treatment was still highly significant, with VPA-treated litters showing reduced scores ( $F_{(1,30)}=57.5$ ;  $p<0.0001$ ), significant effects of strain ( $F_{(1,30)}=5.36$ ;  $p=0.0102$ ) and a significant treatment x strain interaction ( $F_{(2,30)}=3.9$ ;  $p=0.031$ ).

### **Gestational VPA results in malformation of the spine of offspring**

Given the increased incidence of spina bifida in humans exposed to gestational VPA, we were interested in studying the effects of VPA on rat vertebrae in our model. We first measured the average horizontal distance between each vertebral arch (Figure 4A depicts an example of a section of spine measured). Overall, we found that VPA-exposed offspring presented with altered intervertebral distances, compared to control-treated offspring. This was borne out by statistically significant interactions between vertebral location and treatment in all three strains ( $p<0.0001$  for all strains; Figure 4). Broadly speaking, the distances between thoracic and lumbar vertebrae were shorter following VPA exposure, whereas sacral vertebrae were longer. When examining the spines, it became clear that a number of pups had underdeveloped vertebral arches which did not stain for Alcian Blue, and so this was also quantified. We found that, while some control-treated pups showed unstained arches, this number was markedly

increased in VPA-exposed pups from all three strains (GAERS:  $p=0.014$ ; NEC:  $p=0.0007$ ; Wistar:  $p=0.019$ , Table 1). The proportions of pups with underdeveloped vertebral arches in the VPA-exposed groups did not vary between strains ( $p=0.89$ ).

## **Discussion**

Here we report the development and validation of a translational animal model of VPA-induced teratogenicity, reflecting the clinical setting of women becoming pregnant while being chronically treated for epilepsy with VPA. The strengths of this model include the use of genetically epileptic rats, the non-invasive and chronic oral delivery of VPA, the ability to tailor the dose to match blood levels observed in humans, and the demonstration of the therapeutic efficacy of such dosage. The developmental consequences of gestational VPA exposure in our model were marked: specifically, we found dramatically smaller and underdeveloped pups on G21, as well as altered intravertebral distances, and a significant developmental delay of vertebral arches. These deficits were not impacted by rat strain, with the rats having genetic epilepsy not increasing the risk or changing the nature of the adverse outcomes in the pups seen. This model can therefore be used in future research to identify biological mechanisms involved in the pathogenesis of VPA induced teratogenicity, as well as investigate novel therapeutic avenues.

We examined the effect of gestational exposure to VPA using three rat strains in order to explore the potential influence of genetic determinants, in particular those that result in genetic generalised epilepsy. The GAERS and NEC strains are both derived from the same original Wistar rat colony, but were selectively inbred to express, or to not express, absence seizures<sup>18; 26</sup>. This then allowed us to investigate whether the genes that result in genetic generalised epilepsy in the GAERS strain (or the absence seizures themselves) can influence the incidence or severity of VPA-induced effects on the fetus. Interestingly, we did not note any marked differences in any of the consequences of gestational VPA exposure between these strains, suggesting that these factors do not play an important role. The exception was that the size of the NEC rat pups was less affected by VPA exposure than those of GAERS or non-epileptic Wistar mothers. However, this finding should be interpreted with some caution, since the VPA-exposed NEC pups were still significantly smaller than the vehicle-treated NEC pups, but this could suggest that genetic differences may contribute to this phenotype. A previous study made a comprehensive assessment of the effects of genetic background on VPA-induced defects in mice, showing striking differences. However, this previous study used a less

clinically relevant VPA exposure regime: three injections of 400mg/kg delivered on gestational day 9 – a very high dose – which also resulted in variable levels of lethality to different strains<sup>14</sup>. There is some evidence for a genetic component associated with VPA-induced birth defects in humans<sup>27; 28</sup>. However, a study of large international prospective pregnancy registers found no evidence that patients with genetic generalised epilepsy have an increased risk of having a baby with a birth defect when the confounding effect of treatment with VPA is factored into the analysis, suggesting the genes which contribute to GGE with absence seizures may not overlap with those promoting susceptibility to birth defects. In addition to the potential for genetic susceptibility, there is also literature that suggests generalised tonic-clonic seizures (GTCS) *per se* can be dangerous to the developing fetus in humans, resulting from hypoxia, lactic acidosis or bradycardia, although the same does not appear to be true of other seizure types<sup>29</sup>. The GAERS model exhibits absence seizures, but not GTCS, and so the interaction between GTCS and fetal outcomes could not be investigated in this study. However, the lack of difference between vehicle-treated rat strains supports evidence that absence seizures do not contribute to birth defects, and do not interact with the teratogenic effects of VPA. It would be possible to extend our study to explore whether GTCS themselves can cause birth defects, and interact with VPA, using models of acquired epilepsy that exhibit GTCS, such as the post-SE models<sup>30</sup>.

One challenge for developing a valid treatment paradigm in an epilepsy animal model is maintaining sustained exposure to the drug over time in a clinically relevant manner. The majority of AEDs have half-lives of between 8-15 hours in humans and are taken 2 – 3 times per day resulting in peaks and troughs in blood drug levels. In rodents, the half-life of AEDs, including VPA, in the order of no more than 1-2 hours<sup>16</sup>. Therefore, the previous studies that have investigated the effect of gestational exposure by administering high dose intermittent boluses will have resulted in large drug peaks interspersed with long periods with minimal drug levels. Hence, we endeavored to deliver the drug by food in order to achieve more sustained blood concentrations with peaks and troughs related to when the rats ate<sup>21</sup>. Our initial dose-validation study identified 20g VPA/kg food as a target dose, and this converts to an average dose per rat of ~1000mg/kg/rat per day. Although this is numerically similar to doses used in other previous studies resulting in birth defects in mice and rats<sup>13; 31</sup>, the method of delivery is more clinically relevant, spreading the dose over the waking hours. Almost all previous studies used single or repeated injections with the consequential high peak levels are outside the clinical therapeutic range, and result in greater peak dose effects than our oral delivery in the food<sup>15</sup>. Supporting this, we measured VPA blood levels between 9-11 am, and found that this

dose resulted in VPA levels in the mothers' blood approaching human therapeutic levels (ie: ~300-600 $\mu$ mol/L<sup>32</sup>). We attempted to increase the blood levels to be within this human therapeutic window, but the highest dose we attempted to provide to the rats was not consumed. We do not know whether this was because the food was not palatable, or because of adverse drug reactions. If the latter, altering the method of delivery, for example using osmotic minipumps<sup>33; 34</sup> would not have changed the outcome. Nevertheless, we did see significant seizure suppression with a dose of 20g/kg, indicating effective seizure suppression, and validating the dose in our study.

Gestational VPA exposure in this model resulted in striking developmental abnormalities in the pups. Morphological abnormalities were easily identifiable and graded with our scoring scale, and the average weights and lengths of the pups were also significantly reduced in VPA-exposed pups. Although some pups were born severely underdeveloped, no specific obvious external malformations were identified such as external spina bifida (spina bifida aperta), cleft palate or tail malformations. These are types of birth defect commonly observed in previous studies of VPA induced teratogenicity in rodents (eg:<sup>6; 11</sup>). In addition, we found alterations in intra-vertebral distances in VPA-exposed pups. However, the relatively modest changes in this measure - used as a proxy for spina bifida - appears phenotypically quite different from the stark changes reported previously (eg:<sup>13</sup>). This could be attributed to a difference in treatment regime, with the chronic oral dosing regimen used in this study providing an exposure to the developing fetus that is more relevant to that occurring in women with epilepsy treated with VPA. Spina bifida is now a relatively uncommon type of birth defect in pregnant taking VPA, as this is dose-dependent, and so its incidence has dramatically declined as doses of VPA prescribed to pregnant women in clinical practice has declined over the past two decades<sup>35-38</sup>. Not surprisingly, therefore, bolus injections of VPA in pregnant mothers, resulting in large peaks of exposure, appear to result in more substantial teratogenic effects – particularly effecting the neural tube. We also observed a striking reduction in the number of vertebral arches staining for Alcian Blue following in utero VPA exposure, indicative of developmental delay of these structures. Alcian Blue stains for mature cartilage that has developed past the stage of mesenchymal condensation, then releasing GAGs which interact with the dye. Vertebral anomalies have been reported in animal studies of VPA teratogenicity before<sup>14; 39</sup> and is critical to our model validation<sup>10</sup>. It was also interesting to see that a small proportion of control animals had underdeveloped vertebral arches, although this is consistent with literature. For example, one study reported that a proportion of saline-treated mice exhibited vertebral malformations including fused, missing or asymmetrical

arches<sup>39</sup>. While in the current study, we primarily focussed on birth defects associated with spinal development, future studies should also examine other features linked to VPA exposure in humans, such as development of the heart, facial structure, genitals, digits, and skull<sup>9</sup>. In addition, we did not make full characterization of the pregnancy itself, including assessment of number of live and dead fetuses, early and late resorptions, and pre- and post-implantation losses. These would be valuable information to generate in future studies to assess the global effects of VPA exposure on the pregnancy.

The common observations of VPA-induced birth defects, and the importance of VPA in clinical practice as the most effective drug in patients with genetic generalised epilepsy, has promoted speculation about the mechanisms driving such effects. These mechanisms may be unique to this AED, since the associated birth defects are more common than with other AEDs<sup>5</sup>, or they could be common with other AEDs that are also associated with an increased risk of birth defects and neurodevelopmental problems. Epigenetic effects of VPA are a strong candidate, since these mechanisms play important roles during development<sup>40</sup>, and VPA is well-recognised Histone Deacetylase (HDAC) inhibitor<sup>41</sup>. In support of an epigenetic role, one study used methionine – a key methylation precursor – to attempt to prevent VPA-induced birth defects. Treatment of pregnant mice with VPA resulted in spina bifida occulta in the pups, and pretreatment with methionine was able to significantly reduce the incidence of spina bifida and other associated birth defects, suggesting that supplementation with this epigenetic factor was able to overcome the consequences of VPA<sup>42</sup>. In addition, another study examining *Xenopus* and zebrafish showed remarkable overlapping developmental malformations with VPA and other HDAC inhibitors, while VPA analogues without HDAC inhibition did not cause these effects<sup>8</sup>.

To conclude, here we report the development and validation of a novel, clinically relevant, rodent model of VPA-induced birth defects. Rats exposed to therapeutic levels of VPA chronically throughout gestation, by oral delivery through their food, were underdeveloped, morphologically abnormal, and showed significant spinal abnormalities relevant to spina bifida occulta. These effects did not appear to be influenced to genetic differences between the GAERS, NEC and Wistar strains used, including the former which expresses genes for genetic generalised epilepsy. The model is relatively simple to implement, does not require excessive handling or injections of the rats throughout pregnancy, and results in robust, clinically relevant consequences in the offspring. Future studies can use this model and approach to investigate the mechanisms underlying VPA-induced birth defects and developmental abnormalities in humans, and to explore treatment options or strategies to

mitigate the risk of these adverse outcomes occurring in women with epilepsy.

**Disclosure:** The authors have nothing to disclose

**Acknowledgements:** This work was conducted as part of the PhD thesis of Dr. Dana Jazayeri, University of Melbourne, entitled: Antiepileptic drug teratogenicity - A Human and Laboratory Translational Study. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

### **Figure legends**

**Figure 1:** Validation of appropriate VPA dosing. (A) Food laced with varying concentrations of VPA (from 0g – 20g/kg food) did not affect the intake of food in female GAERS (n=5/concentration). Higher doses were not palatable (not shown). (B) Increasing concentration of VPA in food led to linearly increasing blood VPA levels in GAERS. (C) Example of a Spike-and-Wave seizure recorded from GAERS. (D) Epileptic GAERS (n=5) exposed to 20g VPA/kg food exhibit significantly fewer seizures than prior to treatment initiation (\*p<0.05).

**Figure 2:** Effects of gestational VPA on litter sizes in different rat strains. (A) Female GAERS, NEC rats and Wistar rats all show significant blood VPA levels when exposed to 20g VPA/kg food. VPA was never detected in control rats. (B) Litter size was not impacted by gestational exposure to VPA. (C) However, pup length was significantly reduced in all three rat strains exposed to gestational VPA. This was most marked in GAERS and Wistar rats (\*p<0.05; \*\*\*\*p<0.0001). Data generated from n=6 GAERS control litters (total 58 pups), n=6 GAERS VPA-treated litters (54 pups), n=8 NEC control litters (total 81 pups), n=6 NEC VPA-treated litters (54 pups), n=4 Wistar control litters (total 54 pups), n=6 Wistar VPA-treated litters (69 pups).

**Figure 3:** Effect of gestational VPA on body morphology of rat pups. (A) Examples of pups in each of the four scoring categories. (B) In utero exposure to VPA resulted in significantly lower morphological development scores in all three rat strains (\*\*\*\*p<0.0001). Sample sizes as for Figure 2 legend.

**Figure 4:** Effect of gestational VPA on intra-vertebral distances of rat pups. (A) Example

image of rat spine, indicating the locations of unstained and therefore underdeveloped Lumbar (L1-3) and Thoracic (T12-13) vertebrae. Significantly different intra-vertebral distances in GAERS (B), NEC (C) and Wistar (D) pups exposed to gestational VPA, compared with control treated pups (\* $p < 0.05$ ; \*\* $p < 0.01$ ). Data generated from  $n=3$  GAERS control litters (total 16 pups),  $n=3$  GAERS VPA-treated litters (17 pups),  $n=6$  NEC control litters (total 41 pups),  $n=4$  NEC VPA-treated litters (26 pups),  $n=3$  Wistar control litters (total 24 pups),  $n=4$  Wistar VPA-treated litters (27 pups).

### **Table legend**

**Table 1:** Number of rats exhibiting unstained vertebral arches following control or VPA treatment. Total number of rat pups examined is the denominator. GAERS, NEC and Wistar rats exposed to gestational VPA all had significantly higher proportions of offspring with unstained vertebral arches than control-treated rats. The percentage of analysed litters that had at least one pup with an unstained vertebral arch were: GAERS control - 33%; GAERS VPA - 100%; NEC control - 17%; NEC VPA - 100%; Wistar control - 100%; Wistar VPA: 100%. Litter sizes are as for Figure 4 legend.

### **References:**

1. Vajda FJ, O'Brien T J, Hitchcock A, et al. Critical relationship between sodium valproate dose and human teratogenicity: results of the Australian register of anti-epileptic drugs in pregnancy. *J Clin Neurosci* 2004;11:854-858.
2. Vajda FJ, O'Brien TJ, Hitchcock A, et al. The Australian registry of anti-epileptic drugs in pregnancy: experience after 30 months. *J Clin Neurosci* 2003;10:543-549.
3. Tomson T, Battino D. Teratogenic effects of antiepileptic drugs. *Lancet Neurol* 2012;11:803-813.
4. Meador KJ. Effects of in utero antiepileptic drug exposure. *Epilepsy Curr* 2008;8:143-147.

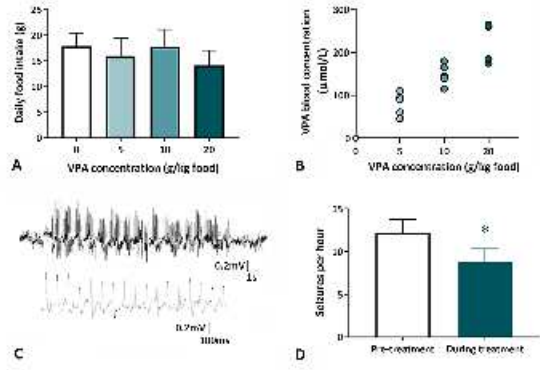
5. Tomson T, Battino D, Bonizzoni E, et al. Dose-dependent risk of malformations with antiepileptic drugs: an analysis of data from the EURAP epilepsy and pregnancy registry. *Lancet Neurol* 2011;10:609-617.
6. Menegola E, Broccia ML, Nau H, et al. Teratogenic effects of sodium valproate in mice and rats at midgestation and at term. *Teratog Carcinog Mutagen* 1996;16:97-108.
7. Hendrickx AG, Nau H, Binkerd P, et al. Valproic acid developmental toxicity and pharmacokinetics in the rhesus monkey: an interspecies comparison. *Teratology* 1988;38:329-345.
8. Gurvich N, Berman MG, Wittner BS, et al. Association of valproate-induced teratogenesis with histone deacetylase inhibition in vivo. *FASEB J* 2005;19:1166-1168.
9. Hill DS, Wlodarczyk BJ, Palacios AM, et al. Teratogenic effects of antiepileptic drugs. *Expert Rev Neurother* 2010;10:943-959.
10. Jentink J, Loane MA, Dolk H, et al. Valproic acid monotherapy in pregnancy and major congenital malformations. *N Engl J Med* 2010;362:2185-2193.
11. Vorhees CV. Teratogenicity and developmental toxicity of valproic acid in rats. *Teratology* 1987;35:195-202.
12. Mahabady M, Varzi H, Ranjbar R, et al. Prophylactic effects of melatonin on sodium valproate-induced neural tube defects and skeletal malformations in rat embryos *American Journal of Applied Sciences* 2011;8:413-419.
13. Ceylan S, Duru S, Ceylan S. Valproic acid sodium-induced spina bifida occulta in the rat. *Neurosurg Rev* 2001;24:31-34.
14. Faiella A, Wernig M, Consalez GG, et al. A mouse model for valproate teratogenicity: parental effects, homeotic transformations, and altered HOX expression. *Hum Mol Genet* 2000;9:227-236.
15. Nau H, Zierer R, Spielmann H, et al. A new model for embryotoxicity testing: teratogenicity and pharmacokinetics of valproic acid following constant-rate administration in the mouse using human therapeutic drug and metabolite concentrations. *Life Sci* 1981;29:2803-2814.
16. Loscher W. The pharmacokinetics of antiepileptic drugs in rats: consequences for maintaining effective drug levels during prolonged drug administration in rat models of epilepsy. *Epilepsia* 2007;48:1245-1258.

17. Powell KL, Tang H, Ng C, et al. Seizure expression, behavior, and brain morphology differences in colonies of Genetic Absence Epilepsy Rats from Strasbourg. *Epilepsia* 2014;55:1959-1968.
18. Casillas-Espinosa PM, Powell KL, Zhu M, et al. Evaluating whole genome sequence data from the Genetic Absence Epilepsy Rat from Strasbourg and its related non-epileptic strain. *PLoS One* 2017;12:e0179924.
19. Jones NC, Salzberg MR, Kumar G, et al. Elevated anxiety and depressive-like behavior in a rat model of genetic generalized epilepsy suggesting common causation. *Exp Neurol* 2008;209:254-260.
20. Dezsi G, Ozturk E, Salzberg MR, et al. Environmental enrichment imparts disease-modifying and transgenerational effects on genetically-determined epilepsy and anxiety. *Neurobiol Dis* 2016;93:129-136.
21. Senn SM, Kantor S, Poulton IJ, et al. Adverse effects of valproate on bone: defining a model to investigate the pathophysiology. *Epilepsia* 2010;51:984-993.
22. Dezsi G, Ozturk E, Stanic D, et al. Ethosuximide reduces epileptogenesis and behavioral comorbidity in the GAERS model of genetic generalized epilepsy. *Epilepsia* 2013;54:635-643.
23. Dezsi G, Sieg F, Thomas M, et al. Disease-Modifying Effects of Neural Regeneration Peptide 2945 in the GAERS Model of Absence Epilepsy. *Neurochem Res* 2017;42:2055-2064.
24. Augustine K. Assessment of the Zebrafish Model for Developmental Toxicology Screening, 2011. Available at: <http://www.diaglobal.org/productfiles/27480/5.2.k.augustine.pdf>.
25. Wassersug RJ. A procedure for differential staining of cartilage and bone in whole formalin-fixed vertebrates. *Stain Technol* 1976;51:131-134.
26. Marescaux C, Vergnes M, Depaulis A. Genetic absence epilepsy in rats from Strasbourg--a review. *J Neural Transm Suppl* 1992;35:37-69.
27. Duncan S, Mercho S, Lopes-Cendes I, et al. Repeated neural tube defects and valproate monotherapy suggest a pharmacogenetic abnormality. *Epilepsia* 2001;42:750-753.
28. Vajda FJ, O'Brien TJ, Lander CM, et al. Teratogenesis in repeated pregnancies in antiepileptic drug-treated women. *Epilepsia* 2013;54:181-186.
29. Battino D, Tomson T. Management of epilepsy during pregnancy. *Drugs* 2007;67:2727-2746.

30. Carron S, Dezsi G, Ozturk E, et al. Cognitive deficits in a rat model of temporal lobe epilepsy using touchscreen-based translational tools. *Epilepsia* 2019;60:1650-1660.
31. Briner W, Lieske R. Arnold-Chiari-like malformation associated with a valproate model of spina bifida in the rat. *Teratology* 1995;52:306-311.
32. Johannessen CU, Johannessen SI. Valproate: past, present, and future. *CNS Drug Rev* 2003;9:199-216.
33. Anderson PM, Pinault D, O'Brien TJ, et al. Chronic administration of antipsychotics attenuates ongoing and ketamine-induced increases in cortical gamma oscillations. *Int J Neuropsychopharmacol* 2014;17:1895-1904.
34. Cardamone L, Salzberg MR, Koe AS, et al. Chronic antidepressant treatment accelerates kindling epileptogenesis in rats. *Neurobiol Dis* 2014;63:194-200.
35. Vajda FJ, O'Brien TJ, Graham JE, et al. Dose dependence of fetal malformations associated with valproate. *Neurology* 2013;81:999-1003.
36. Vajda FJ, O'Brien TJ, Graham J, et al. The Australian Register of antiepileptic drugs in pregnancy: changes over time in the epileptic population. *J Clin Neurosci* 2014;21:1478-1482.
37. Vajda FJE, Graham JE, Hitchcock AA, et al. Antiepileptic drugs and foetal malformation: analysis of 20 years of data in a pregnancy register. *Seizure* 2019;65:6-11.
38. Tomson T, Battino D, Bonizzoni E, et al. Declining malformation rates with changed antiepileptic drug prescribing: An observational study. *Neurology* 2019;93:e831-e840.
39. Downing C, Biers J, Larson C, et al. Genetic and maternal effects on valproic acid teratogenesis in C57BL/6J and DBA/2J mice. *Toxicol Sci* 2010;116:632-639.
40. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001;293:1089-1093.
41. Gottlicher M, Minucci S, Zhu P, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J* 2001;20:6969-6978.
42. Ehlers K, Elmazar MM, Nau H. Methionine reduces the valproic acid-induced spina bifida rate in mice without altering valproic acid kinetics. *J Nutr* 1996;126:67-75.

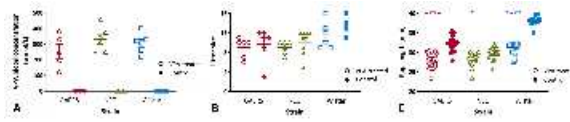
	<b>GAERS</b>	<b>NEC</b>	<b>WISTAR</b>
<b>Control</b>	1/11 (9%)	4/30 (13%)	4/21 (19%)
<b>VPA-treated</b>	12/12 (100%)	19/20 (95%)	16/20 (80%)

Author Manuscript

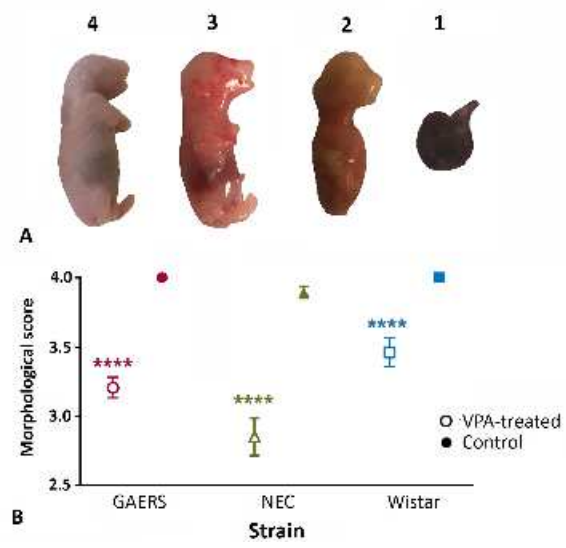


epi\_16536\_f1.tif

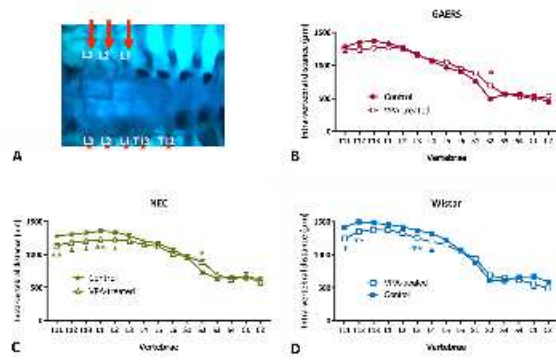
# Author Manuscript



epi\_16536\_f2.tif



epi\_16536\_f3.tif



epi\_16536\_f4.tif