Title: Early physical and motor development of mouse offspring exposed to valproic acid throughout intrauterine development

Author: Jelena Podgorac Vesna Pešić Željko Pavković Ljiljana Martać Selma Kanazir Ljupka Filipović Slobodan Sekulić

PII: S0166-4328(16)30298-4
DOI: http://dx.doi.org/doi:10.1016/j.bbr.2016.05.023
Reference: BBR 10202

To appear in: *Behavioural Brain Research*

Received date: 30-9-2015
Revised date: 6-5-2016
Accepted date: 10-5-2016

Please cite this article as: Podgorac Jelena, Pešić Vesna, Pavković Željko, Martać Ljiljana, Kanazir Selma, Filipović Ljupka, Sekulić Slobodan.Early physical and motor development of mouse offspring exposed to valproic acid throughout intrauterine development.*Behavioural Brain Research* http://dx.doi.org/10.1016/j.bbr.2016.05.023

Early physical and motor development of mouse offspring exposed to valproic acid throughout intrauterine development

Jelena Podgorac[1,*,&], Vesna Pešić[2,*], Željko Pavković[2], Ljiljana Martać[1], Selma Kanazir[2], Ljupka Filipović[3], Slobodan Sekulić[4,5]

[1]Department of Neurophysiology, Institute for Biological Research ‘‘Siniša Stanković’’, University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia

[2]Department of Neurobiology, Institute for Biological Research ‘‘Siniša Stanković’’, University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia

[3]Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia

[4]Medical Faculty, University of Novi Sad, 26 000 Novi Sad, Serbia.

[5]Department of Child Neurology and Epilepsy, Clinic for Neurology, Clinical Center of Vojvodina, Hajduk Veljka 1, 26000 Novi Sad, Serbia.

[*]These authors contributed equally to this work

Running head: Maternal valproate exposure influences early motor response of offspring

[&]Correspondence to:

E-mail: jelenapodgorac@yahoo.com; jelena.podgorac@ibiss.bg.ac.rs

Abstract

Clinical research has identified developmental delay and physical malformations in children prenatally exposed to the antiepileptic drug (AED) valproic acid (VPA). However, the early signs of neurodevelopmental deficits, their evolution during postnatal development and growth, and the dose effects of VPA are not well understood. The present study aimed to examine the influence of maternal exposure to a wide dose range (50, 100, 200 and 400 mg/kg/day) of VPA during breeding and gestation on early physical and neuromotor development in mice offspring. Body weight gain, eye opening, the surface righting reflex (SRR) and tail suspension test (TST) were examined in the offspring at postnatal days 5, 10 and 15. We observed that: (1) all tested doses of VPA reduced the body weight of the offspring and the timing of eye opening; (2) offspring exposed to VPA displayed immature forms of righting and required more time to complete the SRR; (3) latency for the first immobilization in the TST is shorter in offspring exposed to higher doses of VPA; however, mice in all groups exposed to VPA exhibited atypical changes in this parameter during the examined period of maturation; (4) irregularities in swinging and curling activities were observed in animals exposed to higher doses of VPA. This study points to delayed somatic development and postponed maturation of the motor system in all of the offspring prenatally exposed to VPA, with stronger effects observed at higher doses. The results implicate that the strategy of continuous monitoring of general health and achievements in motor milestones during the early postnatal development in prenatally VPA-exposed offspring, irrespectively of the dose applied, could help to recognize early developmental irregularities.

Key words: valproic acid; prenatal exposure; neurodevelopment; mouse model; surface righting reflex; tail suspension test; motor delay

Highlights

1. Chronic prenatal VPA exposure postpones weight gain and eye opening in offspring early in postnatal life.

2. Chronic prenatal VPA exposure delays surface righting reflex and tail suspension responses in offspring early in postnatal life.

3. These motor and somatic effects intensify at higher doses of prenatal VPA, but appear to subside by postnatal day 15.

4. Early motor and physical assessment should be regularly performed in all VPA-exposed offspring.

1. Introduction

The majority of children born to women with epilepsy are normal, but are at increased risk of developing malformations as AEDs have the potential to affect fetal development [1]. Clinical findings have revealed that the rate of birth defects in humans is significantly higher after prenatal exposure to valproic acid (VPA) than with any of other AED currently in use [2]. In children diagnosed with Fetal Valproate Syndrome (FVS), which is characterized by a pattern of major and minor congenital malformations, dysmorphic features and cognitive defects, increased rates of neurodevelopmental disorders have been reported. However, it is not clear whether prenatal exposure to VPA predisposes to problems in psychomotor development in the absence of a full recognizable syndrome, and what parameters could serve as early predictors of developmental impairment [3-7].

Many children prenatally exposed to VPA present impairments in speech, learning and coordination, with relatively high frequencies of maladaptive behaviors [5, 8, 9, 10]. Autism spectrum disorders (ASDs) are shown to be the most frequent neurodevelopmental disorders diagnosed in children of VPA-treated mothers, with the rate of ASD being roughly eight times higher than in the general population [reviewed in 9]. There are findings that gross motor deficits in the first year of life precede the appearance of motor stereotypies [11, 12, 13], the only motor abnormalities currently included in the diagnostic criteria for ASDs [14]. The relationship between motor and language development has been recognized [15, 16]. Recent studies have revealed that the risk of ASD associated with prenatal VPA exposure might persist even in children without congenital malformations [6]. However, studies examining the development of early motor functions in VPA-exposed children that do not appear to have any major or minor

malformations are scant [3]. Low birth weight, a surrogate marker of an infant's general health, was occasionally reported among VPA-exposed newborns [5], while the relationship between maternal weight gain during pregnancy and infant body weight has not been emphasized. This is an important link because, in terms of mammalian developmental biology, the impact of the treatment of maternal general health indirectly influences the development of the offspring [17].

Despite numerous arguments for avoiding treatment with VPA during pregnancy, there are circumstances when VPA cannot be excluded, as in cases of increased seizure occurrence after therapy replacement [18]. To reduce risk to the fetus, application of the lowest possible daily dose of VPA has been suggested, since a positive dose-response correlation between VPA and fetal anomalies has been observed [18]. The risk of major congenital malformations increases significantly at VPA doses of 600-700 mg/day, with the largest attributable risk observed at doses exceeding 1000 mg/day, which causes defects in 15-30% of children [1, 2, 18]. Neural tube defects (NTDs) have been reported as the hallmark of VPA-induced teratogenicity in humans [18, 19].

Animal studies have been carried out in order to mimic the effects of VPA on human development [9, 20]. Dose-related (150-600 mg/kg/day) teratogenicity and behavioral outcomes associated with *in utero* VPA exposure during the second and third weeks of intrauterine development were examined by Vorhees [21, 22]; however, the early motor outcome was not accentuated. The VPA rat model for autism was developed by exposing pregnant dams on gestational days 11.5, 12 or 12.5 to a VPA dose of 350 mg/kg [23], and the use of very high doses (350-800 mg/kg) of VPA during early embryonic development (E11-E12.5) has prevailed in autism research [2, 20, 24]. Behavioral alterations reported in a VPA rodent model of autism

were summarized in a review by Markram et al. [24]. Evaluation of early motor skills has not been assessed to any relevant degree in this model [25, 26].

Considering the remarkable similarities between early postnatal motor development of humans (during the first year) and rodents (during the first two weeks) [11, 27, 28], examination of the motor defects may help in elucidating the early outcome of VPA usage. However, as antiepileptic therapy implies constant, dose-adjusted, daily medication throughout the entire pregnancy, it is difficult to estimate how the application of high doses of VPA at a defined gestational age in rodents, as used to induce the animal model of autism, correlates with VPA treatment in humans. Thus, the present study was aimed at examining the influence of maternal exposure during breeding and gestation to doses (50-400 mg/kg/day) of VPA that mimic the range of doses used in clinical practice [9], on early physical and neuromotor development of the offspring. We assumed that early identification of delayed somatic and motor development could assist in the timely recognition of irregularities induced by prenatal exposure to VPA [29, 30].

2. Material and methods

2.1. Ethics Statement

All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research, University of Belgrade.

2.2. Animals and Treatment

The study was performed on 8-week-old female NMRI mice (N = 50) that were housed under standard conditions (23±2°C, 60–70% relative humidity and a 12-h light/dark cycle), and mated for the first time (2 females and 1 male per cage).

In addition to the control (CTR) group, four additional groups (n = 10 per group) were formed and treated subcutaneously (sc) into the loose skin on the back of the neck with VPA. The sodium salt of VPA (P4543, Sigma Aldrich) was dissolved in saline, to a final volume of 2.5 ml/kg, and VPA doses of 50 mg/kg/day (designated as VPA-50), 100 mg/kg/day (VPA-100), 200 mg/kg/day (VPA-200) and 400 mg/kg/day (VPA-400) were administrated. The CTR group received (sc) the corresponding volume of saline.

Dose translation, which provides conversion of the animal dose to a human dose and, after reversion, conversion of the human dose to an animal dose, is based on body surface area, and was made using the US FDA guideline [31]. Briefly, the Human Equivalent Dose (HED in mg/kg) = Animal Dose (mg/kg) × Animal K_m ÷ Human K_m, where K_m is a correction factor reflecting the relationship between body weight (BW) and body surface area. For a typical adult (BW = 60 kg, body surface area 1.6 $_m^2$), K_m is 37, while the average K_m for the mouse is 3. Thus, the daily doses of VPA used in our study on mice (50, 100, 200 and 400 mg/kg/day) corresponded to human daily doses of 4, 8, 16 and 32 mg/kg/day or to 240, 480, 960 and 1920 mg/day, respectively.

The treatment commenced at the same time as the pairing and was conducted during the whole breeding period and gestation. The animals were treated every morning between 08:00 and 09:00 h, after measuring the BW of females. The treatment was stopped when females gave birth (for those that did not become pregnant the treatment lasted 40 days). Consequently, the offspring were exposed to defined doses of VPA during the whole embryonic development.

The breeding period was not limited because we did not know whether the reproductive capacity of females was influenced by the treatment. The presence of a vaginal plug was not examined as it does not guarantee pregnancy but only indicates that sexual activity has occurred [32]. Examination of vaginal specimens to determine the stage of the estrous cycle was avoided, since pseudopregnancy or damage of the vaginal epithelium can be caused by repeated insertion of the swab used for collecting cells in the course of repeated analyses [33].

Due to the simplicity of the experimental protocol used in our study, all of the changes observed in females could clearly be related to the VPA treatment. The following parameters were noted during the experiment: the number of females that died or did not give birth, the number of days between mate-pairing and birth, BWs, and average litter size at birth.

When females were noticeably pregnant, males were removed. After delivery, each female with its litter was housed individually. For offspring, the day of birth was recorded as day 0.

2.3. Physical measures and behavioral tests

Physical measures and behavioral tests were performed on offspring from 5 litters per experimental group. This decision was made because in the VPA-200 and VPA-400 groups of females, only 5 females per group gave birth and consequently the same number of litters was randomly chosen from the CTR, VPA-50 and VPA-100 groups. Pups were used in physical/behavioral measures without splitting the results by sex. This decision was made because our goal was to avoid artificial selection by means of sampling a limited number of males/females per 5 litters, which might not represent the real state of the group (in this study, the smallest litter represented by 5 pups dictated the sampling). In addition to the BW, VPA-

induced physical developmental deficits were monitored by detection of anterior (anencephaly) and posterior (tail kinks) NTDs [19, 20] and delayed eye opening. Changes in the number of offspring per litter were monitored as well.

Repeated measures on the same animals were performed at PND5, PND10 and PND15. These time-points were chosen since the first two weeks in rodents correspond to the first human year of life regarding postnatal motor development [28]. Only data obtained from offspring that survived the whole examined period were statistically analyzed.

Testing was performed in the following order: physical measurements, the surface righting reflex, the tail suspension test. For physical/behavioral measurements, the duration of separation of each pup from its mother lasted no more than 10 min. Precautions were taken to avoid infanticide/cannibalistic behavior in mothers, i.e. pups were not examined until PND5, when they were marked, weighed and behaviorally tested.

Behavioral testing was performed in a quiet room with non-distracting, homogenous lighting. The experimenter handled each litter separately with a clean pair of surgical gloves. To eliminate odors/traces, the working surfaces were cleaned with 70% ethanol (allowed to dry and evaporate). The data were pooled for male and female pups.

2.3.1. The surface righting reflex (SRR)

SRR testing was based on a previous study [26]. Briefly, each mouse was placed on its back and gently held with all four limbs extended outward. Surface righting, in that all four paws touch the surface, was recorded by video camera, and the time taken to right was calculated using Ulead VideoStudio 11 Video Editing Software. The material was analyzed frame by frame (24 frames per s, 1 frame = 41.667 ms). The cut-off period for successful righting was at 30 sec.

The animals that did not perform surface righting in 30 s at PND5 were not included in the statistical analysis related to SRR activity on this testing day. However, these animals were tested again on PND10 and PND15, and all met the criterion. In addition to the time required to complete the reflex, the posture of the animal during surface righting was also analyzed. The posture during each turn was assigned to one of three categories. Normal development of the surface righting tactics follows an overlapping progression from a U-posture, to corkscrew (CSW), to axial (A) righting. Clear interpretation with the illustrations of surface righting techniques was given previously [34]. U-posture righting is observed in the youngest animals. The animal ventroflexes with quadrupedal extension before rotating its head and body in the same direction. Corkscrew is an intermediate form of righting. The head and forelimbs first rotate in one direction while the hind-limbs rotate in the opposite direction. The hind-limbs then rotate in the same direction as the head and forelimbs to complete the turn. Axial righting is the adult form of righting. The animal rights by cephalocaudal axial rotation. Rotations triggered by snout contact with the ground were included in this category.

2.3.2. Tail suspension test (TST)

In our study, TST was used to assess the motor abilities of the young animals, i.e. to test their motor performance directed toward escape of the imposed position. The animals were suspended 4 cm above the working surface by fixing the end of the tail. Latency to the first immobilization (LFI) in the TST was monitored for 180 s. The animals that were permanently active and whose LFI in the TST was not manifested during the testing period were assigned the maximum score, 180 s. All animals tested were statistically analyzed.

After the testing, the quantitative and qualitative evaluation of the animals' behavior in the TST was performed by watching the recorded material. Qualitative assessment of swinging (the mouse keeps its body straight, continuously moves its paws and moves its body from side to side) and curling (active twisting movements), was performed, since the positioning and activity of the body and limbs during execution of these activities could reflect neurological dysfunction [35].

2.4. Statistical analysis

Statistical analyses were performed using Statistica 6.0. Tabular and graphical presentation of results is given. Qualitative data were expressed as either numbers or percentages, as appropriate, while quantitative data were expressed as mean values ± SEM. The number of animals used in the given analysis is specified within the graph bar.

The effect of VPA treatment on the parameters measured in dams (the number of days between mate-pairing and birth, BW increment) was analyzed by nonparametric Kruskal-Wallis one-way ANOVA. Pairwise comparisons were made by Mann-Whitney *U* test because of differences in sample size. The Wilcoxon test was used to assess the increase in BW during pregnancy (repeated measure).

Although the experimental design suggested that the most appropriate statistical analysis of the physical/behavioral measurements in VPA-exposed pups (PND5 to PND15) would be 2-way ANOVA with the VPA treatment and postnatal age (repeated measure) as factors, we could not perform this analysis due to the extreme non-normality of the data (estimated by Shapiro-Wilk's test, [36]) accompanied by sample size differences. Thus, at each age, the data (litter size, BW of the offspring, the time required to complete the SRR, LFI in the TST) were analyzed by

nonparametric Kruskal-Wallis one-way ANOVA, with VPA dose as a factor. Pairwise comparisons were performed by the *U* test. Friedman's and Wilcoxon's tests were used for repeated measures to assess the influence of the dose of VPA on the parameters (litter size, BW increase, LFI in the TST) measured in the offspring during postnatal development. The results obtained in the SRR testing were unsuitable for statistical analysis with repeated measures across age (some animals did not pass the test criterion at PND5, while the data obtained afterwards reflected the spontaneous redistribution of the animals in accordance with the type of righting).

The accepted level of significance was $p < 0.05$. In the Results section, the exact p value is given, apart from for $p = 0.000$, which was noted as $p < 0.001$.

3. Results

3.1. The highest prenatal dose of VPA has a negative effect on dam health

VPA treatment had a negative effect on dams prior to delivery (Figure 1A), with a higher number of deaths in dams exposed to VPA-400 (5/10) and VPA-200 (2/10), but no effect after exposure to VPA-100 (0/10) and VPA-50 (0/10) in comparison to the CTR (0/10). Moreover, in the VPA-200 group, 3 out of 8 dams that survived the treatment did not give birth (Figure 1A). Consequently, the number of pregnant dams in the CTR and groups exposed to VPA-50, VPA-100, VPA-200 and VPA-400 was 10, 10, 10, 5 and 5, respectively. The small sample sizes and rate events limited the comparison to qualitative observation.

VPA treatment increased the number of days between mate-pairing and birth, as assessed by Kruskal-Wallis ANOVA (Figure 1B, H (4, N = 40) = 9.599, p = 0.047). Compared to the CTR group, a significant increase was observed in the VPA-400 group (* p = 0.022, U test),

borderline significance was observed in the VPA-200 group (p = 0.061), while insignificant changes were detected in the VPA-100 (p = 0.671) and VPA-50 (p = 0.824) groups. Additionally, between-group comparisons revealed significant differences between the VPA-400 and VPA-50 groups (# p = 0.013), as well as between the VPA-400 and VPA-100 groups (& p = 0.039). Other comparisons did not reveal significant differences (VPA-400 *vs* VPA-200 p = 0.749, VPA-200 *vs* VPA-100 p = 0.122, VPA-200 *vs* VPA-50 p = 0.376, VPA-100 *vs* VPA-50 p = 0.742).

Kruskal-Wallis ANOVA confirmed that the dams in all experimental groups had similar BWs at the beginning of the experiment (Figure 1C, H (4, N = 40) = 0.685, p = 0.953) as well as at the end of the experiment (H (4, N = 40) = 6.537, p = 0.162). However, *post-hoc* analysis showed that at the end of the experiment the average BW of the VPA-400 group was significantly decreased in comparison to the CTR (* p = 0.028, U test) and VPA-50 groups (# p = 0.017), while other between-group comparisons revealed insignificant differences (VPA-200 *vs* CTR p = 0.317, VPA-100 *vs* CTR p = 0.272, VPA-50 *vs* CTR p = 0.883, VPA-400 *vs* VPA-200 p = 0.465, VPA-400 *vs* VPA-100 p = 0.206, VPA-200 *vs* VPA-100 p = 0.925, VPA-200 *vs* VPA-50 p = 0.329, VPA-100 *vs* VPA-50 p = 0.291).

The Wilcoxon test confirmed significant increase in BW during pregnancy in all groups of dams, i.e. CTR (§ p = 0.018), VPA-50 (§ p = 0.005), VPA-100 (§ p = 0.002), VPA-200 (§ p = 0.028) and VPA-400 (§ p = 0.033) groups.

The number of pups per litter at birth in CTR, VPA-50, VPA-100, VPA-200 and VPA-400 groups was 7 ± 1, 7 ± 1, 8 ± 1, 7 ± 1 and 6 ± 1, respectively (data not graphed). Kruskal-Wallis ANOVA did not reveal a significant influence of the VPA treatment on average litter size at birth (H (4, N = 40) = 1.468, p = 0.832).

3.2. Prenatal VPA exposure favors the occurrence of tail kinks and reduces the incidence of eye opening

The offspring from 5 litters per group was monitored and analyzed (Table 1). Kruskal-Wallis ANOVA did not reveal a significant influence of the VPA treatment on litter size, as evaluated at PND5 (H (4, N = 25) = 1.933, p = 0.748), PND10 (H (4, N = 25) = 3.447, p = 0.485) and PND15 (H (4, N = 25) = 2.931, p = 0.569).

The Friedman test did not reveal significant changes in the number of pups per litter during PND5-PND15 in any of the experimental groups (CTR group χ^2 (2, N = 5) = 4.000, p < 0.135; VPA-50 group χ^2 (2, N = 5) = 4.000, p < 0.135; VPA-100 group χ^2 (2, N = 5) = 2.000, p < 0.368; VPA-200 group χ^2 (2, N = 5) = 3.000, p < 0.223; VPA-400 group χ^2 (2, N = 5) = 4.000, p < 0.135).

In 2 out of 5 litters from the VPA-400 group we detected that 1 animal per litter was born anencephalic. It is possible that the more severely affected animals died *in utero*, as indicated by the slightly decreased number of pups per litter in the VPA-400 group. Moreover, pups with a kinked tail (assessed at PND15, Table 1) were detected in the VPA-200 and VPA-400 groups (10% and 20% of the total number of pups, respectively). Importantly, prenatal exposure to all tested doses of VPA reduced by more than 50% the incidence of eye opening on PND15 (Table 1), with the greatest reduction observed in the VPA-400 group.

3.3. Prenatal VPA exposure reduces the BW of the offspring during early postnatal development

The BWs of the offspring are presented in Figure 2. Kruskal-Wallis ANOVA revealed the negative influence of prenatal VPA exposure on the measured parameter, as evaluated at

PND5 (H (4, N = 168) = 41.543, $p < 0.001$), PND10 (H (4, N = 168) = 62.668, $p < 0.001$) and PND15 (H (4, N = 168) = 41.804, $p < 0.001$).

At PND5, relative to CTR group, the offspring had a significantly lower average BW after exposure to VPA-400 (* $p < 0.001$, U test) and VPA-200 (* $p < 0.001$); no difference was observed after VPA-100 ($p = 0.239$) and VPA-50 ($p = 0.461$). Between-group comparisons revealed significantly lower BW in the VPA-200 group when compared to the VPA-100 (& $p = 0.001$) and VPA-50 (# $p = 0.001$) groups. Moreover, the VPA-400 group had a lower BW than all of the other groups exposed to VPA, i.e. VPA-50 (# $p < 0.001$), VPA-100 (& $p = 0.001$) and VPA-200 ($ $p = 0.001$). The BWs of offspring in the VPA-100 and VPA-50 groups did not significantly differ ($p = 0.165$).

At PND10, all of the VPA-exposed offspring (VPA-50, VPA-100, VPA-200 and VPA-400) had significantly lower BWs in comparison to the CTR group (* $p < 0.001$). The VPA-400 group weighed less than all other VPA-exposed groups, i.e. VPA-200 ($ $p = 0.038$), VPA-100 (& $p = 0.032$) and VPA-50 (# $p = 0.048$). Other between-group comparisons did not reveal significant differences (VPA-200 *vs* VPA-100 $p = 0.159$, VPA-200 *vs* VPA-50 $p = 0.829$, VPA-100 *vs* VPA-50 $p = 0.281$).

On PND15, relative to the CTR group, the offspring had significantly lower average BWs after exposure to VPA-50 (* $p < 0.001$), VPA-200 (* $p < 0.001$) and VPA-400 (* $p = 0.025$), but not after exposure to VPA-100 ($p = 0.938$). Moreover, compared to the VPA-100 group, all of the other VPA-exposed groups had significantly lower BWs (VPA-50 & $p < 0.001$, VPA-200 & $p < 0.001$, VPA-400 & $p = 0.019$). Other between-group comparisons did not reveal significant differences (VPA-400 *vs.* VPA-200 $p = 0.328$, VPA-400 *vs.* VPA-50 $p = 0.119$, VPA-200 *vs*. VPA-50 $p = 0.115$).

The Friedman test was performed in order to evaluate the increase in BW in offspring during PND5-PND15 (Figure 2). Significant increases in BW were observed in the CTR group (χ^2 (2, N = 35) = 70.000, $p < 0.001$), VPA-50 group (χ^2 (2, N = 35) = 63.727, $p < 0.001$), VPA-100 group (χ^2 (2, N = 38) = 74.053, $p < 0.001$), VPA-200 group (χ^2 (2, N = 33) = 64.061, $p < 0.001$) and VPA-400 group (χ^2 (2, N = 27) = 48.667, $p < 0.001$). In all the groups, *post-hoc* comparisons using the Wilcoxon test revealed significant increases from PND5 to PND10 (§ $p < 0.001$) and PND10 to PND15 (¥ $p < 0.001$). The exact number of animals per group used in the analysis is noted inside each graph bar.

3.4. Prenatal exposure to VPA impairs offspring performance in the SRR at all examined periods of postnatal development

3.4.1. The SRR score at PND5

At PND5, 20% of the CTR group was not capable of performing the SRR within 30 s; however, 80% of the group successfully completed the test by using CSW-type to surface right (Figure 3A). Prenatal exposure to VPA had a negative effect on the ability of the offspring to surface right with respect to the criteria specified in the test (Figure 3A): the lowest number of pups was in the VPA-400 group (37%), followed by the VPA-100 (48%), VPA-200 (61%) and VPA-50 (77%) groups. All the offspring exposed to VPA righted in CSW-type except a part of the VPA-200 group that used CSW- and U-type of righting (28% and 33%, respectively) in almost equal measure. Rate events limited these comparisons to qualitative results.

Kruskal-Wallis ANOVA revealed the negative influence of prenatal VPA exposure on the time required to complete the CSW-type of righting (Figure 3B; H (4, N = 94) = 10.067, p =

0.036). Relative to the CTR group, prenatal exposure to VPA increased the time in VPA-50 (* p = 0.034, *U* test), VPA-100 (* p = 0.027), VPA-200 (* p = 0.033) and VPA-400 (* p = 0.015) groups. No significant changes were detected between the VPA-treated groups (VPA-400 *vs* VPA-200 p = 0.967, VPA-400 *vs* VPA-100 p = 0.334, VPA-400 *vs* VPA-50 p = 0.573, VPA-200 *vs* VPA-100 p = 0.541, VPA-200 *vs* VPA-50 p = 0.326, VPA-100 *vs* VPA-50 p = 0.982).

Offspring in the VPA-200 group that used the U-type of SRR completed the test in 9.271 ± 3.341 s (data not graphed).

3.4.2. The SRR score at PND10

All animals successfully completed SRR testing at PND10 (Figure 4A). The larger part of the CTR group (63%) performed the CSW-type of righting, while a smaller part (37%) used the A-type of righting. Prenatal VPA exposure had a negative effect on the appearance of the A-type of righting, as it decreased in the VPA-50 (20%) and VPA-100 (15%) groups and was completely undetectable (0%) in the VPA-200 and VPA-400 groups. In contrast, the utilization of the CSW-type of righting was favored in all of the VPA-exposed groups (VPA-50, 80%; VPA-100, 85%; VPA-200, 89%; VPA-400, 70%). Importantly, the U-type of righting was detected in a fraction of the VPA-200 (11%) and VPA-400 (30%) groups. Rate events limited these comparisons to qualitative results.

Kruskal-Wallis ANOVA revealed a dose effect of the prenatal treatment to VPA on the time required to complete the CSW-type of righting at PND10 (H (4, N = 130) = 25.956**,** p < 0.001). Relative to the CTR group, prenatal VPA exposure increased the measure in VPA-50 (* p = 0.001, *U* test), VPA-100 (* p = 0.001), VPA-200 (* p = 0.045) and VPA-400 (* p < 0.001) groups. Moreover, the VPA-400 group required more time to complete the CSW-type of righting

than all other groups exposed to VPA, i.e. VPA-50 (# $p = 0.001$), VPA-100 (& $p = 0.001$) and VPA-200 ($ $p = 0.006$). Additional between-group comparisons did not reveal significant differences regarding the CSW-type of righting (VPA-200 *vs* VPA-100 $p = 0.115$, VPA-200 *vs* VPA-50 $p = 0.098$, VPA-100 *vs* VPA-50 $p = 0.588$).

The negative impact of VPA treatment on the time required to complete the A-type of SRR at PND10 was observed (Figure 4C, H (4, N = 26) = 15.415**,** $p < 0.001$). Relative to the CTR group, prenatal VPA exposure increased the measured parameter in VPA-50 (* $p = 0.002$, *U* test) and VPA-100 (* $p = 0.002$) groups, while the differences between the VPA-50 and VPA-100 groups were insignificant (p = 0.432). In the VPA-200 and VPA-400 groups, the A-type of righting was not detected.

The offspring of mice from the VPA-200 and VPA-400 groups that used the U-type of surface righting completed the test in 0.678 ± 0.079 s and 0.841 ± 0.102 s, respectively (data not graphed).

3.4.3. The SRR score at PND15

All animals successfully completed SRR testing at PND15 (Figure 5A). A larger portion of the CTR group (89%) used the A-type of righting whereas a smaller number of animals (11%) used the CSW-type. Prenatal VPA exposure had a negative effect on the appearance of the A-type of righting, decreasing it in the VPA-50 (61%), VPA-100 (63%), VPA-200 (63%) and VPA-400 (44%) groups. In contrast, utilization of the CSW-type of righting was favored in all VPA-exposed groups (VPA-50 39%, VPA-100 37%, VPA-200 37% and VPA-400 56%). Rate events limited these comparisons to qualitative results.

An insignificant influence of the prenatal VPA treatment on the time required to complete the CSW-type of righting at PND15 was revealed by Kruskal-Wallis ANOVA (Figure 5B, H (4, N = 59) = 4.854, p = 0.302).

Prenatal VPA treatment had a dose effect on the time required to complete the A-type of righting at PND15 (Figure 5C, H (4, N = 109) = 44.767, p < 0.001). In comparison to the CTR group, this parameter was increased in the VPA-50 (* p = 0.018, U test), VPA-100 (* p < 0.001), VPA-200 (* p < 0.001) and VPA-400 (* p < 0.001) groups. Moreover, the VPA-400 group required more time to complete the A-type of righting than all the other groups exposed to VPA, i.e. VPA-50 (# p < 0.001), VPA-100 (& p < 0.001) and VPA-200 ($ p = 0.002), as well the VPA-200 group, when compared to the VPA-50 group (# p = 0.016). Other comparisons between the VPA-exposed groups were non-significant (VPA-200 *vs* VPA-100 p = 0.366, VPA-100 *vs* VPA-50 p = 0.354).

3.5. Prenatal exposure to VPA impairs the performance of the offspring in the TST

The negative impact of the VPA treatment on the LFI was observed at PND5 (Figure 6A, Kruskal-Wallis ANOVA: H (4, N = 168) = 27.386, p < 0.001). Compared to the CTR group, significant decreases in the values of this parameter were detected in the VPA-400 ($ p < 0.001, U test) and VPA-200 ($ p < 0.001) groups, but not in the VPA-100 (p = 0.428) and VPA-50 (p = 0.621) groups. Moreover, the VPA-400 and VPA-200 groups displayed a significantly reduced LFI compared to the VPA-50 group (# p = 0.003 and # p < 0.001, respectively) and the VPA-100 group (& p = 0.013 and & p = 0.001, respectively). Additional comparisons between the different VPA groups were non-significant (VPA-400 *vs* VPA-200 p = 0.984, VPA-100 *vs* VPA-50 p = 0.799).

The negative impact of treatment with VPA on the LFI was also observed at PND10 (Figure 6A, H (4, N = 168) = 9.531, p = 0.047). Compared to the CTR group, a significant decrease was detected in the VPA-400 ($ p = 0.045) and VPA-200 ($ p = 0.028) groups, but not in the VPA-100 (p = 0.129) and VPA-50 (p = 0.092) groups. Further comparisons between the VPA-exposed groups did not reveal any significant differences (VPA-400 *vs* VPA-200 p = 0.273, VPA-400 *vs* VPA-100 p = 0.531, VPA-400 *vs* VPA-50 p = 0.337, VPA-200 *vs* VPA-100 p = 0.181, VPA-200 *vs* VPA-50 p = 0.203, VPA-100 *vs* VPA-50 p = 0.567).

The effect of VPA on the LFI in the TST observed at PND5 and PND10, disappeared at PND15 (Figure 6A, H (4, N = 168) = 3.856, p = 0.426).

The Friedman ANOVA revealed a significant increase in LFI in all experimental groups during postnatal development (CTR group χ^2 (2, N = 35) = 6.624, p = 0.036; VPA-50 group χ^2 (2, N = 35) = 17.048, p < 0.001; VPA-100 group χ^2 (2, N = 38) = 12.753, p = 0.002; VPA-200 group χ^2 (2, N = 33) = 26.991, p < 0.001; VPA-400 group χ^2 (2, N = 27) = 15.680, p < 0.001). In the CTR group, the Wilcoxon test revealed a significant increase in LFI in PND10 animals when compared to PND5 animals (* p = 0.017), but a non-significant difference between PND15 and PND10 animals (p = 0.302). However, a different pattern of LFI maturation was observed in the VPA groups, depending on VPA dose range. In the VPA-50 and VPA-100 groups, the parameter changed non-significantly in PND10 compared to PND5 animals (p = 0.815 and p = 0.372, respectively); a significant increase was observed in PND15 in comparison to PND10 animals (¥ p = 0.001 and ¥ p = 0.003, respectively). In the VPA-200 and VPA-400 groups, the LFI significantly increased in PND10 when compared to PND5 animals (* p = 0.003 and * p = 0.005, respectively) as well as in PND15 when compared to PND10 animals (¥ p = 0.022 and ¥ p = 0.022).

Qualitative analysis of the recorded material revealed that the quality of the movements in the TST was similar in the CTR, VPA-50 and VPA-100 groups at all examined ages: at PND5, limb movements were present, body movements manifested as trembling; from PND10 to PND15, limb movements improved in amplitude and frequency, progress in the definition of body movements was evident, and was manifested through shaking, swinging (Figure 6B, panel a) and curling (Figure 6B, panel b). The clasping of the limbs was not observed in either CTR or VPA-50 and VPA-100 groups.

The VPA-200 and VPA-400 groups exhibited movements that were of lower quality, accompanied by a peculiar pattern: at PND5, both limb and body movements were very weak; at PND10 to PND15, the limb and body movements became more apparent than at PND5, but in the majority of the animals they were accompanied by episodes of abnormal posturing (stretched out body, hind limbs flexed, upper limbs outstretched (Figure 6B, panel c), or by dystonic movements during which the limbs were pulled into the body and held and released in short bursts (Figure 6B, panel d).

4. Discussion

The present study is the first experimental approach to focus on the early physical and motor postnatal development of offspring exposed *in utero* to a range of doses of VPA. The results implicate that the strategy of continuous monitoring of general health and achievements in motor milestones during the early postnatal development in prenatally VPA-exposed offspring, irrespectively of the dose applied, could help to recognize early developmental irregularities.

The model of continuous maternal exposure to gradually increasing doses of VPA (50-400 mg/kg/day), which roughly correlate to those used in clinical practice (5-30 mg/kg/day, [9, 20]) was used herein for the first time. We observed maternal toxicity of VPA at higher doses (≥ 200 mg/kg/day) through reduced survival or impacted reproductive capacity in a part of the population of treated dams. Other experimental studies also accentuated maternal toxicity of VPA, with varying dose responses, which were related to treatment duration and the route of administration [20, 21, 37]. Ovarian morphological changes related to VPA exposure are recognized in rodents [38], and help in understanding the lack of pregnancies in some females exposed to VPA, as observed in our study. However, individual sensitivity to the toxic effects of VPA remains to be studied. Diminished BW gain was detected in the group exposed to the highest tested dose (400 mg/kg/day), but prolonged treatment could also contribute to the observed effect. Clinical findings do not consider the dose of 1000 mg/day (simulated in our study by 200 mg/kg/day) harmful to the health of adult patients; however, species differences in the pharmacokinetics of VPA and the daily regimen of administration must be taken into account when comparing human and rodent data [39-41]. VPA usage in women is mainly associated with polycystic ovary syndrome (weight gain, increase in androgen levels) and sub-fertility [42].

The general health of the VPA-exposed offspring has not been examined in detail in experimental studies, and only a few reports have provided information related to the VPA rodent model of autism [20]. We report for the first time that continuous prenatal exposure to VPA affects postnatal BW gain, with clear effects observed with the two higher doses of VPA (200 and 400 mg/kg/day) and postponed effects with the two lower doses (50 and 100 mg/kg/day). These results point to a fine dose-related impact of prenatal treatment with VPA on postnatal BW gain, which could be due to altered feeding abilities of the offspring. Our

presumptions are in agreement with findings described in the VPA rodent model of autism [23], i.e. reduced number of motor neurons in the earliest-forming motor nuclei involved in the control of swallowing. The influence of prenatal VPA exposure on the developmental programming of the metabolic status of the offspring should not be excluded [43]. Moreover, changes in maternal behavior and/or milk production could indirectly contribute to the poor BW gain in offspring, which remains to be examined in detail [17]. In humans, intrauterine VPA exposure has not been recognized as a treatment with a high risk for small birth weight [44]. However, growth retardation and feeding difficulties have been reported in children with FVS [45] and are included in the symptoms of a neonatal withdrawal [46]. Nevertheless, feeding alternatives/supplements available to humans, along with species-specific metabolic characteristics [39], complicate comparisons between human and rodent data.

We observed increased expression of tail kinks in offspring exposed to VPA at doses of 200 and 400 mg/kg/day; this result points to the dose of 200 mg/kg/day as the threshold for NTDs in our model. Importantly, this dose of VPA in mice corresponds to the human dose of 1000 mg/day, which is associated with increased risk of NTDs in humans [2]. Nevertheless, individual susceptibility to NTDs related to VPA exposure should be taken into account [47], as was also revealed in our study.

We detected reduced occurrence of eye opening up to PND15 in all offspring in groups exposed to VPA. Considering that the process of eyelid opening is an external sign of normal maturation of the central nervous system in vertebrates [48], our results indicate that all tested doses of VPA are capable of influencing the maturation of the nervous system. The delay in eye opening has been reported in the VPA rodent model of autism [25], and was explained by the treatment-induced abnormalities in the development of motor nuclei controlling the eye muscles

[23]. It should be noted that in rodents, visual inputs do not play any role in triggering surface righting [49], and therefore the observed delay in eye opening should not *per se* influence the motor outcome in behavioral testing.

Although the literature points to a high prevalence of motor impairments in children with FVS and ASDs [4, 10, 15] and in infants exposed to AEDs who do not appear to have any major or minor malformations [**3**], detailed examinations of early motor development in VPA-exposed infants are not practiced. In the VPA rodent model of autism, transiently impaired swimming performance (PND8 and PND12) and righting reactions (PND5-PND6) have been reported, with greater focus on the behavior of the offspring older than PND10 [25, 26]. The study by Vorhees [21] reported on dose-related behavioral changes in rats tested after weaning.

Our study revealed for the first time that continuous intrauterine exposure to VPA influences early motor development of offspring, i.e. disturbs the rostrocaudal gradient of motor system maturation and affects muscle tone. Precisely, motor impairments in VPA-exposed offspring manifest through the favoring of immature types of SRR, via an increase in the time required to complete the SRR and through a decrease of LFI in the TST.

Low muscle tone was especially evident at PND5 in offspring prenatally exposed to 400 mg/kg/day and 200 mg/kg/day doses of VPA. It is possible that diminished skeletal muscle mass contributes to the weakness/increased fatigue during physical activity in these animals, as they had significantly lower BWs than all other groups at PND5. However, motor delay observed in VPA-exposed pups with control-like BWs pointed to possible defects in the mechanical performance of skeletal muscles, which depends not only on fiber hypertrophy and myogenic differentiation but also on size-independent mechanical function and force generation [50]. The variability of the response in the SRR test, which was particularly evident at PND10, revealed

that animals exposed to VPA have impediments in the dynamic/synchronicity of events directed at improving the tonic component of the motor response [28]. The events of importance are regression of polyneuronal innervation and development of tonic motor-unit activity. These processes *per se* have not been examined in offspring exposed to VPA. However, it is well known that their dynamic is influenced by early motor experience, reflecting the developmental changes in the activity at the neuromuscular junction [50-52].

We showed that in mice prenatally exposed to VPA the delay in rostrocaudal motor maturation and the dose-related impairment of dexterity (the obstacle in reaching the correct result in the SRR) are present at PND15. It is known that proper development of the descending motor pathways, in particular of serotoninergic projections, is important for postural control, interlimb coordination and dexterity of movements [28]. Although disruption of early serotonergic neuronal development following intrauterine VPA exposure has been shown [53], its relation to motor development has not been examined in this model.

The overall score in the TST reflected the improvement of gross muscle activity at PND15. However, atypical posturing (spastic movements) and dystonic movements in offspring exposed to the two higher doses of VPA was a clear reflection of nervous system dysfunction. Spastic movements could be related to dysfunction of pyramidal motor systems, which usually manifests as a combination of weakness and increased stretch reflexes [54]. The dystonic limb movements (limb clasping) in rodents are investigated in the light of difficulties related to goal-orientated behavior and voluntary movements, and are observed in animals with lesions in the cerebellum, basal ganglia and neocortex [35]. The experimental findings available from the VPA rodent model of autism pointed to the abnormalities in a number of regions in the central nervous system, including the brainstem, cerebellum, neocortex, amygdala and superior colliculus [9,

37]. However, as brain development is a dynamic process, with a region-specific timeline for progressive and regressive changes [55], the consequences of the timed exposure to the high doses only during neural tube closure could be different from the effects of continuous prenatal VPA exposure [56].

In view of the above discussed findings, it can be concluded that this study represents a valuable contribution to the understanding the links between prenatal VPA dose and early postnatal development of offspring. Namely, newborn humans and rodents are comparatively immature from a motor point of view at birth, and development occurs along a rostrocaudal axis as follows: (1) in rodents, head lifting appears around postnatal day (PND) 2, which corresponds roughly to the 3rd month in a baby [28]; (2) the shoulders are raised from the ground around PND5 in rodents, which is associated with functional maturation of the forelimbs [27], whereas the human infant starts pushing against the surface with hands and straight elbows by around the 5th month [11]; (3) the hindlimbs are capable of bearing weight around PND10 in rodents, supporting jerky and tremulous walking episodes with the belly free [27], while the human baby stands and starts to walk ("waddling walk") around the 10th month [11]; (4) locomotor performance significantly improves after PND12 in rodents, as does walking in humans after the first year [11, 27, 28]. However, although we used the method of dose translation, conversion alone does not guarantee dosage correspondences from human to mice offspring. Species differences, such as adsorption rates and cross placental capacity should be taken into account, despite their influence still being unclear.

There are some limitations of the present study that should be mentioned. In our experimental protocol, successful pregnancy and delivery dictated the duration of the treatment, and the dams exposed to the highest dose of VPA were treated longer than other groups. The

insufficient maternal BW gain, which has been regarded as a good indicator of toxicity, indicates that the effects of the highest tested dose of VPA on the offspring could, at least in part, be secondary to the maternal toxicity. Thus, the influence of the length of the treatment prior to pregnancy on the parameters measured in both the treated dams and offspring should be additionally assessed in future studies. Considering the challenges in obtaining samples after exposure to teratogens, we pooled siblings from both sexes to maximize the number of used animals. The observation of an effect or lack thereof from such a broad exploratory study does not address the issue of whether one sex or the other is more affected; this should be examined. Regarding data analysis, the absence of corrections for multiple comparisons should be discussed. Namely, we considered the less controlled risk of Type I errors in order to avoid the Type II error. We believe that in the experimental paradigm used in our study, the false negative results could lead to wrong conclusions about the safety of the treatment. Therefore, the obtained results need to be confirmed in further studies examining the robustness of the motor effects after the administration of certain doses and their changes with age.

In conclusion, the results of our study present the first experimental evidence of the effects of continuous prenatal exposure to a wide range of doses of VPA on postnatal physical growth and motor activity of offspring. Delayed somatic development and postponed maturation of the motor system were observed in all of the offspring prenatally exposed to VPA, with the more pronounced effects correlating with the higher doses of VPA. In spite of the improvement in some aspects of behavior, dose-related motor ineptness and irregular movements become obvious during postnatal maturation. Considering the positive correlation between motor activity and the development of motor pathways on the one hand, and high neural plasticity during early postnatal development on the other, the improvement of motor functions provides an opportunity

to facilitate the structural and functional maturation of the nervous system [30]. Moreover, the early detection and correction of inadequate movements are important, because aberrant neural plasticity can develop in response to compensatory motor activities, resulting in further worsening of motor-related behaviors.

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grants ON175006 and ON173056).

Authorship contributions

Conceived and designed the experiments: JP, SS. Performed the experiments: JP, LjM, LjF, ŽP. Analyzed the data: JP, VP, SK, SS. Wrote the manuscript: JP, VP.

Conflict of interest statement

No conflict of interest to declare.

References

[1] Meador KJ, Baker GA, Finnell RH, Kalayjian LA, Liporace JD, Loring DW, Mawer G, Pennell PB, Smith JC, Wolf MC; NEAD Study Group. In utero antiepileptic drug exposure: fetal death and malformations. Neurology 2006; 67: 407-412.

[2] Bath KG, Scharfman HE. Impact of early life exposure to antiepileptic drugs on neurobehavioral outcomes based on laboratory animal and clinical research. Epilepsy Behav 2013; 26: 427-439.

[3] Parisi P, Francia A, Vanacore N, Fiore S, Giallonardo AT, Manfredi M. Psychomotor development and general movements in offspring of women with epilepsy and anticonvulsant therapy. Early Hum Dev 2003; 74: 97-108.

[4] Kini U. Fetal valproate syndrome: a review. Paed Perinat Drug Ther 2006; 7: 123-130.

[5] Thomas SV, Ajaykumar B, Sindhu K, Nair MKC, George B, Sarma PS. Motor and mental development of infants exposed to antiepileptic drug in utero. Epil Behav 2008; 13: 229-236.

[6] Christensen J, Grønborg TK, Sørensen MJ, Schendel D, Parner ET, Pedersen LH, Vestergaard M. Prenatal Valproate Exposure and Risk of Autism Spectrum Disorders and Childhood Autism. *JAMA* 2013; 309: 1696-1703.

[7] Bromley RL, Mawer G, Love J, Kelly J, Purdy L, McEwan L, Briggs M, Clayton-Smith J, Shi X, Baker GA; Liverpool and Manchester Neurodevelopment Group [LMNDG]. (2010) Early cognitive development in children born to women with epilepsy: A prospective report. Epilepsia 51:2058-2065.

[8] Vinten J, Bromley RL, Taylor J, Adab N, Kini U, Baker GA. The behavioral consequences of exposure to antiepileptic drug in utero. Epil Behav 2009; 14: 197-201.

[9] Roullet FI, Lai JK, Foster JA. In utero exposure to valproic acid and autism - a current review of clinical and animal studies. Neurotoxicol Teratol 2013; 36: 47-56.

[10] Ming X, Brimacombe M, Wagner GC. Prevalence of motor impairment in autism spectrum disorders. Brain Dev 2007; 29: 565-570.

[11] Teitelbaum P, Teitelbaum O, Nye J, Fryman J, Maurer RG. Movement analysis in infancy may be useful for early diagnosis of autism. Proc Natl Acad Sci U S A. 1998; 95: 13982-13987.

[12] Bhat AN, Galloway JC, Landa RJ. Relation between early motor delay and later communication delay in infants at risk for autism. Infant Behav Dev 2012; 35: 838-846.

[13] Estes A, Zwaigenbaum L, Gu H, St. John T, Paterson S, Elison J, Hazlett H, Botteron K, Dager SR, Schultz RT, Kostopulos P, Evans A, Dawson G, Eliason J, Alvarez S, Piven J, IBIS network. Behavioral, cognitive, and adaptive development in infants with autism spectrum disorder in the first two years of life. J Neurodev Disord 2015; 7: 24.

[14] Lord C, Jones RM. Annual research review: re-thinking the classification of autism spectrum disorders. J Child Psychol Psychiatry 2012; 53: 490-509.

[15] McCleery JP, Elliott NA, Sampanis DS, Stefanidou CA. Motor development and motor resonance difficulties in autism: relevance to early intervention for language and communication skills. Front Integr Neurosci 2013; 7: 30.

[16] Iverson JM. Developing language in a developing body: the relationship between motor development and language development. J Child Lang 2010; 37: 229-261.

[17] Power M.L., Schulkin J. Maternal regulation of offspring development in mammals is an ancient adaptation tied to lactation. Applied & Translational Genomics 2013; 130: 55-63.

[18] Tomson T, Battino D, Perucca E. Valproic acid after five decades of use in epilepsy: time to reconsider the indications of a time-honored drug. Lancet Neurol 2015; S1474-4422(15)00314-2.

[19] Nau H, Hauck RS, Ehlers K. Valproic acid-induced neural tube defects in mouse and human: aspects of chirality, alternative drug development, pharmacokinetics and possible mechanisms. Pharmacol Toxicol 1991; 69: 310-321.

[20] Favre MR, Barkat TR, LaMendola D, Khazen G, Markram H, Markram K. General developmental health in the VPA-rat model of autism. Front Behav Neurosci 2013; 7: 88.

[21] Vorhees CV. Teratogenicity and developmental toxicity of valproic acid in rats. Teratology 1987; 35: 195-202.

[22] Vorhees CV. Behavioral teratogenicity of valproic acid: selective effects on behavior after prenatal exposure to rats. Psychopharmacology 1987; 92: 173-179.

[23] Rodier PM, Ingram JL, Tisdale B, Nelson S, Romano J. Embryological origin for autism: developmental anomalies of the cranial nerve motor nuclei. J Comp Neurol 1996; 370: 247-261.

[24] Markram H, Rinaldi T, Markram K. The intense world syndrome–an alternative hypothesis for autism. Front Neurosci 2007; 1: 77-96.

[25] Schneider T, Pzewlocki R. Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology 2005; 30: 80-89.

[26] Wagner GC, Reuhl KR, Cheh M, McRae P, Halladay AK. A new neurobehavioral model of autism in mice: pre- and postnatal exposure to sodium valproate. J Autism Dev Disord 2006; 36: 779-793.

[27] Geisler HC, Westerga J, Gramsbergen A. Development of posture in the rat. Acta Neurobiol Exp (Warsz) 1993; 53: 517-523.

[28] Vinay L, Ben-Mabrouk F, Brocard F, Clarc F, Jean-Xavier C, Pearlstein E, Pflieger JF. Perinatal development of the motor systems involved in postural control. Neural Plast 2005; 12: 131-139.

[29] Gajewska E, Sobieska M, Kaczmarek E, Suwalska A, Steinborn B. Achieving motor development milestones at the age of three months may determine, but does not guarantee, proper further development. ScientificWorldJournal. 2013; 2013:354218.

[30] Denckla M. Why assess motor functions 'early and often'? Ment Retard Dev Disabil Res Rev 2005; 11: 3.

[31] U.S. Food and Drug Administration (FDA). Guidance for industry: Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. 2005 (Washington, DC).

[32] Suarez SS, Pacey AA. Sperm transport in the female reproductive tract. Hum Reprod Update 2006; 12: 23-37.

[33] McLean AC, Valenzuela N, Fai S, Bennett SA. Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. J Vis Exp 2012; 67: e4389.

[34] Walton KD, Harding S, Anschel D, Harris YT, Llinas R. The effects of microgravity on the development of surface righting in rats. J Physiol 2005; 565: 593-608.

[35] Lalonde R, Strazielle C. Brain regions and genes affecting limb-clasping responses. Brain Res Rev 2011; 67: 252-259.

[36] Razali NM, Wah YB. Power comparisons of Shapiro-Wilk, Kolmogorov-Smirnov, Lilliefors and Anderson-Darling tests. J Stat Model Anal 2011; 22: 21-33.

[37] Dendrinos G, Hemelt M, Keller A. Prenatal VPA Exposure and Changes in Sensory Processing by the Superior Colliculus. Front Integr Neurosci 2011; 5: 68.

[38] Roste LS, Tauboll E, Berner A, Isojarvi JI, Gjerstad L. Valproate, but not lamotrigine, induces ovarian morphological changes in Wistar rats. Exp Toxicol Pathol 2001; 52: 545-552.

[39] Loscher W. Valproate: a reappraisal of its pharmacodynamic properties and mechanisms of action. Prog Neurobiol 1999; 58: 31-59.

[40] Roma-Giannikou E, Syriopoulou V, Kairis M, Pangali A, Sarafidou J, Constantopoulos A. In vivo effect of sodium valproate on mouse liver. Cell Mol Life Sci 1999; 56: 363-369.

[41] Radatz M, Nau H. Toxicity. In: Loscher W, ed. Milestones in Drug Therapy-Valproate, Basel: Birkhauser Verlag, 1999, 91-128.

[42] Harden CL. Polycystic ovaries and polycystic ovary syndrome in epilepsy: evidence for neurogonadal disease. Epilepsy Curr 2005; 5: 142-146.

[43] Gabory A, Attig L, Junien C. Epigenetic mechanisms involved in developmental nutritional programming. World J Diabetes 2011; 2:164-175.

[44] Veiby G, Daltveit AK, Engelsen BA, Gilhus NE. Fetal growth restriction and birth defects with newer and older antiepileptic drugs during pregnancy. J Neurol 2014; 261: 579-588.

[45] Sargar KM, Herman TE, Siegel MJ. Valproate fetopathy. J Perinatol 2015; 35: 82-83.

[46] Moore SJ, Turnpenny P, Quinn A, Glover S, Lloyd DJ, Montgomery T, Dean JC. A clinical study of 57 children with fetal anticonvulsant syndrome. J Med Genet 2000; 37: 489-497.

[47] Finnell RH, Bennett GD, Karras SB, Mohl VK. Common hierarchies of susceptibility to the induction of neural tube defects in mouse embryos by valproic acid and its 4-propyl-4-penetnoic acid metabolite. Teratology 1988; 38: 313-320.

[48] Stromland K, Pinazo-Duran MD. Ophtalmic involvement in the fetal alcohol syndrome: clinical and animal model studies. Alcohol Alcohol 2002; 37: 2-8.

[49] Pellis VC, Pellis SM, Teitelbaum P. A Descriptive analysis of the postnatal development of contact-righting in rats (Rattus norvegicus) Devel Psychobiol. 1991; 24: 237-263.

[50] Gokhin DS, Ward SR, Bremner SN, Lieber RL. Quantitative analysis of neonatal skeletal muscle functional improvement in the mouse. J Exp Biol 2008; 211: 837-843.

[51] Navarrette R, Vrbova G. Activity-dependent interactions between motoneurones and muscles: their role in the development of the motor unit. Prog Neurobiol 1993; 41: 93-124.

[52] Eken T, Elder GCB, Lomo T. Development of tonic firing behavior in rat soleus muscle. J Neurophysiol 2008; 99: 1899-1905.

[53] Miyazaki K., Narita N., Narita M. Maternal administration of thalidomide or valproic acid causes abnormal serotonergic neurons in the offspring: implication for pathogenesis of autism. Int J Dev Neurosci 2005; 23: 287-297.

[54] Mukherjee A, Chakravarty A. Spasticity mechanisms – for the clinician. Front Neurol 2010;1: 1-10.

[55] Ikonomidou C, Turski L. Antiepileptic drugs and brain development. Epilepsy Res 2010; 88: 11-22.

[56] Frisch C, Hüsch K, Angenstein F, Kudin A, Kunz W, Elger CE, Helmstaedter C. Dose-dependent memory effects and cerebral volume changes after in utero exposure to valproate in the rat. Epilepsia 2009; 50: 1432-1441.

Figure Legends

Figure 1. The highest prenatal dose of VPA has a negative effect on dam health.

A. The highest number of deaths was detected in VPA-400 group, followed by the VPA-200- group, which also includes dams without birth. **B.** The number of days between mate-pairing and birth increased in the VPA-400 group. * $p < 0.05$ *vs* CTR group, # $p < 0.05$ *vs* VPA-50 group, & $p < 0.05$ *vs* VPA-100 group, *U* test. **C.** During pregnancy, the BW increased in all examined groups (§ $p < 0.05$ *vs* the initial BW, Wilcoxon test); the VPA-400 group had a lower final BW than the CTR (* $p < 0.05$, *U* test) and VPA-50 (# $p < 0.05$) groups. Numbers inside the bar graphs denote samples.

Figure 2. Prenatal VPA exposure reduces offspring BW during early postnatal development.

Negative influence of the treatment on the BWs is recognizable with VPA-200 and VPA-400 at PND5, with all doses at PND10 and with VPA-50, VPA-200 and VPA-400 at PND15.

* $p < 0.05$ *vs* age-matched CTR group, # $p < 0.05$ *vs* age-matched VPA-50 group, & $p < 0.05$ *vs* age-matched VPA-100 group, *U* test. Significant BW increase during PND5 to PND10 (§ $p < 0.05$) and PND10 to PND15 (¥ $p < 0.05$) is observed in all experimental groups (Wilcoxon test). Number inside bar graphs denotes samples (5 litters per experimental group).

Figure 3. Prenatal VPA exposure impairs offspring performance in the SRR at PND5.

A. With respect to the criteria specified in the test, delayed righting was observed in a portion of all experimental groups. The CTR group and all the VPA-exposed offspring righted in the CSW-type except a part of the VPA-200 group that almost equally used the CSW- and U-type of righting. The numbers inside the bar graphs denote samples (5 litters per experimental group). **B.** Relative to the CTR group, prenatal VPA exposure increased the time required to complete the righting in CSW-type (* $p < 0.05$, *U* test).

Figure 4. Prenatal VPA exposure impairs offspring performance in the SRR at PND10

A. Prenatal VPA exposure has a negative effect on the appearance of the A-type of righting, while the utilization of the CSW-type is favored. In the VPA-200 and VPA-400 groups, the A-type of righting was completely abolished and the U-type of righting appeared. The numbers inside the bar graphs denote samples (5 litters per experimental group). **B.** Compared to the CTR group, the time required to complete the CSW-type of righting was significantly increased in all of the VPA-exposed groups (* $p < 0.05$, *U* test). Moreover, the VPA-400 group required more time to right than all the other groups (# $p < 0.05$ *vs* VPA-50 group, & $p < 0.05$ *vs* VPA-100 group, $ $p < 0.05$ *vs* VPA-200 group). **C.** Prenatal VPA exposure significantly increased the time required to complete the A-type of righting(* $p < 0.05$ *vs* CTR group, *U* test).

Figure 5. Prenatal VPA exposure impairs offspring performance in the SRR at PND15.

A. Prenatal VPA exposure has a negative effect on the appearance of the A-type righting, while the utilization of the CSW-type is favored. The numbers inside the bar graphs denote samples (5

litters per experimental group). **B.** VPA exposure did not significantly influence the time required to complete the CSW-type of righting. **C.** Compared to the CTR group, the time required to completethe A-type of righting is significantly increased in all of the VPA-exposed groups (* $p < 0.05$, *U* test). The VPA-400 group required more time to complete the A-type of righting than all the other groups exposed to VPA (# $p < 0.05$ *vs* VPA-50, & $p < 0.05$ *vs* VPA-100, $ $p < 0.05$ *vs* VPA-200), as well VPA-200 *vs* VPA-50 group (# $p < 0.05$).

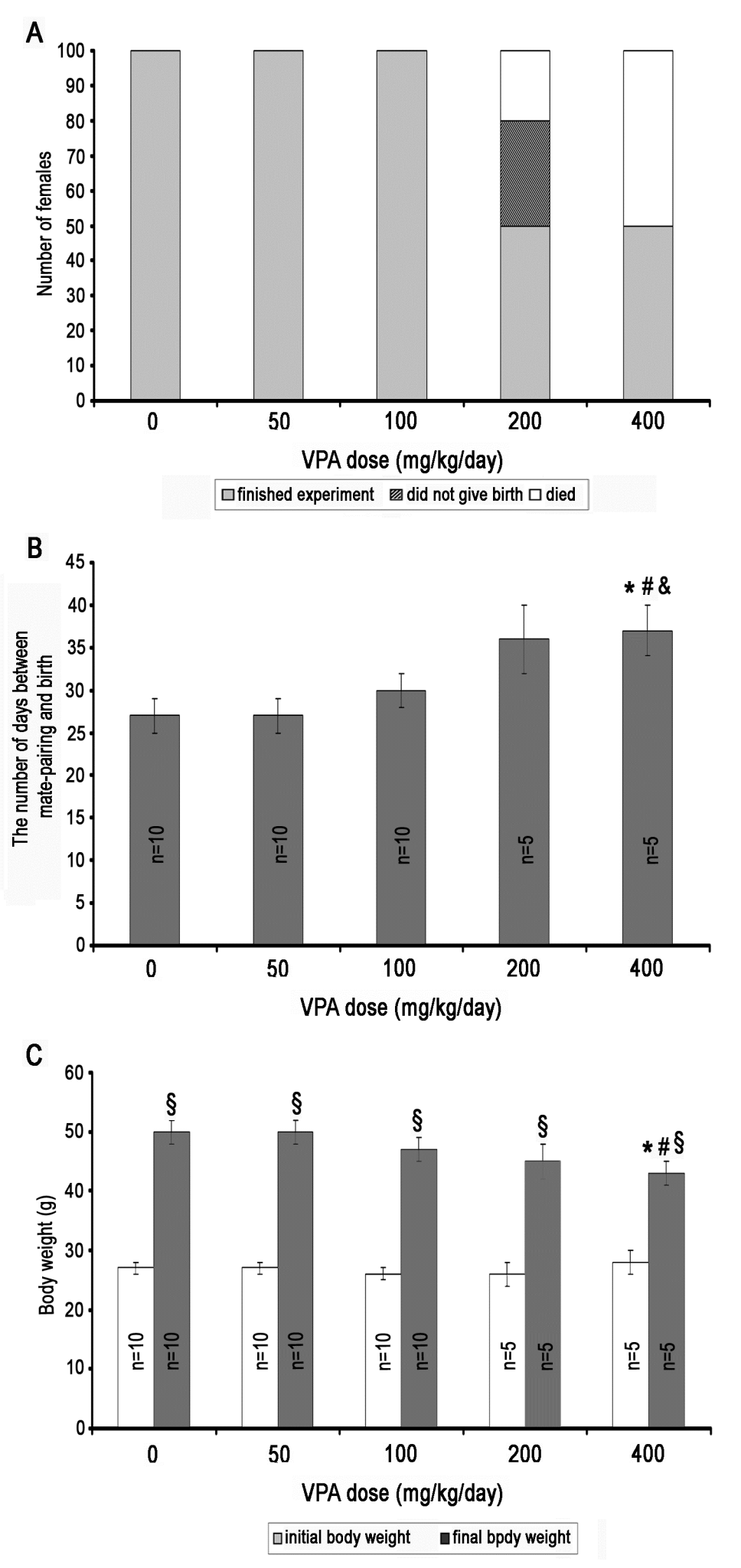
Figure 6. Prenatal VPA exposure impairs offspring performance in the TST.

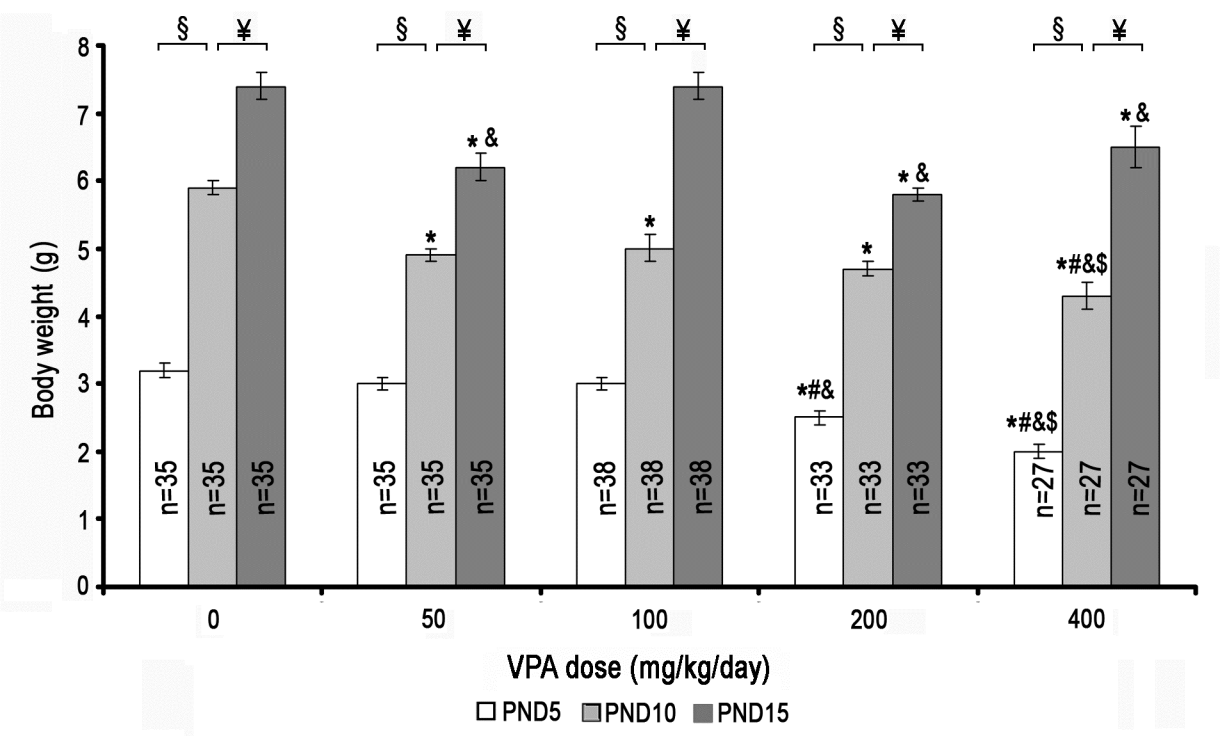
A. The effect of VPA on the latency to the first immobilization observed at PND5 and PND10 disappeared at PND15 ($ $p < 0.05$ *vs* age-matched CTR group, # $p < 0.05$ *vs* age-matched VPA-50 group, & $p < 0.05$ *vs* age-matched VPA-100 group, *U* test). Moreover, the latency to the first immobilization changed in the CTR group and the groups exposed to VPA in a different manner when PND10 was compared to PND5 (* $p < 0.05$, Wilcoxon test), and PND15 to PND10 (¥ $p < 0.05$). **B.** The body/limb movements in the CTR, VPA-50 and VPA-100 groups (a – swinging; b – curling), and in groups exposed to the higher tested doses of VPA (c – abnormal posturing, i.e. stretched out body, hind limbs flexed, upper limbs outstretched; d – dystonic movements of hind- and forelimbs during which the limbs were pulled into the body and held and released in short bursts).

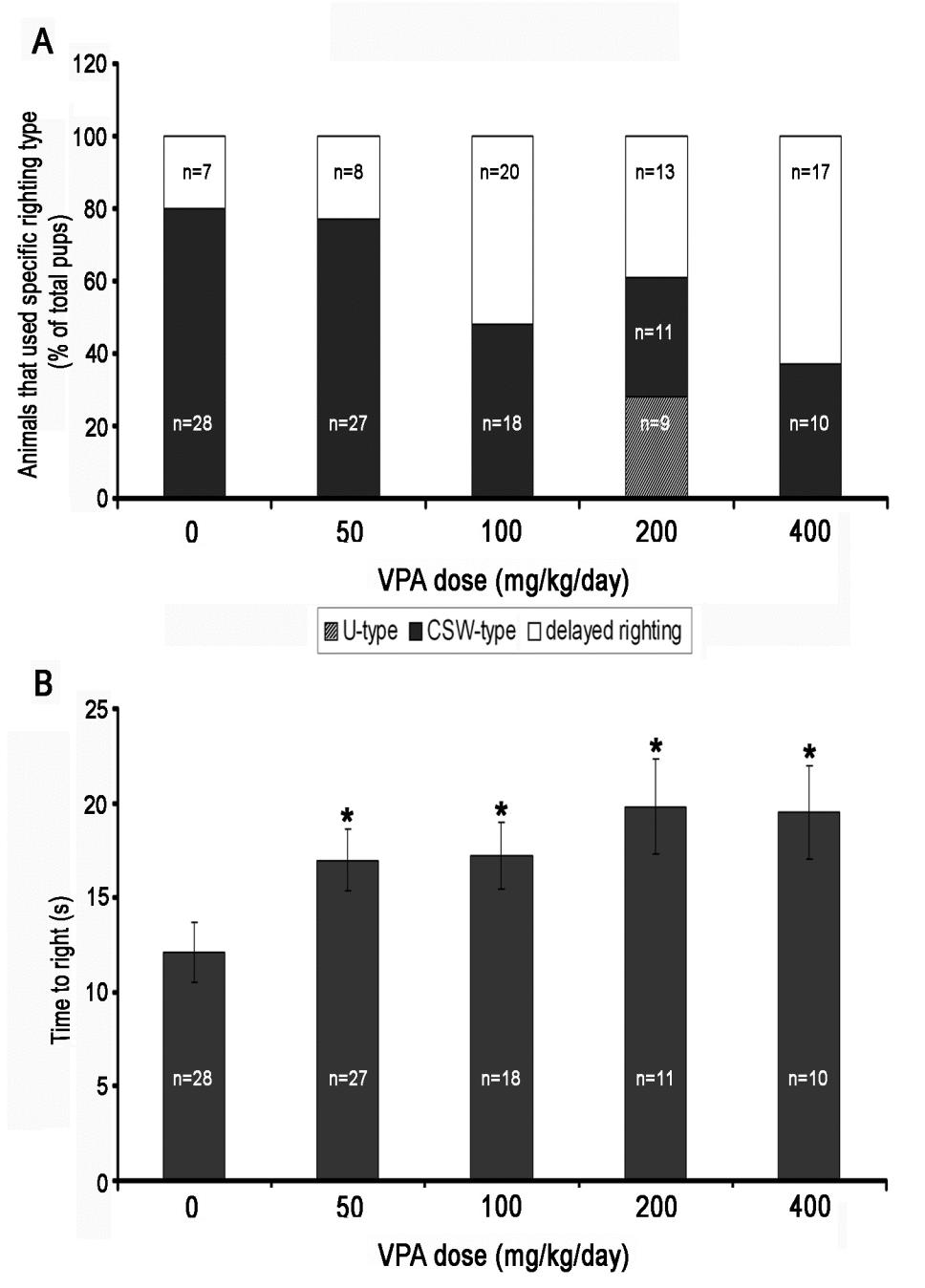
Tables

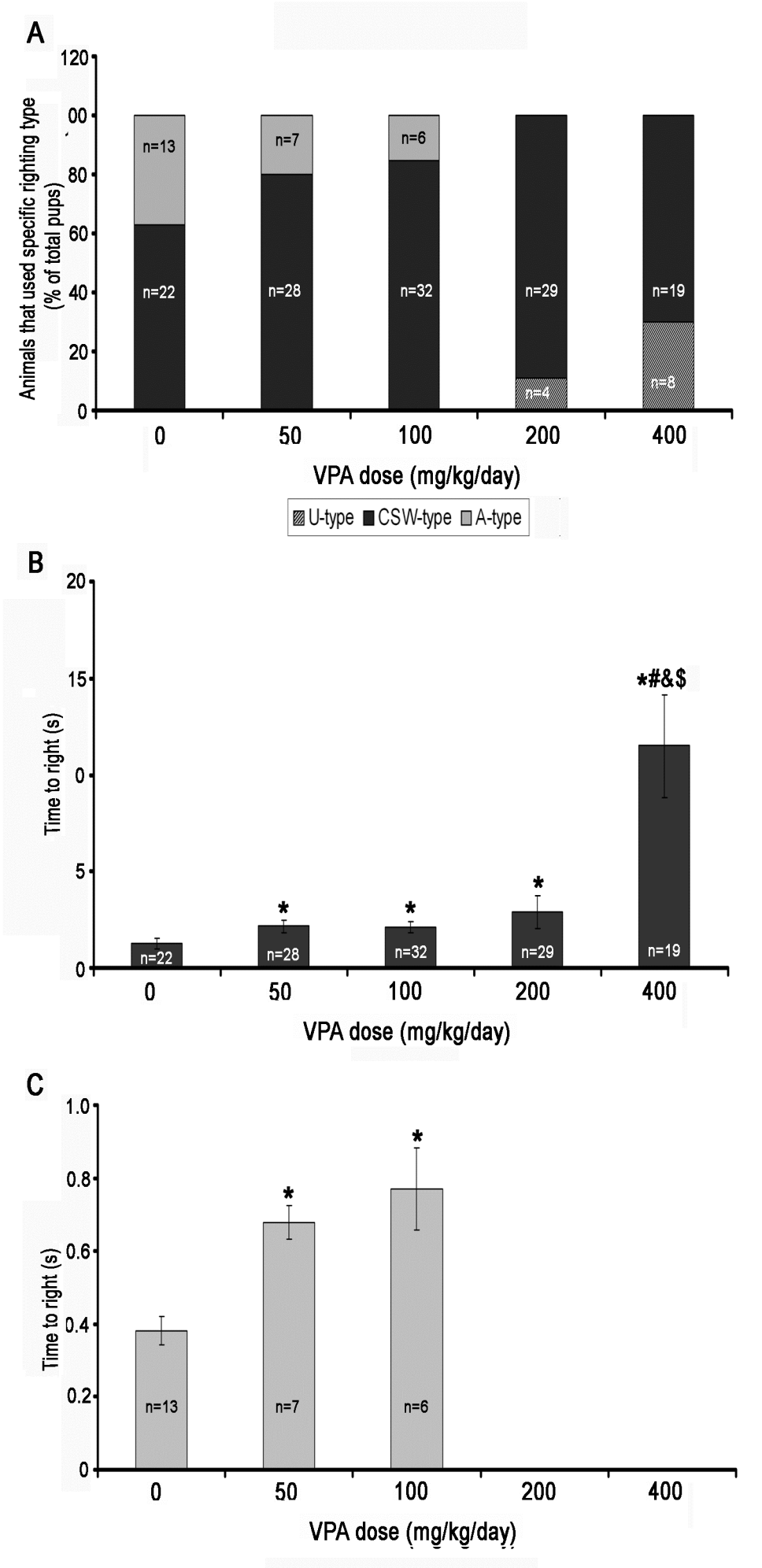
Table 1. The influence of prenatal VPA exposure on physical characteristics of offspring

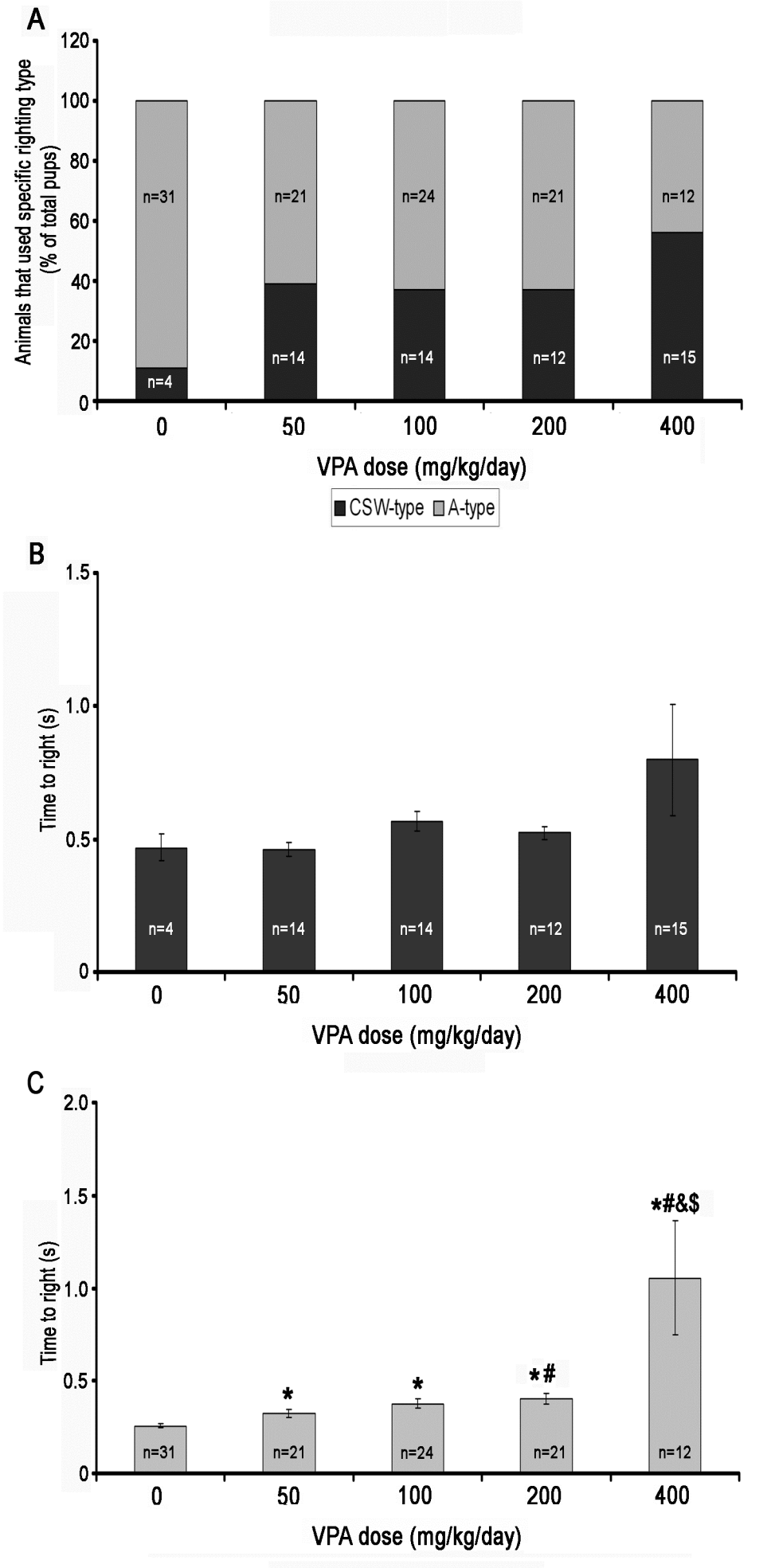
Prenatal VPA dose (mg/kg/day)	Total number of litters	PND5		PND10		PND15			
		Litter size (No. pups)	Total number of offspring	Litter size (No. pups)	Total number of offspring	Litter size (No. pups)	Total number of offspring	Animals with the tail kink (% of total pups)	Animals with both eyes open (% of total pups)
0 (control)	5	7 ± 1	37	7 ± 1	35	7 ± 1	35	0	98
50	5	7 ± 1	37	7 ± 1	35	7 ± 1	35	0	36
100	5	8 ± 1	40	8 ± 1	38	8 ± 1	38	0	24
200	5	7 ± 1	36	7 ± 1	34	7 ± 1	33	10	28
400	5	6 ± 1	30	5 ± 1	27	5 ± 1	27	20	19

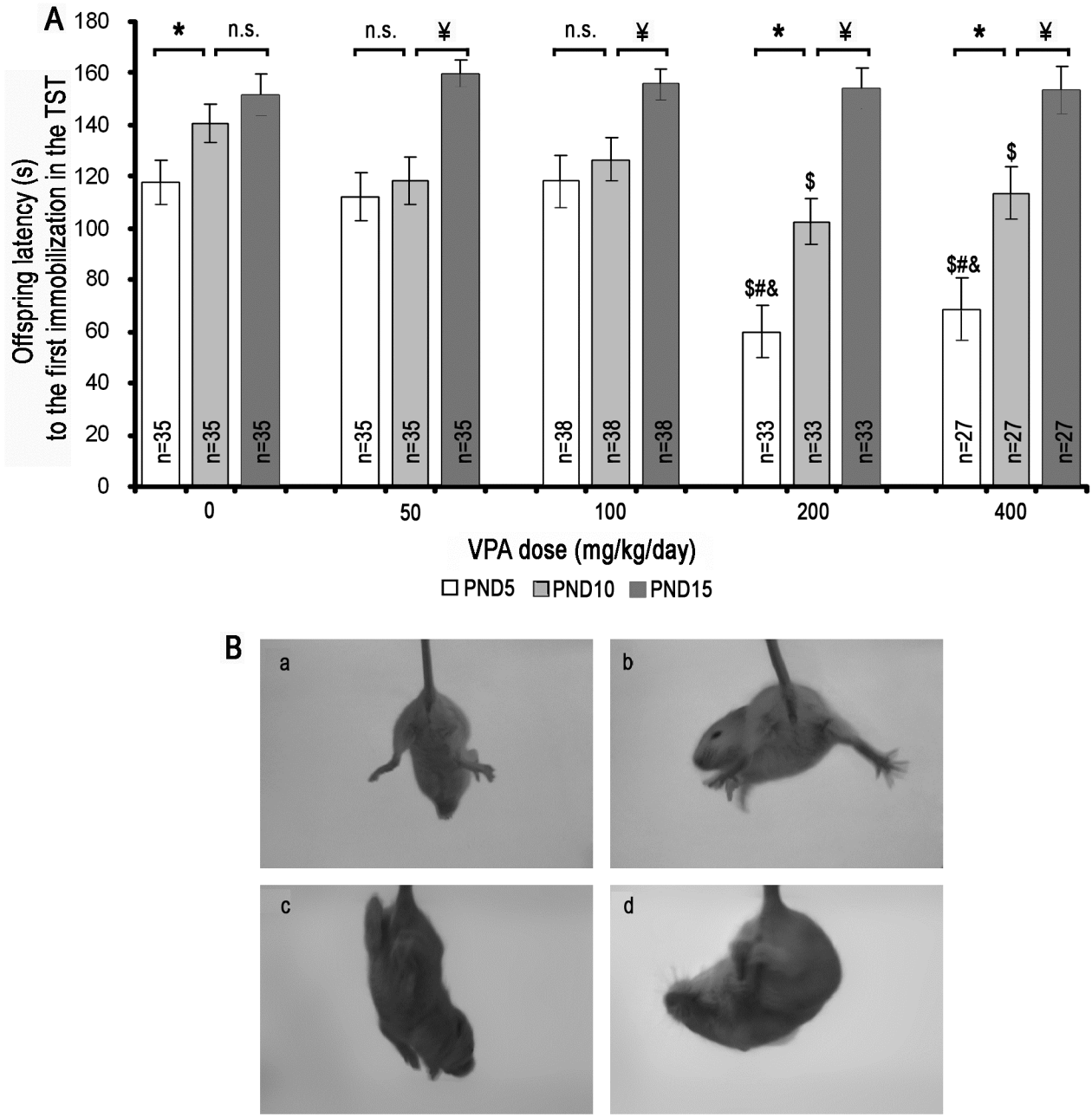

A
Number of females
100
90
80
70
60
50
40
30
20
10
0
0
50
100
200
400
VPA dose (mg/kg/day)
finished experiment
did not give birth
died
B
The number of days between mate-pairing and birth
45
40
35
30
25
20
15
10
5
0
* # &
n=10
n=10
n=10
n=5
n=5
0
50
100
200
400
VPA dose (mg/kg/day)
C
Body weight (g)
60
50
40
30
20
10
0
§
§
§
§
* # §
n=10
n=10
n=10
n=10
n=10
n=10
n=5
n=5
n=5
n=5
0
50
100
200
400
VPA dose (mg/kg/day)
initial body weight
final bpdy weight


Body weight (g)
0
1
2
3
4
5
6
7
8
§
¥
n=35
n=35
n=35
n=35
n=35
n=35
n=38
n=38
n=38
n=33
n=33
n=33
n=27
n=27
n=27
*
*&
*#&
*#&$
0
50
100
200
400
VPA dose (mg/kg/day)
PND5
PND10
PND15


A
120
100
80
60
40
20
0
Animals that used specific righting type
(% of total pups)
n=7
n=8
n=20
n=13
n=17
n=11
n=28
n=27
n=18
n=9
n=10
0
50
100
200
400
VPA dose (mg/kg/day)
U-type
CSW-type
delayed righting
B
25
20
15
10
5
0
Time to right (s)
*
*
*
*
n=28
n=27
n=18
n=11
n=10
0
50
100
200
400
VPA dose (mg/kg/day)


A
Animals that used specific righting type (% of total pups)
120
00
80
60
40
20
0
n=13
n=22
n=7
n=28
n=6
n=32
n=29
n=4
n=19
n=8
0
50
100
200
400
VPA dose (mg/kg/day)
U-type
CSW-type
A-type
B
Time to right (s)
20
15
0
5
0
*
*
*
*#&$
n=22
n=28
n=32
n=29
n=19
0
50
100
200
400
VPA dose (mg/kg/day)
C
Time to right (s)
1.0
0.8
0.6
0.4
0.2
0
*
*
n=13
n=7
n=6
0
50
100
200
400
VPA dose (mg/kg/day)


A
Animals that used specific righting type (% of total pups)
120
100
80
60
40
20
0
n=31
n=21
n=24
n=21
n=12
n=4
n=14
n=14
n=12
n=15
0
50
100
200
400
VPA dose (mg/kg/day)
CSW-type
A-type
B
Time to right (s)
1.5
1.0
0.5
0
n=4
n=14
n=14
n=12
n=15
0
50
100
200
400
VPA dose (mg/kg/day)
C
Time to right (s)
2.0
1.5
1.0
0.5
0
*#&$
*
*
*#
n=31
n=21
n=24
n=21
n=12
0
50
100
200
400
VPA dose (mg/kg/day)


A
Offspring latency (s)
to the first immobilization in the TST
180
160
140
120
100
80
60
40
20
0
*
n.s.
n.s.
¥
n.s.
¥
*
¥
*
¥
$#&
$
$
$#&
n=35
n=35
n=35
n=35
n=35
n=35
n=38
n=38
n=38
n=33
n=33
n=33
n=27
n=27
n=27
0
50
100
200
400
VPA dose (mg/kg/day)
PND5
PND10
PND15
B
a
b
c
d