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Permanent motor activity and learning disorders induced by exposure to phenytoin during gestation and early infancy in the rat

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Abstract

Experimental models and clinical data indicate that the incidence of motor and learning disorders may be increased in children of epileptic mothers taking phenytoin (PHT) during pregnancy. There is little data on the vulnerability of infants to PHT-induced long-term behavioral toxicity after gestational or early life exposure (i.e., infantile convulsion therapy). We examined the persistence of alterations in circling behavior induced by exposure to PHT during gestation, infancy, or both. Pregnant Sprague–Dawley rats were injected IP with saline (SAL) or PHT (30 mg/kg/day) during gestational days (GD) 10–18. The offspring were then administered (IP) SAL or PHT (60 mg/kg/day) during postnatal days (PD) 13–23. Afterward, Circling Training tests were performed at three time points. At PD40 and PD80, the clockwise direction of circling was reinforced. At PD150, counterclockwise circling was rewarded instead. At PD40, all PHT-treated groups demonstrated increased circling velocities compared to saline-treated controls. Higher spatial error rates for direction of circling were also observed in gestation-only and infancy-only exposures. At PD80, groups exposed during gestation had higher circling velocities than control or infancy-only exposed groups. At PD150, increases in circling velocity were apparent for the reverse learning task in groups exposed during gestation. These results indicate that early postnatal exposure to PHT may exacerbate the known long-term behavioral effects of gestational exposure.

Keywords: Phenytoin; Behavioral teratology; Infancy

1. Introduction

The developing brain is highly vulnerable to wide variety of chemicals including pharmaceutical agents and environmental pollutants [38]. Some neuroactive chemicals have been shown to induce long-lasting behavioral alterations, even exposure during critical periods is at low doses and in the absence of acute neurotoxicity [41]. Accordingly, these chemicals have been classified as behavioral teratogens [42,50].

Epilepsy, the second most prevalent neurological disorder [18], affects to about 0.5% of the US population (1996 National Chronic Health Survey, CDC Series 10 (200): 82, Table 57; http://www.cdc.gov/nchswww/data/10_200_1.pdf). Several anticonvulsant drugs have been found to be developmental neurotoxicants [31,49,58] and a primary anti-epileptic drug, phenytoin (PHT) [32], has been characterized as such [43,48]. Data from rats [14,29,36,48], monkeys [35], and humans [1,10,16,46,54] indicate that prenatal exposure to PHT increases the risk for long-term motor, learning and memory disorders in offspring. The effects of PHT exposure on behavioral endpoints such as learning (i.e., water maze) and motor activity in rodent

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models have been associated with a critical period of exposure during gestational days (GD) 11–14. However, a wider critical period (GD 11–18) has been reported for the detection of more profound long-term changes [51]. In addition, higher seizure susceptibility in the offspring of mothers treated with PHT during gestation has been demonstrated [37,47]. The mechanism of PHT-induced behavioral alterations has not yet been elucidated, though oxidative metabolites have been postulated to play a role [23].

Phenytoin is prescribed for infants with seizure disorders [2,7,30]. While the behavioral teratogenicity induced on prenatal exposure to PHT is well established [43,48], the enduring adverse effects of early postnatal administration of PHT, either alone or in combination with prenatal exposure, have not been investigated.

In this study rats were exposed to PHT during both prenatal and postnatal development. Infant rats were treated postnatally during days 13–23, a period corresponding to parturition through ~1.5–3.0 years of age in humans depending on which neurodevelopment landmark is examined in both species [19,38]. Circling Training (CT) in a circular maze apparatus was used to assess long-term effects. CT test has been demonstrated to be useful in assessing motor and associative-spatial behaviors of developing rats under normal conditions [5,22], or after early exposure to neuroactive drugs [6,56].

Following exposure, signs of neurotoxicity may manifest at different times during development due to stage-specific processes in the maturation of the brain [8]. Accordingly, a multistage assessment may be more sensitive in detecting behavioral toxicity than testing at any one particular age [40]. Therefore, the experimental design included circling evaluations at three ages, from puberty to adulthood.

2. Materials and methods

2.1. Animals

Primiparous female Sprague–Dawley rats (local facilities, originally from Holtzman Institute) were used for breeding. Cages containing three females and one male each were placed in a controlled environment [22–24 °C, 12 h dark/light cycle]. The appearance of sperm after vaginal flushing with saline was considered an index for gestational day (GD) 1.

2.2. Doses

Dosages were selected taking into account that humans are generally more vulnerable than experimental animals to the effects of toxic drugs by a factor of 10 [13]. In addition, preliminary work enabled us to determine dose levels that induce long-term circling alterations with minimal acute sedation or ataxia [55]. Accordingly, the PHT doses in this work can be assumed roughly ten-fold higher than a medium dose used in antiepileptic therapy, based on body weight and quoted therapeutic blood level range for humans [25].

2.3. Prenatal treatment

Pregnant rats were randomly divided into two groups. Treatments were administered IP once daily (2.0 ml/kg body weight (bw)) from GD10 to GD18 as follows: saline (SAL, N=7), NaCl 0.9% w/v sterile solution, or sodium diphenylhydantoin (phenytoin, PHT, N=8), 30 mg/kg bw. PHT solution obtained by diluting PHT (Epamin ampoules, 50 mg/ml, from Parke Davis Laboratories, BA) in SAL.

2.4. Postnatal treatment

Stainless steel cages of 22×18×45 cm^3 were used to foster litters. Litters were culled to eight pups by preferential sacrifice of female pups. Pups were randomly relocated at postnatal day (PD) 2 in order to attenuate litter effect. Offspring were then injected IP, once daily, from PD 13 to 23, with 10 μl/g bw of SAL (NaCl 0.9%), or 60 mg/kg bw of PHT diluted in SAL. Since PHT diluted solutions were alkaline (pH=8.3–8.9), SAL was adjusted to an equivalent pH before injecting into SAL-treated animals. Fresh PHT solutions were administered immediately after preparation to prevent precipitation [34].

2.5. Experimental groups

Four treatment groups (each group=four cages; N=7–8 each cage) were designed as follows: control C (SAL in both periods), SAL/PHT (prenatal SAL followed by PHT administration during early infancy), PHT/SAL (prenatal PHT followed by SAL administration during early infancy), and PHT/PHT (exposure to PHT in both periods).

2.6. Perinatal observations and post-weaning conditions

During the first postnatal week the following data were collected: (1) litter size and pup mortality (at PD1), (2) litter sex ratio (at PD2), and (3) maternal behavior. Maternal care was examined twice a day, and five behaviors were observed as follows: breast feeding, mother-pup cannibalism, pup licking, nest maintenance, and ambulatory activity outside the nest area. After weaning (PD23) infants were regrouped, three to four individuals per cage. Two days prior to the first assessment, four cages from each treatment schedule were moved to a testing room with sound-insulated walls, maintained under the same environmental conditions as described above. Before testing, two males from each cage were randomly designated for all behavioral studies included in this work. Non-tested littermates served for controlling body weight loss patterns associated with CT testing conditions [6]. Animals were fed with high protein

food and water ad libitum except when indicated. Body weights were recorded twice a week following pharmacological schedules except when otherwise indicated. Rats were cared for in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

2.7. Behavioral evaluations

2.7.1. Circling training test and apparatus

This assessment consists of training rats deprived of water for 24 h to locomote around a circular maze for a reward. The circling training may be considered a variant of the straight alley maze. In both cases, learning is measured as the rate of approach to asymptote, and is shown to be relatively independent of motivational factors [6,57]. The CT behavioral response (rewarded turns per min) would result from an interaction of two neurological components: the motor ability and the learning process [6].

Two identical CT apparatuses were utilized (for a detailed graphical description see Ref. [5]). A schematic drawing of the circular maze path, as seen from above the apparatus, is shown in Fig. 1. Turns are recorded based on the sequential interruption of four infrared photobeams. Each assessment starts with teaching the associative-spatial conditioning in session zero (S_0). Thereafter, the rat trains one session a day for a series of consecutive days, according to a continuous reinforcement schedule. In this work, a different number of sessions were used at different ages (see below). Each session begins by placing the rat within the circular maze at the reward position (RP), and randomly positioning the head towards either direction in the circling path (see diagram in Fig. 1a). A 60-μl drop of 10% (w/v) sucrose is given for a successful completion of the circle following the reinforced direction of circling. Turn detection, reinforcement delivery (except in S_0), and time are controlled electronically. No spatial references are given during testing, and the apparatus is wiped clean following each session.

2.7.2. First circling training assessment at puberty

Trained animals were subjected to the first CT test (i.e., CT#1) starting at PD40. During CT#1 clockwise circling was reinforced (outlined in Fig. 1b). An unmodified version of the original test was applied [5,6]. The spatial conditioning in S_0 was reinforced by sequential completion of a successful turn as follows: a reward in the first 10 min for a quarter turn, a reward in the next 10 min for a half turn, and a reward for a full turn during the remainder of the session (see Fig. 1a). After S_0, CT#1 testing continued for 7 days (i.e., from session S_1 to S_7). Each session was terminated after 100 turns in $S_{0–1–2–3}$, 150 turns in $S_{4–5–6–7}$, or 30 min of testing in all sessions. Reinforced circling velocity (V_R) is represented by the total turns (1 turn=1 reinforcement) in each session divided by the time required to complete the entire task (i.e., 100 or 150 turns). Accumulated reinforcement (R_a) after each training session S_1 to S_7 is equal to the

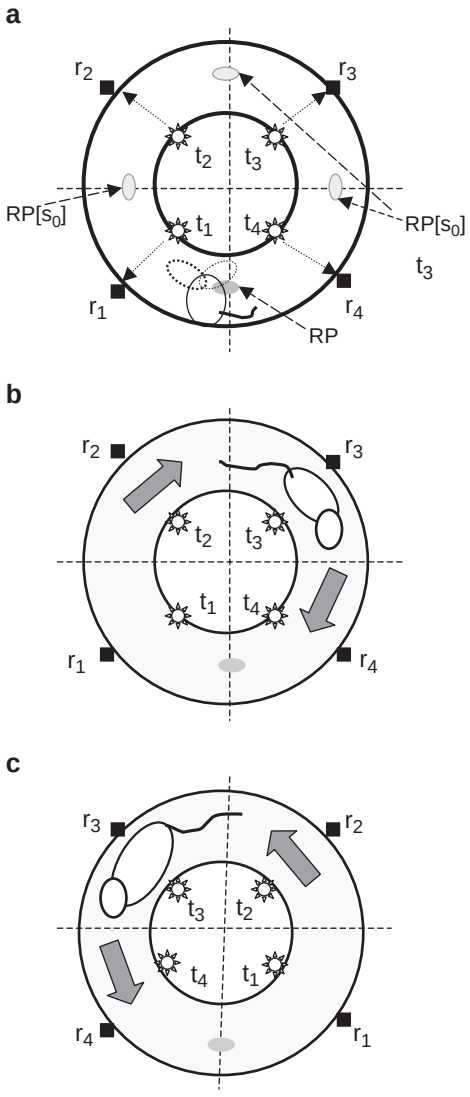

Fig. 1. Schematic diagram of the Circling Training (CT) test apparatus. The CT apparatus consists of two concentrical steel drums (30 cm height) with a circular path in the middle [10 cm width, 1 m length] covered with rubber. Panels a–c depict a view from above the apparatus. In panel a, the rat is placed at the automatic reinforcer delivery point (RP), where each session starts. Reinforcement is manually dispensed at points marked RP[S_0] only during the spatial conditioning session (S_0). Four infrared transmitter/receptor (t/r) pairs allow for recording of speed and direction of circling. Each t/r pair is separated by 90°. The four photobeams the rat has to cross to register a count are represented in panel a by dotted arrows. The direction of circling reinforced during the entire CT scheme is chosen in S_0 by selecting either alternative spatial configuration of t/r pairs, i.e., clockwise or counterclockwise. The receptors are connected to a memory chip, so that the first receptor must have been activated to permit the recorder of the activation of the second one on this chip and so on. Panels a–b show the clockwise setting that was used in CT#1 and CT#2. For CT#1 and CT#2, when photobeam interruptions occurred in the order "1–2–3", a drop of reward was delivered at the reward point (*RP*), but no rewarded turn was counted until a complete series of receptors were activated (i.e., "1–2–3–4"). Each time the counter advances one turn, the memory chip is reset. Panel 3 shows the opposite order of t/r pairs that was set up in CT#3 to assess reversal (i.e., counterclockwise) spatial learning (see Materials and methods and Refs. [5,6] for further details).

summation of rewarded turns for consecutive sessions. Before sessions $S_{0–1–3–7}$, the body weight of trained and non-trained/non-deprived rats was recorded.

During CT#1, we also recorded errors in the direction of circling (i.e., spatial errors) by observing the movement of the rat during S_1, S_3 and S_7, and manually clicking a counter. Four perpendicular marks were drawn on the path floor to divide the circular alley in four equal quarters (see Fig. 1a). Each time all four paws of the animal crossed at least two consecutive marks locomoting in the non-reinforced direction an error was counted. Error rate (E) is the total number of errors. Errors were counted during the first 5 min under the assumption that these initial errors were most likely due to learning and associative effects, while habituation and fatigue may influence circling measurements at longer times [27]. Two individuals conducted spatial assessments in a treatment-blind fashion, and inter-observer bias was not significant. From S_1 to S_3 a steep drop in the number of spatial errors occurs, with a plateau of minimal number of errors occuring during S_4–S_5 [7]. The error count on S_7 was therefore considered an estimation of the asymptotic spatial response. Performance was also examined in sessions S_1, S_3 and S_7 by dividing the sum of incorrect quarter turns by time (i.e. 5 min). This wrong circling velocity (V_W) is expressed as full turns per min. Controls animals for CT#1 were sacrificed at PD70. Trained rats were then held two per cage until the end of the experiment.

2.7.3. Strengthening of clockwise circling training at young adulthood

At PD80, trained rats were again submitted to the CT test (CT#2) under the same conditions applied during CT#1 (outlined in Fig. 1b). Under control conditions, young rats reach the plateau of motor response in a second CT at least two sessions earlier than in a previous assessment. This is so provided that the two consecutive CT schemes are executed at no more than 2 months apart and the direction of reinforced circling (clockwise or counter-clockwise) is the same (unpublished data). Five training sessions (i.e., S_1 to S_5) were carried out at this age. CT#2 strengthened motor/spatial skills and the learning process for clockwise training. Spatial conditioning in S_0 was reinforced by sequential completion of a full turn in the correct direction, as follows: a reward in the first 2 min for a quarter turn, a reward in the next minute for a half turn, and a reward for a full turn during the remainder of the session. Each session was terminated when 100 ($S_{0–1–2}$) or 150 turns ($S_{3–4–5}$) were counted, or after 30 min of testing.

2.7.4. Reversal learning at adulthood

At age PD150, a third CT assessment was performed (CT#3). A counterclockwise direction of circling was rewarded (see Fig. 1c) and three sessions were examined ($S_{0–1–2}$). Spatial conditioning in S_0 was as follows: a reward for a successful quarter turn during the initial eight min, a reward for a successful half turn during the next seven min, and a reward for a full turn for the remainder of the session. Each session was terminated after 30 min or 150 turns. In CT#3, we also obtained a spatial learning ratio (SLR) by dividing V_R by V_W (i.e., $SLR_S=[V_R/V_W]_S$; S=examined session). It was previously observed in naive Sprague–Dawley and Wistar rats submitted to the CT test at puberty that SLR patterns are correlated with spatial performance (unpublished data).

The amount of reinforcement delivered per correct turn was increased to 70 and 90 μl in CT#2 and CT#3, respectively, to help maintain body weight. For all CT assessments, no rat lost more than 12% bw through each testing schedule.

2.8. Statistical analysis

Data transformation was done in most cases. Maternal and litter observations, body weights and behavioral records were analyzed by ANOVA. In the case of weight and motor data from CT#1 and CT#2, whole datasets could not be analyzed by repeated measure (RM) ANOVA because these did not comply with the requirement of sphericity. This requirement was passed when three sessions were included in the RM-ANOVA. V_R, V_W and error rates were therefore analysed by computing sessions S_1–S_3–S_7, S_0–S_2–S_5, and S_0–S_1–S_2, in the cases of CT#1, CT#2, and CT#3, respectively. According to the experience with this circling trained rat model [5,6,22,56], training sessions (i.e., from S_1 to S_7) can be divided into two behaviorally meaningful sets. In the acquisition part (from S_1 to $S_{3–4}$), maximal slopes for the associative-spatial and motor learning processes appear. Maximal spatial and motor performances usually come up from S_5. From this session, the actual V_R is mostly determined by timing management and procedural skills under physiological conditions. Consequently, for circling velocities, we took data from S_1 and S_7, assuming that these sessions can synthesize the response profiles over time for *acquisition* and *maximal performance* phases proposed for this test [6]. In CT#2, S_0 was considered equivalent to S_1 from CT#1 since just 3 min were spent to make rats recall the spatial conditioning learnt in CT#1. In addition, we took into analysis S_3 in CT#1 (or its equivalent in CT#2, S_2) as an inflection point: the rat becomes skilled in solving the spatial challenge, and the motor ability starts to be the principal factor affecting V_R. For V_R recorded in CT#1 we also applied a derivation of Hernstein's Matching Law Equation described by Heyman and Beer [21]. In our case, we used accumulated reinforcers instead of reinforcement frequency [6], and linear regressions between V_R and V_R/R_a, from sessions S_1 to S_7, were computed. The maximal circling velocity (V_M) was estimated for each group by extrapolating the corresponding function to the abscissa (i.e., where y=0). We further calculated V_M by applying a regression model on each individual item of data. V_M values were compared across treatment groups with ANOVA. For

SLR ratios obtained in CT#3, groups were compared by RM-ANOVA. For examining body weights during and after exposure periods, we took into account data from gestation [GDs 5, 10, 15, and 18], infancy [PDs 13, 16, 19, and 23], or infancy-to-puberty [PDs 23, 30, and 37] stages in separate tests. Tukey's test was carried out as multiple comparison method. Findings are noted as statistically significant where $p \leq 0.05$.

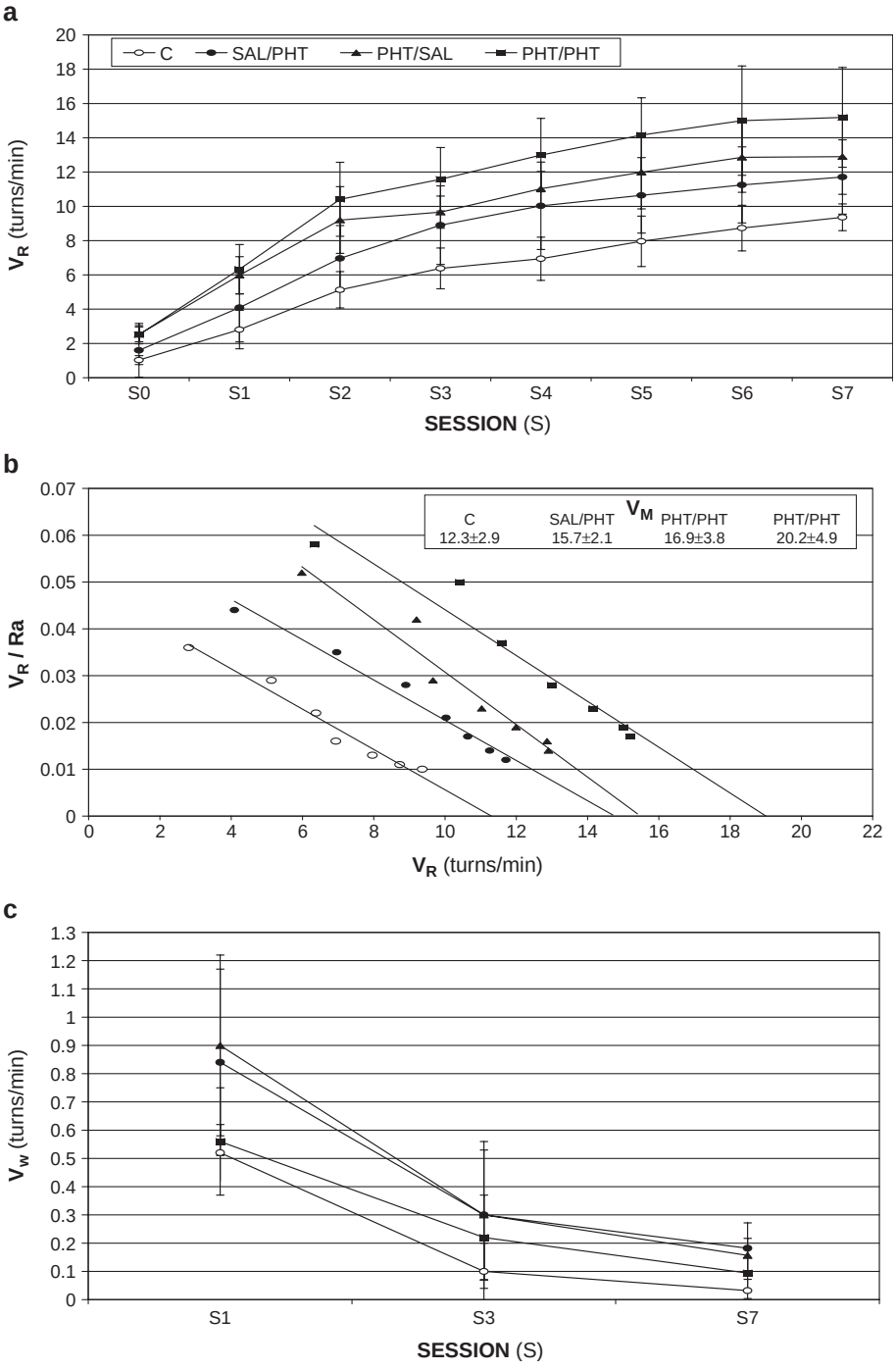

Fig. 2. Motor performance in the Circling Training test at puberty (CT#1). At age PD40, the following groups were assessed in a CT test for the first time (N=8 each): C (control); PHT/SAL (gestation-only PHT), SAL/PHT (infancy-only PHT) and PHT/PHT (exposure during gestation and infancy). Circling activity is expressed as means±SD. Sessions $S_{1–3–7}$ were analyzed by RM-ANOVA. Tukey's test was applied as multiple comparison method. Panel a: Circling velocity in rewarded (clockwise) direction (V_R). Both main effects [T and S] were significant for all pairwise comparisons between PHT-treated groups and control (C), $p<0.05$. Pairing off groups SAL/PHT and PHT/PHT, $p<0.05$. Panel b: Scatchard-like transformation of data shown in panel a. The intersection of the regression with X axis (where $V_R/R_a=0$) represents the theoretical maximal circling velocity (V_M). The inset contains V_M means±SD for each group. The main effect of T was significant. $p<0.05$ for PHT/PHT compared to group C. Panel c: Circling velocity in non-rewarded (wrong) direction (V_W). Both main effects were significant. $p<0.05$ for PHT/SAL and SAL/PHT groups compared to control C. R_a=accumulated reinforcement.

3. Results

3.1. Maternal behavior and gross physical development

No statistically significant change in body weight patterns from PHT-treated groups was found. Further, we did not find treatment-related changes in gestation time. During early postnatal development, the differences among treatment groups in the perinatal and maternal behavior records were not significant. A few treated pups showed moderate sedation during postnatal treatment. After weaning, groups displayed no differences in body weights before and throughout behavioral testing periods (data not shown).

3.2. Circling training test

3.2.1. Motor findings

3.2.1.1. Circling training at puberty. Fig. 2 shows the consequences of early life exposure to PHT on circling speeds as tested in CT#1. Reinforced (clockwise) circling is classified as "right" velocity (V_R, see panel A), and non-reinforced (counterclockwise) circling is classified "wrong" velocity (V_W, see panel C). The main effects of treatment (T, $F_{3,28}$=15.7) and session (S, $F_{2,56}$=399) were significant (p<0.00001). All PHT-treated groups had higher V_R than control C (p<0.05). Groups PHT/SAL and PHT/PHT showed the highest speeds (i.e., for S_5: 50% and 78% above control, respectively). The SAL/PHT group was slower than PHT/PHT (p<0.05). Examination of the last four sessions showed that an asymptotic response appeared from S_5 on.

A Scatchard-like analysis of V_R/R_a as a function of V_R is shown in Fig. 2b. The inset compares estimated maximal velocities (i.e., V_Ms) at the x-intercept (V_R/R_a=0). The main effect of T was significant, $F_{3,28}$=6.8, p<0.002. Treated groups SAL/PHT, PHT/SAL, and PHT/PHT yielded V_M values 28%, 38%, and 65% above control, respectively (p<0.05 for PHT/PHT).

Wrong circling velocities (V_W) are shown in panel C. The main effects of T and S were significant: $F_{3,28}$=8.6, p<0.0005, and $F_{2,56}$=77.5, p<0.00001, respectively. Control C took 79% fewer wrong turns in S_3 as compared to S_1, and showed a further 70% improvement in correct circling activity from S_3 to S_7 (by comparing S_1 vs. S_3, or S_3 vs. S_7, p<0.05). Compared to control C, in sessions S_1, S_3 and S_7, PHT/SAL and SAL/PHT groups had 1.7, 2.8, and 5.0 times higher V_W, respectively (p<0.05).

3.2.1.2. Repetition of clockwise training at young adulthood. CT#2, performed from PD80, showed persistant effects of PHT on circling velocity (see Fig. 3). The effects of T and S and the interaction were significant [T: $F_{3,28}$=28.5, p<0.00001; S: $F_{2,56}$=22.5, p<0.00001; T×S: $F_{6,56}$=3.4, p<0.007]. In the control group, the circling pattern observed in CT#1 was roughly reproduced, but a plateau in V_R appeared earlier in testing (i.e., no difference was found from S_2 on). Both groups exposed to PHT during gestation had higher V_R's with respect to control (p<0.05), and no clear learning curve was observed.

3.2.1.3. Counterclockwise circling training at adulthood. All PHT-treated groups demonstrated higher V_R's during CT#3 at PD150 (see Fig. 4a). All main effects were

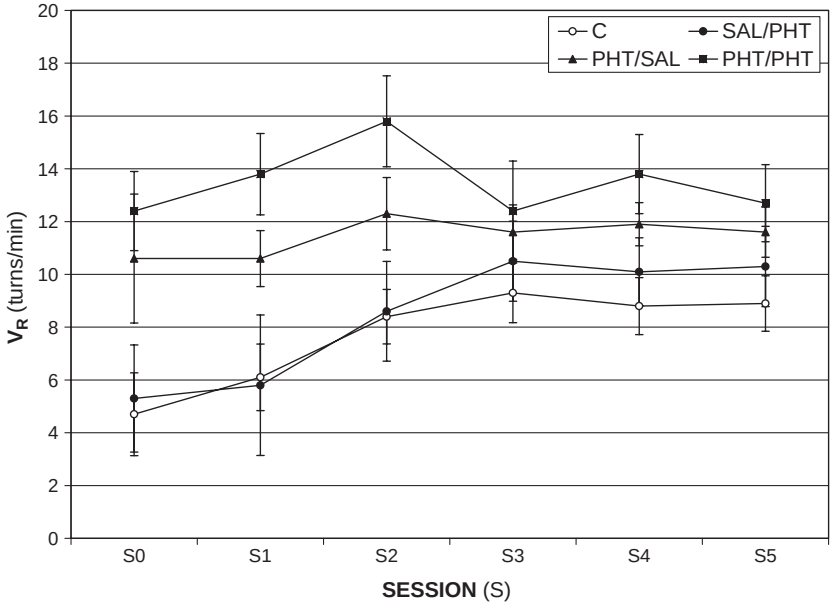

Fig. 3. Motor performance in the Circling Training test at young adulthood (CT#2). At age PD80, the groups in Fig. 2 were tested a second time under the same spatial (clockwise) conditioning in CT#1. Rewarded circling velocity (V_R) is expressed as means±SD. Data from sessions $S_{0–2–5}$ were analyzed with ANOVA. Both main effects and the interaction [T×S] were significant. Pairwise comparison [Tukey's test] of V_R means were signficant (p<0.05) for: PHT/SAL and PHT/PHT groups compared to control for all sessions, and PHT/SAL and PHT/PHT groups compared to SAL/PHT for sessions $S_{0–2}$.

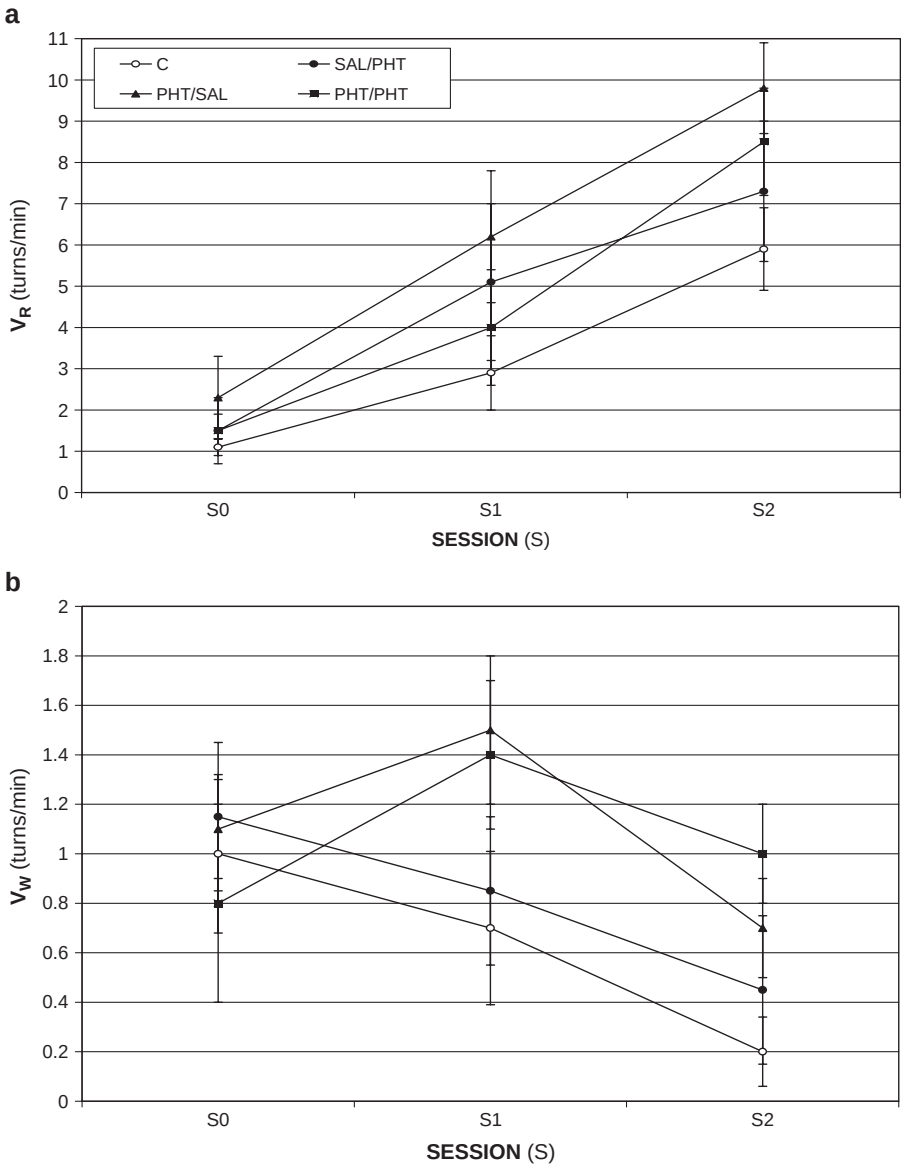

Fig. 4. Motor performance in the Circling Training test at adulthood (CT#3). At age PD150, groups in Fig. 2 were subjected to a third CT assessment. Reversal learning task was then administered. Circling velocity is expressed as means±SD. See panel a for group identification. Panel a: V_R (anticlockwise circling was reinforced). Both main effects and the interaction [T×S] were significant by ANOVA. Multiple comparisons between group means were significant [Tukey's test, $p<0.05$] for: PHT/SAL>C for all sessions, SAL/PHT and PHT/PHT>C in sessions $S_{1–2}$, PHT/SAL>PHT/PHT in S_1, and PHT/SAL>SAL/PHT in S_2. Panel b: Wrong circling velocity (V_W). Both main effects and the interaction [T×S] were significant by ANOVA. Differences among V_W means were significant [Tukey's test, $p<0.05$] as follows: for $S_{1–2}$, PHT/SAL and PHT/PHT>C, and PHT/SAL>SAL/PHT, for S_1, PHT/PHT>SAL/PHT.

significant [T: $F_{3,28}=10.8$, $p<0.0001$; S: $F_{2,56}=397$, $p<0.00001$; T×S: $F_{6,56}=5.6$, $p<0.001$]. The highest activity was observed in group PHT/SAL in which 2.1-, 2.1-, and 1.7-fold the V_R of the control group were measured in S_0, S_1, S_2, respectively. From S_1 to S_2, all PHT-treated groups showed increased circling velocities compared to control C ($p<0.05$).

Non-reinforced circling speeds (V_W) in CT#3 are shown in Fig. 4b. All main effects were significant [T: $F_{3,28}=8.8$, $p<0.0005$; S: $F_{2,56}=34.6$, $p<0.00001$; T×S: $F_{6,56}=7.9$, $p<0.00001$]. The control group showed a steep decrease in time spent in wrong circling (30% and 70% drop in V_W for $S_{0–1}$ and $S_{1–2}$, respectively). V_W for PHT/SAL and PHT/PHT groups remarkably increased from S_0 to S_1. V_W values approximated the level of S_0 in the subsequent S_2 in both these groups (compared to C, $p<0.05$).

3.2.2. Associative-spatial findings

3.2.2.1. Circling training at puberty. Error rates (E) in choosing the direction of circling are presented in Fig. 5. Panel A shows the results obtained in CT#1. Control C had a 58% drop in E from S_1 to S_3, and a 64% decrease between S_3 and S_7. All main effects were significant [T: $F_{3,28}=7.3$,

$p<0.001$, S: $F_{2,56}=89$, $p<0.00001$, T×S: $F_{6,56}=2.4$, $p<0.04$]. PHT/SAL and SAL/PHT groups had tendencies to make more errors than control C ($p<0.05$ in S_1).

3.2.2.2. Reversal circling training at adulthood. The error rates recorded in CT#3, when a new direction of circling was rewarded, are shown in Fig. 5b. The main effects of T [$F_{3,28}=3.5$] and S [$F_{1,28}=51$] were significant ($p<0.03$ and $p<0.00001$, respectively). PHT/SAL and SAL/PHT groups made more spatial errors than control ($p<0.05$). The spatial learning process during CT#3 was also examined by dividing V_R/V_W (i.e., SLR ratios). These results are shown in Fig. 6. The main effect of S was significant [$F_{2,56}=339$, $p<0.00001$]. The control group had a 31-fold improvement in SLR from S_0 to S_2. In addition, SAL/PHT, PHT/SAL and PHT/PHT groups demonstrated 17.8-, 7.4-, and 4.7-fold increase in SLR during the same testing period. Statistical significance was not observed for the effect of T [$F_{3,28}=2.1$, $p=0.12$]. However, the interaction T×S was significant [$F_{6,56}=8.7$, $p<0.00001$]. Prenatal PHT-treated groups showed a higher SLR than control in S_0 ($p<0.05$) followed by a lower SLR than control in S_2 ($p<0.05$). SLR patterns observed in PHT/SAL and SAL/PHT groups were statistically different in S_0 and S_2 ($p<0.05$). In addition, SLR patterns in PHT/SAL and PHT/PHT groups were statistically different in S_2 ($p<0.05$). Finally, the SAL/PHT group had a higher SLR than the PHT/PHT group in S_1 and S_2 ($p<0.05$).

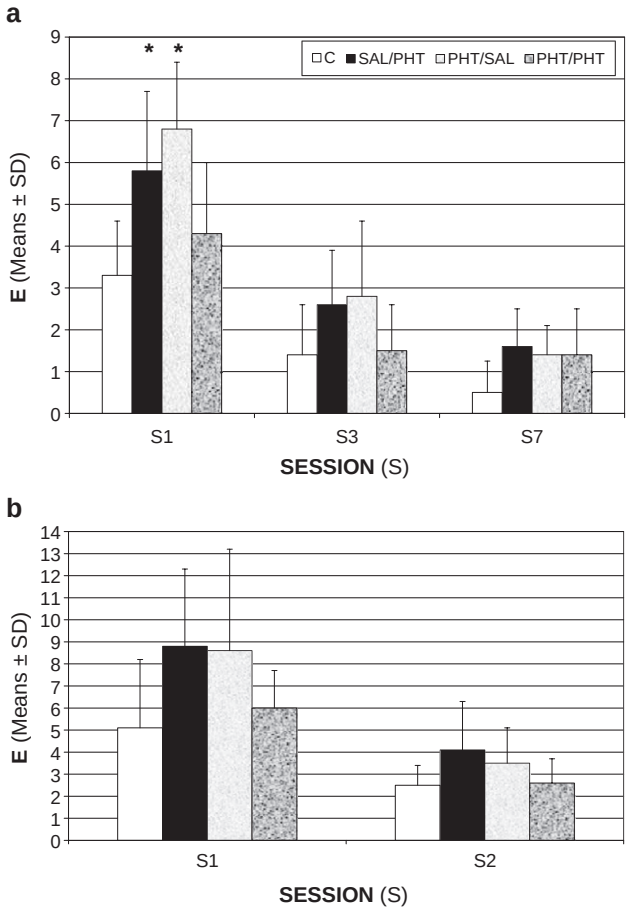

Fig. 5. Spatial performance in the Circling Training test. During the first five min of sessions $S_{1–3–7}$ (CT#1) or $S_{1–2}$ (CT#3) choice of circling direction was examined. Spatial errors are expressed as group means±SD. See bar color coding in panel a for group identification. Data was analyzed using an ANOVA followed by Tukey's test for multiple comparisons. Panel a: Errors in CT#1 (clockwise circling). Both main effects and the interaction were significant. *$p<0.05$ for groups SAL/PHT and PHT/SAL compared to control [S_1]. Panel b: Errors in CT#3 (anticlockwise circling). Both main effects were significant. $p<0.05$ for SAL/PHT and PHT/SAL groups compared to control.

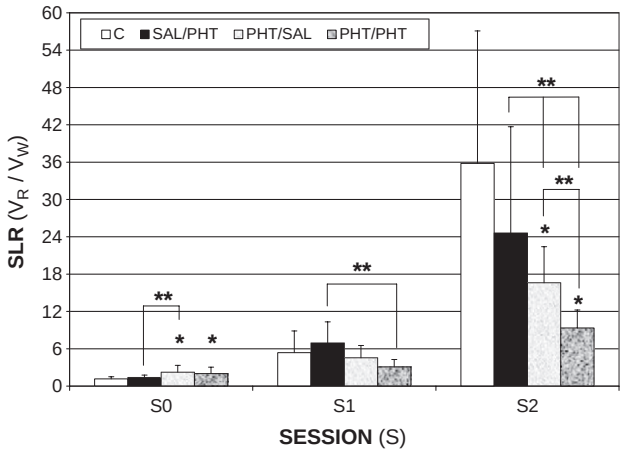

Fig. 6. Spatial learning progress in the Circling Training (CT#3). Spatial learning ratios (SLRs) obtained by dividing V_R by V_W. SLRs are expressed as group means±SD. The same statistical model was applied for data analysis as mentioned in Fig. 4. The main effect S and the interaction [T×S] were significant. (*) Indicates significant differences between PHT-treated groups and control C in the corresponding session. (**) Indicates significant differences between PHT-treated groups.

4. Discussion

In this work we were interested in addressing three questions concerning the behavioral toxicology of anticonvulsant drugs: (1) does the repertoire of PHT-induced developmental effects [48,49] translate into permanent functional disorders?, (2) during infancy, is there a critical period of vulnerability for the aforementioned long-term effects?, and (3) is the long-term behavioral damage attenuated or exacerbated if prenatal exposure to PHT is continued during early postnatal life?. Using the CT test, our findings are consistent with a permanent learning deficit previously reported in rats examined in the Cincinnati maze 17 months after prenatal exposure to PHT [51]. In addition, we found that: (i) hyperactivity was present in groups prenatally treated with PHT through all CT assessments, (ii) administration of PHT during infancy mildly worsens the motor activity alterations induced by prenatal exposure to the same drug, and (iii) the spatial behavior disorder seemed to be more profound after gestation-and-infancy

exposure than in case of an exclusive exposure during either period (see SLR patterns at PD150 in Fig. 6), although spatial error assessments did not support this interpretation.

Group SAL/PHT displayed motor and spatial learning alterations during CT#1, but was undistinguishable from controls in CT#2. We did not examine non-rewarded activity in CT#2. Therefore, it cannot be precluded that a higher total circling activity (e.g., V_R plus V_W) and increased error rate were present in this group during testing at PD80. Actually, in CT#3, a mild functional impairment was still apparent, 4 months after infancy-only exposure to PHT.

We counted errors by tracking the rat's movement about the apparatus during testing. This method enabled us to observe that some PHT-treated rats behaved in the following ways: (i) rats suddenly stopped licking the reward before finishing it and resumed speedy circling, (ii) disorientation, displayed as a spastic change in the spatial orientation of the head or body, and (iii) emotionality, i.e., emitting vocalizations while circling. The role of stress and emotionality on the manifestation of hydantoin-induced developmental neurotoxicity is an important issue that merits further investigation [12,26].

Adaptive stimuli and responses interact to control motion within the CT apparatus [5,52]. Since this circular maze paradigm is designed to reward circling in a particular direction, associative and spatial skills are simultaneously challenged in the context of reward-motivated locomotion. Procedural learning is largely responsible for successful execution of motor tasks during the acquisition phase of maze paradigms. Hippocampal and cerebellar processes have principal roles in modulating maze exploration activity and adjustment of movements while searching for the reward [17,32]. Cellular neurotoxicity and apoptotic neurodegeneration in these brain regions have been found underlying behavioral deficits in rodents treated with PHT during neonatal life [33] or the first month of life [3].

In rats, prenatal exposure to PHT induces hyperactivity, delays in air righting and startle reflex development, and defects in motor coordination [48,49]. The detection of the long-term effects of prenatal PHT on spatial learning has been shown to be dependent upon testing scheme and complexity of the spatial challenge [45,48]. Therefore, we included a reverse learning task in CT#3. This allowed us to evaluate of the permanency of spatial deficits in adulthood following the appearance of higher error rates at puberty in PHT-treated groups. The presence of motor and spatial alterations in prenatally exposed groups (i.e., PHT/SAL and PHT/PHT) is consistent with the teratogenic effects described by Vorhees [48,49] after oral doses of 200 mg/kg daily from GD7 to GD18. In this regard, results observed in the PHT/SAL group represent a positive control in our experimental design, and indicate that different routes of administration did not qualitatively modify the teratogenic effects of PHT as they pertain to behavior. Nevertheless, the lowest behaviorally teratogenic dose using an oral route has been estimated to be ~100 mg/kg in rats [48,49]. We administered nearly one third of this dose (i.e., 30 mg/kg) in the PHT/SAL group and detected neurobehavioral alterations in puberty, young adulthood, and adulthood. This suggests that for PHT the IP route is more potent than the oral route for inducing the behavioral syndrome following developmental exposure.

Other behavioral findings observed after prenatal PHT-exposure are a 20–45% treated offspring displaying spontaneous circling during adolescence and long-term, reference memory-based spatial learning deficits, even in asymptomatic (i.e., noncircling) offspring. This learning disorder is distinct from that observed in littermates exhibiting the circling impairment. In the latter group, impairment of spatial learning is much more profound [39,48]. This study did not discriminate between circlers and noncirclers before CT#1. Even so, we detected alterations in PHT-exposed rats regardless of circling direction rewarded during testing. It follows that results of CT testing would have been more profound if we had focused on spontaneous circling rats. In this work, low doses of PHT and a heterogeneous population (e.g., circlers and noncircler rats pooled) explains some increases in variability within groups and limited statistical consistency in some results.

Locomotor response can influence spatial performance in maze-like paradigms [24]. Particularly, alterations in circling behavior may influence the evaluation of other characteristic motor, learning, and memory disorders following a prenatal exposure to PHT [39,53]. As early as the third week of life, sensorimotor training leads to rapid and accurate performance patterns during a second training schedule following an intermediate retention interval, given that the same testing conditions are used. This has been observed in the rat during spatial navigation tasks [9], and circling training (unpublished work). Besides, it is known from preliminary work that early exposure to PHT induces hightened circling speed at puberty [55]. In order to isolate the spatial learning process from motor function, we computed a spatial learning ratio (i.e., SLR) in CT#3 by comparing reinforced and non-reinforced circling activity. The results in CT#3 suggest that adaptations produced after prenatal exposure to PHT and two CT experiences were not sufficient to attenuate the effects originally observed in motor behavior control and spatial learning in CT#1. Furthermore, SLR analysis suggests the additional induction of a mild learning deficit in rats exposed to PHT both gestationally and postnatally as compared to rats exposed exclusively in the gestational period. Note the control group had the lowest SLR in S_0, while groups treated with PHT prenatally had the highest. The opposite trend was

observed in S_2. Since this was accompanied by a significant interaction of treatment and session, SLR results would be consistent with a PHT-induced spatial learning impairment, not just an effect on hyperactivity during management of the spatial challenge.

Ultimately, we observed a higher error rate in groups treated with PHT in either gestation or infancy, but not in groups exposed during both periods. Phenytoin has a biphasic effect on benzodiazepine-like [^{3}H]diazepam binding in cortical membranes from developing rats. Gallager and Mallorga [15] have reported a reduction in [^{3}H]diazepam binding in rat pup brain tissue following prenatal exposure and a reverse effect (an increase in [^{3}H]diazepam binding) following postnatal exposure. Some benzodiazepines can impair spatial working memory without affecting motor activity or anxiety in the rat [20]. We have recently reported that gestational exposure to haloperidol produces long-term behavioral and neurochemical effects in young Sprague–Dawley rats that are contrary to the effects produced following lactational exposure to the same drug. These differential effects are neutralized when pups are exposed to haloperidol during both periods [56]. Further study is required to discern the relationship between stage-dependent modulation of cortical [^{3}H]diazepam binding and functional neuroteratology produced by PHT after exposure during and beyond gestation.

About 75% of the general population of epileptic patients experience seizures during childhood. In addition, 13.8–28.5% of children with infantile seizures (non-related to CNS infections) have a traceable family history of seizures, e.g., mothers treated with anticonvulsants during pregnancy [37]. This indicates that sequential exposure to hydantoins during both pre- and postnatal development may occur in some cases in clinical practice. In addition, larger doses of anticonvulsant drugs are given during infancy than in adulthood in order to achieve comparable results [11], and 8–15% of infants with neonatal seizures are subject to recurrent seizures later in life [28]. For many parents of treated infants, the most worrisome adverse action of PHT is its effect on various aspects of behavior [7]. About this concern, Bourgeois [4] has found that among the most common dose-related adverse effects associated to antiepileptic drug therapy in children are mental, behavioral, and motor coordination changes that may go unrecognized before adolescence. Furthermore, a multicenter study suggests that medication with PHT during childhood could be related to lowered performance in neuropsychologic tests that assess memory function, and that these effects are detected months after drug withdrawal [44].

Our results combined with existing evidence in children dictate a need for further research concerning the vulnerability of the brain to PHT during postnatal behavioral ontogenesis, particularly in the case of previous anticonvulsant exposure during prenatal development.

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