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Different effects of high- and low-dose phenobarbital on post-stroke seizure suppression and recovery in immature CD1 mice

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Abstract

Neonatal stroke presents with seizures that are usually treated with phenobarbital. We hypothesized that anticonvulsants would attenuate ischemic injury, but that the dose-dependent effects of standard anticonvulsants would impact important age-dependent and injury-dependent consequences. In this study, ischemia induced by unilateral carotid ligation in postnatal day 12 (P12) CD1 mice was immediately followed by an i.p. dose of vehicle, low-dose or high-dose phenobarbital. Severity of acute behavioral seizures was scored. 5-bromo-2'-deoxyuridine (BrdU) was administered from P18-P20, behavioral testing performed, and mice perfused at P40. Atrophy quantification and counts of BrdU/NeuN-labeled cells in the dentate gyrus were performed. Blood phenobarbital concentrations were measured. 30 mg/kg phenobarbital reduced acute seizures and chronic brain injury, and restored normal weight gain and exploratory behavior. By comparison, 60 mg/kg was a less efficacious anticonvulsant, was not neuroprotective, did not restore normal weight gain, and impaired behavioral and cognitive recovery. Hippocampal neurogenesis was not different between treatment groups. These results suggest a protective effect of lower-dose phenobarbital, but a lack of this effect at higher concentrations after stroke in P12 mice.

Keywords

phenobarbital; anticonvulsant; neuroprotection; neurogenesis; behavioral testing; dose-dependence

1. Introduction

Neonatal stroke occurs in approximately one in 4000 term births (Lynch et al., 2002), and about 75% of these have sequelae including cerebral palsy, epilepsy, learning and memory

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problems, and cognitive impairments (Koelfen et al., 1995; Delsing et al., 2001). Neonatal and pediatric stroke frequently presents with seizures, and the occurrence of seizures correlates with the severity of neurologic outcomes (Aden et al., 2002). Administration of anticonvulsants is a common treatment strategy in these cases; therefore evaluation of anticonvulsants as neuroprotective agents has been an area of active research (Trojnar et al., 2002; Calabresi et al., 2003; Leker and Neufeld, 2003; Liu et al., 2004; Traa et al., 2008).

The developing brain differs markedly from the mature brain, requiring a different balance of excitation and inhibition as it forms synapses and neuronal networks. GABAergic synapses, which serve an inhibitory function in the mature brain, are initially excitatory in the immature brain, driving formation of glutamatergic synapses and neural networks (Ben-Ari, 2002). With nascent networks and evolving synaptic connections and ionic profiles, interference with excitation or inhibition through interactions with ion channels, GABA_A receptors, or glutamate receptors could have negative developmental consequences. Many first-line anticonvulsants act on either GABA_A or glutamate receptors, inhibit sodium or calcium channels, or have several of these mechanisms (Trojnar et al., 2002; Leker and Neufeld, 2003; Czapinski et al., 2005). Phenobarbital, the drug of choice at present, potentiates GABA_A receptors, as well as being implicated in inhibition of glutamate-mediated excitation and acting to block sodium channels (Trojnar et al., 2002; Leker and Neufeld, 2003; Czapinski et al., 2005). Phenobarbital's effects on the immature brain, when combined with the effects of neonatal stroke and ischemic seizures, may disrupt ongoing development in the immature brain, resulting in an elevated possibility of unwanted side-effects. Therefore, it is necessary to understand its impact in relevant immature animal models of ischemic brain injury.

Recent reports (Bittigau et al., 2003; Stefovaska et al., 2008) have demonstrated detrimental effects after anticonvulsant administration in the pre-natal and neonatal (postnatal day 0 – postnatal day 14) rodent during the developmental window corresponding to the brain growth spurt (Dobbing and Sands, 1973). At anticonvulsant concentrations, drastically increased apoptosis has been reported and cell proliferation decreased throughout the immature brain after administration of several AEDs including phenobarbital (Bittigau et al., 2003; Glier et al., 2004; Manthey et al., 2005; Schubert et al., 2005; Kim et al., 2007; Stefovaska et al., 2008). Neurotrophins and survival-promoting signaling pathways have been shown to be downregulated (Bittigau et al., 2003; Hansen et al., 2004; Shi et al., 2010). Behaviors have also been affected: phenobarbital specifically has been shown to induce behavioral deficits several months later after 4 doses in the neonatal period (Stefovaska et al., 2008). These effects are dose-dependent, with threshold doses above which detrimental effects have been elicited (Bittigau et al., 2003; Hansen et al., 2004; Shi et al., 2010).

Concerns, therefore, regarding the anticonvulsant's effectiveness, safety, and ability to improve outcome, prompted us to study phenobarbital in the postnatal day 12 (P12) CD1 mouse model of neonatal stroke. This model has been demonstrated to produce acute behavioral seizures, functional deficits, impaired neurogenesis and brain injury (Comi et al., 2004; Kadam et al., 2008; Kadam et al., 2009a; Kadam et al., 2009b). We report here the impact of a single acute dose of phenobarbital (30 mg/kg or 60 mg/kg) upon these endpoints after stroke in the immature brain.

2. Methods

All materials and methods were approved by the Johns Hopkins University Animal Care and Use Committee. Litters of CD1 mice were purchased from Charles River Laboratories Inc. (Wilmington, MA). Pups were housed in polycarbonate cages with the dam on a 12 hour light:dark cycle; food was provided *ad libitum*. Phenobarbital (PB; Sigma-Aldrich Co., St.

Louis, MO, U.S.A.) was reconstituted to 25 mg/ml in sterile dH₂O. Pups from 14 litters were randomly assigned to treatment groups after ligation (refer to Table 1 for details). For serum concentration studies, a total of 4 litters were used. Pups were weighed at P12, P18, P19, P33, and P40. Researchers were blinded to treatment groups.

2.1. Surgery

On the morning of P12, animals underwent permanent unilateral double ligation of the carotid artery as previously published (Kadam et al., 2009b). Immediately thereafter, rectal temperatures were taken, then a single intraperitoneal dose (30 mg/kg or 60 mg/kg PB or 0.9% NaCl) was administered and the pups placed into a 37°C incubator for observation and seizure scoring. Rectal temperatures were also taken 2 and 4 hours post-ligation.

2.2. Acute Seizure Scoring

Seizure activity was scored according to a seizure rating scale as previously reported (Comi et al., 2004; Kadam et al., 2009b). After 4 hours, the mice were returned to the dam and each of their seizure scores was individually summed to produce a total seizure score.

2.3. 5-Bromo-2'-deoxyuridine Administration

From P18-P20, mice received 5 intraperitoneal injections of 50 mg/kg 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog, reconstituted in 0.9% NaCl.

2.4. Behavioral Tests

All behavioral tests were conducted between P32-P37.

2.4.1. T-maze spontaneous alternation—This procedure was carried out in an enclosed “T” shaped maze for a total of 15 trials as described previously (Kadam et al., 2009b). If the mouse did not make a choice within 2 minutes the trial was ended and advanced to the next. At the conclusion of each trial the maze was cleaned of urine and feces. During analysis of this data, one stroke-injured mouse treated with vehicle was excluded because it would not perform the task, i.e., in each of the 15 trials, the mouse did not enter either of the goal arms of the maze within the allotted 2 minute period, thereby not completing any of the trials.

2.4.2. Open field habituation—Testing was carried out in a square open field chamber as previously described to examine activity levels on two consecutive days and to analyze within- and between-session habituation (Kadam et al., 2009b). Data on number of rears and time spent rearing was analyzed for the first 15 minutes of the first session to examine this behavior independent of habituation.

2.4.3. Novel object testing—The first 2 days the mice were allowed to habituate to the square field using the open field procedures described above in section 2.4.2. On the third day, in the sample phase, two identical objects were placed on opposite sides of the arena and equidistant from the walls. Mice were placed in the center of the open field and allowed 10 minutes to explore. Each mouse was then returned to the home cage for a 3 minute inter-phase interval during which one of the objects was replaced with a non-identical object; the mice were then returned to the open-field and given 10 minutes to explore (test phase). The time each mouse interacted with each object was recorded: *Time exploring novel object / (Time exploring novel object + Time exploring familiar object)* quantified object preference.

2.5. Perfusion

On P40, mice were anesthetized with 90 mg/kg chloral hydrate, perfused transcardially with PBS followed by phosphate buffered 10% formalin, and their brains post-fixed in the same fixative, cryoprotected, and snap-frozen and stored at -80°C .

2.6. Immunohistochemistry

A total of 66 brains ($n/n=66/80$) from 8 litters (litters 7 to 14) were stained for BrdU and Neuronal Nuclei (NeuN) ($n=13$ per group for ligation-injured; 7–12 per group for ligation uninjured). Antigen retrieval was performed with 10mM sodium citrate, pH 6.0, with 0.5% Triton-X 100. BrdU (Roche, 1170376) and NeuN (Chemicon, MAB377) IHC and quantification was done as reported previously (Kadam et al., 2008). Slides were examined with an Olympus (Fluoview) confocal microscope using the FV 1000 confocal system based on an Olympus IX81 inverted microscope stand. Counts of BrdU-positive and BrdU/NeuN co-labeled cells were performed on serial coronal sections (atlas coordinates bregma -1.34mm to bregma -2.06mm) (Paxinos and Franklin, 2001).

2.7. Histology and Atrophy Measurement

Using MCID 7.0 Elite (InterFocus Imaging Ltd., Cambridge, UK), hemispheric and hippocampal areas of $40\mu\text{m}$ -thick, Nissl-stained serial coronal sections equally spaced and spanning rostral striatum to caudal hippocampus were measured ($n = 15$ sections per animal) as described previously (Kadam et al., 2009b). Hippocampal and hemispheric atrophy was calculated for each section as $(1 - (\text{area injured side} / \text{area uninjured side})) \times 100\%$. Presence of stroke-injury was determined by microscopic inspection at 4X and 10X for presence or absence of infarct, atrophic malformed structures, gliosis, or pyramidal cell loss in the hippocampus. Dentate gyri were outlined for sections for which counts of BrdU-positive and NeuN-positive cells were performed, and density of BrdU-positive cells in the dentate gyrus (DG) was calculated for each section as $(\text{Number of BrdU-positive cells in the DG} / \text{DG area in mm}^2)$.

2.8. Serum Concentrations- drug administration, sample collection, and analysis

A separate cohort of P12 CD1 mice were injected intraperitoneally with either 30 mg/kg or 60 mg/kg phenobarbital, and samples collected and analyzed as previously published (Markowitz et al., 2010). Blood volumes from two mice were pooled to make each sample (Table 2).

2.9. Analysis

SPSS for Windows (SPSS Inc., Chicago, IL) was used to perform statistical analyses. One-way ANOVAs with post-hoc tests (Bonferroni's multiple-comparisons test) were used to analyze means between treatment groups. Independent sample Student's t-tests were used to analyze means between injured and uninjured animals within treatment groups, and paired sample Student's t-tests to analyze means between repeated measures within specific injury and treatment groups. Mann-Whitney U tests were used to compare seizure grades and seizure scores between treatment groups. Chi-square tests with Fisher's exact test were used to compare seizure occurrence and prevalence of only microscopic injury between treatment groups. Spearman's rho was used for all correlations. $p < 0.05$ was used as the threshold for statistical significance.

3. Results

For each treatment group, ligated mice that fell into either injured or uninjured classifications are described in Table 1. There were no significant differences in mortality

between groups, percent injured, or type of injury (i.e. macroscopic or microscopic), nor were there significant differences in these parameters based on sex. Rectal temperatures showed no significant differences at any timepoint taken.

3.1. Weights

Significant differences in weights between injured and uninjured mice were found at P18, P19, P33, and P40 in both the vehicle ($p=0.004$, 0.003 , 0.015 , and 0.027 respectively) and 60 mg/kg PB groups ($p=0.001$, <0.001 , 0.002 , and 0.011 respectively), while there were no differences in weights at these ages between injured and uninjured mice in the 30 mg/kg PB-treated cohort ($p=0.152$, 0.199 , 0.579 , and 0.135 respectively) (Figure 1). In the ligation-injured group of mice, there was an effect of drug dose on weight at P18 and P19 ($p=0.044$ and 0.023 respectively), driven by the 30 mg/kg PB group weighing more (i.e. similar in weight to their uninjured counterparts) than the 60 mg/kg PB group ($p=0.039$ at P18, $p=0.019$ at P19).

3.2. Acute seizures

Both 30 mg/kg PB (median seizure score=0, range 0–20, $p=0.002$) and 60 mg/kg PB (median seizure score=7.5, range 0–15, $p=0.034$) reduced total seizure activity compared to vehicle (median seizure score=16, range 0–75) (Figure 2). There were also fewer behavioral seizures in the 30 mg/kg PB cohort compared to the 60 mg/kg PB group ($p=0.014$). 30 mg/kg PB and 60 mg/kg PB doses both increased the number of seizure-free epochs ($p=0.002$ and $p=0.041$ respectively); additionally 30 mg/kg PB resulted in more seizure-free epochs than did 60 mg/kg PB ($p=0.014$). The latent period to onset of seizures (i.e., time after ligation until the 1st behavioral seizure was detected) showed an effect of drug dose ($p=0.050$), driven by the increased latency in the 30 mg/kg PB-treated group vs. both vehicle and 60 mg/kg PB-treated injured animals ($p=0.092$ and $p=0.126$ respectively). The 30 mg/kg PB dose had reduced occurrence of grade 3, 5, and 6 seizures compared to vehicle, while 60 mg/kg PB reduced the occurrence of grade 5 and 6 seizures vs. vehicle ($p=0.003$, $p=0.006$, and $p=0.015$ respectively for 30 mg/kg PB; $p=0.014$ and $p=0.025$ respectively for 60 mg/kg PB) (Figure 3). Counts of grade 3 seizures were reduced but not significantly in 60 mg/kg PB vs. vehicle ($p=0.073$); in contrast, there was a significant reduction of grade 3 seizures between 30 mg/kg PB and 60 mg/kg PB ($p=0.035$). Both 30 mg/kg and 60 mg/kg PB completely blocked the occurrence of grade 6 seizures. Comparing seizure occurrence between treatment groups in injured mice revealed a reduction in seizure occurrence in the 30 mg/kg PB group ($n/n=8/24$ with seizures, 33.3%) compared to both the vehicle ($n/n=14/22$ with seizures, 63.6%; 1-sided $p=0.039$) and 60 mg/kg PB groups ($n/n=14/20$ with seizures, 70.0%; 2-sided $p=0.033$).

3.3. Blood levels at P12

Serum concentrations and half-lives for each dose of drug are reported in Table 2. Concentrations maintained therapeutic levels for extended periods for both doses of phenobarbital (Loscher, 2007), and while the 60 mg/kg dose approached levels that could theoretically induce respiratory depression (Yaffe and Aranda, 2005), no significant differences in respiratory rate were observed.

3.4. Injury and Atrophy

Injured mice in each treatment group displayed atrophy ranging from microscopic cell loss to massive infarcts in the ipsilateral hemisphere and hippocampus. Atrophy in the ipsilateral hemisphere ranged from microscopic injury to 100% tissue loss for vehicle-treated mice, from microscopic injury to 55% tissue loss for 30 mg/kg PB-treated mice, and from microscopic to 91% tissue loss for 60 mg/kg PB-treated mice. Atrophy in the ipsilateral

hippocampus ranged from microscopic injury to 100% tissue loss for vehicle-treated mice, from microscopic injury to 85% tissue loss for 30 mg/kg PB-treated mice, and from microscopic injury to 99% tissue loss for 60 mg/kg PB-treated mice. Comparing prevalence of microscopic vs. macroscopic injury between the 3 treatment groups revealed no differences. Within injured mice, both hemispheric and hippocampal atrophy showed an effect of drug dose ($p<0.001$ and $p=0.037$ respectively); 60 mg/kg PB did not affect either hemispheric or hippocampal atrophy, while 30 mg/kg PB reduced hemispheric atrophy compared to both vehicle and 60 mg/kg PB ($p=0.002$ vs. vehicle, $p<0.001$ vs. 60 mg/kg PB) (Figure 4). 30 mg/kg PB additionally reduced hippocampal atrophy compared to the other groups, but significantly only compared to 60 mg/kg PB ($p=0.272$ vs. vehicle, $p=0.037$ vs. 60 mg/kg PB). In injured mice, ipsilateral DG areas for sections in which counts of newborn cells were performed were 0.072 ± 0.053 mm² for vehicle-treated, 0.085 ± 0.064 mm² for 30 mg/kg PB-treated, and 0.037 ± 0.045 mm² for 60 mg/kg PB-treated mice, and not significantly different ($p=0.109$). There was no effect of drug on contralateral hemispheric, hippocampal, and DG areas ($p=0.662$, $p=0.779$, and $p=0.571$ respectively). Significant correlations between seizure score and hemispheric atrophy were found in all injured groups ($\rho=0.785$, 0.706 , and 0.536 ; $p<0.001$, $p<0.001$, and $p=0.015$ for vehicle, 30 mg/kg PB, and 60 mg/kg PB respectively). Correlations also existed between seizure score and hippocampal atrophy for vehicle and 30 mg/kg PB, but significance was lost after 60 mg/kg PB ($\rho=0.806$, 0.649 , and 0.436 ; $p<0.001$, $p=0.001$, and $p=0.054$ respectively).

3.5. Neurogenesis

Differences in newborn cells (i.e. dividing cells labeled with BrdU from P18-P20) were found between injured and uninjured mice in each treatment group on the ipsilateral side ($p=0.001$, $p=0.002$, and $p<0.001$ for vehicle, 30 mg/kg PB, and 60 mg/kg PB respectively), and between ipsi- and contralateral DG in the injured animals in each group ($p=0.008$, $p<0.001$, and $p<0.001$ for vehicle, 30 mg/kg PB, and 60 mg/kg PB respectively), with reduced counts in injured mice compared with their uninjured counterparts. No differences were found on the contralateral side due to injury ($p=0.712$, $p=0.141$, and $p=0.641$ respectively) or due to treatment either ipsi- or contralaterally ($p=0.335$ and $p=0.568$ for uninjured mice and $p=0.131$ and $p=0.420$ for injured mice ipsi- and contralaterally). Differences in counts in the ipsilateral DG between injured and uninjured mice were likely due to the lost tissue in the DG, as there were no differences in densities of newborn cells per unit area found between injured and uninjured mice in each treatment group either ipsi- ($p=0.284$, $p=0.892$, and $p=0.397$ for vehicle, 30 mg/kg PB, and 60 mg/kg PB respectively) or contralaterally ($p=0.338$, $p=0.991$, and $p=0.346$ for vehicle, 30 mg/kg PB, and 60 mg/kg PB respectively). There was also no significant difference in densities of BrdU-positive cells between ipsi- and contralateral DG in any treatment cohort, regardless of injury status. There were no differences in densities of BrdU-positive cells per unit area in uninjured groups due to treatment in either the ipsi- or contralateral DG ($p=0.184$ and $p=0.230$, ipsi- and contralateral respectively). Finally, there was no effect of treatment on densities of BrdU-positive cells per unit area in injured groups in either the ipsi- or contralateral DG ($p=0.926$ and $p=0.448$, ipsi- and contralateral respectively) (Supplemental Figure 1). No significant differences in neuronal commitment indicated by BrdU/NeuN co-labeling were found between treatment groups: percentages of newborn cells committing to the neuronal fate were strong in the DG, similar to previously published data both in rats (Porter et al., 2004) and in mice (Kadam et al., 2008), and were close to 90% in this study regardless of injury or treatment status.

Neurogenesis on the injured side correlated strongly to seizures, atrophy, and ipsilateral DG area. Ipsilateral BrdU-positive counts had negative correlations with seizure score across treatments ($\rho=-0.735$, -0.580 , and -0.733 ; $p=0.004$, $p=0.038$, and $p=0.004$ for vehicle, 30

mg/kg PB, and 60 mg/kg PB respectively) and with hippocampal atrophy ($\rho = -0.796$, -0.907 , and -0.833 ; $p = 0.001$, $p < 0.001$, and $p < 0.001$ for the same). There was a negative correlation between ipsilateral BrdU-positive counts and hemispheric atrophy that was significant in both 30 mg/kg PB- and 60 mg/kg PB-treated mice ($\rho = -0.531$, -0.802 , and -0.563 ; $p = 0.062$, $p = 0.001$, and $p = 0.045$ for vehicle, 30 mg/kg PB, and 60 mg/kg PB respectively). Finally, ipsilateral counts correlated with DG area in all treatments ($\rho = 0.945$, 0.830 , and 0.993 ; $p < 0.001$, $p = 0.003$, and $p < 0.001$ for vehicle, 30 mg/kg PB, and 60 mg/kg PB-treated, respectively), showing a uniformity to the neurogenesis in the surviving neurogenic niche.

3.6. Behavioral Testing

Exploratory behavior was assessed through quantification of rearing activity in the open-field test (Figure 5); in order to examine this data independent of habituation, rearing activity was analyzed on day 1 only. No drug-dependent effects on either number of rears or time spent rearing were noted in the uninjured group. There was a main effect of injury on the number of rears ($F_{1,105} = 11.239$, $p = 0.001$) due to injured mice rearing less than uninjured. There was also a significant Treatment X Injury interaction for the number of rears ($F_{2,105} = 3.276$, $p = 0.042$). Follow up simple comparisons of this data via t-tests revealed that there were significant differences in number of rears between injured and uninjured mice treated with 60 mg/kg PB, with injured mice rearing less ($p = 0.001$). Injured mice treated with 60 mg/kg PB also demonstrated significant rearing depression ($p < 0.03$) compared to all other groups except uninjured mice treated with 30 mg/kg PB ($p = 0.058$) and injured mice treated with vehicle ($p = 0.302$). Number of rears were also depressed in vehicle-treated injured vs. uninjured mice, however these differences were less pronounced ($p = 0.031$). Vehicle-treated injured mice were also significantly depressed compared to 60 mg/kg PB-treated uninjured mice ($p = 0.048$). No difference in number of rears was noted between injured and uninjured mice treated with 30 mg/kg PB.

Time spent rearing (in seconds) was also examined on day 1, and similar to the rearing count data there was a main effect of Injury ($F_{1,105} = 16.983$, $p < 0.001$) due to injured mice rearing less than uninjured, and there was also a significant Treatment X Injury interaction ($F_{2,105} = 4.157$, $p = 0.018$). Simple comparisons of this data via t-tests revealed that there were significant differences in time spent rearing between injured and uninjured mice treated with 60 mg/kg PB, with injured mice rearing less ($p < 0.001$). Injured mice treated with 60 mg/kg PB also spent significantly less time rearing ($p < 0.01$) compared to every other group except vehicle-treated injured mice ($p = 0.259$). Time spent rearing was also diminished in vehicle-treated injured vs. uninjured mice ($p = 0.028$), with injured mice rearing less. Vehicle-treated injured mice also reared for significantly less time than 60 mg/kg PB-treated uninjured mice ($p = 0.016$). Again, no differences were detected due to injury in the 30 mg/kg PB group in rearing activity.

Memory was evaluated through examination of habituation between-sessions (i.e., comparing immediate activity on day 1 with immediate activity on day 2) in the open-field. This was accomplished through evaluation of the distance traveled in the first five minutes on each day of the open field task (Supplemental Figure 2). There were no significant effects, only marginal interactions of Treatment X Injury ($F_{2,105} = 2.766$, $p = 0.068$), Day X Treatment ($F_{2,105} = 2.724$, $p = 0.070$), and Day X Injury ($F_{1,105} = 3.672$, $p = 0.058$). Therefore, between-session habituation (a hippocampal-dependent memory activity) was not significant in this study. However, given the work of Deacon et al., File and Wilks, and Stefovská et al. (see section 4.3), the significant atrophy and depressed rearing activity in the vehicle- and 60 mg/kg PB-treated injured groups compared to the 30 mg/kg PB-treated injured mice, and the marginal Day X Treatment, Day X Injury, and Treatment X Injury interactions, we decided to examine the between-session habituation more closely to see if our data was similar to the

published data. It was found that there was only a main effect of Day for the 30 mg/kg PB-treated group ($F_{1,37}=11.156$, $p=0.002$), indicating that the mice treated with 30 mg/kg PB habituated significantly while the other groups did not.

Evaluation of learning in the open field through examination of within session habituation revealed no main effects of condition or injury demonstrating that all mice habituated over the 30 minute testing period on both days and indicating that all groups learned about the environment during the session (Supplemental Figure 3).

As a measure of anxiety levels, there were no differences in the amount of time groups of mice spent in the center of the field on either day 1 or day 2.

Hyperactivity, a co-morbidity that emerges at an older age in this model (Kadam et al., 2010), had not fully emerged at the time of testing, however one mouse each in both the vehicle and the 60 mg/kg PB-treated groups displayed a hyperactive phenotype (total distance greater than three standard deviations from mean). Three mice in each of these groups, compared to one mouse in the 30 mg/kg PB cohort, also demonstrated a possibility to develop this phenotype, indicated by a greater total distance traveled on both days (more than 10,000 cm in a thirtyminute session).

In the T-maze task there was an effect of Injury ($F_{1,104}=6.873$, $p=0.010$) on alternation rate, due to injured animals alternating less. However, there was no effect of treatment, nor was there an interaction. For the novel object test there were no effects of treatment or injury, nor an interaction between the two.

When behavioral testing was correlated to injury, time spent rearing displayed the strongest relationship to injury severity. Time spent rearing on the first day of open field testing correlated negatively with hippocampal and hemispheric atrophy in both vehicle and 60 mg/kg PB-treated mice ($\rho=-0.498$ and -0.526 ; $p=0.018$ and $p=0.017$, hippocampal; $\rho=-0.505$ and -0.463 ; $p=0.016$ and $p=0.040$, hemispheric). A trend was seen when time rearing on day one was correlated with hemispheric atrophy in the 30 mg/kg PB cohort ($\rho=-0.401$, $p=0.052$); a similar correlation was not noted with hippocampal atrophy in this treatment group. The open-field test was the most sensitive test to injury, demonstrating inhibition of rearing in the injured animals that was mitigated by acute 30 mg/kg PB administration.

4. Discussion

This study revealed the following salient findings– 1: acute seizure scores were lowered after phenobarbital administration, however the lower dosage was more effective as an anticonvulsant than the higher; 2: stroke-related hemispheric and hippocampal atrophy were attenuated after acute administration of the lower, but not the higher, dose of phenobarbital; 3: density of post-stroke neurogenesis in the surviving dentate gyri was not affected by a single dose of phenobarbital given immediately after the ligation-surgery; and 4: impaired weight gain and behavioral co-morbidities related to the stroke were mitigated after acute administration of the lower, but not the higher, dose of phenobarbital.

4.1. Acute-seizure and injury severity

This study demonstrated that 30 mg/kg PB was significantly more effective than 60 mg/kg, in terms of reducing behavioral seizure occurrence and total seizure score. Severity of post-ischemic seizures is considered a good indicator for severity of outcome after a neonatal stroke (Aden et al., 2002). 60 mg/kg PB attenuated seizure activity compared to vehicle (but not as well as the lower dose in this study), however the severity of stroke-injury was similar

to the vehicle-treated groups. Additionally, the loss of correlation between seizures and hippocampal brain injury at the higher dose suggests that anticonvulsant properties of phenobarbital are not the only aspects of the drug relevant to the post-stroke outcome, especially within the pediatric population. Possible other mechanisms for loss of the beneficial effects of phenobarbital demonstrated by the lower dosage may include, but are not limited to, a dysregulation of oxidative stress and apoptosis (Promyslov and Demchuk, 1995; Bittigau et al., 2003; Kaindl et al., 2008).

In the developing brain, phenobarbital has been shown to have dose-dependent effects. Bittigau et al. (Bittigau et al., 2003) demonstrated that a threshold single dose of 40 mg/kg PB was necessary to trigger widespread apoptosis in neonatal rats. This corresponded to plasma concentrations between 25–35 µg/ml for 12 hours; a level produced by the 60 mg/kg but not the 30 mg/kg dose in this study. Additional studies are needed at acute and subacute timepoints to investigate this issue in the CD1 mouse model.

Kaindl et al. (Kaindl et al., 2008) characterized alterations in cerebral cortex proteins at P7, P14, and P35 after two 30 mg/kg PB injections in mice on P6. Dysfunction of proteins involved in oxidative stress and apoptosis was seen. This corroborates a study (Aycicek and Iscan, 2007) which demonstrated an imbalance in serum oxidant/antioxidant concentrations in PB-treated children with epilepsy, which may be associated with side effects of high-dose PB. Lipid peroxide levels quantified one day post-trauma in a head trauma model in rabbits, were not significantly different after acute low-dose PB, demonstrating a lack of effective antioxidant activity of phenobarbital (Promyslov and Demchuk, 1995). Therefore, it is unlikely that the neuroprotective effects seen with the 30 mg/kg dose are due to antioxidant activity. Further studies are needed to investigate the potential mechanisms of this neuroprotection, which in addition to seizure reduction may include a decreased metabolic demand and shunting depolarizing conductances (Staley, 1992).

The use of hypothermia as an adjunct therapy to anticonvulsants is being investigated, and Barks et al. (Barks et al., 2010) recently utilized this therapeutic approach with 40 mg/kg phenobarbital in P7 rats. Improved sensorimotor performance, lower neuropathology scores, and less hemispheric damage were reported with the combination therapy. Hypothermia as a therapeutic option has many diverse effects on metabolism, excitotoxicity, oxidative stress, apoptosis, and inflammation (Gupta et al., 2005; Hemmen and Lyden, 2009; Tang et al., 2009). If the restorative effects of phenobarbital are being negated at higher dosages due to its actions affecting oxidative stress and apoptosis, then hypothermia as an adjunct therapy could help to alleviate these negative consequences, restoring a beneficial effect.

4.2. Post-stroke neurogenesis

Neither neurogenesis nor commitment to the neuronal fate was affected within these treatment regimens. Our data differs from recent publications which demonstrate a depression of neurogenesis in immature rodents after higher doses of phenobarbital. Stefovaska et al. (Stefovaska et al., 2008) reported a decrease in neurogenesis in the subgranular zone after 3 doses of phenobarbital in the neonatal rat. Chen et al. (Chen et al., 2009) corroborated these decreases in hippocampal neurogenesis after chronic administration to neonatal rats, and also reported a conservation of the relative proportions of newborn neuronal and glial cell types. It is possible that these are model-dependent differences: the experiments cited previously were performed in naïve rodents, while our ischemia model has already demonstrated suppressed neurogenesis in the DG after the stroke event (Kadam et al., 2008; Kadam et al., 2009a). It is also possible that a single dose is not enough to induce these changes in neurogenesis in the period investigated in this study.

4.3. Impaired weight gain and behavioral co-morbidities

An alleviation of indicators of functional deficits occurred after administration of the lower but not the higher dose of phenobarbital. The restoration of normal weight gain demonstrated in the 30 mg/kg PB-treated injured mice, as opposed to the impairment in vehicle- and 60 mg/kg PB-treated injured mice, supports overall improvement in animal function necessary to achieve normal feeding and growth.

Behavioral testing, designed to measure post-stroke effect on cognitive ability, demonstrated a pattern of dose response similar to that seen with injury and weight gain profiles. Rearing (an exploratory behavior) was affected by the stroke-injury: vehicle-treated injured mice reared less than their uninjured counterparts. 30 mg/kg PB-treated injured mice exhibited restoration of normal rearing activity, while 60 mg/kg PB-treated injured mice had more depressed rearing activity. Rearing was not, however, affected in uninjured mice after any treatment. While the habituation data reported here was not significant overall, closer examination of the data demonstrates a result similar to the rearing data. Between-session habituation was affected by the stroke-injury: vehicle-treated injured mice did not habituate between sessions in this study, and 60 mg/kg PB treatment had no effect on this consequent elimination of habituation. The 30 mg/kg PB-treated injured mice, however, displayed a similar pattern of activity to the uninjured controls, and together with their uninjured counterparts demonstrated restoration of habituation. Possible attenuation of these deficits in the 30 mg/kg PB group could be attributed to the relative sparing of the hippocampus.

Hippocampal lesions in mice have been shown to produce deficits in exploratory behavior and memory. Deacon, Croucher, and Rollins measured behavioral outcomes in mice with hippocampal lesions (Deacon et al., 2002). Lesioned mice, in an open-field test, displayed significantly less rearing than controls on both days of the trial indicating impaired exploration in that test. Our lab previously demonstrated a deficit in between-session habituation during an open field task in stroke-injured mice, demonstrating a deficit in hippocampal-dependent learning and memory (Kadam et al., 2009b). These stereotypical behaviors after hippocampal lesion were reproduced in the vehicle- and 60 mg/kg PB-treated injured mice. The 30 mg/kg PB-treated injured group however displayed behaviors similar to the uninjured mice, consistent with the decreased hippocampal injury and subsequent improved hippocampal function in the 30 mg/kg group reported here.

Naïve rodents treated with higher dosages of phenobarbital have demonstrated deficits in hippocampal-dependent tasks. File and Wilks examined acute effects of phenobarbital on locomotor activity and exploration in naïve adult mice, and found depression of exploratory head-dipping due to higher but not lower doses (File and Wilks, 1990). Stefovská et al. published an impairment of learning and memory in a water maze task with mature rats treated neonatally with 50 mg/kg PB, with fewer correct findings of the submerged platform on days two and three of the three-day test, and longer latencies to find the submerged platform compared with controls (Stefovská et al., 2008). The lower number of correct findings over subsequent sessions in the water maze task is comparable to the lack of between-session habituation, showing a deficit in hippocampal-dependent memory. While our chronic behavior data does not demonstrate significant impairment of hippocampal function after higher dosages of phenobarbital, it is possible that this discrepancy is due to a single dose not being sufficient to induce these impairments, and/or that the stroke-injury in this model, which already induces a depression in hippocampal function (Kadam et al., 2009b), may overshadow a phenobarbital dose-dependent depression.

4.4. Conclusions

These results demonstrate a mitigation of stroke-injury in both brain atrophy and functional outcome as a result of the 30 mg/kg PB dose, but a loss of this effect with the single 60 mg/kg PB dose. Taking into consideration the seizure reduction after both doses of drug, it is probable that other mechanisms induced by the higher dosage of drug are responsible for the loss of the beneficial effects of anticonvulsant-induced seizure suppression. Significant benefits are witnessed following treatment with the lower dose of drug; therefore, studies are necessary to elucidate the mechanisms eliciting these effects and to determine strategies to maximize these benefits in a translational setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Weights of injured and uninjured mice from time of ligation to time of perfusion

Line graphs of weights of injured and uninjured mice taken on P12, P18, P19, P33, and P40. Weights of injured and uninjured mice administered (A) vehicle, (B) 30 mg/kg PB, or (C) 60 mg/kg PB are compared, with significant differences on P18, P19, P33, and P40 in both the vehicle- and the 60 mg/kg PB-treated mice.

Figure 2. Acute post-stroke seizure suppression following a single i.p. dose of phenobarbital
 Box plot of seizure scores from injured mice administered vehicle, 30 mg/kg PB, or 60 mg/kg PB immediately after ligation. Median seizure scores (dark lines) and interquartile ranges (boxes) are depicted. Seizures were significantly by both 30 mg/kg PB and 60 mg/kg PB compared to vehicle. However, 30 mg/kg PB was significantly more effective in suppressing seizures than 60 mg/kg PB.

Figure 3. Suppression of seizures by severity after acute i.p. phenobarbital administration

Total epochs with each grade of seizure comparing (A) vehicle and 30 mg/kg PB-treated or (B) vehicle and 60 mg/kg PB-treated groups. 30 mg/kg PB significantly reduced the occurrence of grade 3, 5, and 6 seizures compared to vehicle, while 60 mg/kg PB significantly reduced the occurrence of grade 5 and 6 seizures. Both drug doses completely blocked the occurrence of grade 6, severe tonic-clonic, seizures. Reduction of grade 3 seizures in the 30 mg/kg PB group was significantly higher than in the 60 mg/kg PB cohort.

Figure 4. Histology and post-stroke hemispheric and hippocampal atrophy quantification
CV-stained coronal sections representing median injury severity in (A) vehicle-treated, (B) 30 mg/kg PB-treated, and (C) 60 mg/kg PB-treated injured brains, from anterior to posterior coordinates. Scale bar = 1mm. (D) Stroke-related hemispheric and hippocampal atrophy in vehicle, 30 mg/kg PB, and 60 mg/kg PB-treated mice. 30 mg/kg PB significantly reduced hemispheric atrophy compared to both vehicle and 60 mg/kg PB-treated cohorts. 30 mg/kg PB also significantly reduced hippocampal atrophy compared to 60 mg/kg PB.

Figure 5. Effects of stroke-injury and drug administration on rearing activity in the open field
 Number of rears (A) and time spent rearing (B) were examined on the first day of the open field test. Vehicle- and 60 mg/kg PB-treated injured mice both displayed significant depression of both measures of rearing activity on day 1 compared to their uninjured counterparts and other treatment groups. 30 mg/kg PB restored normal rearing activity compared to their uninjured counterparts. No effect of drug was seen on rearing in uninjured mice. * = $p < 0.050$ vs. uninjured counterparts; @ = $p < 0.050$ vs. 60 mg/kg PB-treated injured mice; # = $p < 0.050$ vs. vehicle-treated injured mice.

Table 1

Mice used for Carotid Ligation studies

	Vehicle	30 mg/kg PB	60 mg/kg PB
Ligated and randomly			
	40	42	46
distributed to each group, <i>n</i>			
Females, <i>n</i> (%)	18 (45)	21 (50)	22 (48)
Males, <i>n</i> (%)	22 (55)	21 (50)	24 (52)
Mortality - Females, <i>n</i>	0	0	4
Mortality - Males, <i>n</i>	6	3	4
Surviving to perfusion, <i>n</i>	34	39	38
Injured Females, <i>n</i> (%)	12 (35)	15 (38)	11 (29)
Injured Males, <i>n</i> (%)	10 (29)	9 (23)	9 (24)
Uninjured Females, <i>n</i> (%)	6 (18)	6 (15)	7 (18)
Uninjured Males, <i>n</i> (%)	6 (18)	9 (23)	11 (29)

Table 2

Serum Concentrations

	30 mg/kg PB	60 mg/kg PB
Total mice for each dose, <i>n</i>	20	20
Concentration in Serum at 4hrs (<i>n</i>)	25.4±2.0 (2)	46.9±1.6 (2)
Concentration in Serum at 12hrs (<i>n</i>)	21.4±14.7 (3)	43.2±4.4 (3)
Concentration in Serum at 24hrs (<i>n</i>)	12.9±1.8 (2)	29.6±0.1 (2)
Concentration in Serum at 36hrs (<i>n</i>)	5.7±2.1 (3)	20.7±1.8 (3)
<i>t</i> _{1/2} , hrs	12.6	22.7

* Cardiac blood from two P12 mice was combined to make each sample.

** Concentrations are reported in µg/ml, and the number of samples used to obtain each value is in parentheses.