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Effects of Phenobarbital on Activity and Learning in 6-Hydroxydopamine Treated Rat Pups[1]

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SHAYWITZ, B. A. AND D. A. PEARSON. *Effects of phenobarbital on activity and learning in 6-hydroxydopamine treated rat pups.* PHARMAC. BIOCHEM. BEHAV. 9(2) 173–179, 1978.—Phenobarbital may produce significant behavioral alterations and cognitive deficits in children and impaired brain growth in developing animals. We have investigated the long term consequences of phenobarbital administration on activity levels and cognitive performance in normal developing rat pups and littermates receiving intracisternal injections of 6-hydroxydopamine (6–OHDA) at 5 days of age. In normal rat pups, phenobarbital reduced activity levels at 26 days of age but did not affect avoidance performance. However, in 6–OHDA rat pups, phenobarbital reduced activity at 19 days, increased activity at 26 days of age and significantly impaired both T-maze and shuttle box performance. Phenobarbital concentrations at 35 days (EMIT technique) averaged 15.5 ± 2.01 and 20.2 ± 2.76 μg/ml in control and 6–OHDA rat pups, respectively. Body weight was significantly reduced in 6–OHDA rat pups from Day 8 but there were never significant differences between phenobarbital and control animals. Brain dopamine concentrations in 6–OHDA animals averaged 35.5% of littermate controls and did not differ between phenobarbital and control animals. These findings support the notion that phenobarbital administration may adversely affect activity and cognitive performance in the developing mammalian brain.

Developing rat 6-Hydroxydopamine Behavioral arousal Dopamine Norepinephrine
Phenobarbital Activity Habituation Avoidance performance

EVIDENCE from several lines of investigations supports the belief that sub-narcotic dosages of phenobarbital in mature animals may result in hyperactive behavior. Thus, the acute administration of 100–150 mg/kg of phenobarbital resulted in an increase in locomotor activity in mice and doses of 50 mg/kg produced an increased activity in hamsters [17]. Similar findings of hyperactive motor behavior have been reported after administration of either pentobarbital or phenobarbital after doses of 8–32 mg/kg in mice [34]. Despite a number of clinical studies which indicate that the chronic administration of phenobarbital to children may result in significant behavioral alterations and cognitive deficits [11, 12, 16, 20, 22, 31, 32, 33] few studies have explored the relationship between barbiturates and activity levels and performance in the developing organism. Such studies would be of particular interest in the light of a number of recent investigations which suggest that prolonged administration of phenobarbital to developing animals may profoundly and permanently impair brain growth [7, 8, 23, 24].

During the first weeks of postnatal life, the neonatal rat pup undergoes a remarkable pattern of activity which Campbell and his associates have designated as the development of behavioral arousal. Abundant evidence suggests that this behavior is mediated via central catecholaminergic mechanisms—mechanisms that are also believed to influence the actions of barbiturates as well [1, 6, 13, 14, 19, 34]. In order to explore the effects of phenobarbital and its relationship to brain catecholaminergic systems, we have investigated the consequences of phenobarbital administration on activity levels and cognitive performance in normal developing rat pups and littermates treated with 6-hydroxydopamine to induce a selective depletion of brain dopamine.

METHOD

Animals

Sprague-Dawley rat pups with mother were obtained from Charles River Inc., Wilmington, MA at 24 hr (± 12 hr) of age and individually housed in clean plastic cages (30×32×10 cm) with sawdust bedding. Mothers and pups were housed under fluorescent lighting conditions (16 G.E. 40 W fluorescent bulbs) with 12 hr of light (lights on 0700) and 12 hr of darkness at a temperature of 21°C. Litters were culled to 8–9 pups at 5 days of age. Mothers and pups were housed together for the first 28 days of the experimental period to insure that pups had matured sufficiently. Weights were recorded at the time of intracisternal 6–OHDA adminis-

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tration and again at weekly intervals. Food (Purina Chow) and tap water were available ad lib to the dam and her pups. These experiments included approximately equal numbers of male and female rats. Pups were numbered at the time of intracisternal injection by toe punch and randomized as experimental or control according to a table of random numbers.

Experimental Groups

The animals were divided into four experimental groups as follows: (1) Vehicle-saline (VS)—rat pups treated with intracisternal injections of a vehicle solution consisting of saline and ascorbic acid at five days of age who then received isotonic saline daily beginning at 12 days of age; (2) Vehicle phenobarbital (VP)—rat pups administered intracisternal injections of the vehicle solution at 5 days who then received doses of phenobarbital dissolved in isotonic saline each day beginning at 12 days of age; (3) Treated-saline (TS)—rat pups treated with intracisternal 6-hydroxydopamine (6-OHDA) at five days of age and daily administration of isotonic saline beginning at 12 days of age; (4) Treated-phenobarbital (TP)—rat pups treated with 6-OHDA at five days of age who then received daily doses of phenobarbital beginning at 12 days.

Pharmacological Depletion of Brain Catecholamines with Intracisternal 6-Hydroxydopamine (6-OHDA)

The 6-hydroxydopamine HBr was purchased from Regis Chemical Company (Chicago, IL) and was used without further purification. It was prepared immediately prior to use in a 0.9% isotonic saline solution containing 0.4 mg/ml of ascorbic acid to prevent oxidation of the 6-OHDA. The solution was kept on ice while in use. Intracisternal injections were administered by flexing the neck of the infant rat and injecting 20 μl of solution via a precalibrated microsyringe (Hamilton) with a 27 ga needle inserted immediately beneath the occiput. (Petroleum jelly was applied to the neck prior to injection to minimize leakage.) All pups received intracisternal injections. The experimental group received doses of 6-OHDA and littermate controls received the saline solution containing 0.4 mg/ml of ascorbic acid (vehicle).

Determination of Brain Catecholamines

Rats were sacrificed by decapitation at 33 days of age between 9–11 a.m. in order to minimize brain catecholamine variation due to normal circadian periodicity. Brains were removed and frozen on dry ice within 1 min after death. Frozen brains were stored at -70°C and biochemical determinations performed within 2–3 weeks of sacrifice. Dopamine and norepinephrine were analyzed by fluorometric techniques modified after procedures described previously by Roth and Stone [21] and Boadle-Biber and others [3].

Determination of Activity

Apparatus. The activity was recorded in a soundproof room 2.5×4 meters in size, illuminated by six 150 W infrared heat lamps (General Electric Co.) mounted on tracks 2 meters high on each wall. Ambient temperature ranged between 25–27°C. Activity was recorded via a television camera (Panasonic, Model No. WV0261, equipped with a 12.6 mm F 1.4 lens) that was placed in the center of the room at a height of 2 meters. The camera was coupled to an Hitachi time-lapse tape recorder (Model No. FV-512U), and a Vicon date/time display generator, Model No. 240T, as well as the Setchel-Carlson monochrome video monitor (Model No. 6M-912). Plastic cages, each 20×50×15 cm deep with bottoms painted black with non-reflecting paint and each equipped with a water bottle and food pellets, were placed on the floor of the room so that the entire array of nine cages was viewed simultaneously on the video monitor. When the rat pups reached 2 weeks of age a wire mesh covering was placed over the cages to prevent the animals from escaping.

Procedure. Activity was determined at 8, 12, 19 and 26 days of age and measurements were always performed between 1300 and 1600 hr each day to minimize the variation due to circadian periodicity. Rat pups were randomly assigned to one of the nine plastic cages placed on the floor of the room; recording was begun immediately and continued for at least 1 hr and sometimes as long as 3 hr. At the conclusion of the taping session, the rat pups were replaced in their home cages, and the video tape saved for scoring at a later time. Scoring of activity was accomplished by playing the tape back at a speed equivalent to six times real time, and activity in each rat was determined for alternate 5 min periods throughout the 60 min observation period. For example, we scored the animals' activity from 0–5 min, 10–15 min, 20–25 min, etc. We thus had available for analysis six separate measurements of activity for each animal for the house-long observation period. The mean of these six determinations was then used to obtain the mean activity for the observation period. In addition, the six individual measures of activity were available for use in determining habituation of activity.

Activity was always scored in a blind fashion and thus, activity for any animal was scored without knowledge of whether that animal was a treated or control. This was accomplished in several ways: 1) randomizing the animals as they were placed in the boxes, 2) scoring the rats by number but not breaking the code until the scoring was complete. In addition, the placement of the camera was such that all animals appeared to be the same size though there were reductions in body weight in the dopamine depleted rat pups. This weight difference could not be discerned utilizing this video system, thus adding another control to our experimental design. Activity was scored by the same observer and repeated scoring yielded a reliability of activity measurements with an interobserver correlation of 0.9.

An animal was considered to exhibit activity if any movement of any kind was observed. The duration of these movements was determined by activating an electric timer (standard Electric Time, Model No. 11-2, Springfield, MA) at the onset of any movement, and stopping the timer when the movement ceased. The cumulative duration of movements for each 5 min interval was thus obtained, and the percentage of time that the animal was active during each observation period noted. For example, at the playback speed of our video recorder, 5 min of elapsed time requires 50 sec. If the cumulative duration of activity during this period was 25 sec, the animal's activity is 50%.

Determination of Cognitive Performance

T-maze. Avoidance learning in a T-maze was determined at 20 days of age. The T-maze was constructed of opaque Plexiglas with a floor of stainless steel rods 1 mm dia. The main part of the T was 31 cm long and each cross piece was

16 cm long; all parts of the maze had an internal width of 9 cm and a height of 11 cm, and the 1 mm dia. stainless steel rods were separated by 5 mm. The rat was placed in the long arm of the T and the experiment initiated by raising the starting gate which activated an electromagnetic switch and thus resulted in the conductance of a 2 mA current delivered via a shock generator/scrambler (BRS/LVE Model No. SGS–004). In order to escape the shock, the rat had to traverse the maze into a safe compartment. The safe compartment was on the side opposite to that the rat showed a preference for in a trial run. He was allowed 30 sec to complete this task and if unsuccessful, was manually placed in a safe compartment where he remained for a 30 sec period. The elapsed time in seconds from start to the rat's entry into the safe compartment (the escape latency) was recorded by means of a light switch (Sigma Instrumental 8RC01A, South Braintree, MS). A total of 20 trials was recorded for each rat. Results were coded and analyzed utilizing analysis of variance.

Shuttle box performance. Avoidance learning in a shuttle box was determined at 26 days of age. The shuttle box was constructed of opaque Plexiglas with a floor of stainless steel rods and consisted of two compartments separated by a 5 cm high hurdle, each compartment 20×14×17 cm high. The starting compartment was painted black while the goal compartment was painted white and was illuminated by a flashlight bulb. The experiment was initiated by placing the rat in the black painted compartment. A starting switch was activated which simultaneously sounded a bell for a 1 sec period. Five seconds later a 2.5 mA current was delivered via a shock generator/scrambler (BRS/LVE Model SBS–004, Tech Serve Inc., Beltsville, MD), by way of stainless steel rods 2 mm dia. and separated by 1.5 cm. The floor of the goal compartment was covered by a smooth Plexiglas sheet. If the animal did not cross into the goal compartment within a 25 sec period, he was placed there manually for a 20 sec period. As the animal crossed into the goal compartment, a light switch was activated, stopping the timer (Sigma Instruments, Model B8RC01A). The time required to avoid shock was noted and total of 20 trials performed with each animal. The results were coded and analyzed using analysis of variance.

Phenobarbital Administration

Beginning at 12 days of age, phenobarbital was administered daily in an oral dose of 40 mg/kg. This was accomplished by dissolving the phenobarbital powder in isotonic saline and feeding 50 μl of solution via a Hamilton syringe and plastic PE No. 20 tubing at 1000 hr each day. Control pups received 50 μl of isotonic saline at the same time. Phenobarbital concentrations in serum were determined by the EMIT method [2,4]

RESULTS

Phenobarbital Concentrations in Rat Pups

The concentration of phenobarbital in serum averaged 15.1 ± 2.3 μg/ml in controls and 22.4 ± 2.7 in 6–OHDA treated animals, concentrations which were comparable (mean ± SEM, $p>0.05$, t test).

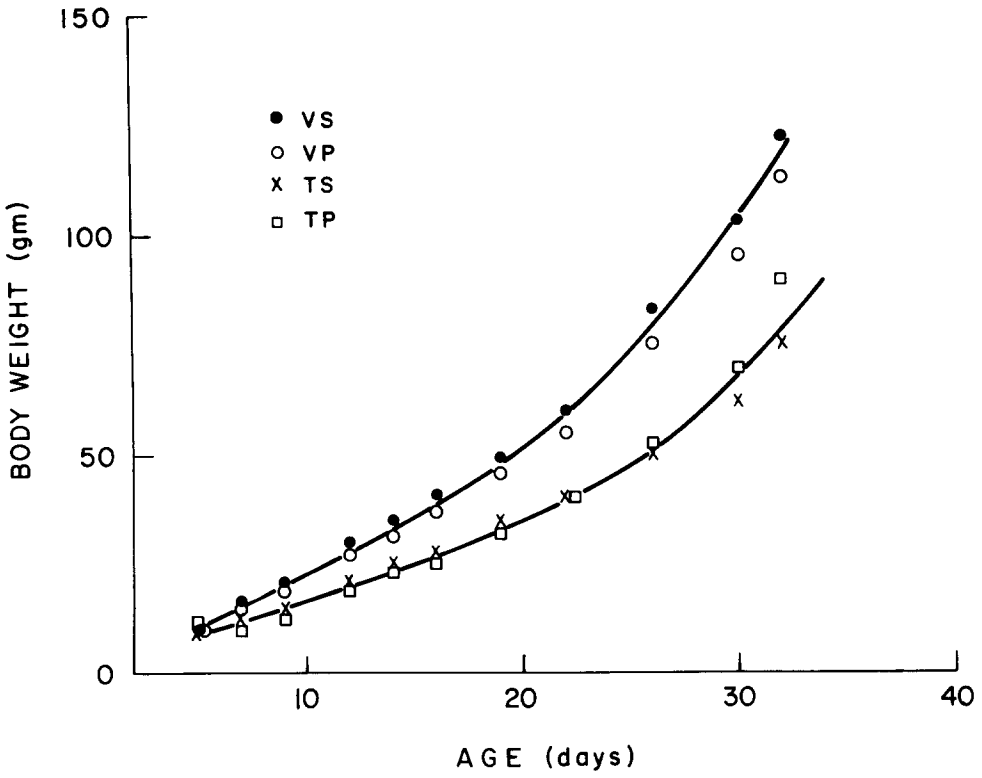

FIG. 1. Body weight in normal developing rat pups given saline daily (VS) or phenobarbital 40 mg/kg (VP), compared to rat pups treated with 6-hydroxydopamine (6-OHDA) at 5 days of age given saline daily (TS) and 6-OHDA treated pups administered phenobarbital (TP). There are no differences between VS vs. VP or TS vs. TP. However, weight differences between V vs. T are significant at all ages.

Effects of Phenobarbital on Body Weight

Rat pups treated with 6-OHDA exhibited a significant reduction in body weight compared to littermate controls an effect noted previously by Smith and associates [29] (Fig. 1). However, normal rat pups treated with phenobarbital did not differ in body weight from those receiving saline. Similarly, dopamine depleted animals treated with phenobarbital did not differ from rat pups treated with 6-OHDA alone.

Effect of Phenobarbital on Activity Levels in Normal and 6-OHDA Treated Rat Pups

Rat pups treated with 6-OHDA as neonates manifest a remarkable activity pattern throughout the first month of life that has been reported by us in previous experiments [25–27] (Fig. 2). Throughout the first 2 weeks of life, 6-OHDA treated animals are similar in activity to controls. However, between 2 to 3 weeks of age they develop a significantly increased motor activity that abates as they mature. Such an effect was clearly demonstrated in this experiment as noted by the analysis of variance within subjects due to 6-OHDA treatments, $F(1,29)=71.5$, $p<0.001$. Age itself even in normal animals produced a significant effect as well, $F(3,665)=8.43$, $p<0.001$. The effect due to phenobarbital was also significant, $F(1,29)=6.10$, $p<0.05$. Such an effect is particularly interesting when examined as an age×treatment interaction. At 8 days, $F(3,179)=0.869$, $p>0.05$, and at 12 days, $F(3,161)=1.5$, $p>0.05$, no significant differences could be discerned between all treatment groups. However, by 19 days significant differences were readily apparent, $F(3,161)=22.1$, $p<0.001$. At this age, phenobarbital had little effect in normal rat pups but resulted in a 35% reduction in activity levels in 6-OHDA treated animals, $F(1,77)=12.4$, $p<0.001$. At 26 days of age, treatment continued to exert significant differences, $F(3,161)=7.66$, $p<0.001$. Here phenobarbital produced a 41% reduction of activity in normal rat pups, $F(1,83)=4.53$, $p<0.005$, but paradoxically increased activity by 40% in 6-OHDA treated animals, $F(1,77)=4.38$, $p<0.05$.

Effects of Phenobarbital and Habituation of Activity in Normal and 6-OHDA Treated Rat Pups

Previous experiments [27] have indicated that habituation of activity over the hour observation period is significantly influenced by depletion of brain dopamine, a finding confirmed in the present investigation. Little difference between the four experimental age groups are seen at 8 and 12 days. By 19 days the inability of 6-OHDA treated animals to reduce their activity is evident. 6-OHDA treated rat pups also treated with phenobarbital are less active but the activity pattern is similar over the 60 min. The increase of activity in the phenobarbital and 6-OHDA treated rat pups compared to 6-OHDA treated alone is evident again at 26 days (Fig. 3). The activity pattern over the hour long observation period suggests some differences but the slowing of the activity profiles for the first 30 min is similar. The effect of phenobarbital on habituation in all animals in all ages does not appear to be significant or different from non-phenobarbital treated animals (Fig. 4).

Relationship Between Activity and Body Weight

Analysis of covariance between body weight and activity by treatments indicated that there was no significant relationship between body weight and activity. The differences between body weight and activity was solely a result of the treatment administered (analysis of covariance indicating $F(3,11)=2.25$, $p>0.1$).

Effects of Phenobarbital of Avoidance Learning

Avoidance learning in a T-maze at 20 days of age was significantly different between experimental groups, $F(1,539)=76.1$, $p<0.001$ (Fig. 5). A predominant effect was the marked increase in the escape latency observed in rat pups treated with 6-OHDA. However, phenobarbital produced an additional detrimental effect in 6-OHDA treated animals increasing escape latency 36%, $F(1,259)=13.9$, $p<0.001$. Similar findings were observed in the shuttle box at

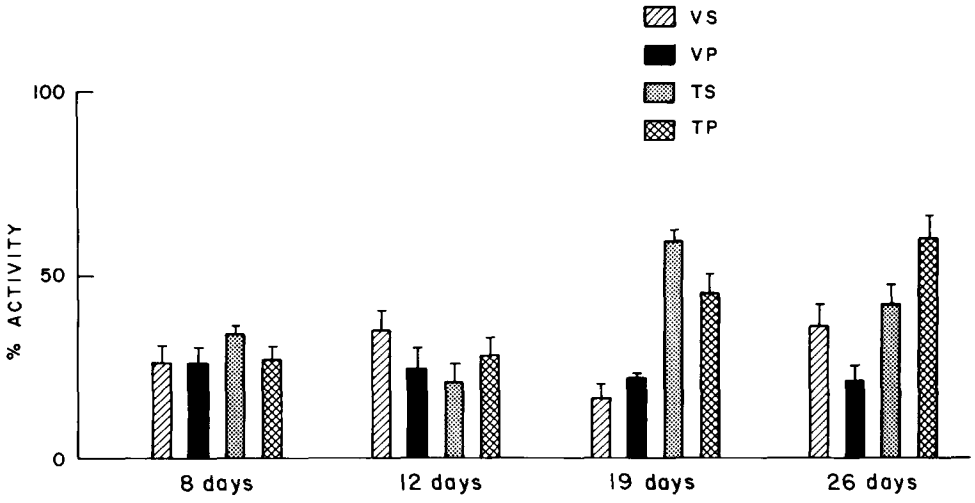

FIG. 2. Activity in normal developing rat pups administered phenobarbital and rat pups treated with 6-OHDA at 5 days of age. At 19 days of age TS exhibit significantly greater activity than TP ($p<0.001$). At 26 days VP rat pups are less active than VS ($p<0.05$) and TP are more active than TS ($p<0.05$).

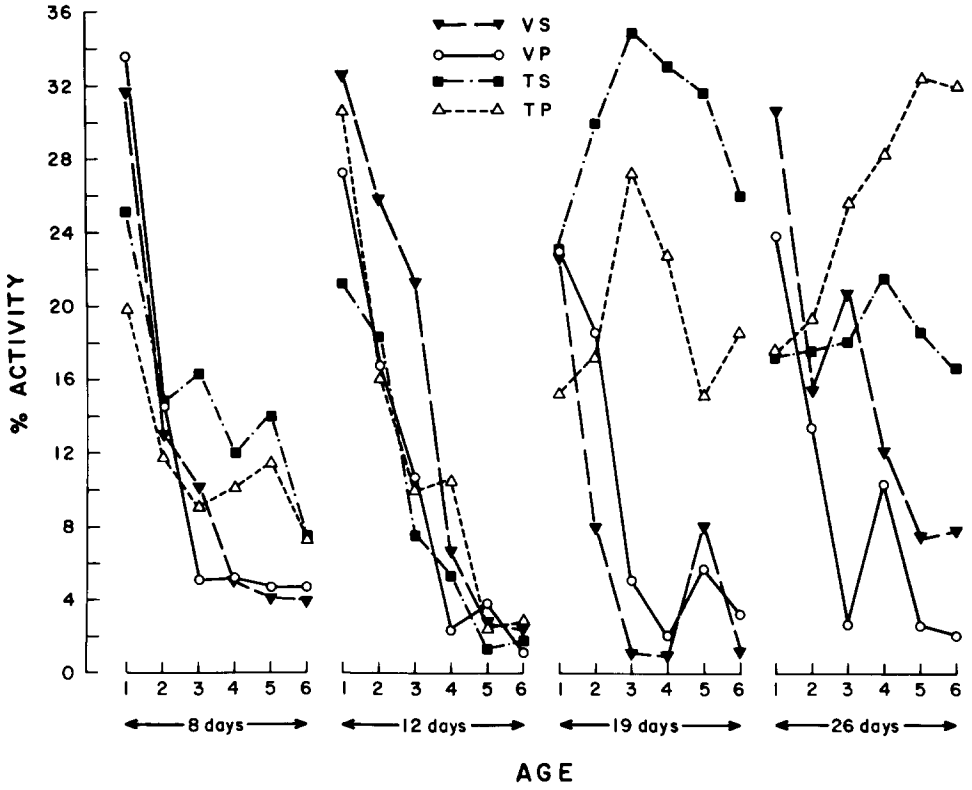

FIG. 3. Habituation of activity by treatment at each age. The numbers 1–6 on the abscissa represent activity in alternate 5 min epochs over the hour. At 19 days both TS and TP rat pups fail to habituate. At 26 days rat pups treated with 6-OHDA and administered daily phenobarbital (TP) actually increase activity at each 5 min epoch. This contrasts with VS and VP pups who show an activity decline and TS rat pups whose activity appears to remain relatively constant over the hour.

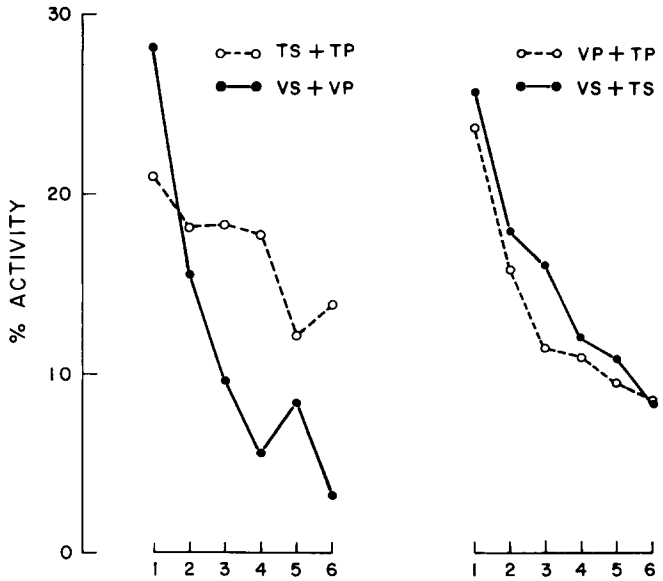

FIG. 4. Habituation of activity by treatment for all ages combined. Numbers 1–6 on abscissa represent activity in alternate 5 min epochs. On the left, normal rat pups (both VS and VP) demonstrate a reduction in activity over the hour observation period. However, rat pups treated with 6-OHDA at 5 days of age (TS and TP) fail to habituate their activity. In the figure on the right, phenobarbital does not appear to alter this habituation.

28 days of age where again the differences between groups were clear, $F(1,539)=133$, $p<0.001$. Here too, the principal effect was observed in the 6–OHDA alone but the combination of phenobarbital and 6–OHDA increased escape latency by 25%, $F(1,259)=16.2$, $p<0.001$.

Brain Catecholamine Concentrations in Animals Treated with Phenobarbital and 6–OHDA

As noted by us on previous occasions, the administration of 6–OHDA as described, results in a profound and permanent reduction in brain dopamine while norepinephrine is unaffected [25–27]. Phenobarbital administration had no significant effect on the catecholamine concentrations in either the normal rats or rat pups treated with 6–OHDA (Table 1).

DISCUSSION

Despite the extensive utilization of phenobarbital for the treatment of seizure disorders in infants and children, little is known of the chronic effects of this agent in the brain of the developing organism. Anecdotal evidence suggests that phenobarbital may precipitate or exacerbate hyperactive motor behavior in children [5,18] but corroboration of such observations has not been reported. In fact a number of investigations suggest that phenobarbital produces no adverse effects on learning or behavior [15, 30, 35]. In contrast to

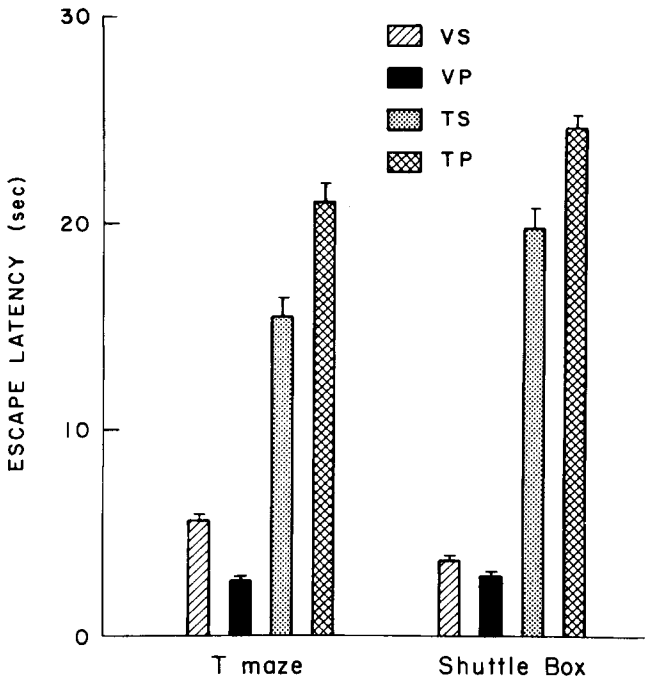

FIG. 5. Escape latency in T-maze at 20 days and shuttlebox at 28 days. In both tasks, rat pups treated with 6-OHDA at 5 days and administered daily phenobarbital (TP) exhibit escape latency significantly greater than TS ($p<0.001$.) Normal rat pups receiving phenobarbital demonstrate escape latency no different or less than controls.

such a sanguine view of the chronic effects of phenobarbital, are the results of more recent studies which suggest that this agent may indeed adversely influence performance [11, 12, 20, 22, 31, 32, 33].

These investigations suggest that the chronic administration of phenobarbital may result in subtle impairment of neurological function and are supported by a number of investigations in animals. Thus, Schain and Watanabe [23,24] administered phenobarbital to developing rats and observed a reduction in brain weight. In more recent investigations employing an artificial feeding technique Diaz and Schain [7,8] administered daily subcutaneous injections of phenobarbital at a dose of 60 mg/kg. Such dosages resulted in serum concentrations between 7–13 μg per ml 30 hr after

TABLE 1

BRAIN CATECHOLAMINE CONCENTRATION

	Dopamine	Norepinephrine
VS	608 ± 34.4 (9)	317 ± 10.9 (8)
VP	588 ± 27.2 (8)	306 ± 12.4 (8)
TS	292 ± 55.5 (9)	288 ± 9.35 (9)
TP	305 ± 62.4 (8)	295 ± 14.2 (8)

Results as mean ± SEM (ng/g wet weight)
( )=numberof animals
VS=controls given water daily
VP=controls given phenobarbital daily
TS=6-OHDA pups given water daily
TP=6-OHDA pupsgiven penobarbital

injection, and rat pups treated in this manner exhibited reductions in DNA, RNA, protein, and cholesterol in both cerebral hemispheres and cerebellum as well as a reduced brain weight.

In our experiment, we administered phenobarbital orally and began at 12 days of age. Pilot experiments had indicated to us that such a technique would prevent the reduction of body weight observed by Schain and Watanabe when the drug was administered from 5 days of age and thus we should be able to circumvent the difficulties in separating the effects of malnutrition from those of phenobarbital. That we did achieve such comparability in weight is shown in Fig. 1. Thus the difference in body weight were noted in 6-OHDA and sham treated animals. However, there were no significant differences between body weight in those rats receiving phenobarbital and those rats receiving saline in each group. Furthermore, analysis of covariance demonstrated that the changes in activity observed by us were the result of treatment (6-OHDA or phenobarbital) and not an effect of body weight.

In previous publications, we have demonstrated that rat pups receiving intracisternal administration of 6-OHDA at 5 days of age exhibit an increase in motor activity between 15–22 days which abates as the animals mature [25,26], impaired habituation to a novel environment [27] and a profound inability to perform T-maze and shuttle box tasks [28]. Phenobarbital appears to exacerbate many of these phenomenon resulting in a still greater increase in motor activity, impaired habituation, and further decrements in performance on avoidance tasks.

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