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p-Chloroamphetamine: Evidence Against a Serotonin Mediated Learning Deficit in PKU[1]

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SCHAEFER, G. J., R. J. BARRETT, E. SANDERS-BUSH AND C. V. VORHEES. *p-Chloroamphetamine: evidence against a serotonin mediated learning deficit in PKU.* PHARMAC. BIOCHEM. BEHAV. 2(6) 783–789, 1974. – Rats were injected with 5.0 mg/kg of p-chloroamphetamine (PCA) or saline from 1–28 days of age. Groups treated with PCA showed hypoactivity in the open field at 29, 50 and 75 days of age when compared to rats treated with saline and facilitated avoidance in Y-maze acquisition at 57–61 days of age. At 36 and 68 days of age, at the time of behavioral testing, brain serotonin (5HT) was reduced slightly or not at all. However, in separate groups, brain 5HT was reduced 23% and 35% in 14 and 26 day-old rats after only a single treatment with PCA, suggesting that 5HT was reduced during the treatment period. At 66–68 days of age, 5HT turnover was also unaltered. These results suggest that the behavioral effects were due to neonatal 5HT depletion during a critical period of development rather than a concurrent 5HT depletion at the time of behavioral testing. Moreover, these data are at variance with the concept that neonatal 5HT depletion impairs learning ability and is the underlying CNS defect in phenylketonuria.

p-Chloroamphetamine Serotonin Phenylketonuria Shock avoidance learning Open field Behavior

THE genetically determined biochemical defect in phenylketonuria (PKU) is reduced or inactive hepatic phenylalanine hydroxylase [17]. This biochemical lesion produces, directly, a block in the conversion of phenylalanine to tyrosine, and an accumulation of phenylalanine and its metabolites in plasma and urine. Indirectly, the accumulation of phenylalanine and phenylpyruvic acid produces a decrease in serotonin (5HT) in rat brain [4, 5, 10, 20, 35, 40], apparently by impairing the uptake of 5HT precursors [22, 30, 39]. It has been proposed that this indirect effect of reduced cerebral 5HT during some critical stage of early development may be the basis of the severe mental retardation associated with PKU [37,38]. Experimental attempts to induce PKU have traditionally involved chronic neonatal administration of excess phenylalanine [15] or p-chlorophenylalanine (PCPA) [14, 16, 25, 31, 36], a phenylalanine and tryptophan hydroxylase inhibitor, or combination of both [1, 6, 7, 29, 34]. Only the latter model has been consistently successful in producing a permanent behavioral change after the treatment regimen has been discontinued; however, all of these PKU models fail to adequately separate the effects of reduced 5HT from other potential behavioral effects of excess phenylalanine. This is further complicated in cases where PCPA is used, since as noted above, PCPA inhibits 5HT synthesis thereby confounding the direct effects produced by PCPA with the

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indirect effects produced by phenylalanine or its metabolites on 5HT metabolism. What is needed, therefore, is a drug which inhibits phenylalanine hydroxylase without directly affecting 5HT synthesis, or conversely, a drug which depletes 5HT without elevating phenylalanine.

A second consideration involves the nature of the learning impairment associated with induced PKU. Few of the investigations have attempted to provide multiple converging measures of learning in order to insure that the behavioral changes observed constitute a general model of mental retardation [15].

Thus, the present investigation was undertaken in order to: (1) determine more specifically the long term behavioral consequences of neonatal 5HT depletion, and (2) provide multiple behavioral measures in order to better differentiate associative versus performance factors and to more accurately evaluate the pattern of behaviors resulting from neonatal 5HT depletion.

In order to accomplish the first objective p-chloroamphetamine (PCA) was employed. PCA offers two advantages compared to PCPA: (1) in adult rats PCA has been shown to cause a long-lasting decrease in the activity of tryptophan hydroxylase, extending up to four months [27] and (2) PCA is a specific in vivo inhibitor of brain tryptophan hydroxylase and therefore causes a selective reduction of brain 5HT [26,28], without affecting phenylalanine hydroxylase [12]. In order to accomplish the second objective both open field activity and Y-maze discriminated avoidance tasks were used. The open field was used to assess general locomotor activity, and was chosen for two reasons: (1) activity changes associated with the induction of PKU and with 5HT depletion have not been consistent [1, 29, 34], but may be important when considered together with other measures in defining a specific pattern of behaviors related to the effects produced by either phenylalanine or 5HT changes. (2) The open field was used as a general screening procedure, especially in determining the duration of the behavioral effects. The Y-maze was used in preference to shuttle box avoidance because the avoidance response alone is not adequate for differentiating associative and motivational influences. In the Y-maze an animal must learn where to run, i.e., to discriminate the safe stimulus, reflecting primarily associative factors, in addition to learning when to run, i.e., avoiding or escaping, reflecting primarily motivational factors. For example, it has been shown that poor avoidance performance does not necessarily reflect a general learning deficit, since some strains of rats that do not learn to avoid (due to shock-induced response suppression, such as freezing and crouching, which are incompatible with avoiding), nevertheless, readily learn to discriminate the safe stimulus [2]. Therefore, the Y-maze represents a more powerful tool for delineating between various behavioral factors.

EXPERIMENT 1

To determine if reduced 5HT during neonatal development produced detectable behavioral effects and, if so, the time course of those effects, open field activity was measured at varying intervals after the termination of treatment. The range of treatment-to-test intervals used was arbitrary (2, 23 or 47 days after treatment), but was designed to include possible cumulative effects of the 28-day treatment regimen as well as possible long-term effects produced during some critical stage of early development.

Method

Animals. The animals were offspring of Sprague-Dawley rats (Zivic-Miller Laboratories, Glenshaw, Penn.). The females were obtained at 14 days of gestation and housed individually in standard (18 × 18 × 24 cm) wire mesh cages under normal laboratory conditions. Both dams and offspring were allowed free access to food and water throughout the experiment.

Apparatus. The open field apparatus consisted of a black wooden box. It measured 81 × 81 cm with walls 27 cm high and with the floor marked off into 20.5 × 20.5 cm squares with white lines. The room which contained the open field was sound attenuated with masking noise and lighting was provided by a single 40 W red light bulb suspended 110 cm above the center of the field.

Procedure. The preweaning treatment was the same in all experiments and was as follows. Offspring were assigned by litter to either the drug or vehicle control group. Drug and vehicle administration began on the day of birth and lasted for 28 days. In all cases the animals received the drug or vehicle subcutaneously. The PCA rats were injected with 5 mg/kg of p-chloroamphetamine hydrochloride (Regis Chemical Company, Chicago, Ill.), expressed as the free base in 1 ml/kg of normal saline. The control group received an equal volume of saline. The day after the last injection all animals were weaned and housed two per cage of like sex.

Separate groups (n = 6–8 per group) of males and females treated with either saline or PCA were tested in the open field at the following ages: 29, 50 and 75 days of age.

Results

No signs of PCA-induced toxicity occurred as evidenced by weight changes during the 1–28 day period of administration. The mean weight on the last day of injection was not significantly different (saline = 67 ± 1.5 g vs PCA = 69 ± 1.4 g, $t = 1.16$, $df = 98$, $p > 0.10$). In addition, the hyperthermic response typically noted with adult PCA injections was not observed until the pups were approximately 25 days old.

A 2 (sex) × 2 (treatment) × 5 (days) analysis of variance was performed on the open field data at each age. All significant results are reported at $p < 0.05$.

As can be seen in Fig. 1 both male and female PCA groups were significantly less active than their respective saline controls at all three ages tested, $F(1,20) = 26.98$, $F(1,24) = 9.34$, $F(1,28) = 10.03$, respectively. Further, the females were more active than males regardless of treatment, although this effect was not significant for the 29-day-old animals (50-day groups, $F(1,24) = 12.19$; 75-day groups, $F(1,28) = 6.24$).

Discussion

The hypoactivity seen in the open field suggests that early 5HT depletion during a critical stage of development leads to an apparently permanent reduction in exploratory behavior. Unfortunately, consistent activity changes have not been reported from studies attempting to model PKU, even those using phenylalanine in combination with PCPA [1, 29, 34]. Given this inconsistency and lacking additional

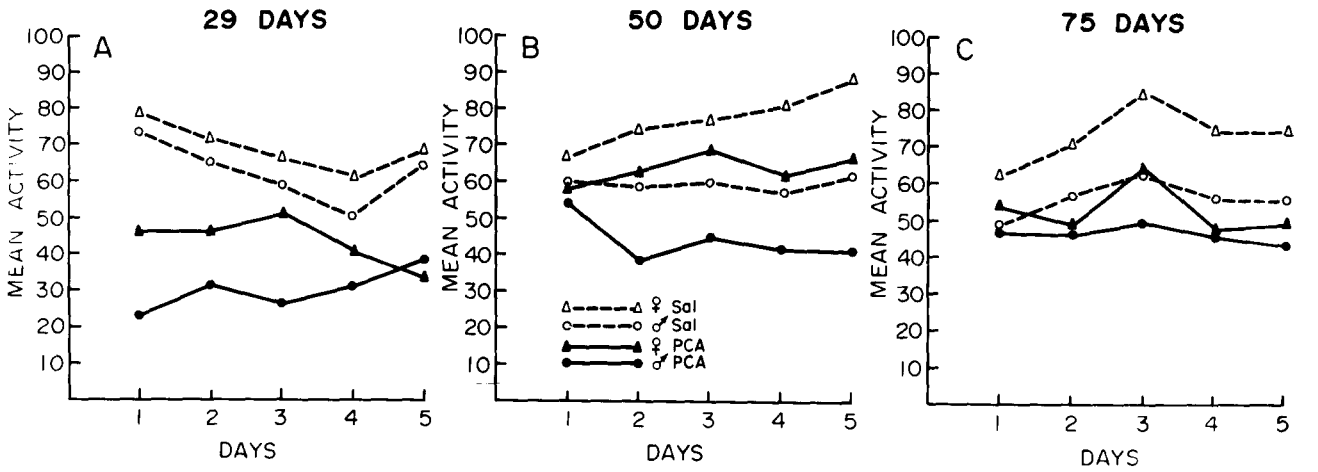

FIG. 1. Mean activity in the open field over 5 daily sessions in separate groups of animals tested at 29, 50 and 75 days of age as a function of sex and drug treatment.

and more specific behavioral measures for comparison, it is difficult to draw any meaningful conclusions about a measure as nonspecific as activity. This led to the use of the Y-maze in Experiment 2.

EXPERIMENT 2

Since the activity data were consistent at the three ages tested, avoidance behavior was measured at an intermediate interval after weaning. It was also reasoned that the activity differences resulting from PCA administration might affect performance in an active avoidance task withoug modifying the organism's ability to learn a light–dark discrimination. The second study was conducted to assess this possibility.

Method

Animals. The animals used in this experiment had previously been tested in the open field at 50–54 days of age. Testing in the Y-maze was begun when these animals were 57 days of age.

Apparatus. Three fully automated symmetrical Y-mazes were used for active avoidance testing and have been described elsewhere [8,32]. Briefly, the mazes consisted of three arms each measuring 27.9 × 17.5 × 15.2 cm with a 17.5 cm triangular choice area. The grid floor consisted of stainless steel bars 0.3 cm in dia. suspended from Plexiglas strips mounted on the side walls. A 7 W bulb positioned behind a translucent Plexiglas panel at the end of each arm served as the cue light. Foot shock was 1.0 mA 60 Hz a.c. delivered to the grid floor through a scrambler (Forringer, Model No. 1925) and regulated by an autotransformer. A fixed resistor (270 kΩ) in series with the animal provided a relatively constant current. Avoidance and discrimination responding were monitored by means of photoelectric cells activated when an animal entered 8.9 cm into an arm. Standard relay switching circuitry, timers and digital counters were used to program the behavioral contingencies and differentiate responding into various categories. The mazes were housed in a darkened room adjacent to the programming equipment and masking noise was used to eliminate extraneous auditory stimuli.

Procedure. Trials in the Y-maze were programmed on a constant 30 sec intertrial inverval and consisted of switching the safe stimulus (light onset) in a random order to one of the previously dark arms. Entry into the safe arm within 10 sec successfully avoided shock. Failure to avoid within 10 sec resulted in the onset of shock after which only escape responses were possible. Entering a dark arm during the intertrial interval turned shock on everywhere in the maze except in the safe arm. The animals were given 25 trials per day for 5 days. The following response measures were recorded during each session. (1) Avoidances: entry into the lighted arm any time during the 10 sec prior to shock onset. (2) Correct discriminations: the number of trials on which the initial response (whether avoidance or escape) was to run into the lighted (safe) arm. (3) Response latency: the time elapsed between onset of a trial and the animal's entry into the lighted arm.

Results

Separate analyses of variance were performed on each of the response measures recorded in the Y-maze. The left panel of Fig. 2 presents the mean number of avoidances made during the 5 daily sessions of 25 trials. As can be seen the PCA animals avoided significantly better than saline groups irrespective of sex, $F(1,24) = 5.41$.

Mean correct discriminations are presented in the right panel of Fig. 2. All groups regardless of treatment or sex made a comparable number of correct discriminations.

Response latencies are not shown because they essentially reflect the avoidance results. PCA animals of both sexes had significantly shorter latencies than the saline animals, $F(1,24) = 5.41$.

Discussion

There are two important points to be derived from the Y-maze data. First, avoidance acquisition was facilitated rather than impaired as would be expected if neonatal 5HT depletion produced a learning deficit related to PKU. Second, it is clear from the lack of differences in discrimination acquisition in the present study and based on several recent analyses of active avoidance acquisition [2,8] that the avoidance response is not a valid indicator of learning in and of itself. Avoidance acquisition is also related to the animal's predisposition to run in response to shock, i.e.,

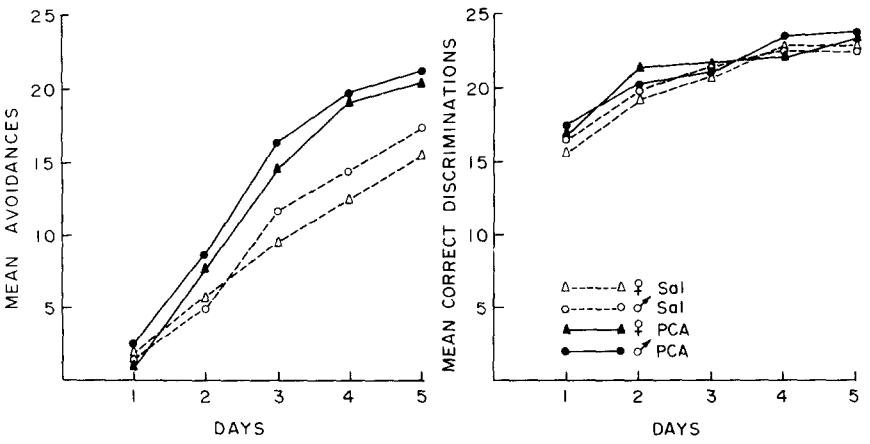

FIG. 2. Mean number of avoidances and correct discriminations over 5 daily sessions as a function of sex and drug treatment.

shock induced activity changes. The validity of this interpretation has been demonstrated elsewhere [2,8]. However, these considerations raise some doubt about previous studies of PKU in which active avoidance acquisition was used as an index of learning ability [13, 16, 19, 31]. While an avoidance deficit occurs in the PKU model, it is not necessarily indicative of a learning deficit in a more general sense. The discrimination measure circumvents this problem and clearly shows in the present context that the learning ability of the PCA animals was not changed in any general sense of the word.

EXPERIMENT 3

In order to determine the degree to which a reduction in brain 5HT at the time of testing may account for the behavioral changes noted above, the levels of 5HT in whole brain were determined. Since a previous investigation with adult rats demonstrated long-term 5HT depletion after a single injection of PCA [27], it was thought that long-term 5HT depletion might also result from chornic neonatal PCA administration. In addition, both raphé lesions, which destroy 5HT cell bodies [32], and PCA administration in adult rats [33] have been shown to produce Y-maze avoidance facilitation and corresponding 5HT depletion.

Method

Animals. Four rats from each sex and treatment which had been tested in the open field at 29–33 days of age were sacrificed at 36 days of age. In addition, six male saline and five male PCA treated animals which were left undisturbed from the time of weaning were sacrificed at 68 days of age.

Procedure. Whole brains were assayed for 5HT levels according to the procedure described by Bogdanski *et al.* [3].

Results

Approximately a 10% reduction in 5HT levels in both male and female rats treated with PCA was found at 36 days of age (Table 1); however, this difference did not reach a conventional level of statistical significance, $F(1,12) = 3.92$, $p = 0.069$. No significant differences between sexes was found. In the PCA group killed at 68 days of age, a 10% reduction in the brain levels of 5HT was found; this difference was significant ($t = 2.54$, $df = 9$, $p < 0.05$).

Discussion

The lack of profound 5HT depletion found in the present experiment suggests that the long-term behavioral effects may not be related to 5HT depletion at the time of testing. In contrast to previous experiments showing avoidance facilitation following raphé lesions or PCA administration associated with substantial reductions in 5HT (67–70%) [32,33], in the present report, PCA was given chronically to developing organisms resulting in avoidance facilitation associated with little or no 5HT reduction at the time of testing. Thus, the 10% reduction, although statistically significant at 68 days, is apparently not functionally significant. This raises the question of whether PCA is capable of reducing 5HT in neonates. In order to get at this problem additional data were collected on brain 5HT levels at selected ages of early development. A single dose of 5 mg/kg of PCA administered to 14 or 26 day-old rats produced 23% and 35% reductions in cerebral 5HT, respectively, measured 48 hours after treatment. These results suggest that PCA did reduce cerebral 5HT during the treatment period and that the observed avoidance facilitation and reduced open field activity is not related to 5HT depletion at the time of testing.

EXPERIMENT 4

An alternative explanation of the results still relying upon 5HT might be that 5HT turnover was reduced in the PCA-treated animals at the time of behavioral testing, long after PCA treatment had stopped, even though 5HT levels were not substantially altered. Indeed, turnover more closely reflects the functional state of the 5HT system than do levels. Thus, in the final experiment, 5HT turnover was measured.

Method

Animals. Six male saline and seven male PCA-treated animals which were left undisturbed from the time of weaning served as the animals.

TABLE 1

WHOLE BRAIN 5HT LEVELS

Age at Assay*	Sex	Treatment PCA	Treatment SAL	Percent of Saline
36	M	0.26 ± 0.02 (4)†	0.28 ± 0.02 (4)	93
	F	0.25 ± 0.01 (4)	0.29 ± 0.01 (4)	86
68	M	0.41 ± 0.01 (5)	0.46 ± 0.01 (6)	89‡

*Rats assayed at 36 days were injected with either 5 mg/kg of p-chloroamphetamine or saline from birth to 28 days of age and tested in the open field from 29–33 days of age. Rats assayed at 68 days were prepared similarly but were not tested in the open field.

†Values are group means and are expressed as μg/g ± SE, with the number of animals per group in parentheses.

‡$p<0.05$

Procedure. At 61 days of age, a polyethylene cannula was implanted into the lateral ventricle by the method of de Balbian Verster *et al.* [11]. Five to seven days after placement of cannulas, the animals were gently restrained and 20 μc of tryptophan-H^3 were administered intraventricularly in a volume of 10 μl. Fifteen minutes after the intraventricular administration, all animals were sacrificed by decapitation. The brains were rapidly removed and frozen. The specific activity of 5HT and tryptophan in whole brain was measured according to a modification of the procedure described by Neff *et al.* [24]. For each sample, a conversion index [9] estimating the in vivo conversion rate of tryptophan into 5HT was calculated.

Results

A two-tailed *t*-test was performed on the conversion indexes for each sample. No significant difference ($t = 1.21$, $df = 11$, $p>0.10$) between the saline (2.0 nmol/g/hr) and PCA-treated (2.4 nmol/g/hr) animals was found.

Discussion

Failure to find a significant alteration in brain 5HT turnover at a time after treatment comparable to the age at which behavioral testing in the Y-maze was undertaken suggests that the avoidance facilitation was not due to changes in the 5HT system at the time of testing, but rather to a change produced by neonatal 5HT depletion at a critical period of development.

GENERAL DISCUSSION

It has been suggested that the biochemical lesion responsible for the mental retardation associated with PKU is a reduction in cerebral 5HT [37,38]. Support for the 5HT depletion hypothesis has been indirect and consists of the following observations. (1) High doses of phenylalanine administered to rats produces a depletion of cerebral 5HT that may be associated with certain learning deficits [20]; (2) in humans with PKU a reduciton of 5HT in blood is found [17]; (3) preliminary data indicate a reduction in the levels of 5HT and catecholamines in brains of human patients [21]; and (4) elevated serum phenylalanine and reduced cerebral 5HT are associated with behavioral learning deficits in rats administered PCPA neonatally [16, 25, 31]. The latter findings are particularly suspect, however, because of the very slight elevation of plasma phenylalanine levels after treatment with PCPA. These levels never approach those seen in human PKU, but such elevations are generally considered a prerequisite to PKU by the very definition of the disease [17].

The present investigation was undertaken in an attempt to obtain a more direct assessment of the 5HT depletion hypothesis. The results suggest that the underlying biochemical defect in PKU is not a neonatal depletion of cerebral 5HT during some critical period of development, since neonatal animals treated chronically with the 5HT depletor, PCA, not only failed to show a behavioral pattern consistent with a PKU model (no deficit in avoidance or discrimination learning), but actually showed a facilitation in avoidance acquisition.

The failure of these data to support the 5HT depletion hypothesis are more significant when contrasted to various PKU models. (1) Models employing neonatal administration of excess phenylalanine have demonstrated a variety of behavioral learning deficits [15], including deficits in active avoidance acquisition [13,19], and in some instances a concomitant reduction in cerebral 5HT [20]; however, these models have failed to demonstrate a causal relationship between the 5HT reduction and the behavioral changes, given the unknown contributions of excess phenylalanine and its metabolites on other CNS systems. (2) Models employing PCPA, a phenylalanine hydroxylase inhibitor, have also shown behavioral learning deficits [16, 25, 36], again, including deficits in active avoidance acquisition [16,31], but PCPA is also a potent inhibitor of tryptophan hydroxylase. Inhibition of tryptophan hydroxylase leads to a depletion of 5HT, thereby making it impossible to separate the relative contributions stemming from phenylalanine hydroxylase inhibition and tryptophan hydroxylase inhibition to the behavioral changes. (3) Obviously, combined models employing both excess phenylalanine and PCPA are doubly confounded with regard to the role of 5HT depletion. By contrast, only one

of these factors was manipulated in the present investigation, namely, 5HT depletion using PCA. Since PCA apparently does not inhibit phenylalanine hydroxylase [12] the behavioral results seen here are not attributable to the effects of excess phenylalanine or its metabolites, but are more parsimoneously related to neonatal 5HT depletion per se. That neonatel 5HT depletion, independent of the phenylalanine effects, produces a facilitation in avoidance acquisition suggests that the behavioral deficits seen with phenylalanine and phenylalanine-PCPA models are more closely related to phenylalanine than to 5HT depletion. The relationship between PCPA models and associated behavioral learning deficits are even less clear, if the above argument is true, since they cannot be related to either 5HT depletion or to phenylalanine. However, these behavioral effects have been shown to be reversible within several weeks following the termination of treatment [25,36] and may be related to the reduction of norepinephrine after PCPA [23]. In any case the fact that the behavioral effects are reversible means that they are inadequate as models of PKU on behavioral grounds alone.

Interpretation of the open field data is less clearcut, at least within the realm of PKU models, primarily because these models make no obvious predictions about activity changes. Empirically, PKU models have been reported showing both hypo- [29,34] and hyperactivity changes [1], further complicating interpretation of the present open field results. But rather than comparing activity in isolation from other behavioral measures, it may be more profitable to consider changes in activity as a function of task requirement and the relationship of these activity changes to the behavioral deficits predicted by the PKU model.

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