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Induced PKU in Rats: Effects of Age and Melatonin Treatment[1]

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BUTCHER, R. E., C. V. VORHEES, C. W. KINDT, K. J. KAZMAIER-NOVAK AND H. K. BERRY. *Induced PKU in rats: effects of age and melatonin treatment.* PHARMAC. BIOCHEM. BEHAV. 7(2) 129–133, 1977. – Newborn rats injected on Days 1–8 of life with L-phenylalanine (2 g/kg) and p-chlorophenylalanine (80 mg/kg) displayed biochemical symptoms analogous to human phenylketonuria (PKU) and maze learning impairments. The behavioral effects were less evident in rats treated on Days 9–16 or 17–24. None of the symptoms observed were alleviated by simultaneous administration of melatonin (10 mg/kg/day).

Phenylketonuria Hyperphenylalaninemia p-Chlorophenylalanine Melatonin Phenylalanine Serotonin

BOTH Woolley and van der Hoeven [28] and Polidora [16] have reported that the administration of melatonin (N-acetyl-5-methoxytryptamine) prevents or attenuates learning impairments in rodents made artificially phenylketonuric. The results of these studies appeared quite general in that entirely different species, methods for the production of artificial phenylketonuria (PKU), and treatment periods were used. These findings were consistent with the hypothesis forwarded by Woolley and van der Hoeven [28,29] that reduced levels of brain serotonin (5-hydroxytryptamine) or its derivatives during development leads to the mental defect in PKU. Melatonin administration was thought to restore serotonin to normal levels and prevent behavioral impairment. The major elements of this hypothesis have received some support by other investigators. Decreased serotonin metabolism has been demonstrated in human phenylketonurics [14,18], reduced cerebral serotonin has been shown to have behavioral effects (e.g., [24,26]), and melatonin administration elevates brain serotonin levels [2].

This apparently promising hypothesis for the mechanism of the mental deficit in PKU, has not been vigorously explored. In part this absence of research is attributable to the limitations imposed by past animal models of PKU. As asserted by Karrer and Cahilly [11], animals made phenylketonuric by the administration of excess L-phenylalanine (Phe) do not in general display an adequate number of the biochemical or behavioral symptoms found in human phenylketonuria.

A more recent series of animal studies, however, indicates that a model of PKU bearing a closer resemblance to the biochemical characteristics of the human disease can be created by the joint administration of p-chlorophenylalanine (PCPA) and Phe to rodents [15]. The biochemical manifestations induced by this model include inhibition of phenylalanine hydroxylase, elevated Phe levels in blood and brain, substantially increased Phe/tyrosine (Tyr) ratios, and the appearance of abnormal Phe metabolites in the urine [1, 4, 23]. Importantly, the learning impairments and other behavioral abnormalities which result from early exposure to this treatment persist, even after periods of recovery from the immediate effects of the treatment [1, 5, 7, 23, 25]. Using this animal model the present studies were undertaken to reexamine the effects of melatonin administration on the biochemical and behavioral consequences of induced PKU in rodents.

It should be noted that attempts have been made to induce PKU using PCPA and Phe alone rather than in combination. The successful modeling of PKU with PCPA has met with very limited success. Even when administered at a very early age and in quantities substantially greater than those used in this laboratory, PCPA has not been shown to reliably mimic either the biochemical symptoms or enduring behavioral characteristics of the human disease [7, 10, 12, 19, 27]. The use of Phe alone has produced a more complicated set of results. When fed in the diet to weanling animals the biochemical abnormalities are modest and the behavioral effects reversible [16,17]. When, however, very young animals received Phe by gavage the biochemical changes are more dramatic and the behavioral effects endure beyond the immediate treatment period [21,22]. Since the literature presents evidence that some methods of early Phe administration produce a reasonable model of PKU, a Phe group was included in the present study to determine the effects of this substance alone under circumstances of very early administration.

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Finally, we were interested in the possible interaction of age at treatment and subsequent behavioral effects, since previous investigations suggested that timing was important in the few successful phenylalanine only models [21,22].

METHOD

Using a split litter procedure, the offspring of 16 Sprague-Dawley dams were assigned to one of four treatment groups which received: (1) L-Phe (Nutritional Biochemicals), 2 g/kg/day and PCPA (Sigma), 80 mg/kg/day in Ringers solution: PKU Group; (2) L-Phe, 2 g/kg/day, PCPA, 80 mg/kg/day and melatonin (Sigma), 10 mg/kg/day, in Ringers solution: MEL Group; (3) L-Phe, 2 g/kg/day in Ringers solution: Phe Group; and (4) an equivalent volume of Ringers Injection Solution: RIS-C Group.

An entire litter received its treatments (SC) in two equal daily doses on Days 1 through 8 (Period 1), 9–16 (Period 2) or 17–24 (Period 3) following birth (Day 0). All animals were also injected twice daily with Ringers Injection Solution on the 16 nontreatment days. Thus, each animal received 48 injections (16 treatments and 32 Ringers) from Day 1 through 24 of life. To insure survival of animals in the MEL and PKU groups, the heaviest animals in each litter were assigned to these groups. Randomly selected animals from each group were lightly anesthetized with ethyl ether and blood samples were drawn from their tails at 4 or 6 hr following each animal's last treatment injection. Samples were centrifuged and plasma Phe and Tyr values were determined by quantitative thin layer chromatography with a Schoeffel Spectrodensitometer. This method produces separate values for Phe and PCPA [3].

Three PKU group animals, 2 MEL animals, and 1 Phe animal died prior to weaning (25 days). AFter weaning all animals were housed individually with food (Purina Lab Chow) and water available ad lib in quarters providing alternating 12 hr periods of light and dark.

At 50 days of age animals were weighed and tested for swimming ability in a water filled straight channel 127 cm long. The animals were then required to learn to escape from a water filled multiple T-maze. A detailed description of this apparatus is provided elsewhere [6]. Five trials per day for 2 days were administered, and two measures of maze performance were recorded: (1) elapsed time between entry into the water and contact with the exit ramp, and (2) errors (whole body entries into blind alleys). To provide a more difficult learning task, the start and goal areas of the maze were reversed for three additional days of testing and the subjects were required to learn the backward path through the maze. The backward path is more difficult primarily because the arrangement of blind alleys is such that the probability of making errors is 2–3 times greater than for the forward path, plus the contribution of negative transfer due to prior learning of the forward path. In the backward phase 2 trials per day were run; time and errors were again recorded. To prevent exhaustion, no animal was allowed to remain in the maze for more than 6 min on any trial. If an animal failed to solve the maze within the maximum time, the trial was terminated by guiding the animal to the exit ramp. The animal was then given an error score of 56 for that trial (one more than the largest number of errors scored by any animal in less than 6 min).

RESULTS

Phe and Tyr values obtained from the 4 and 6 hr blood samples are displayed in Table 1. The regimen of combined PCPA and Phe administered to both the PKU and MEL groups induced the plasma abnormalities characteristic of human phenylketonuria. Regardless of the period of treat-

TABLE 1

PLASMA PHENYLALANINE AND TYROSINE CONTENT FROM RATS TREATED DAYS 1–8 (1), 9–16 (2) OR 17–24 (3)

		Plasma Amino Acid Analysis*							
		4 Hr (μmoles/ml)				6 Hr (μmoles/ml)			
Period	Treatment	Phe	Tyr	Phe:Tyr Ratio	N	Phe	Tyr	Phe:Tyr Ratio	N
1	PKU	4.10 ± 0.07	0.68 ± 0.11	6.03	3	1.31 ± 0.21	0.42 ± 0.08	3.12	3
	MEL	3.85 ± 0.39	0.75 ± 0.20	5.13	3	1.01 ± 0.21	0.34 ± 0.05	2.97	3
	PHE	2.68 ± 0.31	0.78 ± 0.15	3.44	3	0.08 ± 0.02	0.47 ± 0.05	0.17	3
	RIS-C	0.07 ± 0.01	0.16 ± 0.05	0.44	3	0.06 ± 0.00	0.06 ± 0.00	1.00	3
2	PKU	1.70 ± 0.16	0.19 ± 0.06	8.95	3	1.14 ± 0.11	0.17 ± 0.03	6.70	6
	MEL	1.00 ± 0.17	0.09 ± 0.02	11.11	3	0.94 ± 0.16	0.20 ± 0.04	4.70	6
	PHE	0.46 ± 0.22	0.45 ± 0.12	1.02	3	0.15 ± 0.04	0.19 ± 0.06	0.79	6
	RIS-C	0.06 ± 0.00	0.13 ± 0.02	0.46	3	0.08 ± 0.02	0.07 ± 0.01	1.14	5
3	PKU	0.91 ± 0.21	0.42 ± 0.01	2.17	2	0.61 ± 0.08	0.05 ± 0.00	12.20	3
	MEL	0.72 ± 0.06	0.36 ± 0.08	2.00	2	0.81 ± 0.16	0.07 ± 0.01	11.57	3
	PHE	0.04 ± 0.00	0.05 ± 0.00	0.80	2	0.06 ± 0.00	0.06 ± 0.00	1.00	3
	RIS-C	0.06 ± 0.00	0.06 ± 0.00	1.00	1	0.06 ± 0.00	0.06 ± 0.00	1.00	3

*Each value represents averaged determinations ± SE.

TABLE 2

WEIGHT AND MAZE PERFORMANCE OF RATS TREATED DAYS 1–8 (1), 9–16 (2) OR 17–24 (3)

			Mean ± SE			
			Weight (g) at 50 Days		Biel Maze Total Errors	
Period	Treatment	N	Male	Female	Forward Phase	Backward Phase
1	PKU	12	161.8 ± 5.0*	125.9 ± 7.0*	32.5 ± 4.6	105.7 ± 22.3*
	MEL	12	145.2 ± 9.9*	124.8 ± 8.9*	33.4 ± 4.2	118.8 ± 23.7*
	PHE	14	173.0 ± 5.5	143.5 ± 4.8	48.8 ± 6.4	55.4 ± 10.4
	RIS-C	15	181.7 ± 9.7	150.5 ± 2.7	43.5 ± 6.5	69.7 ± 14.1
2	PKU	13	135.1 ± 5.1*	119.5 ± 8.1*	56.0 ± 12.3	89.4 ± 8.7
	MEL	14	140.5 ± 6.1*	123.5 ± 6.6*	51.5 ± 10.2	108.8 ± 18.9
	PHE	17	161.5 ± 4.2	138.3 ± 2.8	36.4 ± 5.2	68.1 ± 10.4
	RIS-C	14	151.0 ± 5.2	133.2 ± 3.9	44.7 ± 5.2	79.0 ± 18.4
3	PKU	13	133.4 ± 2.5	132.2 ± 2.1	43.7 ± 7.5	62.3 ± 13.3
	MEL	13	140.6 ± 3.6	124.6 ± 3.9	39.5 ± 5.0	90.5 ± 17.4
	PHE	12	147.3 ± 6.8	138.0 ± 4.7	53.6 ± 6.4	62.1 ± 10.3
	RIS-C	11	148.3 ± 3.2	129.8 ± 6.6	52.0 ± 7.4	61.8 ± 14.0

*$p<0.05$.

ment, an elevation of Phe in blood was observed in these groups together with an increased Phe:Tyr ratio. Phe alone produced lesser plasma abnormalities when administered to the youngest animals, and blood Phe levels approached normality by six hr posttreatment with a subsequent decline in Tyr levels. The PKU and MEL animals in Period 1 were found to experience the most dramatic increase in absolute levels of Phe, but this elevation was not reflected in a Phe:Tyr ratio substantially different from that of subjects similarly treated during Period 2 due to the substantial elevations in Tyr of Period 1 animals. The largest Phe:Tyr ratios actually appeared among Period 2 PKU and MEL groups due to their lower Tyr levels. No significant mitigation of the plasma abnormalities resulted from the administration of melatonin; in each treatment period comparable increases in the Phe level and Phe:Tyr ratios were found in the PKU and MEL groups.

A similar pattern of effects is suggested in the weights of the animals at 50 days of age (Table 2) in that the PKU and MEL group subjects were lighter compared to Phe and RIS-C animals. Analyses of variance performed on these weight data indicated a significant effect of both the treatment administered and the period of administration in both male ($p<0.01$) and female ($p<0.05$) subjects. Scheffé analyses [8] indicated that the weights of the PKU and MEL males and females differed significantly ($p<0.01$) from the Phe and RIS-C animals for Periods 1 and 2, but not for Period 3. Although treatment during Periods 1 and 2 was associated with the largest relative reduction in PKU and MEL weights, the clarity of this effect is diminished by the unexpected reduction in the weight of all groups treated in Periods 2 and 3. The cause of the lower overall weights in Period 2 and 3 animals is uncertain, but this may be an effect of a shorter recovery time in these groups, a result of the older age at which the heavier animals were selected for assignment to the PKU and MEL groups, or an interaction of these factors.

An analysis of variance performed on the data from the pretest swimming speed trials indicated no significant differences attributable to treatment or period of administration. Preliminary analyses of maze error and time data also revealed no significant differences in the maze performance of male and female animals and correlations between the weight of animals and maze performance proved to be small and nonsignificant (forward phase $r = +.002$, backward phase $r = -.207$). Sex and weight of the animal were, therefore, disregarded in subsequent analyses of variance examining the effects of treatment and period upon forward and backward maze performance. No significant effects were found in the analysis of the forward path data (Table 2). A significant effect of treatments was evident in both error and time data, however, for learning of the more difficult backward path through the maze. As shown in Table 2, the PKU and MEL animals treated during the first 8 days of life (Period 1) displayed the greatest difficulty in learning the maze. Animals in these treatment groups made progressively fewer errors when treatments were administered during Periods 2 and 3. The statistical significance of this effect was confirmed by individual Scheffé analyses [8]; the comparison of PKU and MEL groups with the Phe and RIS-C groups was significant ($p<0.05$) for Period 1 both in number of errors and maze time during the backward phase (PKU and MEL groups did not differ from one another nor did the Phe and RIS-C groups). An apparent and similar difference between PKU-MEL and Phe-RIS-C in Period 2 approached, but did not achieve statistical significance ($p<0.07$). There were no significant treatment effects for Period 3. In a few instances Phe groups were observed to make fewer errors than RIS-C controls in learning the maze and MEL to make more errors

than the PKU group. However, neither of these effects approached statistical significance.

The data from Period 1 were further scrutinized to determine whether influences other than a learning impairment might have produced the observed increase in backward path error rates in the PKU and MEL groups. The possibility of negative transfer from forward path training (in which these groups showed some trend toward reduced errors) was considered. To test this possibility, a Scheffé test was made on forward errors between the combined PKU and MEL groups and the Phe and RIS-C groups, even though the original ANOVA was not significant. This comparison did not approach statistical significance ($t = 0.06$, $df = 51$). In addition, the possibility of a negative correlation between forward and backward phases which might have been expected if there had been a negative transfer effect, was looked for. The actual correlation ($r = +.15$), however, was small and positive, thereby failing to support the negative transfer concept.

DISCUSSION

Unlike the previous reports [16,28], no evidence indicating that melatonin treatment modified the behavioral consequences of induced PKU could be found in the present study. Indeed, the maze performance of the MEL group was slightly inferior to that of the PKU animals. The contrast between the results of this study and those reporting a beneficial effect of melatonin treatment may be attributed either to the absence of a causal relationship between reduced brain serotonin and behavioral deficits in induced PKU or to methodological differences between this and the prior studies. Yuwiler and Louttit [30] have reported reduced brain serotonin levels following the prolonged feeding of rats with L-Phe (5 g/kg) but found neither a relationship between learning performance and serotonin levels nor improvement with isocarboxazid feeding which significantly increased brain serotonin. They concluded that the decrease in serotonin is an auxillary phenomenon in PKU. In human trials, iproniazid increased serum serotonin in eight PKU patients, but no benefit of this treatment was observed in the general behavior, IQ, or electroencephalographic pattern of these children [13].

The methodological differences between this report and previous investigations are numerous; indeed, as noted above, the two earlier studies differ markedly from one another. The differences between the present study and what is common to the others are the duration of treatment and the substances used to induce PKU-like symptoms. In this study animals were tested following a substantial period of recovery from a brief treatment period. In both the prior studies [16,28] a longer treatment period was used and was continued throughout behavioral testing. It may be that melatonin administration moderates some functional abnormality which occurs concurrently with the elevation of serum Phe rather than affecting the enduring, perhaps structural, deficits that persist after treatment is ended. In the present study the addition of PCPA which is a direct inhibitor of tryptophan hydroxylase as well as of phenylalanine hydroxylase may prevent the appearance of a beneficial effect from melatonin treatment by requiring substantially larger quantities of melatonin to restore serotonin levels to normal. Thus, the potential involvement of serotonin in PKU cannot at present be resolved. However, there is recent evidence that neonatal serotonin depletion using p-chloroamphetamine, which inhibits only cerebral tryptophan hydroxylase, produces behavioral effects that do not resemble PKU [20]. Further direct evidence on this topic may be forthcoming through the behavioral examination of animals treated with α-methylphenylalanine, an agent which increases plasma phenylalanine without effecting cerebral serotonin content [9].

The finding that diminished maze learning ability was related to the animal's age at administration is striking in view of the relatively short term of treatment and the small differences between treatment periods. We have previously found enduring impairments with longer treatments begun with considerably older animals [7], so the early age must be considered a relative rather than an absolute period of vulnerability. None of the biochemical measures taken appear related to the behavioral differences found among the treatment periods except for the increased concentration of plasma Phe and Tyr in the youngest animals. This relationship suggests that the mechanism for the diminished maze learning ability is related to the amount of these amino acids or their metabolites. If so, the greater vulnerability of the youngest animals would be a result of the immaturity of enzyme systems responsible for the metabolism and/or excretion of Phe and Tyr. It may also be, however, that the rat brain, because of its immaturity, particularly in enzymes of the tricarboxylic acid cycle, would be vulnerable early in development to even moderate elevations in plasma Phe concentrations.

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