NEUROCHEMICAL AND BEHAVIORAL STUDIES ON THE EXPERIMENTAL PHENYLKETONURIC RATS

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ABSTRACT

Neurochemical changes and discriminative learning ability were investigated on experimental phenylketonuric rats (PKU rats). PKU rats were fed a diet containing 7% L-phenylalanine from 22nd day of age for several months. Some rats were fed the high phenylalanine diet from 22nd day of age up to 3 months of age, and thereafter they were fed a normal diet for appropriate period (Rehabilitated PKU rats).

Gain in body weight was clearly lowered in the PKU and Rehabilitated PKU rats (R-PKU rats), but the DNA content per cerebrum did not differ from that of the controls. The concentration of 5-hydroxytryptamine (5-HT) and 5-hydroxyindol acetic acid (5-HIAA) in the cerebrum decreased significantly in the PKU rats, while the R-PKU rats showed normal values. The activity of 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase) decreased significantly in the cerebrum of the PKU and R-PKU rats. Operant brightness discriminative learning ability of the PKU and R-PKU rats was found to be disturbed compared to that of the controls.

It was suggested that the poor learning ability in the PKU rats was more closely related to the dysmyelination in the cerebrum than other neural elements such as DNA and monoamine contents.

INTRODUCTION

Phenylketonuria (PKU) is an inherited disorder of phenylalanine metabolism. The well defined biochemical changes resulting from single gene defect cause an abnormal cerebral development, and in human, severe mental retardation has been recognized. Many investigators have attempted to study the mechanism resulting in behavioral abnormality owing to the metabolic changes using experimental phenylketonuric animals. Pathological examination revealed that the decreases in brain weight and myelination were the prominent mani-
festations of brain damages. Several kinds of biochemical abnormalities were reported such as inhibition of protein synthesis in the brain, and abnormal composition of myelin lipid in relation to dysmyelination on the PKU. Abnormal metabolism of 5-hydroxytryptamine (5-HT) was also observed, and this, together with the dysmyelination, was thought to be one of the possible mechanisms causing mental retardation in the PKU.

In this paper, operant brightness discriminative learning ability and certain neurochemical changes in the brain were investigated using experimental phenylketonuric rats.

**MATERIALS AND METHODS**

*Experimental animals*

Pregnant wistar albino rats were purchased from animal dealer (Japan Laboratory Animals Inc.). After delivery, eight pups were nursed by each dam for 21 days. After weaning, the pups were divided into two groups at random. One group of pups was fed a normal diet for several months (control rats), and the other group of pups was fed a diet containing 7% L-phenylalanine (high phenylalanine diet) for several months (PKU rats) according to the procedure of Tsukada. Some pups were fed the high phenylalanine diet from weaning up to 3 months of age, and thereafter they were fed the normal diet for appropriate period (R-PKU rats). The normal and high phenylalanine diets were purchased from Oriental East Co. L-phenylalanine was a kind gift of Ajinomoto Co., Japan.

*Biochemical analyses*

After decapitation, brain was rapidly removed and separated into several brain regions (pallium cerebri, brain stem and cerebellum) under the cold. Blood was collected from the abdominal artery.

Amino acid concentration in the pallium cerebri and plasma was measured according to the procedure as follows. The pallium cerebri and plasma were homogenized in 10 volumes of 75% ethanol and centrifuged at 800 g for 10 min. The supernatant was dried up and then 0.01N-HCl was added to extract the free amino acids. The extracted solution was washed with water-saturated chloroform and centrifuged at 800 g for 15 min. The squeeous phase was applied to an automated amino acid analyser (JLC-6AH, Nihon Denshi Co.).

DNA concentration in the cerebrum (pallium cerebri and brain stem) was measured by the method of Burton after the fractionation of DNA and RNA according to the method of Schmidt & Thannhauser and Schneider.

The concentration of 5-HT and 5-HIAA in the cerebrum was measured by
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a fluorometric method of Fischer et al.\textsuperscript{14}

The activity of CNPase (EC 3.1.4.37) was assayed according to the method of Kurihara & Tsukada.\textsuperscript{15} Protein concentration was measured by the method of Lowry et al.\textsuperscript{16} using bovine serum albumin as a standard.

Operant brightness discriminative learning

When the experimental rats reached 4 months of age, a brightness discriminative learning was performed. Rats were housed individually and the amount of the diet was restricted maintaining 80\% body weights of their free feeding weights throughout the learning experiments.

On the first day of the learning experiment, rats were conditioned to press a bar under a continuous reinforcement schedule (CRF-80) in which bright light (8000 lux) was displayed on the front wall of a Skinner box using a food pellet (36 mg) as a reinforcer. From the 2nd day of the experiment, they were trained under a variable interval extinction (VI) schedule. In this training, when bright light (8000 lux) was displayed on a stimulus panel as a positive stimulus (S\textsuperscript{+}), bar-pressing response was reinforced under VI schedule. On the other hand, when dim light (80 lux) was displayed as a negative stimulus (S\textsuperscript{-}), reinforcement was not available even by the bar-pressing response. Each daily session consisted of 20 S\textsuperscript{+} and 20 S\textsuperscript{-} presentations, one presentation being 30 seconds. These conditioned stimuli were presented at random in accordance with the Gellerman series. The length of the variable interval (VI) of reinforcement was gradually increased from 5 seconds to 30 seconds. The correct response ratio (R\textsuperscript{+}/R\textsuperscript{+}+R\textsuperscript{-}) was calculated from the number of correct response (R\textsuperscript{+}) during S\textsuperscript{+} presentation and the number of incorrect response (R\textsuperscript{-}) during S\textsuperscript{-} presentation in each session. The criterion of the discriminative learning was set at a correct response ratio of 85\% in three successive sessions. The training was continued until the rats were able to attain the criterion of the learning or for 40 sessions. The operation of the learning experiment was controlled by a microcomputer (UP-8, Unitec Electronics Co.).

RESULTS

Amino acid concentration in the pallium cerebri and plasma

Table 1 showed amino acid concentration in the pallium cerebri and plasma of 50-day-old rats. In the PKU rats, high phenylalanine concentration was observed in both the pallium cerebri and plasma. Tyrosine concentration also increased significantly in the PKU rats. The concentration of other amino acids, such as aspartate, glutamate and GABA, decreased or tended to decrease in the
Amino acid concentration in the pallium cerebri and plasma of 50-day-old rats

<table>
<thead>
<tr>
<th></th>
<th>Pallium cerebri (μmol/g.w.w.)</th>
<th>Plasma (μmol/ml)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control (4)</td>
<td>PKU (4)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>3.41±0.09</td>
<td>*1.79±0.16</td>
</tr>
<tr>
<td>Glutamate</td>
<td>8.82±0.29</td>
<td>*6.45±0.22</td>
</tr>
<tr>
<td>GABA</td>
<td>2.53±0.25</td>
<td>1.86±0.18</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.10±0.03</td>
<td>*0.67±0.17</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.10±0.02</td>
<td>*0.73±0.17</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E.M. with the number of rats shown in parentheses. Values with asterisks are significantly different from the controls by Student's t-test with two-tailed distributions (P<0.01).

Table 2 showed the body weight, cerebral weight and DNA content per cerebrum at the age of 50 days and 7 months. At both ages, the body weight gain was significantly lower in the PKU rats compared to the controls. The body weight of the R-PKU rats was decreased compared to the controls but it

Body weight, cerebral weight and DNA content per cerebrum

Table 2 showed the body weight, cerebral weight and DNA content per cerebrum at the age of 50 days and 7 months. At both ages, the body weight gain was significantly lower in the PKU rats compared to the controls. The body weight of the R-PKU rats was decreased compared to the controls but it

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Cerebral weight</th>
<th>DNA content/cerebrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>50-day-old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (4)</td>
<td>210±17</td>
<td>1.17±0.04</td>
<td>1.08±0.03</td>
</tr>
<tr>
<td>PKU (4)</td>
<td>* 84±9</td>
<td>1.06±0.01</td>
<td>1.05±0.01</td>
</tr>
<tr>
<td>7-month-old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (4)</td>
<td>374±14</td>
<td>1.33±0.03</td>
<td>1.16±0.03</td>
</tr>
<tr>
<td>PKU (4)</td>
<td>*265±6</td>
<td>1.21±0.03</td>
<td>1.13±0.02</td>
</tr>
<tr>
<td>R-PKU (4)</td>
<td>*311±15</td>
<td>1.28±0.05</td>
<td>1.13±0.04</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E.M. with the number of rats shown in parentheses. Values with asterisks are significantly different from the controls by Student's t-test with two-tailed distributions (P<0.01). R-PKU; Rehabilitated PKU rats.
was clearly restored from that of the PKU rats at the age of 7 months. The cerebral weights of the PKU and R-PKU rats did not differ from that of the controls at either ages.

5-HT and 5-HIAA concentration in the pallium cerebri and brain stem

5-HT and 5-HIAA concentration in the pallium cerebri and brain stem was measured at the age of 50 days and 4 months (Table 3). At both ages, the concentration of 5-HT and 5-HIAA in the pallium cerebri and brain stem decreased remarkably in the PKU rats. However, the concentration of 5-HT and 5-HIAA in the cerebrum had been restored to normal level in the R-PKU rats at the age of 4 months.

| Table 3 |

Concentrations of 5-HT and 5-HIAA (µg/g, wet wt.) in the cerebrum of 50-day-old and 4-month-old rats

<table>
<thead>
<tr>
<th></th>
<th>Pallium Cerebri</th>
<th>Brain Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-HT</td>
<td>5-HIAA</td>
</tr>
<tr>
<td>Control</td>
<td>0.79±0.01</td>
<td>0.56±0.02</td>
</tr>
<tr>
<td>PKU</td>
<td>*0.33±0.05</td>
<td>*0.11±0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-PKU</td>
<td>0.90±0.05</td>
<td>0.41±0.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E.M. with the number of rats shown in parentheses. Values with asterisks are significantly different from the controls by Student's t-test with two-tailed distributions (P<0.01).

R-PKU; Rehabilitated PKU rats.

2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase) activity

The activities of CNPase in the various brain regions were measured as a marker of myelination in the central nervous system (Table 4). In the PKU rats, a significant decrease of CNPase activity was observed in the pallium cerebri and brain stem at the age of 4 months and 8 months, but there was no change of the activity in the cerebellum. In the R-PKU rats, the CNPase activity still remained at a low level in the pallium cerebri and brain stem at the age of 7 months.
Table 4
2’,3’-cyclic nucleotide 3’-phosphohydrolase activities (μmol/mg protein/min) in several brain regions of 4-month-old, 7-month-old and 8-month-old rats

<table>
<thead>
<tr>
<th></th>
<th>4-month-old</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>8-month-old</td>
<td></td>
<td>Cerebellum</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7-month-old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>R-PKU</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E.M. with the number of rats shown in parentheses. Values with asterisks are significantly different from the controls by Student's t-test with two-tailed distributions (P<0.01).
R-PKU; Rehabilitated PKU rats.

Brightness discriminative learning ability

Fig. 1 showed the changes of correct response ratios during the learning experiment. In the control rats, the correct response ratio increased gradually by daily training and reached the criterion of the learning until 40 sessions. In the PKU and R-PKU rats, their correct response ratios also increased gradually with training, but they could not attain the criterion of learning within 40 sessions. Their correct response ratio at 40th session was 75-80%.

DISCUSSION

In the present paper, the correlation between discriminative learning ability and neurochemical changes such as the lowering of CNPase activity and the abnormal metabolism of 5-HT in the cerebrum was investigated on the PKU rats. The PKU rats were produced by subjecting rats to a diet containing 7% L-phenylalanine from the 22nd day of age for several months according to the method described by Tsukada. At the age of 50 days, a high accumulation of phenylalanine was observed in both the plasma and the cerebrum of the PKU rats (Table 1). On the contrary, the concentration of aspartate and glutamate in the pallium cerebri decreased significantly and GABA tended to decrease. This might be due to the inhibition of carbohydrate metabolism by the accumulation...
Fig. 1 Changes of correct response ratio. Each points represents the mean value of correct response ratio with the number of rats in parentheses.

of phenylalanine itself or its metabolites such as phenylpyruvate or phenyl lactate.\(^{17}\) Urinary excretion of phenylpyruvate, which is an abnormal metabolite of phenylalanine and is excreted in the urine of the PKU patients, was measured
using phenytip (Dai-ichi Pure Chemicals Co.). About 5 mg/dl of phenylpyruvate was excreted in the urine of the PKU rats throughout the period during which they were being given a high phenylalanine diet. These biochemical features of the PKU rats were almost identical to those of the PKU patients except for high level of tyrosine in the plasma and the pallium cerebri.

In the PKU rats, the gain in body weight and cerebral weight was considerably low, but the DNA content per cerebrum did not differ from that of the controls. It is believed that in the rat brain, neuronal cells proliferate in the pre-natal period, whereas glial cells proliferate in the early postnatal period. Then, in the PKU rats, the proliferation of neuronal and glial cells was assumed to be unaffected, but slight decrease in the cerebral weight might indicate that the cell size is smaller than that of the controls due to the retardation of cell maturation.

5-HT is thought to be one of the neurotransmitters in the central nervous system. Many investigators have reported the decrease of 5-HT concentration in the plasma and the brain of both the PKU animals and PKU patients, and it has been assumed to be one of the factors involved in the mental retardation in the PKU subject. The mechanism of the decrease of 5-HT in the brain has also been studied by number of investigators. It is now believed that the decreases of tryptophan concentration and tryptophan hydroxylase activity in the cerebrum cause the depletion of 5-HT in the brain. In the present study, we have also shown that 5-HT and 5-HIAA concentration in the pallium cerebri and brain stem were decreased in the PKU rats at the age of 50 days and 4 months (Table 3). In the case of the R-PKU rats, however, the concentration of 5-HT and 5-HIAA in the pallium cerebri and brain stem showed normal values at the age of 4 months. An abnormal metabolism of 5-HT in the cerebrum of the PKU rats could be restored by feeding a normal diet due to the normalization of phenylalanine concentration in the cerebrum.

CNPase is a marker enzyme of myelin sheath in the central nervous system, and the activity reflects well the degree of myelination in the central nervous system during the course of development. In the PKU patients or PKU animals, dysmyelination has been suggested by pathological and biochemical studies. To investigate the degree of myelination in the cerebrum of the PKU rats, CNPase activity in the several brain regions was examined. A decrease of CNPase activity in the pallium cerebri and brain stem was observed in the PKU rats at both ages of 4 months and 8 months. This result might indicate that myelination in the pallium cerebri and brain stem of the PKU rats was disturbed. It is believed that oligodendroglia which form myelin sheath in the central nervous system proliferate actively during the early postnatal devel-
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opment. If it is taken into account that the DNA concentration in the cerebrum showed a normal value in the PKU rats, the proliferation of oligodendroglia should be normal. However, the activity of CNPase showed lower level which might indicate that the maturation of oligodendroglia seems to be disturbed by the accumulation of phenylalanine during the postnatal development. This possibly causes the dysmyelination in the cerebrum. Whereas, in the cerebellum, CNPase activity showed no difference between the PKU and control rats. It seemed that cerebellar myelination is less sensitive to environmental factors than other parts of the brain. In the R-PKU rats, the activity of CNPase was still lower in the pallium cerebri and brain stem at the age of 7 months. It is suggested that the disturbance of myelination caused by amino acid imbalance during early postnatal period could not be restored by feeding a normal diet after the age of 3 months. In addition, the effect of the accumulation of tyrosine in the cerebrum on the dysmyelination should be considered. Grundt & Hole reported that the incorporation of radioactive fatty acid into myelin fraction was suppressed in the PKU rats which were produced by the injection of phenylalanine and p-chloro-phenylalanine and having a low level of cerebral tyrosine. This result strongly suggested that the accumulation of phenylalanine but not tyrosine in the cerebrum is at least one of the factors involved dysmyelination.

The learning ability of the PKU animals has been examined by many investigators using T-maze learning, water maze learning, active avoidance learning or passive avoidance learning. There has been no report, however, concerning operant discriminative learning ability in the PKU animals. In the present study, operant brightness discriminative learning ability was examined on the PKU and R-PKU rats. The control rats were able to achieve the criterion of discriminative learning within 40 sessions, while the PKU and R-PKU rats could not attain this criterion within 40 sessions (Fig. 1). It was concluded that the learning ability of the PKU and R-PKU rats was disturbed. Andersen & Guroff reported that hypersensitivity was observed in the PKU rats under the open field test for motor activity or the activity wheel test. However, on our discriminative learning test, the total number of bar-pressing response in one session, which is thought to be an indicator of emotional activity of the rats, showed no difference between the PKU and control rats.

The decrease of cerebral 5-HT concentration is thought to be one of the mechanisms of mental retardation in the PKU subjects. However, it has been reported that in the PKU rats of which the cerebral 5-HT concentration has been increased by giving monoamine oxidase inhibitor, the learning ability on T-maze test was also poor. This result suggested that there is no correlation
between the decrease of cerebral 5-HT and the learning disability. The results shown in this paper also supported this view, because the R-PKU rats could not attain the criterion of discriminative learning, even though they had a normal 5-HT concentration in the cerebrum. On the other hand, CNPase activity in the cerebrum of both the PKU and R-PKU rats was decreased significantly, and the learning abilities were also poor. It has been previously reported\textsuperscript{36,37} that the experimental hypothyroid and neonatally hydrocortisone-treated rats showed a significant lower activity of cerebral CNPase and their discriminative learning abilities were also poor. These results strongly suggest that the poor learning ability in terms of operant discriminative learning was more closely related to the dysmyelination in the cerebrum than other neural elements such as DNA content and monoamine contents.

ACKNOWLEDGEMENTS

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