Enhancement of Ambulation-Increasing Effect of Methamphetamine by Peripherally-Administered 6R-L-Erythro-5,6,7,8-Tetrahydrobiopterin (R-THBP) in Mice

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Abstract—Behavioral effects of 6R-L-erythro-5,6,7,8-tetrahydrobiopterin (R-THBP), a co-factor for tyrosine hydroxylase and tryptophan hydroxylase, were investigated by means of ambulatory activity in mice. Single administration of R-THBP (50 and 100 mg/kg, s.c.) showed no significant effect on the mouse's ambulatory activity for 5 hr. The ambulation-increasing effect of methamphetamine (2 mg/kg, s.c.) was dramatically enhanced and prolonged by the pretreatment with R-THBP (100 mg/kg, s.c.) 0, 2, 6, 12 and 24 hr before, but not 18 or 36 hr before, the methamphetamine administration. However, when combined administration of R-THBP (100 mg/kg, s.c., 2 hr before) with methamphetamine (2 mg/kg, s.c.) was repeated at intervals of 3-4 days, the enhancement by R-THBP of the methamphetamine effect was observed only in the 1st and 2nd administration, but not in the later administration. The pretreatment with R-THBP (100 mg/kg, s.c., 2 hr before) enhanced the ambulation-increasing effect of ephedrine (80 mg/kg, i.p.), but failed to modify those of cocaine (20 mg/kg, s.c.), mazindol (2.5 mg/kg, s.c.), bromocriptine (8 mg/kg, i.p.), morphine (20 mg/kg, s.c.) and scopolamine (0.5 mg/kg, s.c.). It is noteworthy that R-THBP differentially modifies the ambulation-increasing effect of the above-mentioned drugs.

It is known that 5,6,7,8-tetrahydrobiopterin (THBP) is a natural co-factor for tyrosine hydroxylase and tryptophan hydroxylase, rate-limiting enzymes in the biosynthesis of catecholamines and indoleamine, respectively. It has been reported that an alteration of THBP metabolism is responsible for the rare childhood disease of THBP-deficient hyperphenylalaninemia, which is also related to an atypical phenylketonuric disease (PKU) (3). Additionally, deficits of THBP metabolism have been demonstrated in various types of neurological and psychiatric diseases including Parkinson's disease (4, 5), familial dystonia (6, 7), endogenous depression (8), infantile autism (9), etc.; and therapeutic trials with peripheral administration of THBP have been initiated in these diseases with limited success (3-9).

In animal studies, the THBP content in the whole rat brain increases for 2 hr after i.p. administration of a comparatively higher dose of THBP, although only a small percentage of peripherally-administered THBP enters into the brain (10). A natural isomer of THBP, 6R-L-erythro-5,6,7,8-tetrahydrobiopterin (R-THBP), has been demonstrated to penetrate the blood-brain barrier more effectively than THBP (11, 12). It has been also reported that intraventricular injection of R-THBP elicits not only significant increases in the activities of both tyrosine hydroxylase and tryptophan hydroxylase, but also increases in dopamine and serotonin metabolisms (11). In these respects, R-THBP seems to play an important role in the function of the central nervous system. However, characteristics of the central effect of R-THBP have not been known when this drug is peripherally administered.

The purpose of this research was to con-
duct a behavioral study on the effects of R-THBP by means of ambulatory activity in mice. In this experiment, R-THBP was administered singly or repeatedly and combined with several central-acting drugs, all of which increase mouse’s ambulatory activity through direct or indirect stimulation of central catecholaminergic systems.

Materials and Methods

1 Animals

The experimental animals used were male mice of the ddY strain (Japan Laboratory Animal), which were provided at 5 weeks of the age. The groups of 10 mice had been housed in aluminum cages of 30 (W) x 20 (D) x 10 (H) cm with a wooden-flake floor mat (White Flake: Charles River Japan, Inc.) and given food and tap water ad libitum until the start of the experiment. The breeding room was controlled in illumination (light period: 6:00–18:00) and temperature (22±2 °C). The experiment was conducted when the mice were 7 weeks of age and weighed 28–35 g.

2 Apparatus

The apparatus for measurement of the mouse’s ambulatory activity was a tilting-type ambulometer (AMB-10: O’hara & Co.) (13). The apparatus consisted of 10 bucket-like activity cages, each of which was made of plexiglas and had a diameter of 20 cm. Therefore, the ambulatory activities of 10 mice could be measured individually at the same time.

3 Drugs and administration schedules

The drugs used were R-THBP (Suntory), methamphetamine HCl (Philopon, Dainippon Pharm.), ephedrine HCl (Dainippon Pharm.), cocaine HCl (Takeda Chem.), mazindol (Sandoz), bromocriptine mesylate (Sandoz), morphine HCl (Takeda Chem.) and scopolamine HBr (Sigma Chem.). Except for mazindol and bromocriptine, the drugs were dissolved in physiological saline. Mazindol was first dissolved in a small volume of 0.1 N HCl solution, then diluted by physiological saline. Bromocriptine was dissolved in physiological saline with one drop of Tween 80 per 3 ml. The concentration of each drug solution was adjusted so that each volume administered was always constant at 0.1 ml/10 g body weight regardless of the drug doses. Since R-THBP dissolved in water was unstable, the solution was used within 1 min after its preparation.

1) Single administration

R-THBP alone: Mice were individually put into activity cages, and after an adaptation period of 30 min, R-THBP at 50 or 100 mg/kg or physiological saline was administered s.c. The ambulatory activity of each mouse was observed for 5 hr thereafter.

Combination of R-THBP with methamphetamine: Four experiments were conducted to observe interactions between R-THBP and methamphetamine.

a) R-THBP at 50 or 100 mg/kg or physiological saline was administered s.c. to mice; and 2 hr later, the mice were treated with methamphetamine at 2 mg/kg, s.c.

b) R-THBP at 100 mg/kg or saline was first administered to mice, and 2 hr after the dosing, the mice were treated with methamphetamine at 0.5, 1 or 2 mg/kg, s.c.

c) R-THBP at 100 mg/kg or saline was first administered to 14 groups of mice. Each group of mice treated with R-THBP or saline were given methamphetamine at 2 mg/kg with a time span of either 0 (simultaneous administration), 2, 6, 12, 18, 24 or 36 hr. To avoid a circadian variation in the methamphetamine sensitivity (14), the time-of-day of R-THBP administration was adjusted so that the mice were always given methamphetamine between 12:00–12:30 of the light period.

d) Mice were first treated with methamphetamine at 2 mg/kg, s.c.; and 4 hr after the dosing, they were given R-THBP at 100 mg/kg, s.c.

Combination of R-THBP with other central-acting drugs: R-THBP at 100 mg/kg or saline was first administered s.c.; and 2 hr after the dosing, it was followed by treatment with second dosing of one of ephedrine, 80 mg/kg, i.p.; cocaine, 20 mg/kg, s.c.; mazindol, 2.5 mg/kg, s.c.; bromocriptine, 8 mg/kg, i.p.; morphine, 20 mg/kg, s.c.; and scopolamine, 0.5 mg/kg, s.c. After the second dosing, the mouse’s ambulatory activity was observed for 2 to 6 hr depending on the drugs. The doses and administration routes of methamphetamine, ephedrine, cocaine, mazindol, bromocriptine, morphine and scopolamine employed in this experiment were taken to be optimum to adequately increase ambulatory activity in
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mice according to our previous investigations (13, 15-18).

2) Repeated administration

R-THBP alone: R-THBP at 50 or 100 mg/kg or saline was administered s.c. 5 times at intervals of 3-4 days, and the mouse's ambulatory activity was observed for 5 hr after each administration.

Combination of R-THBP with methamphetamine: Repeated methamphetamine administration elicits a reverse tolerance to its ambulation-increasing effect. We studied the effect of R-THBP on the reverse tolerance to methamphetamine. Mice were first treated with R-THBP at 50 or 100 mg/kg or saline, s.c.; and 2 hr later, they were given methamphetamine at 2 mg/kg, s.c. The same treatment was conducted 5 times at intervals of 3-4 days.

4 Urinary pH level

Just before and 5 hr after the administration of R-THBP (100 mg/kg, s.c.) or saline, the urinary pH levels of mice were measured by bromothymol blue test-paper (Toyo Filter Paper). During the observation period, food and water were removed from the cages.

5 Statistical analyses

Statistical comparisons of the mean ambulatory activity counts or mean urinary pH levels were conducted using Student's t-test. The number of mice that died after R-THBP were compared using the x-square test. When P values were equal to or less than 0.05, they were taken to indicate a significant difference.

Results

1) Single administration

R-THBP alone: The administration of R-THBP (50 or 100 mg/kg) alone produced no significant change in the mouse's ambulatory activity for 5 hr (data not shown), because the baseline activity of saline-treated control mice showed much lower activity throughout the observation period.

Combination of R-THBP with methamphetamine: a) Figure 1 shows the time-course of changes in the ambulatory activity in the mice that were treated with the combination of R-THBP and methamphetamine. In the saline-pretreated control mice, methamphetamine increased the ambulatory activity for 3 hr, with the maximum effect at about 1 hr after the administration. The pretreatment with R-THBP at 50 mg/kg failed to modify the ambulation-increasing effect of methamphetamine (MAP: 2 mg/kg, s.c.) in the mice that were pretreated with R-THBP (0, 50 or 100 mg/kg, s.c.) 2 hr before MAP administration. * indicates significant difference as compared with the ambulatory activity counts in the control mice administered the saline vehicle (P<0.05, Student's t-test).

Fig. 1. Time-course changes in mean ambulatory activity counts after administration of methamphetamine (MAP: 2 mg/kg, s.c.) in the mice that were pretreated with R-THBP (0, 50 or 100 mg/kg, s.c.) 2 hr before MAP administration. * indicates significant difference as compared with the ambulatory activity counts in the control mice administered the saline vehicle (P<0.05, Student's t-test).
amine. However, the effect of methamphetamine was markedly enhanced and prolonged by R-THBP at 100 mg/kg.

b) Figure 2 shows the mean overall ambulatory activity counts after administration of methamphetamine at 0.5, 1 and 2 mg/kg to the mice that were pretreated with R-THBP at 100 mg/kg or saline 2 hr before. Methamphetamine scarcely increased the mouse's ambulatory activity at 0.5 mg/kg, while it dose-dependently increased the activity at 1 and 2 mg/kg. Although R-THBP at 100 mg/kg did not modify the effect of methamphetamine at 0.5 mg/kg, it enhanced the ambulation-increasing effect of methamphetamine at 1 and 2 mg/kg.

c) Figure 3 shows the mean overall ambulatory activity counts for 5 hr after administration of 2 mg/kg of methamphetamine to mice that had been pretreated with R-THBP at 100 mg/kg or saline at 0, 2, 12, 18, 24 or 36 hr before methamphetamine. There was no significant difference in the ambulatory activity counts among the 7 saline-treated groups. The pretreatment with R-THBP at 0, 2, 6, 12 and 24 hr before, but not at 18 and 36 hr before, were effective for enhancing the ambulation-increasing effect of methamphetamine. The maximum enhancement was observed when R-THBP was pretreated at 2 hr before.

d) The ambulation-increasing effect of methamphetamine almost abolished 4 hr

**Fig. 2.** Mean overall ambulatory activity counts for 5 hr after administration of methamphetamine (0.5, 1 or 2 mg/kg, s.c.) in the mice that were pretreated with R-THBP (100 mg/kg, s.c.) or saline 2 hr before the methamphetamine administration. *: significant difference compared to the saline-pretreated control value (P<0.05). Each figure in parenthesis indicates number of mice used.

**Fig. 3.** Mean overall ambulatory activity counts for 5 hr after administration of methamphetamine (MAP: 2 mg/kg, s.c.) in the mice that were pretreated with saline or R-THBP (100 mg/kg, s.c.) at 0, 2, 6, 12, 18, 24 or 36 hr before MAP administration. *: significant difference compared to the saline-treated control value (P<0.05). Each figure in parenthesis indicates the number of mice used.
after the administration. When R-THBP at 100 mg/kg was administered 4 hr after methamphetamine, the mice did not show any increase in the ambulatory activity (data not shown).

**Combination of R-THBP with centrally acting drugs:** Figure 4 shows time-course changes in the ambulatory activity counts after the administration of ephedrine, 80 mg/kg (i.p.); cocaine, 20 mg/kg (s.c.); mazindol, 2.5 mg/kg (s.c.); bromocriptine, 8 mg/kg (i.p.); morphine, 20 mg/kg (s.c.); and scopolamine, 0.5 mg/kg (s.c.) in the mice that were pretreated with R-THBP at 100 mg/kg (s.c.) or saline (s.c.) 2 hr before each drug administration. The doses of these drugs were taken from our previous experiments (13-16). Although the grade and duration of the effects were different among the drugs, all of them increased the mouse's ambulatory activity. R-THBP specifically enhanced the ambulation-increasing effect of ephedrine, but those of cocaine, mazindol, bromocriptine, morphine and scopolamine were not modified by R-THBP.

2) Repeated administration

**R-THBP alone:** Repeated administration of R-THBP at 50 and 100 mg/kg produced no remarkable change in the mouse's ambulatory activity throughout the 5 times administration (data not shown).

**Combination of R-THBP with methamphetamine:** Repeated administration of methamphetamine at 2 mg/kg, s.c. produced no remarkable change in the mouse's ambulatory activity throughout the 5 times administration (data not shown).
reverse tolerance, an enhancement of the sensitivity, to the ambulation-increasing effect. R-THBP at 50 mg/kg hardly modified the process of reverse tolerance. In the case of the combination of 100 mg/kg of R-THBP and methamphetamine, the activity counts in the 1st and 2nd administrations were significantly higher as compared with methamphetamine alone. However, the activity counts in the later administrations were almost the same as those of the mice given methamphetamine alone (Fig. 5).

3) General symptoms

Table 1 shows occurrences of dead mice within 24 hr after the drug treatments. R-THBP at 100 mg/kg, but not 50 mg/kg, showed a lethal effect, eliciting the death of 4/30 (13%) mice. The occurrence of dead mice distributed between 0% (treatment with R-THBP+MAP, 0.5 mg/kg; R-THBP+

![Fig. 5. Changes in mean overall ambulatory activity counts in mice during the 5 hr observation after repeated combined administration of R-THBP (0, 50 or 100 mg/kg, s.c.) and methamphetamine (MAP: 2 mg/kg, s.c.). The experiment was conducted 5 times at intervals of 3–4 days. In each experiment, saline and R-THBP were administered 2 hr before MAP administration. *: significant difference as compared to the saline-pretreated control value (P<0.05).]

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Occurrence</th>
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<tbody>
<tr>
<td>Saline alone</td>
<td>0/20</td>
</tr>
<tr>
<td>R-THBP, 50 mg/kg, alone</td>
<td>0/20</td>
</tr>
<tr>
<td>R-THBP, 100 mg/kg, alone</td>
<td>4/30</td>
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<tr>
<td>R-THBP, 100 mg/kg + methamphetamine, 0.5 mg/kg</td>
<td>0/10</td>
</tr>
<tr>
<td>R-THBP, 100 mg/kg + methamphetamine, 1 mg/kg</td>
<td>5/20</td>
</tr>
<tr>
<td>R-THBP, 100 mg/kg + methamphetamine, 2 mg/kg</td>
<td>4/20</td>
</tr>
<tr>
<td>R-THBP, 100 mg/kg + ephedrine, 80 mg/kg</td>
<td>7/20</td>
</tr>
<tr>
<td>R-THBP, 100 mg/kg + cocaine, 20 mg/kg</td>
<td>2/20</td>
</tr>
<tr>
<td>R-THBP, 100 mg/kg + mazindol, 2.5 mg/kg</td>
<td>0/10</td>
</tr>
<tr>
<td>R-THBP, 100 mg/kg + bromocriptine, 8 mg/kg</td>
<td>1/20</td>
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<tr>
<td>R-THBP, 100 mg/kg + morphine, 20 mg/kg</td>
<td>3/13</td>
</tr>
<tr>
<td>R-THBP, 100 mg/kg + scopolamine, 0.5 mg/kg</td>
<td>0/10</td>
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Time interval between the administrations of R-THBP and other drugs was 2 hr.
mazindol; and R-THBP+scopolamine) and 35% (R-THBP+ephedrine). However, these occurrence rates were not significantly different from that observed after treatment with R-THBP (x-square test). The surviving mice also exhibited severe general symptoms that were characterized by a decrease in body weight, a mild tremor, a bad coat of hair, etc. These symptoms attained a maximum level at 18–24 hr and persisted for 2–3 days.

The control mice showed a body weight gain of 5.6 g during the experimental period of repeated administration. The mice repeatedly treated with R-THBP at 50 mg/kg showed no remarkable change of their body condition throughout the administration period. However, at 100 mg/kg, 4/20 and 1/16 mice died after the 1st and 2nd administrations, respectively, but after the 3rd–5th ones, no mice died. Although the surviving mice failed to gain body weight after the 1st and 2nd administrations, their body weight tended to be at the initial level until the 4th administration.

4) Urinary pH level
R-THBP did not modify the mouse’s urinary pH level (data not shown).

Discussion
R-THBP is the natural isomer of THBP, a co-factor for tyrosine hydroxylase and tryptophan hydroxylase (10, 12). Therefore, it is primarily expected that a single dosing of R-THBP may markedly stimulate the central catecholaminergic systems and is effective to increase the mouse’s ambulatory activity like that observed after stimulant drugs. However, the first experiment of our study showed that R-THBP alone failed to modify the ambulatory activity at 100 mg/kg, but demonstrated a lethal effect. Miller et al. (19) reported that the administration of THBP at 20 mg/kg, i.v. did not modify the metabolism of monoamines in the brain of the monkey, although the drug was detected for 15 hr with the maximum concentration at 1.5–2 hr in the cerebrospinal fluid. Levine et al. (20) also observed a similar result in rats at 50 mg/kg, i.p. It might be therefore reasonable that peripherally administered R-THBP alone scarcely shows a clear central effect even at sublethal to lethal doses. The lethal effect may be caused by non-specific action.

To investigate the possibility that R-THBP may have a central effect, we conducted combined administration of R-THBP with typical central-acting drugs. The pretreatment with R-THBP at 50 mg/kg scarcely modified the ambulation-increasing effect of methamphetamine. However, it is notable that R-THBP at 100 mg/kg enhanced the effect of methamphetamine. This result indicates that a comparatively higher dose of R-THBP is required to modify the ambulation-increasing effect of methamphetamine and that R-THBP’s effect persists approximately 24 hr. The present experiment demonstrated that the effect of R-THBP attained the maximum level at about 2 hr after its administration. This result is consistent with the metabolic data on THBP by Miller et al. (19).

It has been well-known that a change in the urinary pH level is closely related to the sensitivity to methamphetamine (21, 22), because urinary excretion of methamphetamine is strongly dependent on urinary pH level. However, mice treated with 100 mg/kg maintained the baseline level of urinary pH. This finding indicates that the change in the urinary excretion of methamphetamine is scarcely involved in the interaction between R-THBP and methamphetamine.

It is unclear why the effect of R-THBP transiently disappeared when it was administered 18 hr before methamphetamine. The body condition of mice became worse during the time period of 18–24 hr after R-THBP. The time period corresponded to that for observing the sensitivity to methamphetamine in the mice that had received R-THBP 18 hr before, suggesting a non-specific change. However, a further study including pharmacokinetic and neurochemical examinations is required to elucidate the time-dependent interactions between R-THBP and methamphetamine.

On the other hand, R-THBP failed to increase the mouse’s ambulatory activity when it was administered 4 hr after methamphetamine. The time span might be enough to abolish the main effects of methamphetamine in mice (23). The pretreatment with R-THBP at 100 mg/kg did not potentiate the effect of methamphetamine at 0.5 mg/kg. R-THBP at
50 mg/kg also failed to modify the effect of methamphetamine at 1 and 2 mg/kg. These results indicate that the enhancement of the ambulation-increasing effect of methamphetamine by R-THBP is produced when sufficient amounts of the two drugs exist in the brain at the same time.

Methamphetamine is considered to cause the release of newly synthesized, but not stored, catecholamines, in particular dopamine, because the central stimulant effect is blocked by pretreatment with α-methyl-p-tyrosine, but not with reserpine (24, 25). It is therefore postulated that R-THBP enhances the methamphetamine effect through its action as a co-factor for tyrosine hydroxylase, i.e., eliciting an enhancement of catecholamine synthesis. In agreement with this consideration, R-THBP enhanced the ambulation-increasing effect of ephedrine, which is thought to show a similar neurochemical action to methamphetamine (24).

It is also noteworthy that pretreatment with R-THBP at 100 mg/kg hardly modified the ambulation-increasing effect of cocaine, mazindol, bromocriptine, morphine and scopolamine. In our pilot study, R-THBP was ineffective to modify the ambulation-increasing effect of apomorphine and methylphenidate in dd strain mice (H. Kuribara, unpublished data). Cocaine and mazindol inhibit reuptake of catecholamines at presynaptic terminals (25, 26). Methylphenidate releases catecholamines from storage sites, which is sensitive to reserpine but insensitive to α-methyl-p-tyrosine (24, 25). Bromocriptine and apomorphine directly stimulate postsynaptic dopamine receptors (27, 28). Catecholaminergic systems are indirectly stimulated through agonistic action on opioid receptors by morphine (29) and antagonistic action on muscarinic-cholinergic systems by scopolamine (30). Lee and Mandel (31) demonstrated that repeated dosing of amphetamine decreased the THBP level in the rat whole brain, while Brautigam et al. (32) reported that apomorphine produced no significant change in THBP level in the corpus striatum. These neurochemical data are consistent with our results that R-THBP enhances the ambulation-increasing effect of methamphetamine, but not those of bromocriptine and apomorphine.

The effects of methamphetamine and cocaine on animal behaviors, particularly on ambulatory activity, are quite similar to each other. It is therefore noteworthy that the behavioral characteristics of methamphetamine and cocaine can be differentiated by using R-THBP.

When methamphetamine is repeatedly administered to the mouse, the ambulation-increasing effect is progressively enhanced, showing reverse tolerance (33). The present experiment also confirmed the reverse tolerance to methamphetamine in the saline-treated control group. It is, however, notable that although R-THBP at 100 mg/kg enhanced the effect of methamphetamine in the 1st and 2nd administration in the combined repeated administration regimen, the effect was abolished in the 3rd and later dosing. The ambulatory activity counts in the 3rd–5th administration were almost comparable between R-THBP-treated and saline-treated groups. However, the mechanism is not elucidated yet, and further investigations are required.

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References
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