A Supplemental Measure of Stay-Times in the Light Box on Step-Through Passive Avoidance

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ABSTRACT—The avoidance behavior of rats in a typical step-through passive avoidance task was evaluated by using two types of time measurements that differed from traditional step-through latency (STL). The total stay-time in the light box (TL) was calculated as an index of the dark box avoidance throughout the retention trial, counting the time before and after the first step-through. The total stay-time in the far area of the light box (TF) was calculated as an index of the tendency to keep away from the dark box. TL, TF and the STL increased with electrical shock intensity at acquisition trials and gradually decreased through an extinction procedure. The discrepancy between our new measures and the STL was observed when the effects of a new drug, RS-8359, which was reported to ameliorate ischemic brain damage, were examined in ischemic animals. TL, TF and STL decreased in rats receiving brain ischemia, and we found that after treatment with RS-8359, TL and TF increased, but no effect was observed on STL. The discrepancy observed suggests that a short STL does not necessarily imply a loss of avoidance behavior. Passive avoidance tests can be more revealing about a drug's effects when stay-time measures are used.

Keywords: Passive avoidance, Step-through latency, Stay-time, Memory impairment, MAO-A inhibitor

Step-through type passive avoidance is one of the most popular methods to test memory and learning, especially in pharmacological studies (1–3), but it has not proved to be a fully reliable method for selecting a medicine to improve memory impairment. We observed the behavior of animals in a passive avoidance apparatus to determine whether improvements could be made in this method.

Avoidance behavior during a retention trial is used as an index of memory and usually measured by the time between the start of the retention trial and entrance into a dark box, called the step-through latency (STL). Although STL is thought to be a function of the intensity of avoidance, animals often show characteristic avoidance responses that are not reflected in this measure. First, STL does not distinguish between animals that jump out of the dark box immediately after the first entrance and animals that remain inside. Second, among the animals that do not enter the dark box at the retention trial, some animals keep away from the dark box, while others approach and peep into the dark box repeatedly.

Instead of STL, time measures such as duration of dark compartment entries (4) or neutral area stay time (5, 6) were used as measures in a few studies, but we could find no report that made comparisons of such stay-time meas-

ures with STL. Moreover, few reports have analyzed the basic behavioral pattern observed in the passive avoidance task (7–9). In the present study, we recorded the movements of the animals during the retention trial to obtain two measures, total time staying in the light box (TL) and total time staying in the half area of the light box opposite to the dark box (stay-time in the far area: TF).

To examine whether these stay-time measures are associated with the degree of learning, the effects of varying foot shock intensity and the effect of an extinction procedure on these measures were tested. The amnesic effect of forebrain ischemia was also examined. Furthermore, we found an interesting discrepancy between STL and TL in an experiment using RS-8359, a new A-type monoamine oxidase specific inhibitor that is reported to ameliorate ischemic amnesia (10).

MATERIALS AND METHODS

Subjects

We used ten-week-old Male Wistar rats (Clea Japan, Tokyo) weighing about 300 g. The rats were maintained in our colony for at least 1 week prior to the beginning of

each experiment. They were housed in groups of three to five in a metal cage under 12 hr of light from 7:00 to 19:00. All behavioral experiments and ischemic operations were performed between 8:00–14:00.

Apparatus

We used a typical apparatus for step-through passive avoidance (Type PA-R; O'hara & Co., Tokyo) which consists of a light box (20 cm H $\times$ 24 cm W $\times$ 40 cm L) and a dark box (20 cm H $\times$ 14 cm W $\times$ 20 cm L). The light box was marked down the middle to distinguish the near and far areas from the dark box (Fig. 1A). The dark box was separated from the light box with a guillotine door. A grid floor consisting of 2-mm stainless steel rods spaced 1 cm apart extended throughout the bottom of the boxes.

An electric foot shock of 0.33 mA AC was given via the grid floor of the dark box by a 1 kV AC power supply through serially sequenced 3.0 Mohm resistance. During the initial experiment, the current was varied between 0.125–0.5 mA using 8.0–2.0 Mohm resistance. The current intensity between the grids was checked before each acquisition trial with a current meter.

Procedure

Habituation trials and acquisition trials were performed on the first experimental day and the subjects were tested for retention of passive avoidance response 24 hr later. The habituation trial was performed 30–60 min before the acquisition trial, wherein a rat was placed in the far area of the light box and the guillotine door

Fig. 1. Apparatus and the typical patterns of movement of rats during the retention trial. A: The apparatus used in this study. B to D: Typical patterns of movement of rats at each time in the 10-min retention trial. The location of the animals represented by the shaded areas. B: Ambulatory pattern of a normal rat that received no foot shock in the acquisition trial. C and D: The ambulatory patterns of two rats that received 0.33 mA current during the acquisition trial. The shadows across the near area and dark box portions of the graphs represent occasions when the rats place both forepaws inside the dark box.

opened 5 sec later. The animal was allowed to explore the apparatus freely, and the door was gently closed just after the animal put all four paws into the dark box. After being confined in the dark box for 5 sec, the animal was returned to its home cage where it remained until the acquisition trial.

During the acquisition trial, the rat was again placed in the far area of the light box facing away from the dark box and the door opened. The door was closed just after the animal put all four paws inside the dark box and then the electric foot shock was given for 5 sec. Pain reactions, vocalization, urination and defecation induced by the shock were recorded as indicators of its effect. Before the next trial, the grid floor was cleaned to adjust the current. The small number of animals that showed a latency of over 60 sec at the acquisition trial were omitted from further experiments.

The day after the acquisition trials, retention trials were performed to test the influence of the foot shock given during the acquisition trials. Animals were put in the far area of the light box and the door opened as previously, but this time the foot shock was turned off and the door left open, and the movements of the animals were recorded for 10 min. All the crossings between the far and near areas and between the light box and dark box were recorded.

Forebrain ischemia

A number of animals were subjected to transient forebrain ischemia by 4-vessel occlusion (11) just after the acquisition trial. One day before the acquisition trial, the animal's vertebral arteries were cauterized bilaterally by using an electric coagulator (Sunstar SES-01, Osaka), under pentobarbital anesthesia. The following day, the rats that could not move around freely during the habituation trial were excluded from the subsequent experiments. Each animal was placed in a gas chamber filled with anesthetic gas of 1.5% halothane in 67% N₂O and 33% O₂, and moved on a board with a mask of halothane gas to expose the common carotid arteries. Three minutes after the acquisition trial, the carotid arteries were occluded bilaterally with aneurysm clips, and the halothane mask was then removed. After 10 min occlusion, the clips were removed and the arteries were perfused. The animals that recovered the righting reflex within 3 min of the perfusion were eliminated. To observe neuronal cell death after ischemia, brain sections were prepared. Eight days after ischemia, rats were anesthetized with pentobarbital and perfusion-fixed with 4% paraformaldehyde in 50 mM phosphate buffer, pH 7.4, and paraffin sections of the dorsal hippocampus were stained with hematoxylin and eosin. The number of cells with clear nuclei within 1-mm length of the middle of the CA1 pyramidal cell layer were

counted, and the average number of cells of the left and right CA1 in each rat was used for further analysis.

Experiment 1

The effect of reinforcement was examined by varying the foot-shock: 0, 0.125, 0.25 and 0.5 mA.

Experiment 2

The effect of extinction of passive avoidance was examined. Retention trials, where animals explored the apparatus for 10 min without foot shocks, were repeated daily for 5 days. The same procedure was also carried out with six animals receiving no acquisition trial for comparison.

Experiment 3

The amnesic effect of transient forebrain ischemia was examined by giving 4-vessel occlusion for 10 min or sham-operation just after the acquisition trial.

Experiment 4

As RS-8359 is known to ameliorate ischemic damage of the brain (10, 12, 16, 17), the effect of the drug on ischemic amnesia was examined where we found a discrepancy between STL and TL. RS-8359, ($\pm$)-4-(4-cyanoanilino)-7-hydroxycyclopenta (3, 2-*e*) pyrimidine, was suspended in 0.5% CMC and orally administered three times: just after perfusion, during the evening (18:00–18:30) following the acquisition trial and 1 hr before the retention trial. A solution of 0.5% CMC was administered to the control animals. RS-8359 is known to have little effect on brain temperature (12).

Statistics

Analysis of variance (ANOVA) and Student's *t*-test (two-tailed) were used for most statistical analyses of data. When the variance was significantly different between two groups, the *U*-test was used instead of the *t*-test. Correlation coefficients were obtained by the least squares method.

RESULTS

Typical ambulatory patterns of the rats during the retention trials are shown in Fig. 1. Animals not receiving a foot shock (Fig. 1B) entered the dark box immediately and then repeatedly exited and reentered thereafter. Animals that received a 0.33 mA foot shock during the acquisition trial (Fig. 1, C and D), however, showed quite a different behavior pattern. Typically, they at first froze in the far area, facing the dark box, and then slowly started moving to the near area, directing their noses towards the doorway to the dark box. While repeating movements

back and forth between the far and near areas, their speed gradually increased, and the rats began to display sniffing and rearing. Behavior such as biting around the opening and peeping into the dark box was frequently observed. As peeping became more frequent, the animals ventured further and further into the dark box. Finally, they placed both hind-limbs into the dark box, which we defined as step-through. The door was left open and the rat's behavior after step-through was also recorded. Although a foot shock was never given during the retention trials, several animals jumped out of the dark box immediately after entrance, and kept away afterwards (Fig. 1D).

These observations show that the characteristic behavior induced by foot shock is not only inhibition of the first entrance into the dark box. To more fully represent the rat's actual behavior, we calculated two values. The total stay-time in the far area of the light box (TF) was calculated as an index of the tendency to keep away from the dark box. The total stay-time in the light box (TL) was calculated as an index of the dark box avoidance throughout the 10 min retention trial, counting the time before and after the first step-through.

Experiment 1

The effect of the intensity of the foot shock was examined to evaluate whether TL and TF were suitable as indices of avoidance learning (Fig. 2). While the animals without foot shock stayed in the light box for about half of the retention trial, the animals that received 0.25–0.5 mA stayed in the light box for more than three quarters

Fig. 2. Effect of foot shock intensity during the acquisition trial on performance in the retention trial. Wide columns show mean light box stay-time (TL) and unshaded areas of the columns show mean far area stay-time (TF). Adjacent narrow columns show the mean step-through latency. Numbers of animals are shown in parentheses below. Bars on the columns show standard errors of the mean.

of the retention trial. ANOVA showed the effect of foot shock was highly significant in TL ($F(3,13)=10.41$, $P<0.01$) and STL ($F(3,13)=10.71$, $P<0.01$). Positive correlations with the intensity of electrical foot shock were seen in TL ($r=0.79$, $P<0.01$) as well as in STL ($r=0.81$, $P<0.01$). Although ANOVA fell short of significance for TF ($F(3,13)=3.16$), it did, however, significantly correlate with foot shock intensity ($r=0.73$, $P<0.01$).

In the following experiments, a current intensity of 0.33 mA was used because of its demonstrable but minimum aversive effect on the animals. All animals receiving 0.33 mA shock showed pain reactions, but some of the animals receiving 0.25 mA did not.

Experiment 2

The extinction of the passive avoidance was examined. TL and TF were seen to diminish during this extinction procedure (Fig. 3, A and B) and comparable changes were observed on STL (Fig. 3C). ANOVA showed that the individual daily changes in TL ($F(4,48)=20.12$, $P<0.01$), TF ($F(4,48)=20.92$, $P<0.01$) and STL ($F(4,48)=36.30$, $P<0.01$) were highly significant. The differences between rats that did and did not undergo acquisition trials observed in TL ($F(1,12)=7.23$, $P<0.05$), TF ($F(1,12)=8.25$, $P<0.05$) and STL ($F(1,12)=14.27$, $P<0.01$) were significant. After 2 trials, the differences in TL and TF between the two groups were statistically indistinguishable. The differences in the STL were statistically indistinguishable after 4 trials. The division of the 10 min retention trial into four 150-sec blocks shows that TF was high in the early phase of the first retention trial (Fig. 3B), consistent with Fig. 1, C and D. The changes in TL ($F(3,36)=6.97$, $P<0.01$) and TF ($F(3,36)=9.43$, $P<0.01$) between the blocks were significant in ANOVA. The graph of TL on day 2 is V-shaped because many animals stayed in the far area at first, gradually approached and explored the dark box during the middle period of the trial, and finally went back to the far area and stayed there.

Experiment 3

The effect of brain specific damage was examined using transient forebrain ischemia. Ten-minute ischemia was applied just after the acquisition trial. As shown in Table 1, TL and TF were significantly reduced as was STL.

Experiment 4

RS-8359 prolonged the reduced TL caused by ischemia ($P<0.05$, Student's *t*-test), although it had no effect on STL (Table 1). The increase in TL was mainly due to the rat's behavior in keeping away from the dark box, because TF also tended to increase ($0.05<P<0.06$). Some ambulatory patterns of ischemic animals are shown in

Fig. 3. Extinction of the passive avoidance. Ten-minute retention trials were repeated once daily for 5 days after the acquisition trial using 0.33 mA. Black squares or columns show the acquisition group ($n=8$) and the white ones show no-acquisition group ($n=6$). Data represent means $\pm$ S.E.M. A: TL values obtained each day are plotted side-by-side. Each 10-min period is divided into four 150-sec blocks for further comparison. B: Daily changes in TF. C: Daily changes in STL. The asterisks in A and B indicate the difference between two groups at each time point. *: $P<0.05$, **: $P<0.01$, by Student's t -test. In C, the U -test was used because the variance of STL was very different between the two groups. *: $P<0.05$, **: $P<0.01$.

Table 1. Effect of forebrain ischemia and effect of RS-8359

Ischemia	Drug	n	TL	TF	Latency
Exp.3					
Sham	—	6	568.9 $\pm$ 25.7	381.1 $\pm$ 41.7	536.0 $\pm$ 42.1
Isc	—	12	415.4 $\pm$ 39.2*	226.3 $\pm$ 32.9*	192.3 $\pm$ 67.9**
Exp.4					
Isc	vehicle	10	314.6 $\pm$ 41.0	146.8 $\pm$ 21.4	111.6 $\pm$ 35.8
Isc	RS-8359	12	431.0 $\pm$ 37.6 [#]	249.5 $\pm$ 42.2	130.0 $\pm$ 49.7

Values are means $\pm$ S.E.M. Two-tailed Student's t -test was used for comparison between groups. *: $P<0.05$, **: $P<0.01$ vs sham. The ischemic rats were given RS-8359 at 30 mg/kg, p.o. or 0.5% CMC solutions (0.3 ml/300 g-weight) three times. [#]: $P<0.05$ vs CMC-treated ischemic group.

Fig. 4. One vehicle-treated animal stayed mainly inside the dark box (Fig. 4C), while a drug-treated animal kept away from the dark box after entering (Fig. 4E). Another drug-treated animal (Fig. 4F) entered the dark box with a short STL, but it immediately jumped out. This animal often displayed partial entrance behavior, which was only observed in foot shock-trained animals (see Fig. 1, C and D). A vehicle-treated animal, shown in Fig. 4D, exhibited comparable STL to a drug-treated animal (Fig. 4F), but its behavior after its first entrance into the dark box was indistinguishable from that of the no foot-shock animal (see Fig. 1B).

To illustrate the discrepancy between TL and STL more clearly, the relationships between TL and STL for each animal are shown in Fig. 5A. Although the latencies

Fig. 4. Examples of the movement of ischemic rats during the retention trial. A and B: Patterns of movement of sham-operated rats. C and D: Movement of rats that received 10-min cerebral ischemia and afterwards given only CMC solution. E and F: Movement of rats that received 10-min cerebral ischemia and given RS-8359 at 30 mg/kg, p.o. three times. Other details are the same as in Fig. 1.

of most ischemic animals were lower than 200 sec, drug-treated animals (white squares) showed higher TL than vehicle-treated animals (black squares).

Because the STL is almost equal in both groups, the light box stay-times before the first step-through are equivalent. In order to analyze the avoidance behavior af-

ter the first entrance, the stay-time in the light box within 1 min of the first step-through was calculated for each animal (Fig. 5B). The stay-time in the light box just after first entrance was significantly prolonged by the drug.

To illustrate the effect of RS-8359 on rat's preference for dark, normal rats were applied on acquisition trials

Fig. 5. The effect of RS-8359 in ischemic animals. A: Relationships between TL and STL of ischemic animals treated with CMC (black squares) or RS-8359 (white squares). Ordinates give TL and abscissas give STL. The upper cross shows the mean and S.E.M. of the RS-8359 group. The lower cross shows that of the CMC group. The two groups were significantly different in TL but not in STL. B: Stay-time just after the first step-through. Stay-time in each area within 1 min after the first step-through were calculated for each animal. Columns show the mean light box stay-time and unshaded areas of the columns show the mean far area stay-time. Numbers of animals are shown in parentheses below. Bars on the columns show standard errors of the mean. Only animals whose STL was less than 9 min were used for this analysis. *: $P < 0.05$, CMC-treated ischemic group vs RS-8359-treated ischemic group for light box stay-time, Student's *t*-test (two-tailed).

without foot shock, and RS-8359 30 mg/kg (p.o.) or vehicle were given three times as in Exp. 4. There were no differences between CMC ($n=13$) and drug-treated ($n=12$) animals in TL (264.6 ± 11.7 and 272.4 ± 14.1), TF (116.2 ± 6.0 and 120.6 ± 7.5) or STL (17.5 ± 3.9 and 19.4 ± 4.1).

TL, TF and STL were not correlated with the degree of hippocampal cell death. The correlation value of the number of remaining CA1 cells and TL, TF and STL ($r=0.012$, 0.082 and 0.135 , respectively) were not significant. There was no difference between CMC (51.00 ± 10.47 cells/mm, $n=10$) and RS-8359 groups (59.96 ± 9.65 cells/mm, $n=12$), while the sham-operated animals had 130.33 ± 1.76 cells/mm ($n=3$). Regardless of taking STL or stay-time measures, deficits in step-through passive avoidance may not be dependent on hippocampal damage. However, it is difficult to discuss the correlation between the hippocampal delayed neuronal death which begins more than 3 days after ischemia and the behavior in the extinction trial one day after ischemia.

DISCUSSION

The basic characteristics of stay-time measures were examined. The stronger the stimuli were to the animals, the greater the TL and TF they showed, whereas the extinction procedure had the opposite effect. In the next experiment, the amnesic effect of ischemic brain damage was examined. There have been several studies reporting the amnesic effect of forebrain ischemia in step-through passive avoidance tasks (13) and other memory tasks (10, 14). As shown in Table 1, TL and TF were reduced by forebrain ischemia. These results indicate that TL and TF can be used as indices of memory and learning.

A discrepancy between TL and STL was observed when we examined the effect of RS-8359, a reversible and specific inhibitor of A-type monoamine oxidase (15), which is reported to improve ischemic amnesia in gerbils (10), and several impairments following forebrain ischemia (12, 16, 17).

We found that this drug prolonged the reduced TL of ischemic animals but had no effect on STL. Why did this discrepancy between STL and TL emerge? Figures 4 and 5B indicate that many of the drug-treated animals avoided the dark box more intensely than the vehicle-treated animals after entrance into the dark box in the retention trial. The discrepancy between the STL and the stay-time measures suggests that a short STL does not necessarily imply a loss of avoidance behavior.

We mentioned above that drug-treated animals continued avoidance behavior after step-through, but it might be more accurate to say that animals occasionally stepped through to the dark box during continued

avoidance behavior, due to a kind of motor error. Animals are thought to be drawn to the opening of the dark box by an exploratory tendency and a preference for the dark. However, the avoidance of the dark box based on the memory of the electric foot shock may conflict with the tendency to go inside (8). The short STL of the drug-treated ischemic animals can be due to a failure of stopping at the opening, because enhanced motor activity after brain ischemia (18, 19) may exclude the conflict mentioned above, regardless of the memory effect. If we can remove the assumed stopping failure, for example by making a low wall across the doorway, STL may indicate the difference as well as TL.

TL can be more sensitive than STL in cases where dark box avoidance is evident after the first step-through. TF can also be useful in cases where many animals do not enter the dark box during the retention trial. In this case, STL reaches the maximum value and TF is the only index that can show the difference in avoidance behavior. We have often experienced such "ceiling effects" in step-through tasks using rats and mice.

Some past reports (20–22) showed that after several weeks of recovery from ischemic damage, rats in the passive avoidance task did not display memory deficits, but did so in the radial maze task. These results imply that passive avoidance is less useful than the radial maze task to detect changes in learning and memory. However, one-trial passive avoidance has great advantages because of its convenience. Food control is not required. It takes only a few minutes per animal per day, and needs only two days for one experimental session. Moreover, normal rats have no difficulty in completing this task. As demonstrated in this study, we can get more information from passive avoidance if the behavior of the animals used is carefully observed and analyzed.

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