Role of Cholinergic Neurotransmission in the Amygdala on Performances of Passive Avoidance Learning in Mice

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To clarify the role of cholinergic neurons in the amygdala on learning and memory, scopolamine was injected topically into the bilateral amygdala of mice, and the ability to perform two types of passive avoidance tasks (step-through and step-down) was investigated. On the first day mice performed the learning trial and on the second day their retention was tested. Scopolamine (0.5 or 1 μ g/site) was injected bilaterally into the amygdala 30 min before or immediately after the learning trial or 30 min before the testing trial. Scopolamine impaired the performance ability of the mice dose-dependently only when it was injected 30 min before the learning trial. The results coincided well with the effect of scopolamine injected intraperitoneally. Taken together, these results suggest that the site of action for scopolamine to cause anterograde amnesia is the amygdala, and that cholinergic neurons projecting to the amygdala play an important role in memory acquisition in the two passive avoidance tasks.

Keywords cholinergic neuron; amygdala; scopolamine; passive avoidance task

Numerous studies have suggested that cholinergic systems in the central nervous system play an important role in memory in human and animals.¹⁾ Scopolamine, a muscarinic receptor antagonist, causes amnesia in human,^{2,3)} which can be cured by administration of the analog of acetylcholine.⁴⁾ The reduction of presynaptic cholinergic markers such as the activity of choline acetyltransferase in the cortex, amygdala and other regions has been observed in Alzheimer's disease patients.⁵⁾ However, muscarinic receptors are found in virtually every region of the central nervous system, and the net level of muscarinic blockade would not be universal throughout the brain. It is not known which cholinergic neurons account for the inhibitory effect of scopolamine injected systemically. Local application of muscarinic agents should be useful in defining the still mysterious functions of central cholinergic neurons.¹⁾

The amygdala receives cholinergic projections from the nucleus basalis of Meynert as well as cortex. Several recent findings from our laboratory indicate that 1) the cholinergic system in the amygdala is involved in the memory, 2) parameters of cholinergic neuronal activities in the amygdala change after the performance of learning tasks,⁶⁻⁹⁾ 3) lesion of the amygdala impairs the performing ability of mice, and 4) the deteriorated ability of basal forebrain-lesioned mice in two passive avoidance tasks was improved by administration of carbachol into the amygdala (Segawa *et al.*, unpublished data).

In the present study, we examined the effects of scopolamine administered directly into the amygdala at various times in two types of passive avoidance tasks, and compared them with the results from systemic injection.

MATERIALS AND METHODS

Animals Male Std-ddY mice, 8 weeks-old (SLC, Hamamatsu, Japan), were housed in groups of 5—7 in metal cages (30 × 20 × 10 cm) in a temperature- (22 ± 1 °C) and humidity-(55 ± 5%) controlled room. Food (CE-2, Clea Japan, Inc., Tokyo, Japan) and water were provided

ad libitum.

Implantation of the Cannulae Approximately one week after their purchase, mice were anesthetized with a combination of ketamine (80 mg/kg, i.m.) and xylazine (7 mg/kg, i.m.) and fixed to a stereotaxic apparatus. Stainless-steel cannulae (19 gauge) were implanted bilaterally into the amygdala (AP -0.8 mm from the bregma, LM: 2.7 mm from the median line, DV: 0 mm from the ear 0). A small stainless-steel screw was implanted over the right frontal skull and served as an anchor. The cannulae and anchor were glued to the skull with dental cement. Fourteen days after the surgery, experiments of two passive avoidance tasks and measurement of motor activity were begun.

Step-Through Test A two-compartment step-through type passive avoidance apparatus (PA M1, O'hara & Co., Ltd., Tokyo, Japan) was used. In the learning trial, a mouse was placed in the illuminated front chamber. When it entered the dark rear compartment, it received a 60 V footshock through the grid floor. The time that elapsed before the mouse entered the dark compartment was recorded. Twenty-four h after this learning trial, the mouse was submitted to a testing session. In the testing trial, a mouse was placed in the lighted compartment, and the latency to its first entrance into the dark compartment was measured with a ceiling score of 300 s. The punishing footshock was always given to the mouse that stepped into the dark compartment.

Step-Down Test The apparatus of the step-down test consisted of a chamber equipped with a grid floor and an escape platform located in the corner of the chamber. In the learning trial, the mouse was placed on the platform and allowed to explore for 10 min. When the mouse got off the platform, it received a 60 V footshock through the floor grid. During the latter half (5 min) of the learning, the number of shocks received per mouse and the number of punished mice were counted. Twenty-four h after this learning trial, the testing session was begun, in which the number of descents was counted for 3 min. The punishing footshock was always given to a mouse that stepped down.

Measurement of Motor Activity After each passive avoidance test, motor activity of each mouse was measured using a tilting-type ambulator (O'hara & Co., Ltd., Tokyo, Japan) for 30 min.

Intra-amygdala or Intraperitoneal Injection of Scopolamine Saline or scopolamine solution was injected bilaterally into the amygdala 30 min before or immediately after the learning trial or 30 min before the testing trial. A 27-gauge injection needle connected to a 10 μ l Hamilton microsyringe via a piece of 15 cm PE-20 polyethylene tube was inserted through the guide cannula for the injection. The injection needle tip protruded approximately 1.0 mm beyond the tip of the cannula. Scopolamine hydrobromide (Wako) was dissolved in saline. One half microliter of the solution was injected slowly into the bilateral amygdala over a period of 3 min for each side.

After completion of the experiment, all mice were killed and the brains were dissected, frozen and serial sections made. The position of the cannula was checked to make

sure saline or the drug had actually been injected into the amygdala. In five animals blue ink was injected into the amygdala by the same method as used for scopolamine injections. Location of the blue ink was checked 40 min after the administration; the ink did not diffuse to other regions of the brain but remained in the injected area.

In a separate experiment, scopolamine or saline was injected intraperitoneally as in the case of intra-amygdala injection. The effects of systemically injected scopolamine were compared with those of the topical injection.

Statistical Analysis Mann-Whitney's *U*-test was used to test statistical significance of the mean latency and the mean number of descents. Analysis of variance (ANOVA) followed by Duncan's new multiple range test was used to analyze motor activity.

RESULTS

Scopolamine Administration 30 min before the Learning Trial Effects of scopolamine injected into the amygdala on memory acquisition in the two passive avoidance tasks were studied. Figure 1 shows the results of the two passive

Fig. 1. Effects of Scopolamine Administered into the Bilateral Amygdala 30 min before the Learning Trial in the Step-Through Test (A), the Step-Down Test (B), and the Motor Activity (C)

Open columns indicate the results of the learning trial, and crosshatched columns the results of the testing trial. Heights of each column and vertical bar represent mean $\pm$ S.E.M. from 12 to 15 mice. The ordinates are latency to enter the dark chamber (A), number of descents (B) and number of spontaneous movements (C). Intact animals received neither the injection nor implantation. Statistical significance by Mann-Whitney's *U*-test (A and B) or Duncan's new multiple range test (C) is shown as follows: a) ($p < 0.05$), b) ($p < 0.01$) vs. intact group; c) ($p < 0.05$), d) ($p < 0.01$) vs. saline-treated group.

Fig. 2. Effects of Scopolamine Injected Intraperitoneally 30 min before the Learning Trial in the Step-Through Test (A), the Step-Down Test (B), and the Motor Activity (C)

The ordinates, abscissas, columns, vertical bars and statistical symbols are as described in the legend of Fig. 1. Hatched column shows testing trial. The number of mice was 8 to 10.

avoidance tasks and motor activity when scopolamine was administered 30 min before learning trial. Scopolamine significantly shortened the latency in the step-through test and increased the number of errors in the step-down test. The impairing effect of scopolamine was dose-dependent. However, the motor activities were not affected by scopolamine injected into the amygdala.

Similar results were obtained when scopolamine was injected intraperitoneally (Fig. 2). The spontaneous motor activities were increased after the injection of 2 mg/kg scopolamine.

Scopolamine Administration Immediately after the Learning Trial In studies on the effects of scopolamine injected into the amygdala on memory consolidation in the two passive avoidance tasks, scopolamine was administered into the amygdala immediately after the learning trial (Fig. 3). In the step-through test, 1 μ g of scopolamine decreased performing ability significantly, but 10 μ g of scopolamine was not effective. On the other hand, the effects of scopolamine on performance abilities were not significant in the step-down test, while the motor activity was increased significantly.

Except for the increase of motor activity, systemically

injected scopolamine did not affect the performing abilities in either of the two tasks (Fig. 4).

Scopolamine Administration 30 min before the Testing Trial The third experiment investigated the effects of scopolamine on memory retrieval in the two passive avoidance tasks. Mice were trained for 7 d in these two passive avoidance tasks, then scopolamine was administered 30 min before the testing trial. Performance ability in the step-through and step-down test and the motor activity are shown in Fig. 5. The motor activities were not changed by intra-amygdala injection of scopolamine. There was no significant effect of scopolamine on the performance of mice which had been trained well in the step-through test. The number of errors during the step-down test in the group which had received 1 μ g scopolamine tended to increase, however, the increase was not statistically significant.

Intraperitoneal administration of scopolamine was also ineffective in assuring the performance of the two tasks (Fig. 6), although the motor activities were significantly augmented by the drug.

Fig. 3. Effects of Scopolamine Injected into the Bilateral Amygdala Immediately after the Learning Trial

The ordinates, abscissas, columns, vertical bars and statistical symbols are as described in the legend of Fig. 1. The number of mice was 13 to 16.

Fig. 4. Effects of Intraperitoneal Scopolamine Injected Immediately after the Learning Trial

The ordinates, abscissas, columns, vertical bars and statistical symbols are as described in the legends of Figs. 1 and 2. The number of mice was 8 or 9.

Fig. 5. Effects of Scopolamine Administered into the Bilateral Amygdala 30 min before the Testing Trial

The ordinates, abscissas, columns, vertical bars and statistical symbols are as described in the legend of Fig. 1. The number of mice was 13 to 15.

DISCUSSION

The intra-amygdala injection of scopolamine decreased the performance ability of mice when injected 30 min before the learning trial in both step-through and step-down tests. The motor activity of the mouse was not changed by scopolamine injected into the amygdala; therefore, the deteriorative effect of scopolamine is not due to reduced motor activity. As mentioned in Materials and Methods, the effect of scopolamine is probably restricted to the amygdala region. These results suggest that scopolamine blocks muscarinic receptors in the amygdala and decreases the memory acquisition of mice in the two passive avoidance tasks. Conventionally, both step-through and step-down tests are assumed to be in the same category, passive avoidance learning. In the present study, scopolamine affected both tests similarly, however, Segawa *et al.* have pointed out that the tests are not identical in a strict sense.^{8,9)} They divided mice into good and poor learners on the basis of their performance abilities in step-through and step-down tests, and measured parameters for cholinergic neurotransmission (contents of acetylcholine, activities of acetyltransferase and binding

Fig. 6. Effects of Intraperitoneal Scopolamine Injected 30 min before the Testing Trial

The ordinates, abscissas, columns, vertical bars and statistical symbols are as described in the legend of Figs. 1 and 2. The number of mice was 8 to 10.

site for muscarinic receptor). They found that changes of cholinergic transmission in certain brain areas are not necessarily identical in the two types of tests. In the hippocampus, there was no difference in cholinergic transmission between good and poor learners in the step-through test, but in the step-down test, good learners had more acetylcholine and less muscarinic binding sites than poor ones, suggesting an increase of cholinergic neurotransmission in good learners. Cholinergic transmission in the amygdala of good learners compared to poor ones, however, was similarly activated in both tests. Therefore, our results that intra-amygdala injection of scopolamine similarly affected both tests may be reasonable.

We would be able to confirm the role of amygdala if we could compare the results in which scopolamine was not injected into the amygdala because the guide cannula was not in the proper position. However, this occurred in only one or two animals in each experiment, a number too small to be conclusive. We have not tested whether or not unilateral injection of scopolamine was effective.

Intraperitoneal injection of scopolamine also impaired the performance in the two tasks. Although there was no

apparent dose-dependency in the step-down test, the effects were very similar to those after the intra-amygdala injection. Two mg/kg scopolamine increased the motor activity of mice on the day the drug was injected.

Scopolamine did not affect performance abilities in the step-through and step-down tests when administered immediately after the learning trial or 30 min before the testing trial. Neither administration route was effective. Intra-amygdala injection of 1 μ g scopolamine impaired the performance ability more in the step-through test than in the saline-treated group; however, the effect was neither dose-dependent nor manifest in the step-down test, and therefore has not significant meaning. We do not know why the motor activities measured on the first day (10 μ g) or both days (1 or 10 μ g) were increased in the scopolamine-treated groups (Fig. 3). The drug was not injected on the second day. The motor activity is generally divided into exploratory movement when animals are placed in a new environment and spontaneous movement. If the exploratory movement on the first day is not enough and the mouse does not become acclimated to the apparatus, it will explore on the second day also, and the motor activity will be increased. However, this does not seem to be the case since 10 μ g scopolamine increased the motor activity on the first and second days.

These results suggest that scopolamine selectively impairs memory acquisition without affecting consolidation or retrieval, which are consistent with the results of systemically injected scopolamine. In the present study, we showed that intra-amygdala injection of scopolamine can mimic the systemic injection-induced deteriorative effects. Therefore, the amygdala is at least partly, if not solely, the site of action for scopolamine. Blocking of the muscarinic receptors in the amygdala can cause anterograde amnesia.

Liang *et al.* demonstrated that the amygdala has an important role in the retention and/or retrieval of memory

processes.¹⁰⁾ However, in their experiments, the amygdala was lesioned after the learning trial. Non-cholinergic neurons in the amygdala may be involved in retention and retrieval of memory.

Several studies have shown the indispensable role of the amygdala in emotion,¹¹⁻¹³⁾ an important factor for memory performance.¹⁴⁻¹⁸⁾ In the present study, we showed that cholinergic neurons projecting to the amygdala are important for memory acquisition using passive avoidance tasks. However, these tasks can test only part of the function of learning and memory. Therefore, further studies are necessary to clarify the contribution of the cholinergic systems in the amygdala in memory and learning.

REFERENCES

- 1) H. C. Fibiger, *Trend Neurosci.*, **14**, 220 (1991).
- 2) M. M. Ghoneim, S. P. Mewald, *Psychopharmacol.*, **52**, 1 (1977).
- 3) R. C. Petersen, *Psychopharmacol.*, **52**, 283 (1977).
- 4) N. Sitaram, J. C. Gillin, *Science*, **201**, 274 (1978).
- 5) P. Davies, A. J. F. Malony, *Lancet*, ii, 1403 (1976).
- 6) M. Segawa, H. Saito, N. Nishiyama, *Biogenic Amines*, **7**, 171 (1990).
- 7) M. Segawa, H. Saito, N. Nishiyama, *Biogenic Amines*, **7**, 181 (1990).
- 8) M. Segawa, H. Saito, N. Nishiyama, *Biogenic Amines*, **7**, 191 (1990).
- 9) M. Segawa, H. Saito, N. Nishiyama, *Biogenic Amines*, **7**, 199 (1990).
- 10) K. C. Liang, J. L. Jr. McGaugh, R. A. Jensen, B. J. Vasquez, R. B. Messing, *Behav. Brain Res.*, **4**, 237 (1982).
- 11) L. Schreiner, A. J. P. Kling, *Am. J. Physiol.*, **184**, 486 (1956).
- 12) J. Iwata, J. E. LeDoux, M. P. Meeley, S. Arnertic, D. J. Reis, *Brain Res.*, **383**, 195 (1986).
- 13) J. E. Ledoux, P. Cicchetti, A. Xagoraris, L. M. Romanski, *J. Neurosci.*, **10**, 1062 (1990).
- 14) M. Mishkin, *Nature (London)*, **273**, 297 (1978).
- 15) D. Gaffan, S. Harrison, *J. Neurosci.*, **7**, 2285 (1987).
- 16) L. Lenard, Y. Oomura, Y. Nakano, S. Aou, H. Nishino, *Brain Res.*, **500**, 359 (1989).
- 17) D. Gaffan, A. Murray, *J. Neurosci.*, **10**, 3479 (1990).
- 18) E. A. Murray, M. Mishkin, *Science*, **228**, 604 (1985).