PHYSIOLOGY

Training of Mice in the Y Maze Using Drinking Reinforcement and Aversive Olfactory Signal: Facilitatory Effect of Ginsenosides

S. A. Chepurnov, N. E. Chepurnova, S. S. Kholmanskikh, R. K. Berdiev, J. K. Park,* and Jae-Oh Sohn*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 122, No. 9, pp. 253-257, September, 1996
Original article submitted December 12, 1995

The learning process is studied in mice in the Y maze using drinking as reinforcement. Fright caused in drinking mice by benzaldehyde odor at the site of reinforcement (which prolongs the latency of approach to the drinking bowl in 55% of the mice) is used as a detector of learning. Mice administered ginsenosides derived from the Korean red ginseng show significantly improved memory and learning. The percentage of animals frightened by the repellent odor increases with improved learning. Mice injected MK-801, an antagonist of NMDA receptor, more rapidly find the drinking bowl in the maze arm with repellent odor.

Key Words: *maze; drinking reinforcement; olfaction; ginseng ginsenosides; NMDA receptors*

Behavioral tests can be used to objectively evaluate brain processing of signals induced by opposite motivations [7] and to determine how the functional state of limbic structures is affected by the "collision" between aversive sensory signals and efferent impulses generated by memory retrieval. Concerning conditioned reflexes, the processes of such a "switching" [2] were helpful for understanding the functional significance of major limbic structures (amygdala and hippocampus [6]) and were applied by Bures for developing a model of aversive drinking [4].

In this study mice were trained in the Y maze, where drinking reinforcement was combined with a repellent signal in the same arm of the maze on one of the days of testing. Our objective was to study changes in the response to aversive signal as a function of learning period and the memory of the two opposite reinforcements. More than two decades ago, such tests were described by Academician I. S. Beritashvili as eliciting an emotion of fear from memory [3].

Chair of Human and Animal Physiology, Department of Biology, Moscow State University; *Institute of Ginseng and Tobacco, Taejon, Korea

MATERIALS AND METHODS

The study included two series of tests on 26 ICR mice and 67 house mice bred for large brain weight at the Laboratory of Genetics and Behavioral Physiology, Moscow State University (all mice were provided by Drs. N. V. Popova and I. I. Poletaeva [5]).

The Y maze used was made of black plastic and had a wall 160 mm high and three arms 140 mm wide and 260 mm long. All arms were blind. A mice was placed in the starting arm, and the drinking bowls were put in the two other arms: an empty one in the left arm and a bowl filled with water in the right, where they were left for the entire learning

0007-4888/96/0009-0872$15.00

period. Since the mice had been accustomed to receive water from above and drank standing on the hind legs, the drinking bowls were attached 80 mm above the maze floor. The maze was placed on a 900×900 mm reflecting black plastic plate. There was a spare "bottom" to rapidly remove dirt.

In the first test series, ICR mice maintained on the standard lighting schedule (12 h light—12 h darkness) were randomly assigned into control (n=11) and test (n=15) groups, placed in individual cages without water, and fed the standard dry diet *ad libitum*. During a 3-day period before learning, the mice were allowed to drink for 20 min at 10:30 (the time when the tests began) from the drinking bowls they used for drinking before. The same bowls were then placed in the maze. All cages with mice were simultaneously brought to the experimental room illuminated with a faint red light (from two 40 W bulbs) for adaptation. The time during which the mice remained in the maze for drinking did not exceed 5 min. The latency period preceding the start of drinking was measured. Each test was performed once daily at the same time.

Control mice were injected normal saline (0.2 ml intraperitoneally). Experimental mice received 2 mg ginsenosides complex (fraction II) from Korean red ginseng (*Panax ginseng* C. A. Mey) in 0.2 ml normal saline. This fraction contains Rd ginsenoside and unidentified ginseng saponins [12] and improves learning and spatial memory [11]. It was administered starting from the first day of the test period after a learning session. Benzaldehyde (Sigma), a compound with almond odor, was used as the olfactory stimulus. It was applied in a volume of 0.1 ml to two pieces of filter paper fixed to the maze wall on both sides of the drinking bowl.

In the second test series, control mice were injected normal saline (0.1 ml intraperitoneally). Experimental mice were given the NMDA receptor antagonist MK-801 (RBI, 0.01 mg/kg body weight in 0.1 ml normal saline) 30 min before placing in the maze. Mice from both groups were injected on the day when the repellent odor was presented. A learning session was started after 14:00. The latency of drinking was measured and the number of correct and incorrect entries into the maze arms was counted, as was the number of attempts to "drink" from the dry bowl.

The results were statistically analyzed by the Mann—Whitney U test.

RESULTS

On day 1 of the learning period, ICR mice found the drinking bowl in most instances with a latency

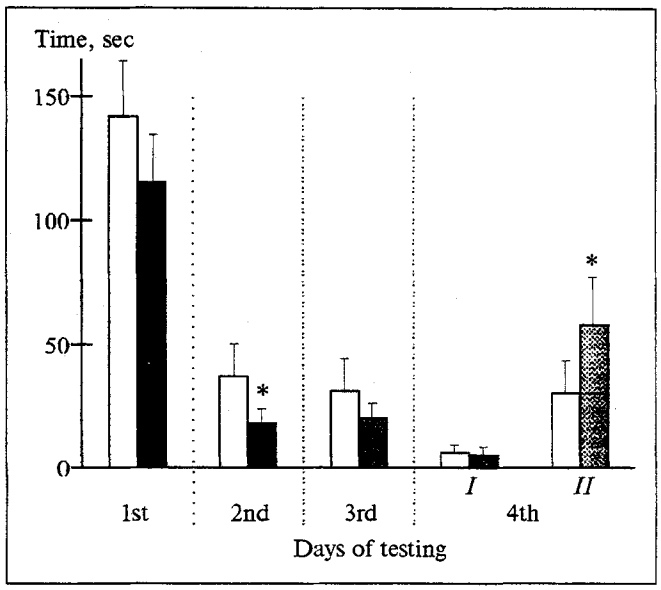

Fig. 1. Variations in the latency of searching for the drinking bowl by mice during learning in the Y maze. Days 1 and 2: latency of the correct bowl selection; day 3: test with the repellent benzaldehyde odor; day 4: test in clean maze, with the mice of both groups divided into two subgroups: with (*II*) and without (*I*) neophobia to the odor perceived on the preceding day. White bars: saline-injected (control) group; black bars: ginsenoside-injected group. *p<0.05 in comparison with the control group.

of more than 1.5 min (Fig. 1). On day 2, it took them significantly less time to locate the bowl, the time being shorter in the mice given ginsenosides after the learning session. The mean intergroup differences were statistically significant (Fig. 1), indicating accelerated learning in the test group. Test mice never or very rarely entered the maze arm with the empty drinking bowl. The ginsenosides, therefore, made it easier for the mice to remember where the bowl with water was located. On the day when the repulsive odor of almond emanated from the site of drinking reinforcement, 4 out of 11 control mice and 6 out of 15 test mice showed a longer latency of approach to the drinking bowl. Under these conditions, they first went to the arm with the empty bowl. In the presence of the aversive olfactory stimulus, individual variations in the time of approach to the bowl were observed: from 4 to 25 sec, from 12 to 59 sec, and from 23 to 52 sec, being never longer than 60 sec. On day 3, the indicators of learning did not differ from those on day 2, despite the presence of repellent odor (Fig. 1). After the test on day 3, administration of ginsenosides was continued to stimulate the recall of bowl location and to enhance the memory traces for the aversive odor. Day 4 of the experiment was decisive for evaluating the complex behavioral reaction of the animals. The latency of approach to the drinking bowl was measured in a clean maze without

any repellent odor. By that time the animals had experienced increasing water deprivation against the background of the trace which the repellent odor had formed in their memory.

In order to confirm the stimulatory effect of ginsenosides on memory, they were administered ginsenosides in the same dose 30 min prior to learning session. The mice were frightened when the repellent odor was first presented and, retaining the memory of this odor, were expected to approach the drinking bowl with a greater latency (even in the clean maze — according to the contextual afferent impulse traffic [7]).

Only one mouse from the experimental group out of 10 mice with retained "neophobia" (both in experimental and control groups) exhibited increased latency on the following day. By contrast, the mice that did not respond to the aversive odor went to the empty bowl, avoiding the arm where they had been exposed to aversive stimulus one day earlier.

Ginsenoside-treated mice were then divided into two equal groups, one of which showed a markedly reduced latency (7 sec). While retaining the memory of aversive odor, these mice went for the water as a result of the dominating drinking motivation. In mice of the other group, the memory of the repellent odor at the site of drinking reinforcement predominated, and the latency of the reaction was markedly increased, being significantly longer in the ginsenoside-treated animals than in the control ones (Fig. 1).

The methodology of research into memory phenomena under changing conditions of reinforcement has a long history. As noted by I. S. Beritashvili [3], the perception of the site where food is located is a complex process, and the memory of complex perception of a food item is much stronger than its separate perception via receptor systems of different (gustatory, visual, auditory, and olfactory) modalities. Delayed reactions were shown to be shorter and long-term memory to be stronger when gustatory and olfactory stimuli are perceived simultaneously. In such situations, sensory signals activating positive food reinforcement were involved. The situation arising in the experimental paradigm we used was similar to the reaction to aversive drinking [8].

Several years ago the use of ginseng and its components was limited because the mechanisms of its action on the central nervous system were not well understood and its components were found to be incapable of crossing the blood-brain barrier. Molecular and psychopharmacological studies of ginsenosides show that they act directly on the central synaptic transmission by modulating specific binding of classic neurotransmitters to their receptors and affecting second messengers. Ginseng components used in this study inhibit the absorption of neurotransmitters by brain synaptosomes in the following decreasing order: GABA>norepinephrine>dopamine>serotonin>glutamate [15]. Ginsenosides modulate specific binding of $GABA_A$ and $GABA_B$ receptors, thus influencing GTP proteins and the adenylate cyclase system [10]. Although no definite conclusions can be made regarding ginsenoside neurochemistry, it seems clear that the steroid-like effect of ginseng components plays an important role in the central effects produced by this plant. It can be concluded that the fright experienced by mice at the time of reinforcement in response to the aversive olfactory stimulus did not interfere with subsequent learning in the Y maze on the basis of drinking motivation. The learning scheme we used for ICR mice in the Y maze permits the detection of a positive (facilitatory) influence of ginsenosides on both spatial and olfactory memories.

The second series of experiments was performed on mice (n=68) of the 13th-14th generation of selective breeding [5]. It was designed to find out how the strengthening of habit acquired in the Y maze would influence the reaction to aversive olfactory signal and to elucidate the role of NMDA receptors in this reaction.

In simple (T or Y) mazes, responses can be learned by animals quite rapidly with a minimal latency for correct responses by day 4. Indeed, the learning rates were equal in nearly all mice, the minimal latency of searching for the drinking bowl being achieved by day 4 or 5. No further shortening of latency was observed when the learning period was prolonged to 7 or 13 days.

Mice presented with the repellent odor showed a statistically significant (p<0.01) increase in the latency of searching for the drinking bowl and drank water for a much shorter time or refused to drink at all. The latter is of special interest. Only 50% of mice responded negatively to the olfactory stimulus (benzaldehyde) in the preceding test series, where the learning period was 3 days, while identical negative responses to this stimulus were observed in >80% of mice after the habit had been strengthened in the maze for 7 or 13 days.

The role of NMDA receptors was evaluated with the use of their antagonist MK-801 in low doses. Preliminary analysis showed that the behavior of mice given MK-801 intraperitoneally in a dose of 0.01 or 0.03 mg/kg in 0.1 ml saline 30 min before the maze test did not differ from that of control mice on the day of aversive odor presentation. Higher doses of MK-801 (0.05 mg/kg) impaired motor coordination of the hind limbs and increased excitability immediately postinjection; 0.1-0.5 mg/kg

MK-801 induced profound changes in motor activity and movement coordination. For this reason, the near-threshold dose of 0.1 mg/kg was used in subsequent tests.

A pronounced significant decrease in the latency of searching for the drinking bowl when the aversive odor was presented was observed in mice given MK-801 before the Y maze test, the mean latency being 46.6±20.0 sec in the experimental group (n=9) and 108.6±43.0 sec in the control group (n=8; p<0.05) after 7 days of learning and 10.8±2.6 sec (n=8) and 50.7±22.9 sec (n=7; p<0.05), respectively, after 13 days of learning.

After entering the maze arm with the repellent odor, the mice, as a rule, left this arm, made no attempt to find the drinking bowl, or began running alternately into the maze arms instead of manifesting the acquired habit of approaching the bowl and drinking from it. Quite often the mice attempted to drink from the empty bowl, which they never did under ordinary experimental conditions.

The presence of the second (empty) bowl could provoke the mice to visit the not reinforced maze arm, which would result in more time being "wasted" and influence the length of the latent period (which is a measure of memory consolidation) of the drinking reaction. In tests where the aversive odor was presented for the first time, only 6 out of 19 MK-801-treated mice (32%) tried to drink from the empty bowl *vs.* 15 of the 16 mice (94%) in the control group. Similar results (5/19 *vs.* 15/16) were obtained when the same repellent odor was presented 3 days later. These findings indicate that NMDA receptors are involved in the formation of memory traces. Thus, it was shown that the number of mice reacting to the aversive benzaldehyde odor for the first time and the memory trace are influenced by the length of the learning period.

A conclusion regarding the role of subthreshold MK-801 concentrations in altering the reaction should be made taking into consideration the possible psychotropic action of MK-801, especially in low doses [9].

The fact that memory in tests with various reinforcements [1] and in emotional stress [6] has specific neurochemical features as well as different degrees of involvement of brain structures responsible for the studied types of memory should be taken into account. For example, the hippocampus is associated with spatial memory, the medial part of the cingulate gyrus (in rats) is responsible for lateralization when the side of reinforcement is selected in the Y maze, while the piriform and frontal cortices and the cortical region of the amygdala are associated with olfactory memory. The mechanisms of spatial and olfactory memories can be separated: when the memory of avoidance is blocked, the olfactory memory may remain unaffected [12]. However, disorders of learning when olfactory signals are discriminated results from impairments of the piriform cortex or hippocampus, since the NMDA receptors of these structures are involved in both types of memory [14]. Consequently, these structures may be involved in memory facilitation under the influence of systemically administered ginseng ginsenosides.

We wish to thank Drs. O. Litvin and D. Sokolov for providing us with useful guidelines.

This work received financial support from the International Science Foundation (Grant NB 1000).

REFERENCES

1. I. P. Ashmarin and R. I. Kruglikov, *Neirokhimiya*, **2**, No. 3, 327-341 (1983).
2. E. A. Arsatyan, *Fiziol. Zh. SSSR*, **30**, 13 (1941).
3. I. S. Beritashvili, *Memory in Vertebrates: Characteristics and Origin* [in Russian], Moscow (1974).
4. J. Bures, O. Buresova, and J. P. Houston, *The Brain and Behavior Research: Procedures and Basic Experiments*, A. S. Batuev (ed.) [Russian translation from English], Moscow (1991).
5. N. V. Popova and I. I. Poletaeva, *Vestn. Mosk. Universiteta, Ser. Biol.*, 30-34 (1983).
6. P. V. Simonov, in: *Experimental Neurophysiology of Emotions* [in Russian], Leningrad (1972), p. 93.
7. K. V. Sudakov, *Systemic Mechanisms of Emotional Stress* [in Russian], Moscow (1981).
8. O. Buresova and J. Bures, *Behav. Brain Res.*, **1**, 299-312 (1980).
9. L. L. Iversen, *Neurotransmission*, **10**, No. 1, 1-4 (1994).
10. T. Kimura, P. A. Saunders, H. S. Kim, *et al.*, *Gen. Pharmacol.*, **25**, No. 1, 193-199 (1994).
11. J. K. Park, K. Y. Nam, H. C. Hyun, *et al.*, *J. Korean Ginseng Sci.*, **18**, No. 1, 32-38 (1994).
12. U. Staubli, R. Faraday, and G. Linch, *Behav. Neural Biol.*, **43**, 287-297 (1985).
13. U. Staubli, O. Thibault, M. DiLorenzo, and G. Lynch, *Behav. Neurosci.*, **103**, No. 1, 54-60 (1989).
14. D. Tsang, H. W. Yeung, W. W. Tso, and H. Peck, *Planta Med.*, **3**, 221-224 (1985).