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Research article

Length of the memory retention period depends on the extent of protein synthesis in the terrestrial slug *Limax*

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HIGHLIGHTS

- Onset timing of amnesia shifts depending on the timing of anisomycin injection.
- Amnesia appears early if anisomycin is injected close in time to conditioning.
- Amnesia appears late if intervals are given between the injection and conditioning.
- The LTM phase is more graded than previously thought.

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ABSTRACT

The terrestrial slug *Limax* can form an odor-aversion memory by the single simultaneous presentation of a food odor and an aversive stimulus. We have previously demonstrated that the long-term retention of this memory was impaired by a high-dose injection of a protein synthesis inhibitor 30 min prior to the conditioning. However, the onset of amnesia was delayed if the dose of the inhibitor was reduced or a less potent protein synthesis inhibitor was used. We thus speculated that the persistence of memory depends on the amount of newly synthesized protein following learning. In the present study, we further elaborated on this idea by injecting a high dose of anisomycin at different timings before or after conditioning, and tested the memory retention at 1, 2, 3, 7, or 14 days after the conditioning. We found that the injection of anisomycin 6 h before, or 1 h after the conditioning had no effect on memory retention for 7 days, and an injection at 30 min before and just following the conditioning impaired the memory retention at 3 days. Interestingly, the injection at 3 h before and 30 min after the conditioning did not impair the retention at 3 days but did impair retention at 7 days. Taking into account the time course of protein synthesis inhibition in the brain, our results further support the idea that the memory retention period is dependent on the amount of protein synthesized following memory acquisition.

1. Introduction

Long-term memory (LTM) is defined as the memory phase dependent on newly synthesized protein/mRNA following memory acquisition [1]. Many of the early studies investigated the protein synthesis-dependent phase of memory at 24 h or earlier following conditioning [2–10]. However, there are also several studies reporting a delayed appearance of amnesia caused by protein synthesis inhibitors [11–14].

The terrestrial slug *Limax* can form an odor-aversion memory once presented with an aversive stimulus (e.g. the bitterness of quinidine sulfate solution) in combination with a food odor (e.g. carrot juice). The memory can be established by a single conditioning trial and lasts for several weeks [12,15]. Therefore, the odor-aversion conditioning of *Limax* is an ideal model experimental system to investigate the effects of artificial manipulation, such as protein synthesis inhibition or rapid cooling delivered at different timings before or after the memory formation, with a fine time resolution [12,16–18].

Pre-conditioning injection (30 min before the conditioning) of anisomycin into *Limax* impaired memory retrieval 2 days following the conditioning, but not at 1 day (Fig. 1A, [12]). Injection of cycloheximide, which inhibits protein synthesis in the brain less potently, caused amnesia at day 7 but not at day 2 post-conditioning (Fig. 1B, [12]). In Fig. 1, the curves of the protein synthesis inhibition

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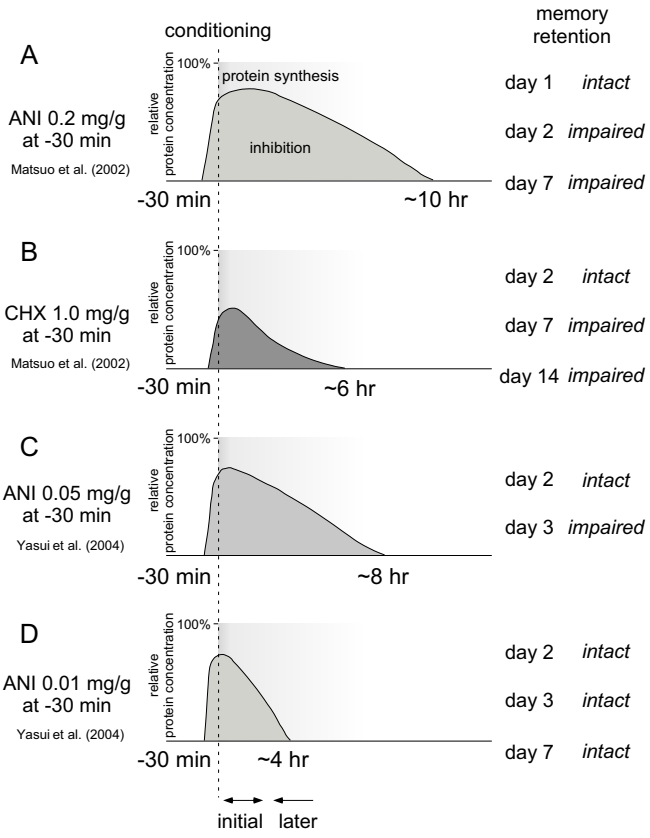

Fig. 1. Schemas explaining the protein synthesis/inhibition events hypothesized to occur in the previous studies. A vertical broken line indicates the timing of the conditioning. The pale and dense gray protuberances indicate protein synthesis inhibition with anisomycin and cycloheximide, respectively. The shapes of these curves are based on the time course of the reduction of ^{35}S-labeled Met/Cys incorporated into the protein fraction (i.e.% inhibition [12,16]). Learning-induced protein synthesis is expressed as gradations because its actual time course is not clear. The phases of initial and later protein synthesis are provisionally indicated below. The results of the memory retention are indicated on the right. (A) An injection of 0.2 mg/g.b.w. anisomycin impaired memory retention at 2, and 7 days, but not at 1 day. (B) An injection of 1.0 mg/g.b.w. cycloheximide impaired memory retention at 7, and 14 days but not 2 days. (C) An injection of 0.05 mg/g.b.w. anisomycin impaired memory retention at 3 days, but not 2 days. (D) An injection of 0.01 mg/g.b.w. anisomycin did not affect memory retention at 2, 3, or 7 days. ANI, anisomycin; CHX, cycloheximide.

were based on our previous experiment using ^{35}S-labeled Met/Cys in [12] and [16]. These observations led to the idea that the onset timing of amnesia is dependent on the extent of protein synthesis following the conditioning. Indeed, we showed that amnesia appeared as late as 3 days (but not at 2 days) after the conditioning if a lower dose of the anisomycin (reduced to 25%) was injected 30 min before the conditioning (Fig. 1C, [16]).

However, the above idea is derived from our previous data that were not systematically analyzed with respect to the mechanism or level of protein synthesis inhibition. Moreover, what happens if a later phase of the learning-induced protein synthesis is inhibited remains an open question. In the work of Yasui et al. [16], reducing the dose of anisomycin caused more rapid decay of the protein synthesis inhibition in the brain, where the early phase of protein synthesis was expected to be inhibited (up to ~30 min following the conditioning, Fig. 1C). Therefore, it is possible that the early phase of protein synthesis is more important for the long term retention of memory. However, further reduction in the dose of anisomycin, which exhibits a more rapid decay of the inhibitory effects, failed to affect LTM even at 7-day (Fig. 1D, [16]). Therefore, the suppression of only the very initial phase of protein synthesis seems to be ineffective.

These observations prompted us to investigate more thoroughly the relation between the extent of protein synthesis inhibition and the onset timing of amnesia, and to ask whether LTM is impaired if the early or late phase of protein synthesis is suppressed. In the present study, we injected anisomycin at different timings with respect to the conditioning, and analyzed its effects on the retention of memory.

2. Materials and methods

2.1. Animals

Terrestrial slugs, *Limax valentianus*, have been maintained in our laboratory at 19 °C for at least 27 generations as a closed colony. They were fed a diet of humidified powder mixture, consisting of 521 g of rat chow (Oriental Yeast, Tokyo, Japan), 500 g of potato starch, and 21 g of mixed vitamins (Oriental Yeast). The body weight of the slugs was between 0.2 and 0.7 g (average 0.39 g) at the time of the conditioning.

2.2. Preparation of anisomycin

Anisomycin powder was purchased from Sigma (#A9789), and was dissolved in water by adding 1 M HCl. One-twentieth volume of 20 × slug physiological saline (1.4 M NaCl, 40 mM KCl, 94 mM $MgCl_2$, 98 mM $CaCl_2$, 100 mM glucose, and 100 mM HEPES, pH 7.0) was then added and the volume was adjusted with water to a concentration of 2 mg/ml (the final pH was approximately 7.5). This concentration of anisomycin was the same as that used previously [12].

2.3. Odor-aversion conditioning and memory retention test

The slugs were conditioned and tested for memory retention using a shading box as described previously [12,19]. Briefly, the slugs were individually weighed and placed in a plastic container (90 × 70 × 23 mm) where a moistened filter paper was laid. The slugs were conditioned using carrot juice (as a conditioned stimulus) and 1% quinidine sulfate (as an unconditioned stimulus, Wako, Osaka, Japan). Anisomycin (0.1 ml/g.b.w. [gram body weight], i.e. 0.2 mg/g.b.w.) or saline (0.1 ml/g.b.w.) as a control was injected into the body cavity before or after the conditioning with several time intervals between the conditioning and the injection. The injection volume was almost equivalent to one-tenth of the slug's body mass. The needle was injected into the middle of the body cavity from the dorsal surface of the slug at just caudal part of the mantle. In the conditioning, 1 ml of carrot juice was placed on a glass plate in the shape of a half circle (radius 6 cm), and a slug was placed at the center of the half circle. One ml of 1% quinidine sulfate solution was applied to the mouth when the slug touched the carrot juice. The slug was kept in the mixture of carrot juice and quinidine sulfate for 90 s, and then submerged in water for 60 s to remove carrot juice/quinidine sulfate. We excluded from further experiments those slugs that did not reach the carrot juice within 3 min during conditioning. Memory retention (either at 1, 2, 3, 7, or 14 days following the conditioning) was tested in a blind manner with respect to which solution (anisomycin or saline) was injected before or after the conditioning. Each animal underwent only one memory retention test, and was never used for further experiments. The slug was considered to retrieve the memory if it took longer than 3 min to reach the carrot juice placed in the shape of a half circle (radius 9 cm) after starting from the center of the circle, whereas if the slug reached the carrot juice within 3 min, it was considered to have lost the memory [12]. The memory score was expressed as the ratio (%)