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Onset of Amnesia Is Delayed with a Decrease in Inhibition of Protein Synthesis during Odor-taste Associative Learning in the Terrestrial Slug *Limax valentianus*

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ABSTRACT—Slugs can retain odor-taste associative memory for several weeks, and this requires protein synthesis. We examined the dose-dependency of the onset time of amnesia caused by the protein synthesis inhibitor anisomycin, and showed that with reduced dose, the onset is shifted from 2 days to 3 days after conditioning; we could not shift the onset delay later than 3 days. Our results suggest that the mechanism underling memory retention is different in the period up to 3 days, *versus* the period later than 3 days. Our results also suggest that sustained inhibition of protein synthesis in the period from zero to 3 hr after conditioning is necessary to cause amnesia.

Keywords: long-term memory, onset of amnesia, protein synthesis inhibition, anisomycin, *Limax*

INTRODUCTION

The terrestrial slug *Limax* is known to acquire odor-taste associative memory. Slugs can learn to avoid the odor of innately preferred food such as carrot juice after it is presented in combination with quinidine, a substance whose taste typically provokes aversive behavior in *Limax* (Gelperin, 1975; Sahley *et al*., 1981). This associative memory can be established by one-trial conditioning, and thus we can ask precise questions about the time-dependent processes of memory consolidation (Yamada *et al*., 1992; Sekiguchi *et al*., 1997; Matsuo *et al*., 2002). Slugs can retain such memories for several weeks and we reported in a previous study (Matsuo *et al*., 2002) that protein synthesis is required for long-term memory formation, as has been shown in many other species with various paradigms (Davis and Squire, 1984; Stork and Welzl, 1999). Unexpectedly, slugs retained this type of memory for one day after condioning even when they were injected with a considerable dose (0.2 mg/g body weight) of the protein synthesis inhibitor anisomycin (ANI) 30 min before conditioning, although memory retention was not possible on day 2 after the conditioning (Matsuo *et al*., 2002). Moreover, they retained the memory even on day 2 when injected with another protein synthesis inhibitor, cycloheximide (Matsuo *et al*., 2002). Such delayed onset of amnesia by protein synthesis inhibition has rarely been reported, except in a few cases (Tully *et al*., 1994, Wüstenberg *et al*., 1998). In many species, memory can be maintained for no more than several hours if protein synthesis is inhibited (Stork and Welzl, 1999).

We hypothesized that the above-mentioned delay in the onset of amnesia might be caused by insufficiency of protein synthesis inhibition. With less inhibition, more protein synthesis can occur, and this may play a role in the temporary memory retention we observed. Specifically, our hypothesis leads to the prediction that the greater the protein synthesis inhibition, the earlier the onset of amnesia. Matsuo *et al*. (2002) determined the amount of [^{35}S]Met/Cys incorporated in the brain of the slugs injected with protein synthesis inhibitor and of the saline-injected slugs, and then calculated the difference in [^{35}S]Met/Cys incorporation between the two groups, which serves as an index of the extent of protein synthesis inhibition. Actually, in the results above, ANI inhibited protein synthesis by over 60% for 6 hr, but cycloheximide inhibited it by 40% or less and the effect was shorter in duration (Matsuo *et al*., 2002). To test this hypothesis, we here injected ANI at three doses (high, medium and low) and examined the onset of amnesia.

MATERIALS AND METHODS

The terrestirial slug, *Limax valentianus* (0.17–1.00 g, 0.45 g on average, 9–16 weeks after hatching) was maintained in our laboratory at 20°C. They were fed only on a diet of moistened powder (Nakaya *et al*., 2001) and have never eaten carrot. Starved slugs (5–24 days of starvation) were placed individually in plastic contain-

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ers, and were injected into their body cavity with saline (70 mM NaCl, 2 mM KCl, 4.7 mM $MgCl_2$, 4.9 mM $CaCl_2$, 5 mM glucose, 2.36 mM HEPES and 2.64 mM HEPES-Na, 100 μl/g b. w.) or with anisomycin (ANI, Sigma) dissolved in saline, 30 min prior to conditioning. The odor-taste associative conditioning and retention tests were performed as described (Matsuo *et al.*, 2002). All the retention tests were carried out in a blind manner; the experimenters did not know which slugs had been injected with ANI and which with saline. Solutions with three different concentrations (2.0, 0.5, and 0.1 mg/ml in saline) of ANI were prepared. An ANI solution of 2.0 mg/ml represents almost the maximum possible concentration with respect to solubility in saline. The measurement of protein synthesis inhibition was performed as described (Matsuo *et al.*, 2002) with minor modifications as follows. After 300 μl trichloroacetate was admixed, the tube was maintained at –20°C for about 20 min. The volume of Clear-sol I (Nakalai-Tesque) that was added also was changed, from 1 ml to 0.9 ml. A χ^2-test was done to analyze the behavioral data, and one-tailed *t*-test was applied to compare the extent of protein synthesis inhibition.

RESULTS AND DISCUSSION

As shown in Fig. 1, the slugs injected with ANI at 0.2 mg/g 30 min before conditioning showed amnesia on day 2 (χ^2=6.27, p<0.05, d.f.=1 in all cases), consistent with the previous results (Matsuo *et al.*, 2002). There was no difference in the survival rate between saline-injected group and anisomycin-injected group. When the dose of ANI was reduced to 0.05 mg/g, the amnesia was observed later, on day 3 (χ^2=8.04, p<0.01), but not on day 2 (p>0.05). We further reduced the dose of ANI to 0.01 mg/g, but this failed to cause amnesia by either 3 days or even by 7 days after conditioning (p>0.05). In our preliminary study, slugs injected with ANI at an intermediate dose between 0.01 and 0.05 mg/g showed no sign of amnesia in 4 days, in a week, or even 2 weeks after conditioning (data not shown). Therefore, it seems that amnesia cannot be induced unless it occurs within 3 days; if slugs maintain the memory on day 3, then they establish a full long-term memory that persists for several weeks.

Next, we quantified the extent of inhibition of protein synthesis by ANI in the cerebral ganglion of the slug (Fig. 2). Injection of ANI at 0.2 mg/g suppressed protein synthesis by approximately 80% at maximum (which occurred 3.5 hr after the injection), consistent with our previous results (Matsuo *et al.*, 2002). The inhibition of synthesis ended sooner as we reduced the dose of ANI. The effect of ANI at 0.01 mg/g at 3.5 hr and 6 hr post-injection was significantly less than that of higher doses, 0.05 mg/g (p<0.05) and 0.2 mg/g (p<0.005), although this difference was not yet observed at 1 hr post-injection. This suggests that because of the shorter duration of inhibition or the weaker inhibition at its maximum (lower than 80% inhibition) or both, slugs can synthesize a certain amount of protein at a dose of 0.01 mg/g, which allows them to form an intact long-term memory without amnesia. The inhibition produced by ANI at 0.05 mg/g was significantly less than that of 0.2 mg/g at 3.5 hr (p<0.005) or at 6 hr (p<0.05) post-injection, although there was no significant difference at 1 hr and at 10 hr (p=0.104). This suggests that injection of ANI at 0.05 mg/g allowed a greater amount of protein to be synthesized than at 0.2 mg/g, and these proteins were utilized for the retention of memory for one more day, but were not sufficient to establish an intact long-term memory. These results suggest at least two possibilities: (1) the extent of inhibition at around 3-6 hr after conditioning may determine the onset time of amnesia; or (2) the total

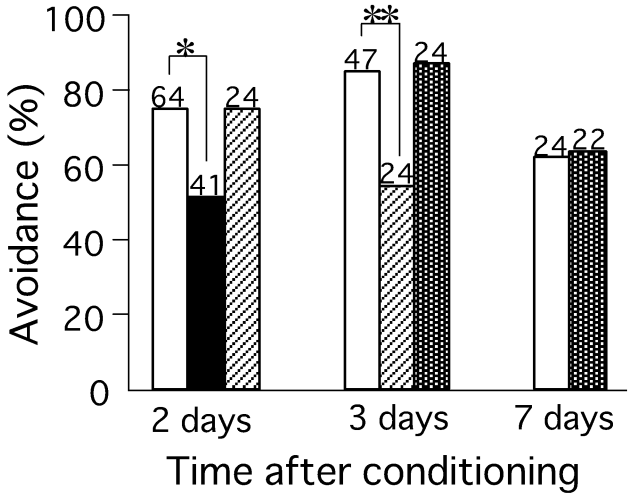

Fig. 1. The effect of pre-conditioning injection of ANI on memory retention examined on day 2, 3, or 7. The ordinate represents the relative number (%) of slugs with intact memory retention. The numerals above the columns indicate the number of slugs used. * p<0.05, ** p<0.01 by χ^2-test. □, saline; ■, 0.2 mg/g; ▨, 0.05 mg/g; ▩, 0.01 mg/g.

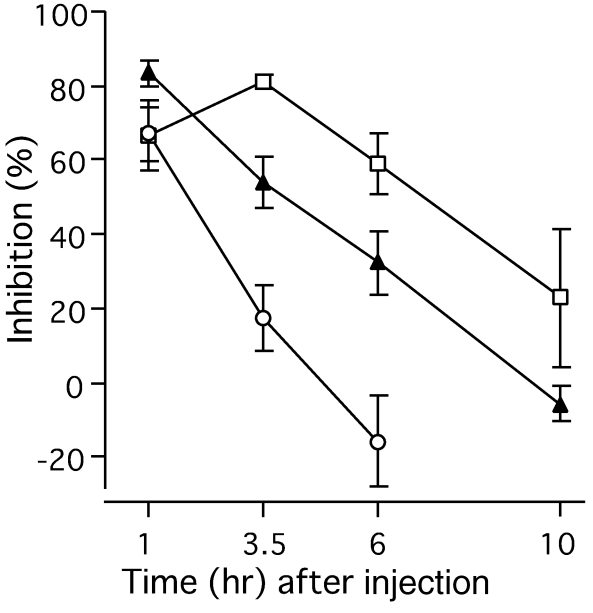

Fig. 2. Time course of protein synthesis inhibition in the slug brain using three different doses of ANI. Inhibition (%) is the percentage of decrease in the incorporation of L-[^{35}S] Met/Cys into a trichloroacetate-insoluble pellet of the brain homogenate derived from ANI-injected slugs relative to that from saline-injected slugs. Error bars indicate SEM across each 4-8 independent measurements. —□—, 0.2 mg/g; —▲—, 0.05 mg/g; —○—, 0.01 mg/g

amount of protein synthesized during and after conditioning is the major determinant.

We noticed that the decay curves for 0.05 mg/g and 0.2 mg/g overlapped each other when the curve for 0.05 mg/g was shifted to the right by 2.5 hr (Fig. 2). When ANI at 0.05 mg/g was injected 2 hr after conditioning instead of 30 min before conditioning, the inhibition-decay curve of the 0.2 mg/g injection was almost replicated. Thus, 2 hr post-conditioning injection of ANI at 0.05 mg/g and retention tests on day 2 and on day 3 will serve to examine the first possibility. But this experiment actually resulted in no amnesia being observed either on day 2 or day 3 (data not shown), indicating that protein synthesis inhibition that began 2 hr after conditioning was not sufficient to cause amnesia. We next asked if post-conditioning injection of ANI at 0.2 mg/g b.w. had any effect on memory retention. When we used a 2 hr post-training injection, 16 out of 18 slugs injected with ANI retained the memory on day 3, while 14 out of 17 saline-injected slugs did, and there was no significant difference between the two groups ($p>0.05$ by Fishers exact test). With a 6 hr post-training injection, all 18 slugs injected with ANI retained the memory on day 3, while 13 out of 18 saline-injected slugs did. In the latter case, the ANI-injected group showed slightly better retention ($p<0.05$ by Fishers exact test). More investigation will be required to clarify the latter paradoxical result, but at least it will be safe to say that slugs had probably completed learning-induced protein synthesis by 2 hr after conditioning. Insects, however, have relatively wide time window of susceptibility to translational or transcriptional inhibition: long-term olfactory memories of crickets and honeybees are inhibited by respective injections of ANI and actinomycin D 6 hr after training (Matsumoto *et al*., 2003; Wüstenberg *et al*., 1998). There may be a great variation in the time window of susceptibility among species.

We have observed impairment, by pre-conditioning injection of ANI, of long-term memory on day 2 or on day 3, but not later. A previous study using *Limax* has shown that presentation to a conditioned slug of a conditioned stimulus followed immediately by cooling induces amnesia if such an operation is done within about 3 days after conditioning, but is less effective if done later than 3 days (Yamada *et al*., 1992). Both results strongly support the notion that the mechanism underlying memory retention differs between up to 3 days and thereafter, and that the memory state becomes stable within 3 days.

We also showed that the onset of amnesia could be delayed 1 day by reducing the dose of ANI from 0.2 mg/g to 0.05 mg/g. Other investigators have reported that the number of amnesiac animals increases with longer durations of protein synthesis inhibition (Flood *et al*., 1973). Our results confirm this pattern if we focus on the results of the retention tests on day 2 and day 3 (Fig. 1). A few studies have examined the relationship between impairment of memory and the dose of a protein synthesis inhibitor (Flood *et al*. 1975; Rainbow *et al*., 1980; Meiri and Rosenblum, 1998; Naghdi *et al*., 2003), but, as far as we know, it has not previously been reported that the duration of memory retention varies with differential protein synthesis inhibition.

Although ANI at 0.2 mg/g inhibited protein synthesis for a longer period than ANI at 0.05 mg/g, at both 0.2 and 0.05 mg/g ANI was an effective inhibitor up to 2 hr post-conditioning (Fig. 2). Given that protein synthesis 2 hr after conditioning is not necessary for long-term memory, what causes the difference in the onset of amnesia? We hypothesize the following. A long period of protein synthesis inhibition is said to be required for strongly conditioned animals to lose their long-term memory (Flood *et al*., 1973). That is, strongly conditioned animals can resume learning-related protein synthesis when the effect of an inhibitor wears off. How long animals retain the capacity for this synthesis seems to depend on the strength of the training paradigm (Flood *et al*., 1973). If our conditioning paradigm is strong, then injection of ANI at 0.05 mg/g allows greater amounts of learning-related protein synthesis to occur than ANI at 0.2 mg/g during 3-6 hr post-conditioning. During the consolidation period, the structural changes are in an unstable state and are vulnerable to disruption. A certain amount of protein must be synthesized after conditioning to stabilize memory into a permanent form. If the amount of synthesized proteins is insufficient, however, these proteins might play only a temporary role during the consolidation period, structural changes would not be completed, and the memory would decay before undergoing the customary transition to a more stable form (Flood *et al*., 1972; Matsuo *et al*., 2002).

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