Mechanisms of Amnesia Induced by Impairment of Long-Term Memory Reconsolidation in Edible Snail

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Involvement of neurotransmitter receptors and translation and transcription processes in reconsolidation of conditioned food aversion memory was investigated in experiments on edible snails. Injections of neurotransmitter receptor antagonists and protein synthesis inhibitors before the reminder session were found to induce amnesia that was characterized by the possibility of memory recovery in repeated training and under the effect of mnemotropic agent D-cycloserine (early stage of amnesia) or by resistance to the mentioned actions (late stage). It has been shown that amnesia induction by memory reconsolidation impairment by neurotransmitter receptor antagonists depends on synthesis of specific proteins and mRNA, similar to the cases of induction of other adaptive brain modifications.

Key Words: *memory reconsolidation; amnesia; neurotransmitters; serotonin and glutamate receptor antagonists; protein and mRNA synthesis inhibitors*

Consolidated long-term memory can again become labile (destabilize) during learned skill performance and then consolidate repeatedly (reconsolidate) [6,7,10,13]. Discovery of reconsolidation is of inestimable importance for the development of one of fundamental problems in neurobiology – investigation of mechanisms of long-term memory storage, as well as development of amnesia when reconsolidation is impaired. A number of key mechanisms of memory reconsolidation was investigated in experiments conducted on animals situated at different evolutionary stages and using different types of training [6,7,10,12,13]. In addition, the mechanisms of amnesia-induced by reconsolidation impairment remain virtually uninvestigated. In discussion of this problem, many authors [7,13] point that there is still no answer to the fundamental question: whether experimental amnesia that develops in consolidation and reconsolidation impairments is a consequence of retrieval inhibition or the consequence of memory trance erasure.

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We investigated the mechanisms of amnesia resulted from memory reconsolidation impairment and the dynamics of amnesia development in edible snails using conditioned food aversion paradigm.

MATERIALS AND METHODS

Edible snails (*Helix lucorum*) were trained in food aversion model using the method described elsewhere [2]. Three days before training and before the test of the learned skill, the animals were deprived of food. The snails were tethered by the shell allowing them to crawl relatively freely on a plastic ball floating in water. Banana and boiled carrots were used as the conditioned (CS) and differentiating stimuli (DS), respectively. Alternating current (50 Hz, 300 msec, 1.2 mA) was applied to the food and snail body at the time of first consummatory reactions. Food presentation to the animals was limited by 120 sec. Food presentations were combined with electric shock every 15-20 min. There were three training sessions: daily for 3 days. CS was presented 12-16 times, DS was presented 6-10 times. Experimental groups included from 8 to 16 animals.

For testing memory storage, the snails were placed into the training context (plastic balls) for 30 min, and were presented CS and DS with 15 min intervals. The latent periods of consummatory reactions were measured for 120 sec. The snails were injected with the solutions 2 days later and the reminder session was conducted 15-20 min after that: the animals were placed into training context and CS was presented 3 times over 120 sec with 15 min intervals. If animals tried to eat the food during test or reminder session, it was taken away without applying of reinforcement stimulus. We investigated effects of NMDA antagonist MK-801 ((+)-MK-801 hydrogen maleate) and APV (DL-2-amino-5-phosphonopentanoic acid); partial agonist of NMDA-receptor glycine site D-cycloserine; nonselective serotonin antagonist methiothepin; protein synthesis inhibitors cycloheximide and anisomycin; mRNA synthesis inhibitors actinomycin D and DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole). Compound solutions (0.25 ml per snail) were administered into the snail body cavity. Control snails were injected with saline before the reminder procedure.

Results were averaged, standard error of the mean was calculated ($M \pm SEM$). One-way ANOVA was used to assess the level of significance.

RESULTS

The snails demonstrated defensive and avoidance reactions to CS for at least 1-2 months following the training. When the snails were tested within that period, latent periods for the consummatory reactions to CS were 100-120 sec and were significantly higher than those in responses to DS and reactions to banana in naïve snails (20-40 sec; $p<0.0001$).

Serotonin receptor antagonists and NMDA antagonists selectively impaired memory reconsolidation. Injections of methiothepin (5 mg/kg), MK-801 (0.25 mg/kg), or APV (15 mg/kg) combined with reminder resulted in altered reproduction of the skill. Testing 2 weeks after the exposure to antagonists and reminder demonstrated that latencies of the responce to CS were shorter than in control snails ($p<0.0001$) and had no differences from those in response to DS or to banana presentation in naïve animals ($p>0.05$, Fig. 1, *a*). To evaluate memory storage in snails that exhibited amnesia, repeated avoidance training was carried out using the same type of food that was used in initial training (banana). Repeated training 2 weeks following amnesia induction with methiothepin/reminder resulted in memory recovery: reaction latency to CS approximated the control level ($p>0.05$, Fig. 1, *b*). At the same time, if skill reproduction was impaired by MK-801/reminder or APV/reminder, repeated training resulted in no skill formation: no differences were observed in latencies of the responce to CS and DS ($p>0.05$, Fig. 1, *b*). The effect was selective, since the same snails were able to acquire memory when were trained with new type of food (cucumber).

According to generally accepted ideas, amnesia following long-term memory impairment may appear as a consequence of either suppression of retrieval or erasure of memory trace, which does not exclude the possibility for acquirement of the same skill in repeated training [6,13]. However, we discovered new, previously non-described phenomenon: repeated training

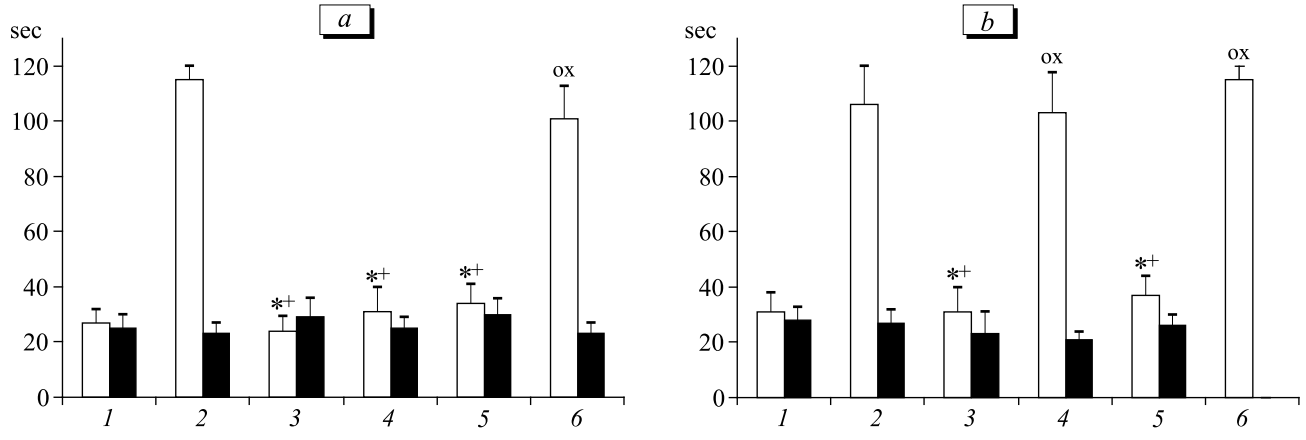

Fig. 1. Effects of neurotransmitter antagonists and protein and mRNA synthesis inhibitors on reconsolidation of food aversion memory in edible snail. *a*: test 2 weeks after compound administration and reminder session. Reactions to food stimuli in native snails (*1*); snails injected with saline before the reminder session (control; *2*); MK-801 (*3*); methiothepin (*4*); cycloheximide (*5*); actinomycin D (*6*). *b*: test following repeated training of the snails, exhibited amnesia 2 weeks after exposure to compounds/reminder (*1-5* as seen on fragment *a*). *6*) reaction to cucumber following aversive training with this type of food in snails exhibited amnesia following exposure to MK-801/reminder. Here and at Fig. 2, 3: ordinate: mean latencies of consummatory reactions to food stimuli ($M \pm SEM$). Light bars: snail responses to CS (banana) dark bars – responses to DS (boiled carrot). $p>0.05$ in relation to $^{+}$responses to DS and reactions to banana presentation before initial training (Group 1), ^{o}reactions to CS in control animals. $p<0.0001$ in relation to *reactions to CS in control animals (Group 2), ^{x}responses to DS and reactions to banana presentation before initial learning.

of the snails with NMDA-dependent amnesia resulted in no skill acquirement [4]. Memory consolidation impairment was selective, since memory for another type of food (cucumber) was successively formed in the same snails. The mechanisms of impairment the training ability remain unclear. One of the reasons of "irreversible" amnesia may comprise changes in molecular-genetic processes in the neurons that result in "destruction" of morphological "carrier" of engram, for example due to elimination of between-neuron synaptic connections functionally essential for memory trace storage [11,15]. Mechanisms of "serotonin-dependent" amnesia are apparently associated with impairment of memory retrieval or with partial damage to memory trace, which can be recovered in repeated training. The findings are consistent with the fact that the glutamate system is important for consolidation and stabilization of certain types of memory, whereas the monoaminergic systems are preferentially involved in modulation of these processes [1,9,14].

Stages of amnesia development following impairment of memory reconsolidation. In further experiments we investigated the dynamics of amnesia development at earlier terms following impairment of memory reconsolidation using MK-801. Three and 10 day after the exposure to MK-801/reminder, the snails demonstrated amnesia: latencies for CS and DS were similar ($p>0.05$, Fig. 2, *a*). Repeated training 3 days after exposure to MK-801/reminder recovered the memory: latencies of reactions to CS in experimental and control snails were similar ($p>0.05$) and higher than latencies in responses to DS ($p<0.0001$, Fig. 2, *a*). Repeated training 10 days after exposure to MK-801/reminder did not result in skill formation: latencies for reactions to CS and DS were similar ($p>0.05$, Fig. 2, *a*).

In further experiments, we studied the possibility of memory recovery by mnemotropic agent, agonist of NMDA receptor glycine site, D-cycloserine, at different stages of amnesia induced by MK-801 administration before the reminder session. It was found that on day 3 after the amnesia induction, D-cycloserine injections (20 mg/kg) in combination with reminder resulted in memory recovery: the latencies of the reactions to CS increased up to the level similar to that in control snails ($p>0.05$, Fig. 2, *b*). D-cycloserine injections and reminder 12 days after amnesia induction had no effect on its development: latencies for reactions to CS and DS were similar ($p>0.05$, Fig. 2, *a*).

Thus, we found that impairment of conditioned food aversion memory by NMDA-antagonist was followed by amnesia that comprised two stages [5]. Early amnesia (<10 days) was characterized by the possibility of memory recovery in repeated training or D-cycloserine treatment. The late stage occurred 10 days after amnesia induction and was characterized by loss of ability for memory recovery in repeated training and exposure to D-cycloserine.

Exposure to protein synthesis inhibitors before reminder session. Cycloheximide (100 mg/kg) or anisomycin (75 mg/kg) injections before reminder session resulted in amnesia that lasted at least 1-1.5 months. Latent periods of the reactions to CS at that time were substantially shorter that in control animals ($p<0.0001$) and were similar to latencies of the reaction to DS and reactions to banana in naïve snails ($p>0.05$, Fig. 1, *a*). Repeated training 2 weeks after amnesia induction did not result in skill formation: no differences were detected in latencies of the reaction to CS or DS ($p>0.05$, Fig. 1, *b*). However, these snails learned to reject new type of food: fresh cucumber. Cycloheximide injections 3 h after the reminder session had no effect on

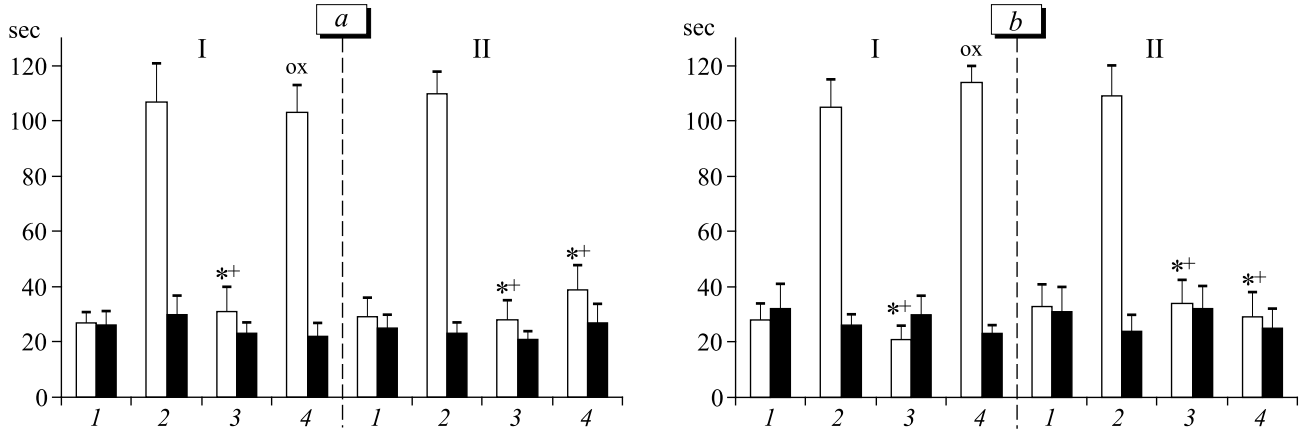

Fig. 2. Stages of amnesia development following impairment of memory reconsolidation. *a*: repeated training 3 (I) and 10 (II) days after amnesia induction by MK-801/reminder. *1*) naïve snails; *2*) saline injection before the reminder session; *3*) reactions to food stimuli following exposure to MK-801/reminder; *4*) test 24 h after repeated training of the snails from 3 groups. *b*: D-cycloserine injections in combination with reminder 3 (I) and 10 (II) days after amnesia induction by MK-801/reminder. *1-2*) see a; *4*) test of snails from 3 groups 24 h after exposure to D-cycloserine/reminder.

memory preservation (Fig. 1, *a*). Thus, the time window for the development of memory reconsolidation on translation processes was less than 3 h.

Combined administration of MK-801 and cycloheximide or APV and anisomycin before the reminder session. Two weeks after combined exposure to MK-801 and cycloheximide or APV and anisomycin, latencies of the reactions to CS were similar to those in control snails ($p>0.05$) and were higher than latencies of the reaction to DS ($p<0.0001$, Fig. 3, *a*). Thus, no disturbances in skill reproduction were detected following combined injections of NMDA-antagonists and translation inhibitors before the reminder session.

Cycloheximide administration 3, 6 or 9 h after the exposure to MK-801/reminder. Test 2 weeks after exposure to MK-801/reminder and subsequent (3 h or 6 h later) cycloheximide injection revealed that latencies of the reactions to CS were longer than in response to DS ($p<0.01$), but shorter than latencies of the reactions to CS in control animals ($p<0.01$; Fig. 3, *b*). Test 2 weeks after exposure to MK-801/reminder and subsequent cycloheximide administration 9 h later demonstrated that latencies of the reactions to CS and DS were similar ($p>0.05$). Thus, cycloheximide injections 3 and 6 h after exposure to MK-801/reminder prevented amnesia, whereas complete amnesia was observed following cycloheximide administration 9 h later.

Effects of DRB and actinomycin D administered before reminder session. Test 2 weeks after exposure to actinomycin D (1 mg/kg) and reminder or DRB (50 mg/kg) and reminder demonstrated (Fig. 1, *a*) that latencies of the reactions to CS did not differ from that in control animals ($p>0.05$) and were longer than latencies of the responses to DS ($p<0.0001$) and reactions to banana in naïve snails ($p<0.0001$). Thus, actinomycin D or DRB injections before the reminder session had no effect on skill performance.

Combined effects of MK-801 and DRB, MK-801 and actinomycin D, or APV and actinomycin D before the reminder session. Test 2 weeks after exposure to MK-801 and actinomycin D, MK-801 and DRB, or APV and actinomycin D revealed (Fig. 3, *c*) that latencies of the reactions to CS did not differ from that in

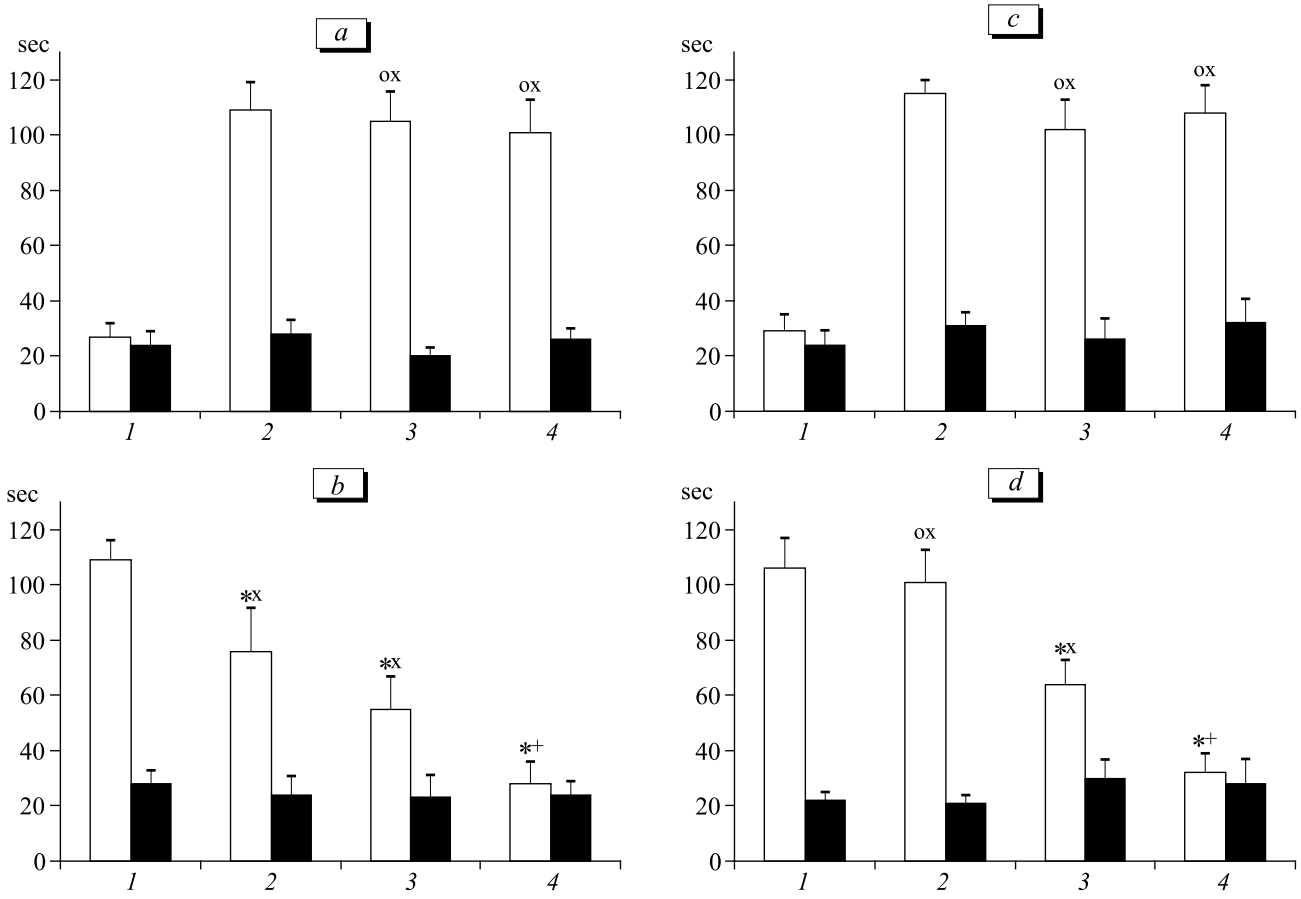

Fig. 3. Amnesia induction by exposure to neurotransmitter antagonist/reminder depends on both protein and mRNA synthesis. *a*: native snails (*1*), saline (*2*), MK-801 and anisomycin injections (*3*) or methiothepin and anisomycin injections (*4*) before the reminder session; *b*: cycloheximide injection followed MK-801 administration with 0h (combined injections; *1*); 3 h (*2*); 6 h (*3*); 9 h (*4*) interval; *c*: *1-2* see *a*, APV and actinomycin D injections (*3*) or MK-801 and DRB injections (*4*) before the reminder session; *d*: actinomycin D administration followed MK-801 injection with 0 h (combined injections; *1*), 3 h (*2*), 6 h (*3*), or 9 h (*4*) interval.

control snails ($p>0.05$) and were longer than latencies of the reactions to DS and reactions to banana in naïve animals ($p<0.0001$). Thus, no disturbances in representation of conditioned food aversion were noted following combined injections of NMDA-antagonists and transcription inhibitors.

Actinomycin D administration 3, 6, or 9 h after exposure to MK-801/reminder. Test 2 weeks after exposure to MK-801/reminder and subsequent (3 or 6 h later) actinomycin D injection demonstrated that latencies of the reactions to CS were longer than in responses to DS ($p<0.0001$, Fig. 3, *d*). Test 2 weeks after exposure to MK-801/reminder followed by actinomycin D administration 9 h later revealed similar latencies of the reaction to CS and DS ($p>0.05$). Thus, actinomycin D injection 3 and 6 h after exposure to MK-801/reminder prevented amnesia, whereas complete amnesia was observed when actinomycin D was administered 9 h latter.

Thus, in snails trained in conditioned food aversion paradigm, the reminder procedure (presentation of conditioned stimulus) against the background of exposure to protein synthesis inhibitors and NMDA receptor antagonists resulted in stable amnesia [2,4,5]. Time window for memory reconsolidation dependence on translation was less than 3 h. In addition, injections of mRNA synthesis inhibitors before the reminder session had no effect on memory preservation in snails. Thus, protein synthesis inhibitors impaired reconsolidation of long-term food aversion memory, whereas mRNA synthesis inhibitors had no effect on this process. We proposed that the proteins essential for memory reconsolidation may be translated from previously synthesized mRNA stored in a silent state [3]. It was assumed that silent mRNA is reactivated when synapses are stimulated, and translated proteins become involved in specific morphofunctional modification of synaptic connections, that underlies the mechanisms of long-term memory storage [8,11].

Mechanisms of amnesia induced by impairment of reconsolidation of long-term food aversion memory are essentially different. We revealed that isolated NMDA receptor antagonist injections before the reminder session resulted in amnesia, whereas combined administration of MK-801 or APV and protein or mRNA synthesis inhibitors before the reminder did not result in impaired skill representation. These findings indicated that as distinguished from memory reconsolidation, amnesia induction mechanisms depend on both protein and mRNA synthesis. In addition, we revealed that the time window of NMDA-dependent amnesia sensitivity to protein or mRNA synthesis inhibitors was about 9 h: cycloheximide and actinomycin D prevented amnesia development when were administered 3 h and 6 h after, but not 9 h after exposure to MK-801/reminder. We supposed that memory reconsolidation and amnesia require synthesis of process-specific proteins, which have different metabolism dynamics [2,3].

Thus, for the first time in snails trained in conditioned food aversion paradigm we revealed selective mechanisms of long-term memory reconsolidation and amnesia induced by reconsolidation impairment. Memory reconsolidation impairment by neurotransmitter antagonists or protein synthesis inhibitors induced amnesia characterized by the possibility for memory recovery in repeated training and exposure to D-cycloserine (early stage) or by resistance to mentioned procedures (late stage). Apparently, late stage is associated not with retrieval impairment or memory erasure, by with its inability to consolidate. In addition, we demonstrated that memory reconsolidation depends on translation, but not transcription, whereas amnesia induced by exposure to glutamate receptor antagonists during the reminder session depends on both translation and transcription processes. These findings indicate that amnesia induction, like inductions of other long-term adaptive brain reorganizations, depends on synthesis of specific mRNA and proteins.

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