Role of DNA Methylation in Mechanisms of Anterograde Amnesia

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Abstract

Previously, we found that impairment of conditioned food aversion memory consolidation or reconsolidation in snails by NMDA glutamate receptors antagonists led to the induction of amnesia changing over time. In particular, at the later amnesia stages (10 or more days), repeated aversion training for the same food type that was used in the initial training did not induce long-term memory formation. In these animals, long-term aversion memory for a new food type was formed. We characterized this amnesia as specific anterograde amnesia. In the present work, using DNA methyltransferases (DNMT) inhibitors, the DNA methylation processes role in mechanisms of anterograde amnesia and recovery from amnesia was investigated. It was found that in amnestic animals, DNMT inhibitor administration before or after repeated training led to the rapid long-term conditioned food aversion memory formation. It depended on proteins and mRNA synthesis at certain time windows. Thus, protein synthesis inhibitors administration before or immediately after repeated training, or RNA synthesis inhibitor injection after training, prevented memory formation induced by the DNMT inhibitor. The effects of DNMT inhibitors were specific for certain conditioned stimulus, since these inhibitors did not affect amnestic animals training for a new food stimulus. DNMT inhibition during second training removed blockade of these genes' expression, opening up access to them for transcription factors synthesized during training. Thus, this work was the first to study the molecular mechanisms of anterograde amnesia, as well as memory recovery, which can be important for search for pharmacological correction of this neuropsychic pathology.

Introduction

The fundamental task of neurobiology and medicine is to study the mechanisms of amnesia occurring after long-term memory impairment. Central to this task is the question of what lies at the heart of amnesia: impairment of retrieval or memory “trace” storage [1, 2, 3, 4, 5].

Researches used different experimental approaches to answer this question. After long-term memory consolidation or reconsolidation impairment, the possibility of spontaneous memory recovery, its recovery after the presentation of conditioned or unconditioned stimuli, when moving animals to a new context, was studied [2, 6, 7, 8, 9]. In addition, it was found that in amnesic animals, memory can be restored during optogenetic stimulation of cells containing specific information about memory (engram cells) [10]. Some researches have also shown that the use of psychostimulant drugs (in particular, substances that modulate the action of monoamines) before testing can also lead to memory recovery [11, 12, 13, 14]. The memory recovery process correlated with activating molecular cascades in neurons, including some early genes transcription and protein synthesis [15].

Based on the results obtained, it was suggested that memory expression after these manipulations demonstrates that amnesia resulted from difficulties in the retrieval and memory trace impairment in some part. When the memory was not expressed, it was assumed that memory was “erased” [2, 5, 8, 16, 17].

Repeated training is another experimental method to study amnesia mechanisms [4]. It makes sense to expect a direct correlation between the number and severity of memory trace impairments, as well as the number of repeated trainings required for memory formation [18]. A smaller number of unconditioned and conditioned stimuli in repeated training than the initial training could indicate partial memory trace impairment which is compensated by repeated training, Ebinhaus's saving effect [2, 4, 7, 18]. If the required number of stimuli for memory formation is the same during repeated and initial training, it could be that the memory was “erased” and reformed during subsequent training.

At the same time, we discovered a new, unexpected result of repeated training. We examined the characteristics of amnesia following the conditioned food aversion memory reconsolidation impairment. As an animal model we used grape snails. We studied the features of amnesia after the impairment of conditioned food aversion memory reconsolidation in grape snails. It was found that administration of NMDA glutamate receptors antagonist or inhibitor of protein synthesis prior to conditioned stimulus reminder led to the development of amnesia, during whose early stage (up to day ten after induction) with repeated training the likelihood of memory recovery slows down [19, 20, 21, 22]. However, it was discovered that repetition of training does not result in formation of long-term memory at the late amnesia stages (after 10th day). Also, the same animals developed an aversive memory for a new food type [19, 21, 22]. Based on these and other results, we hypothesized that memory reconsolidation impairment can cause amnesia. This is an active time-evolving process that leads to specific anterograde amnesia [19, 20, 21, 23].

Studies have shown that anterograde amnesia can be found in other animals than snails. Storozheva et al. found that rats with impaired gustatory aversion memory reconsolidation by an NMDA glutamate antagonist show amnesia. This was characterized by impairment of long-term memory formation during the second training session [24]. An inhibitor of glycogen synthase kinase 3 in rats caused impairment of spatial memory reconsolidation during the Morris water maze [25]. In studies on chickens, Tiunova et al. found that memory impairment could be caused by the administration of NMDA receptor antagonists or protein synthesis inhibitors before training or memory reconsolidation. The second training, however, did not result in long-term memory formation [26]. These facts indicate that anterograde amnesia may occur when memory is impaired in any of the three species (birds, mammals, and mollusks). However, further research is required to establish the boundaries for anterograde amnesia in different animal types. It is possible that the discovery of anterograde amnesia in a specific animal species and the creation of an experimental model to explain its formation could be of practical value for understanding the mechanisms and pharmacological correction tools development.

The extreme resistance of specific anterograde amnesia to memory restoring procedures suggests that persistent changes in neural and synaptic processes may mediate it. Among these processes, genetic and epigenetic processes stand out; they can lead to long-term, specific and stable rearrangements of nerve cell gene expression [7, 18, 27, 28].

The epigenetic mechanisms significance, including methylation of DNA and histones, as well as DNA and histones phosphorylation and acetylation is widely studied in learning processes in different animal species [29, 30, 31]. Highly conserved enzymes mediate DNA methylation, DNA methyltransferases (DNMT), present in invertebrates together with vertebrates [32, 33, 34]. Inhibition of DNMT in the model of rat brain studies led to impairments in formation of spatial long-term memory and conditioned memory of fear reflex [35, 36, 37, 38]. The DNMT inhibitor disrupted object recognition memory performance [39]. A DNMT inhibitor counteracted scopolamine-induced amnesia in an object recognition test [40]. In bees, DNMT inhibitors did not affect the learning dynamics of appetizing olfactory conditioning and memory strength [32]. At the same time, inhibitors reduced the olfactory discrimination ability between conditioned and new odor stimulus [32]. In the mollusk, Aplysia, inhibition of DNMT during training blocked the long-term non-associative and associative memory consolidation and synaptic relief underlying memory [41]. In a study on the pond snail, Lukowiak et al. and Rothwell et al. found that DNMT inhibition doesn't change not enhanced operant conditioned memory expression; however, after this manipulation there was no memory enhancement caused by stress or pharmacological agents [42]. Memory maintenance also influenced by DNA methylation. Some researches have shown that injections of DNMT inhibitors into the brain structures of rats or aplysia mollusk long after training caused impairments in long-term memory reconsolidation [18, 31]. DNA methylation seems to be a conservative mechanism for long-term memories in different species.

We have previously investigated DNA methylation participation long-term conditioned food aversion memory formation in animal model using snails [43]. Solntseva et al. found that learning under the action of a DNMT inhibitor caused memory formation stronger than memory formed without the use of the inhibitor. This strong memory was resistant to reactivation and amnestic agents.

Another study was done to determine the role of DNA methylation in the amnesia mechanisms following aversion memory reconsolidation impairment using NMDA glutamate receptors antagonists [44]. In the early amnesia stages (up to 10 days), pairings of DNMT inhibitors injections with a conditioned reminder caused recovery of memory. We proposed the hypothesis according to which reminder presented during the amnesia development induces DNA methylation-dependent reactivation and labilization of the amnesia processes. DNMT inhibition at this time caused amnesia process disruption and recovery of memory. At the same time, in the late amnesia stages (from the 10th day), pairings of DNMT inhibitor and reminder had no effect on its characteristics. Thus, at these times, the reminder does not seem to cause amnesia reactivation dependent on DNA methylation.

However, these results do not mean that anterograde amnesia cannot be reversed in another experimental paradigm. As noted above, a stable change in transcription processes due to methylation/demethylation of specific genes, which prevents the conditioned food aversion memory formation in case of repeated training, can be the basis for specific anterograde amnesia. The DNMT inhibitor use during repeated training can lead to the derepression of genes involved in memory mechanisms and contribute to memory recovery or formation.

This assumption served as the basis for planning the present study, in which methylation of DNA and its involvement in the specific mechanisms of anterograde amnesia and reversal of this amnesia type were studied. Disruption of memory reconsolidation process caused by antagonist of NMDA glutamate receptors induced amnesia in mollusk's conditioned food aversion model. At different periods after amnesia induction (10 or 45 days), repeated training for aversion of the same conditioned food type stimulus as in the initial training under the action of DNMT inhibitors was performed. In addition, we studied the possible mechanisms of the DNMT inhibitors' effects and their relationships with translation and transcription processes.

Methods

Object

We conducted all the experimental procedures on grape snails (Helix Luculum L.) of 30 and 35 g weigh. Their dwelling during not less than three weeks up to experimental procedures and in between them were "home boxes" providing animals with a vegetable diet. Three days of starvation was administered to the animals before they were trained. The experimenters also fed the animals if there was sufficient time between experiments to prevent them from being starved for three days.

Training procedure

Conditioned food aversion was developed as previously described [19]. Mollusk studies widely use this learning model. The snail's shell was used as a place for bracket fixation in order to let animals move somewhat free over a ball on the physiological saline surface. Food fragment 2 – 3 g in weigh was as 0.5 cm as near to the snail's head on a mechanical manipulator. The CS was raw carrots, and an electric current (50 Hz, 300 ms, 1.2 mA) was an reinforcing unconditioned stimulus (US). An electric shock was applied in case animal tried to eat the food. Food fragments and physiological saline were the points of electrode fixation. Experimenters used the ball made from plasmas and wrapped in metal foil. Consummatory reactions were disrupted by electric shock with a withdrawal response, when snail hides in sink. In addition, the experimenters presented an unpaired differentiating stimulus (DS, boiled carrots) without the application of an electric current. A video recorder and a PC fixation was applied for the latencies of the onset of consummatory reactions. The researcher performed the paired presentation of raw carrots and unconditioned electric stimulus (CS+US) every 20 – 30 min. In consecutive three days snails got one training a day. In the day of training, the animals were presented with 4 CS alternated with 4 DS. The interval between CS and DS varied from 8 to 15 minutes. The absence of consummatory reactions in 2 min period was followed by the fact, that researcher removed the food and did not apply electric shock. Reactions latencies growth from 20 – 35 s in intact snails up to 100 – 120 s or raw carrots refusal in 120 s period together with significant CS and DS reactions latencies differences were the key points for the food conditioned aversive reaction revealing. On average, snails received 5 – 6 CS and US pairings.

Substances and injections

Researchers studied the influence of antagonist of NMDA glutamate receptors MK-801, the inhibitor of protein synthesis cycloheximide (CYH), the inhibitor of RNA synthesis actinomycin D (ACD), DNMT inhibitors zebularine, RG108 (N- Phthalyl-1-tryptophan), and 5-AZA (5-Aza-2'-deoxycytidine) (Sigma-Aldrich, USA). The solvent for neurotransmitter receptors antagonist was saline. Protein synthesis inhibitors, RNA synthesis, and DNMT inhibitors were dissolved in DMSO and then diluted in saline. The volume of the solution administered to the snails was 0.2 ml/snail. The content of DMSO in the solution was 1%. The solutions were injected into the body cavity using a syringe through the skin of the leg insensitive part under the mantle roll. The dose of MK-801 was 0.25 mg/kg, cycloheximide – 100 mg/kg, actinomycin D – 1 mg/kg, zebularine – 10 mg/kg, 5-AZA – 5 mg/kg. RG108 was usually used at a dose of 3 mg/kg. In a separate experiment, we studied the dose dependence of the RG108 effects: we administered the inhibitor to snails at doses of 1 or 6 mg/kg. At the indicated doses, the substances were used to study the processes of learning and memory in different animals, including mollusks [18, 19, 21, 37, 42]. Researchers injected control snails with saline before the reminding procedure and with DMSO solution before repeated training. A "blind" method was used in work – different researchers carried out solutions injections into snails and memory testing.

The DNMT inhibitors used have different mechanisms of action. 5-AZA works as cytosine analog disrupting DNMT function by covalently binding to the enzyme's catalytic site and may require incorporation into the DNA strand to exert its inhibitory effect. Zebularin is also a cytosine analog, an inhibitor of cytidine deaminase, and a DNA demethylation agent. Zebularin was developed to address the disadvantages of 5-AZA, particularly its cytotoxicity, instability, and short half-life. In contrast, RG108 is a non-nucleoside inhibitor that acts by reversibly blocking the active DNMT site without the need for incorporation into the DNA strand. In addition, most researchers suggested RG108 is less likely to exhibit nonspecific effects as it was explicitly modeled to fit the active pocket of DNMT. A DNMT-like enzyme was identified in the mollusk Aplysia [34], and Rajasethupathy et al. shown that RG108 blocks the catalytic site of DNMT [41].

Testing

Responses to food stimuli were tested one h before training, 1, 10, and 45 days after training, and at different times after repeated training (Figs. 1–4). During testing, we placed the snails in a training environment for 40 – 50 min, presented the CS and DS with an interval of 15 – 20 min, and measured the latencies of consummatory reactions for 120 s. If animals tried to eat food, we stopped testing. During testing, electroshock was not applied.

Reminder and amnesia induction

Two days after training, researchers injected snails with MK-801 or saline in a neutral context (i.e., on a glass plate) (Fig. 1). Then the researchers placed animals in a training context (i.e., on plastic balls), and after 30 minutes, performed a reminder procedure as described below: presented CS (raw carrots) three times with an interval of 15 minutes. The latencies of consummatory reactions were recorded for 120 s. If

the animals tried to eat the food, we removed it. We did not apply US during the remainder. The reminder was followed by snails removed from their balls to their home boxes within an hour.

Repeated training and training for a new type of food

10 or 45 days after the amnesia induction, researchers injected the snails with solutions of substances and carried out the repeated training for aversion of the same type of food as in the initial training (raw carrots) or training for aversion of a new type of food (apple) using the methods described above. The memory was tested 1 and 10 days after repeated training.

Uncombined presentations of CS and US

Ten days after the amnesia induction, we presented 4 CS, 4 US, and 4 DS to the animals unpaired. The intervals between stimuli were 8-14 minutes.

Studied groups of animals

Snails that received saline+reminder or MK-801+reminder 2 days after training and tested 10 and 45 days later;

animals that were injected with a DNMT inhibitor (RG108, 5-AZA or zebularine) 10 or 45 days after amnesia induction (MK-108+reminder) and researchers carried out repeated training for aversion of the food used in the initial training (raw carrots) 1 hour later;

animals in which, ten days after amnesia induction, researchers carried out repeated training and 15 minutes or 4 hours after its completion, RG108 was administered;

snails which were co-injected with RG108 and an inhibitor of protein or RNA synthesis ten days after amnesia induction 1 hour before repeated training;

snails injected with RG108 10 days after induction of amnesia 1 hour before retraining, and an inhibitor of protein or RNA synthesis was injected 15 minutes or 4 hours after training;

animals ten days after amnesia induction presented with CS and US in an uncoupled manner;

snails injected with different doses of RG108 (1, 3, or 6 mg/kg) before repeated training (determination of dose-dependence of effects);

snails which ten days after amnesia induction were injected with RG108 and presented with a single combination of CS+US;

animals injected with RG108 10 days after the amnesia induction and after one h trained to aversion of a new type of food (apple) for one day.

Researchers used each animal in only one series of experiments.

Data analysis

Classic parametric tests must produce accurate results if their assumptions (e.g. normality and heteroscedasticity) are met. Semiquantitative data, such as 120 s cut off latency, was used. This required only nonparametric analysis methods. We used nonparametric criteria for analysing the data. Researchers averaged the data and calculated the standard deviation of the mean. Researchers compared the latencies for consummatory reactions to CS presentation in animals injected before the reminder with the latency of responses to DS, raw carrot presentation prior to training in the same animal group, as well as the latencies for responses to CS in snails injected before the reminder. The Mann-Whitney rank sum test was used to compare the latencies between the different animal groups. To compare data from the same snails (CS vs DS), we used the Wilcoxon signed rank test.

Results

Conditioned food aversion long-term memory formation and amnesia after memory reconsolidation impairment

Untrained snails had average consummatory reactions times of 20 – 35 s to raw and boiled carrots (Fig. 1B; Test 1). We observed the superiority in CS reactions time compared to the same in DS ($p<0.05$, Wilcoxon test) and in CS recorded the day before ($p<0.05$, Wilcoxon test). Consummatory responses to CS (raw carrots) were significantly slower than those to DS (boiled carrots; $p<0.0001$; Wilcoxon test) and to raw carrots presented before training ($p<0.0001$; Wilcoxon test). Five to six presentations of unconditioned and conditioned stimuli were required for long-term memory formation in three days.

Two days after training, snails of the control group (n=20) were injected with saline and presented with a reminder. Animals tests 10 and 45 days after injections paired with the reminder showed (Fig. 1, B, T3, and T4) the superiority in CS reactions time compared to the same in DS (after 10 and 45 days $z>5.1$, $p<0.0001$; Wilcoxon test) and in raw carrots prior to training procedure (after 10 and 45 days: $z>5.03$ $p<0.0001$; Wilcoxon test).

To snails of another group (n=16), we injected MK-801 2 days after training and performed a reminder procedure (Fig. 1, A). Testing snails 10 or 45 days after pairing of MK-801 and reminder revealed the amnesia development (Fig. 1, B, T3, and T4): in both time intervals, responses to CS were significantly slower than those to DS (for 10 and 45 days: $z<0.84$, $p>0.22$; Wilcoxon test) and faster than the those to CS in control snails ($z>4.9$, $p<0.0001$; Mann-Whitney test).

Thus, not only long-term conditioned memory, but also amnesia occurred after disruption of memory reconsolidation persisted in snails for at least 1.5 months. We obtained similar results earlier [19, 21, 45].

Ten or forty-five days after induction of amnesia caused by pairings of NMDA glutamate receptors antagonist+reminder, no long-term memory was revealed

Two days after training, we administrated two groups of snails with MK-801+reminder. Animals of one group (n=24) after ten days, and the other group after 45 days, underwent repeated aversion training for

same CS (raw carrots) used during the first training session (Fig. 2, A). We injected the snails with a DMSO solution 1 hour before each training session. It was found the superiority in CS reactions time compared to those in previous day during each of the three days of repeated training and to the same parameter in DS (Fig. 2, D) ($p<0.05$, Wilcoxon test). However, on each next day, responses times to CS were the same as those to DS ($p>0.2$, Wilcoxon test). Thus, memory of short-term type still was untouched in the amnestic snails. At the same time, one day after repeated training we showed (Fig. 2, B and C, T3) that there was no memory of long-term type – it was a reduction in CS reactions time compared to the same in control animals (for 10 and 45 days: $z=4.9$, $p<0.0001$; Mann-Whitney test) and no distinction to the same parameter in DS ($z=0.91$, $p>0.16$; Wilcoxon test). During repeated training ten days after initial training, the number of CS and US pairings was more significant compared to the first training session: 9.7±0.7 and 6.1±0.4, respectively ($z=2.9$; $p=0.0019$, Wilcoxon test); after 45 days, the corresponding values were: 10.1±0.8 and 6.3±0.5 ($z=2.8$; $p=0.008$, Wilcoxon test).

Thus, repeated training at different time intervals after the induction of NMDA-dependent amnesia led to formation of short-term memory not long-term memory transforming. We have previously shown that impairment of conditioned food aversion memory by another NMDA glutamate receptor antagonist or protein synthesis inhibitor also led to the development of anterograde amnesia [19, 20, 21]. Amnesia was specific since amnestic snails were able to be trained for aversion to a new type of food for them [19, 21]. According to the indicated features of amnesia, we characterized it as specific anterograde amnesia.

Ten or forty-five days after amnesia induction injections of DNMT inhibitors before repeated training-induced memory formation

Two groups of snails ten days (n=24) or 45 days (n=12) after amnesia induction (MK-801+reminder) were injected with RG108 and repeatedly trained after one h (Fig. 3, A). In both time intervals, we found the progressive growth in CS reactions time during the first day of training (Fig. 3, B and C). By the fourth pairing of CS+US, consummatory responses to CS rose up to 110 – 120 s and were slower than those to DS (for 10 or 45 days: $z>3.6$; $p<0.0001$; Wilcoxon test). The CS and US stimuli pairings as much as 2.5±0.12 were required for the formation of long-term memory in repeated training after ten days and 2.3±0.21 in repeated training after 45 days. On days 2 and 3, repeated training was not performed since all animals trained during the first day retained aversive responses to CS: during testing 1 and 10 days after repeated training (Fig. 3, B and C, T3 and T4) there was no distinction in CS reactions time compared to the same parameter in control trained snails (for 10 or 45 days: $z <0.11$; $p> 0.42$; Mann-Whitney test) and there was superiority in CS reactions time compared to the same in DS ($z>3.6$; $p<0.0001$; Wilcoxon test).

Snails of the other two groups, as after induction of amnesia ten days passed (MK-801+reminder), got zebularine (n=10) or 5-AZA (n=11) injections and were repeatedly trained (Fig. 4, A). As in the experiments described above, both snails groups showed aversion to the CS as the first day of training finished. After 1 and 10 days after this shortened repeated training, we found (Fig. 4, B and C, T3 and T4) the consummatory reactions to CS were the same as those to CS in control trained animals (for zebularine

and 5-AZA, after 1 and 10 days: z <1.8; p> 0.14; Mann-Whitney test) and were slower than those to DS (z>2.75; p<0.0019; Wilcoxon test).

One group of snails (n=8), as after induction of amnesia (MK-801+reminder) ten days passed, was injected with RG108, and, one h later, one pairing of CS+US was presented (Fig. 5, A). After 24 h, we found (Fig. 5, B) the reduction in CS reaction time compared to control trained animals (z=3.2, p=0.002; Mann-Whitney test) but also the superiority in CS reaction time compared to the same parameter in DS (z=2.45, p=0.007; Wilcoxon test). However, after ten days, CS reactions were the same as in DS (z=0.59, p=0.57; Wilcoxon test).

Thus, repeated training for aversion of raw carrots under the action of a DNMT inhibitor led to the rapid long-term memory formation within one day of training, and snails retained this memory for at least ten days. For the memory formation during repeated training, four pairings of CS+US are required, a single pairing of CS+US induced memory expression, which was testable after 24 h, but not after ten days.

Interestingly the described DNMT inhibitors effects are not related to the peculiarities of the taste and smell of the food used. In an additional series of experiments using a banana as the CS, we found that injections of the DNMT inhibitor RG108 before repeated training of the amnestic animals caused rapid long-term memory formation, similar in dynamics to those described for the raw carrots CS. In addition, it was found that if it passed ten days as amnesia started because of APV and reminder pairing, injections of RG108 before repeated training also caused a rapid long-term memory formation. Given the fundamental similarity of the results obtained, we do not provide specific data on these experiments.

Injection of DNMT inhibitors before the unpaired CS and US presentation brings no memory recovery (unpaired control)

As after induction of amnesia (MK-801+reminder) ten days passed, snails (n=11) got the RG108 injections and unpaired CS, DS, and US after one h with an interval of 7 – 14 min. We presented each stimulus four times a day (Fig. 5, A). 24 h after administration of RG108 before unpaired control, it was found (Fig. 5, C) the reduction in CS reactions time compared to the same parameter in control trained animals (n=16) (z=5.21, p<0.0001; Mann-Whitney test) and no distinction in CS reactions time compared to the same parameter in DS (z=0.56, p=0.33; Wilcoxon test).

Thus, injections of the DNMT inhibitor prior to the unpaired presentation of CS or US did not result in formation of long-term memory.

Dose-dependent effects of DNMT inhibitors during repeated training

In most experiments, we used RG108 as a DNMT inhibitor with an inhibitor dose of 3 mg/kg. We also conducted experiments to determine the dose dependence of the RG108 effects (Fig. 6, A). As after induction of amnesia (MK-801+reminder) ten days passed, we injected snails (n=8) with RG108 6 mg/kg and did a repeated training. We found (Fig. 6, B) that the consummatory reactions to CS and DS became

slower on the day of training. On the next day, as repeated training passed (Fig. 6, B, T3), there was no distinction in CS and DS reactions times compared to each other ($z=0.54$; $p=0.51$; Wilcoxon test) or to CS reaction time in control trained snails ($z=62$; $p=0.33$; Mann-Whitney test). In the next ten days, consummatory reactions to DS became as slow as those in control animals (Fig. 6, B, T4) ($z=1.17$; $p=0.15$; Mann-Whitney test). At the same time, consummatory reactions to CS were still the same as those in control snails ($z=0.62$; $p=0.21$; Mann-Whitney test). Thus, the administration of RG108 high dose before retraining led to impairment of the snail's discriminatory ability to distinguish between CS and DS, which persisted for several days. It is interesting to note that similar results were obtained in studies on bees, in which the use of a DNMT inhibitor during olfactory learning caused impairment of odor discrimination on the CS and odor on new food [32].

Another group of snails (n=10), as after induction of amnesia (MK-801+reminder) ten days passed, got 1 mg/kg RG108 injections and were subjected to repeated training one h later (Fig. 6, A). 24 hours and ten days after repeated training, it was found (Fig. 6, C, T3, T4) the reduction in CS reactions time compared to the same parameter in control trained animals ($z>4.7$, $p<0.0001$; Mann-Whitney test) and no distinction compared to the same parameter in DS ($z<2.5$, $p>0.7$; Wilcoxon test). Thus, using RG108 at a 1 mg/kg dose prior to repeated training did not cause memory formation.

Time windows of facilitating effects of DNMT inhibitors on memory recovery during repeated training

In previous experiments, we found that injections of DNMT inhibitors before repeated training led to rapid memory formation. It was interesting to test whether DNMT inhibitors would have a memory-facilitating effect on memory formation in case of injection after repeated training. As after induction of amnesia (MK-801+reminder) ten days passed, the dependence of the effects of RG108 on memory formation upon its administration (Fig. 7, A) was investigated in one group of animals (n=15) 15 min after repeated training and in another group (n=14) 4 hours after training. DNMT inhibitor administration in each of the time intervals led to the long-term memory formation: the consummatory reactions to CS 1 and 10 days as training passed were slower than those to DS (Fig. 7, B and C, T3, T4) ($z>3.1$, $p<0.0001$; Wilcoxon test) and were the same as consummatory reactions to CS in control snails ($z<1.7$, $p>0.12$; Mann-Whitney test).

Thus, the data presented above and in this section show that DNMT inhibitor injections to snails with amnesia developed contribute to the formation of long-term memory in all studied time intervals – 1 h before repeated training, and 15 min and four h after its completion.

Dependence of the DNMT inhibitors effects on protein and RNA synthesis

Many experiments have shown that protein and RNA synthesis inhibitors can impair long-term memory consolidation [46]. We have studied the conditioned food aversion memory formation dependence on the translation and transcription processes induced by the administration of DNMT + repeated training in

amnestic snails. In this series of experiments, six groups of animals were studied (Fig. 8, A). All snails were injected with RG108 10 days after amnesia induction 1 hour before repeated training. The RG108 combined with cycloheximide (n=22) or actinomycin D (n=12) were injected to animals of the two groups. Snails of the other two groups got cycloheximide (n=14) or actinomycin D (n=12) 15 min after repeated training. We injected the snails of the remaining two groups with cycloheximide (n=12) or actinomycin D (n=15) 4 hours after repeated training.

In snails that received RG108+cycloheximide before repeated training or cycloheximide 15 min after administration of RG108 + repeated training, there was a fast growth of CS reactions time followed by aversive responses to CS by the animals as day of training fininshed (Fig. 8, B and C). However, as after induction of amnesia one day passed (Fig. 8, B and C, T3, T4) there was the absence of long-term memory: consummatory reactions to CS were the same as those to DS ($z<0.24$; $p>0.41$; Wilcoxon test) and consummatory reactions to CS were slower than those to CS in control snails ($z>3.6$; $p<0.0001$; Mann-Whitney test). At the same time, the injections of cycloheximide four h after the RG108 and training pairing could not prevent formation of long-term memory: testing after 1 and 10 days revealed (Fig. 8, D, T3, T4) no distinction in CS reaction time compared to the same parameter in control snails ($z<1.8$; $p>0.14$; Mann-Whitney test) and superiority in CS reaction time compared to the same parameter in DS ($z>3.0$; $p<0.0001$; Wilcoxon test).

In animals that were administrated with RG108+actinomycin D before repeated training, no impairments in formation of long-term memory were found: consummatory reactions to CS became slower during repeated training and were the same as those to CS in control snails during testing 1 and 10 days after training (Fig. 8, E, T3, T4) ($z<1.06$; $p>0.47$; Mann-Whitney test) and consummatory reactions to CS were slower than those to DS ($z>3.02$; $p<0.0001$; Wilcoxon test).

In snails that received actinomycin D injections 15 min or four h after RG108 + repeated training administration, memory formation impairment was found (Fig. 8, F and G, T3, T4): testing after 1 and 10 days showed no distinction in CS reaction time compared to the same parameter in DS (for both tests: $z<1.6$; $p>0.09$; Wilcoxon test) and inferiority in CS reaction time compared to the same parameter in control animals ($z>4.8$; $p<0.0001$; Mann-Whitney test).

Thus, the protein synthesis inhibitor completely suppressed the formation of long-term memory induced by RG108 upon injections of the inhibitor at two-time intervals – before training and 15 min after it. At the same time, the administration of RNA synthesis inhibitor before training was not effective, whereas its injections 15 min or four h after training suppressed long-term memory formation. Interestingly, both inhibitors did not affect the growth in CS reaction times during training. This fact indicates that during repeated training during the action of the DNMT inhibitor, the expression of aversive responses to CS did not depend on the proteins and RNA synthesis. According to these features, we can characterize the facilitation of aversive reactions on the day of training as short-term memory.

Specificity of the DNMT inhibitors effects concerning CS

Previously, we found that conditioned food aversion training to a new food stimulus caused the formation of long-term memory as after induction of NMDA glutamate receptor antagonist or protein synthesis inhibitor caused amnesia 10 days passed [19, 21]. Thus, anterograde amnesia was specific to the baseline CS. We were interested in whether the DNMT inhibitor will facilitate the accelerated aversive learning of amnestic snails to a new food stimulus for them.

Snails (n=17) were initially trained to conditioned food aversion for raw carrots. As after induction of amnesia (MK-801+reminder by CS1 raw carrots) ten days passed, one-day training for aversion to a new type of food – an apple (CS2, four pairings of apples and electroshock). Animals were injected with RG108 1 hour before training. Consummatory reactions of intact snails to an apple were 26±3 sec. During training, the growth in CS2 (apple) reaction time during the first day of training was found. By the fourth pairing of CS2+US, the latencies of responses to CS2 reached 115 s and were slower compared to the same parameter in DS ($z=3.49$; $p<0.0001$; Wilcoxon test). The number of CS2+US paired stimuli received by the animals on the training day was 2.7+0.16. As after repeated training one day passed it was found that consummatory reactions to CS2 were slower than those to DS ($z=3.5$; $p<0.0001$; Wilcoxon test). However, as after training ten days passed no distinction in CS2 and DS reactions times were found ($z=1.5$; $p=0.09$; Wilcoxon test).

Thus, as after induction of amnesia ten days passed, the training of snails for an apple as a new food type under the DNMT inhibitor action led to the conditioned aversive memory formation for an apple, which, however, was retained for less than ten days. The results obtained indicate the features of the effects of the long-term memory formation facilitation induced by the DNMT inhibitor concerning the initial CS.

Discussion

Earlier and in the present work, we found that disrupted reconsolidation of conditioned food aversion memory after NMDA receptors antagonist+reminder administration caused amnesia development. During the later stages of development, amnesia was characterized by extreme resistance to different memory recovering procedures, including repeated training. In particular, ten or more days after the amnesia induction, no long-term memory consolidation, but short-term memory formation after second food aversion training for the same food type compared to the initial training within three days. In other words, the animals developed anterograde amnesia.

In the present study, we have shown that the processes of DNA methylation/demethylation can underlie anterograde amnesia. We found that DNMT inhibitor (RG108, zebularine, or 5-AZA) and repeated training pairing caused rapid formation of long-term memory within one day of training. The aversive reaction to CS persisted for more than ten days. Only RG108 injections and three or four pairings of CS+US induce memory formation, while formation of long-term memory after single CS+US pairing wasn’t found. The unpaired presentations of CS and US after DNMT inhibitor injections also did not induce formation of long-term memory. The effect of DNMT inhibitors was featured to the stimulus (raw carrots), applied

during first training session and in reminding CS in memory reconsolidation impairment. When amnestic snails were trained for aversion of a new type of food (apple) within one day under the action of a DNMT inhibitor, they retained memory for less than ten days.

The critical question for interpreting amnesia processes is what lies on an amnesia basis – whether it is a consequence of impaired memory trace storage or impaired retrieval [9, 47, 48, 49]. As noted above, DNMT inhibitors in amnestic snails eliminated the blockade of long-term memory formation and significantly accelerated training compared with the initial training. The phenomenon of accelerated memory formation during repeated training is known in neuroscience and psychology and is called the Ebinghaus saving effect [7, 9]. Some researchers believe that repeated training facilitates memory formation by increasing residual memory [4]. Thus, with the development of anterograde amnesia, memory is not erased; rather, snails can partially preserve the memory for the CS. Moreover, the mechanism for preserving this memory is very stable over time. In particular, we showed that administering the DNMT inhibitor + repeated training both ten days and 45 days after the anterograde amnesia induction led to equally effective accelerated memory formation.

Protein and mRNA synthesis processes involvement in anterograde amnesia mechanisms

The present study showed that for the reversal of anterograde amnesia and long-term memory formation, along with changes in DNA methylation, macromolecules are required. What is the relationship of DNA methylation processes with the processes of protein and RNA synthesis? Some researchers believe that if synthesis of plasticity proteins requires a change in DNA methylation, this would mean that a prerequisite for this synthesis is a change in the activity of one or several genes [41, 50]. On the other hand, the change in DNMT activity may depend on protein synthesis. Moreover, finally, protein synthesis may be necessary both for activating the RNA transcription processes and for changing DNA methylation.

The results we have obtained allow us to determine some regularities in the relationships between these processes. We found that paired injections of cycloheximide and RG108 to amnestic animals before repeated training did not prevent the expression of aversive responses to CS on the day of training. The memory retained on the day of training and not dependent on protein synthesis can be characterized as short-term memory. However, testing 1 and 10 days after training revealed impairment of long-term memory formation. Cycloheximide injections 15 min after RG108 + repeated training also led to long-term memory impairment. At the same time, injections of cycloheximide four h after RG108 + repeated training did not suppress the long-term memory formation. The administration of RNA synthesis inhibitor actinomycin D before training under the action of RG108 did not affect the formation of both short-term and long-term memory. Injections of actinomycin D 15 min or four h after administration of RG108 + repeated training suppressed long-term memory formation. The effect of DNMT inhibitor was manifested when it was administered both before repeated training and 15 minutes and 4 hours after it.

Thus, protein and mRNA synthesis inhibitors and DNMT inhibitors manifested the effects when administered at different time intervals. The synthesis of proteins involved in long-term memory formation was required several hours earlier than mRNA synthesis. From this fact, it follows that the synthesized proteins can be transcription factors that activated genes and mRNA transcription, involved in the mechanisms of long-term memory. In addition, synthesized proteins can regulate the activity of DNMT and other epigenetic factors. On the other hand, protein synthesis does not depend on DNMT activity – the DNMT inhibitor facilitated the formation of long-term memory when it was injected four h after training, i.e., after the end of the "time window" of the effectiveness of inhibitor of protein synthesis. Thus, with a certain degree of confidence, it can be argued that protein synthesis precedes and is necessary for the activation of gene transcription processes and DNA methylation/demethylation processes.

Literature data giving preference to any variant of the proposed schemes of molecular events for the formation of long-term memory in amnestic snails are incredibly scarce. Usually, researchers suggest two periods of mRNA and protein synthesis, which are necessary for long-term memory consolidation – the first during training and the second 3 – 6 hours later (Alberini and Kandel 2014). At the same time, data show the outrunning involvement of protein synthesis and delayed RNA synthesis in the plasticity mechanisms. In particular, Vickers et al. (2005) showed that late LTP in neurons of hippocampal slices was prevented by translational inhibitors after about 1 – 2 hours, whereas inhibitors of RNA synthesis were effective in blocking LTP starting after about 4 – 6 hours. Similar results were obtained earlier by us during studying the neural mechanisms of formation of conditioned food aversion long-term memory in snails [51]. Some studies have also shown that changes in DNA methylation can precede changes in transcription and be responsible for subsequent genomic regulation by transcription factors [52, 53]. On the other hand, studies on the culture of rat cortical neurons revealed that an inhibitor of RNA synthesis suppressed the increase in synaptic strength induced by RG108 [54].

One of the few works on the problem under discussion was carried out on the Aplysia mollusk. In particular, the model of behavioral sensitization has shown that the consolidation of long-term memory requires epigenetic regulation of the one or more genes transcription, mediated by DNA methylation [18, 41]. The authors point to the exciting possibility that this regulation may depend on early protein synthesis. It was found that the Piwi protein expression regulates methylation of the promoter of *the CREB2* early gene [41]. Early synthesis of this protein may provide long-term memory consolidation. In addition, DNA demethylation in the mammalian hippocampus requires the activity-dependent induction of the specific gene *Gadd45b* [55], which is consistent with the proposed idea.

The role of repressed mRNA in the mechanisms of anterograde amnesia

The fact that earlier induction of the protein's synthesis is necessary for memory formation during repeated training than the mRNA synthesis that we discovered requires a more detailed discussion. This result suggests that previously synthesized and translationally repressed mRNAs, which are involved in

the mechanisms of memory formation, should exist in neurons. Some researchers believe that mRNAs involved in the maintenance of long-term memory and synaptic plasticity are under strict spatial control, including the intracellular transport of translationally repressed mRNAs deposited in ribonucleoprotein particles [56]. The activation of individual synapses leads to the fixation of ribonucleoprotein particles and subsequent local translation of mRNA. Once released from granules, mRNAs can be repackaged in them, indicating that specific mRNAs undergo multiple cycles of translation and repression depending on synapse activity [56].

One of the possible mechanisms of local limitation of the proteins and mRNA functions during training can be the mechanism of synaptic tagging, the molecular identity of which has not yet been established [57]. Tagged synapses can selectively co-opt (synaptic capture) mRNA and newly synthesized proteins (plasticity-related proteins) required to induce long-term functional and structural rearrangements of synapses. However, we cannot apply the hypothesis of synaptic tagging as a mechanism for memory maintenance. In particular, in synaptic tagging, the half-life of the tag is several hours, whereas the half-life of the tag would have to be much longer for it to play a role in memory maintenance. Sossin proposed molecular complexes present in memory synapses for the role of a long-term synaptic tag that ensures long-term maintenance of synaptic modifications [58]. These molecular complexes retain their identity despite the ongoing molecular turnover. In addition, the complexes have specific properties due to a specific set of molecules being their constituents and allowing selective co-optation of transcription products. However, verifying these hypothetical molecular complexes is a problem for future experimental studies [58].

Based on the preceding, we can assume that proteins translated from the previously synthesized and translationally repressed mRNAs are involved in the mechanisms of long-term memory formation in amnestic snails. The inducer of the mRNAs reactivation can be synapses stimulation due to the CS and US paired presentation during repeated training.

We have previously obtained results that support the hypothesis of mRNA repression as one of the molecular substrates for long-term memory storage. In snails trained for conditioned food aversion, we found that protein synthesis inhibitors injections caused impairment of reminder-induced memory reconsolidation [23]. At the same time, injections of RNA synthesis inhibitors before the reminder did not cause memory impairment. We concluded that in the absence of de novo mRNA synthesis, preexisting repressed mRNAs are the matrices for translating proteins required for memory reconsolidation [23].

From the obtained results, it follows that two populations of mRNAs are involved in the processes of memory formation in amnestic animals. The mRNA represses one population – the matrix for early protein synthesis, which is transcription factors. These proteins activate the transcription of another mRNA population, which is synthesized after repeated training and is involved in the mechanisms of long-term memory formation. It is tempting to assume that one of the transcribed mRNAs is identical to that repression in ribonucleoprotein particles and can replace the mRNA “depleted" during training. This assumption is consistent with the hypothesis according to which, to maintain long-term memory and the

strength of the synapses, it is necessary to maintain a molecular turnover based on positive feedback, including transcription factors that increase their transcription, as well as the transcription of other genes, which leads to an increase in the synthesis of proteins associated with synaptic plasticity [58, 59].

The role of DNA methylation in the mechanisms of anterograde amnesia

Our studies have shown that the mechanisms of anterograde amnesia and memory recovery depend on DNA methylation. How does this methylation occur and develop? As indicated above, we found that the initial conditioned food aversion training in snails under the action of DNMT inhibitor led to the formation of stronger memory than the memory that was formed in control animals without the use of the inhibitor [43]. We cannot rule out that strong memory could result from a lower level of DNA methylation of genes (hypomethylation) involved in training processes than in control animals.

In the early amnesia stages (before day 10), the administration of a DNMT inhibitor+reminder caused memory recovery [44]. We hypothesized that the presentation of a reminder caused reactivation of amnesia processes dependent on DNA methylation, and DNA demethylation, as a result of the DNMT inhibitor use, led to impairment of these processes and memory recovery.

In the later stages of development, amnesia was resistant to both DNMT+reminder administration and repeated training. However, repeated training under the action of DNMT inhibitors led to long-term memory formation. We can assume that at the later stages of amnesia, DNA methylation reached a high level (hypermethylation), which impeded the processes of memory formation. This hypermethylation is not permanently stable. Presentation of CS+US pairing led to the activation of DNA methylation/demethylation processes. Experimental interference in these processes due to the DNMT inhibitors administration can lead to genes demethylation, creating the possibility of their activation by transcription factors and memory formation. In general, our studies show that DNA demethylation is one of the conditions for forming strong memory, memory recovery upon presentation of a reminder, and the formation of long-term memory during anterograde amnesia. Thus, memory formation or development of amnesia was determined by multidirectional changes in DNA methylation. Over time, there may be a shift in the threshold of genes expression associated with plasticity, supported by epigenetic modifications.

The idea that DNA methylation usually correlates with transcription repression and demethylation with its activation, proposed by us, is the basis for explaining the obtained results [60]. However, the DNA methylation status can have a more complex effect on transcription processes. The productive activity of genes under the influence of epigenetic regulation may result from the combined effects of hypomethylation of memory “enhancer genes” and hypermethylation of “repressor genes” [37, 61]. In addition, we cannot exclude the paradoxical possibility that DNA methylation can activate gene transcription [62]. It is also suggested that gene activity depends not only on gene promoter’s methylation but also on gene bodies methylation [63]. In the process of training and memory maintaining, differential methylation/demethylation can occur in many genes [64]. Considering mentioned above, one would expect that using a DNMT inhibitor would cause multiple changes in the methylation of promoters and

bodies of different genes in functionally different cells, which would lead to complex, ambiguous and challenging to interpret behavioral effects. However, studies show that the effects of DNMT inhibitors can be pretty unambiguous. For example, in mammals, DNMT inhibition caused impairments in the formation of long-term spatial memory [35], conditioned reflex fear [37, 38, 61], associative reward learning [62]. In the mollusk Aplysia, DNMT inhibitors impaired the consolidation and reconsolidation of non-associative and associative memory [18, 41, 59], while in the pond snail, inhibitors did not affect the formation of operant conditioning memory, but they suppressed strengthening of memory caused by stress or pharmacological agents. So that some researchers suggested that memory formation and preservation require the coordination of precise and temporarily coordinated changes in gene expression, activation and inactivation of transcription factors, bidirectional changes in the activity of chromatin and DNA-modifying enzymes [61]. This assumption, however, does not explain how changes in gene activity are coordinated in functionally different cells and what specific mechanisms underlie them.

The results of our studies indicate that clear direction of the effects also characterizes the administration of DNMT inhibitors in different experimental paradigms – for the formation of memory, its consolidation and recovery, it is necessary to suppress DNA methylation, and, on the contrary, the development and maintenance of anterograde amnesia require DNA methylation. We know that our ideas about the epigenetic mechanisms of memory and anterograde amnesia simplify the obtained results. However, it does not inconsistently include the obtained results and can be a starting point for the subsequent study of the molecular and cellular mechanisms of learning, memory, and amnesia.

Conclusion

The obtained results suggest that the critical mechanism of anterograde amnesia is methylation-dependent repression of specific neuronal genes, the activation of which is necessary for the formation of long-term memory. The DNMT inhibitor's action during repeated training in amnestic animals leads to demethylation of these genes promoter and opening of access to them for transcription activator proteins, which are translated from the preexisting mRNA during training. On the other hand, animal retains residual memory during anterograde amnesia. Thus, the preexisting, repressed mRNA can be one of the memory retention mechanisms, and its reactivation and protein synthesis during repeated training induce the expression of genetic and epigenetic programs underlying long-term memory [7]. It is important to note that the described effects of DNA methylation changes are specific to a particular signaling stimulus. Indeed, experiments have shown that DNMT inhibitors eliminated the blockade of long-term memory consolidation concerning the studied CS and significantly accelerated memory formation. At the same time, DNMT inhibitors did not affect training for a new food stimulus. These results suggest that specific molecular, synaptic, and genetic mechanisms can be the basis for anterograde amnesia and memory recovery processes.

At the same time, many questions remain unclear concerning the obtained results and their interpretation. One of the main is the biological and medical significance of the anterograde amnesia described by us. Could it be a helpful adaptive brain response to a negative stimulus? Or is this type of amnesia a

consequence of pathological processes in the nervous system caused by brain damage or disease? We discussed this issue in detail in our early work [20]. We should also note here that the memory recovery from anterograde amnesia due to relatively simple pharmacological and physiological manipulations can indicate the “normal” functional significance of this amnesia type.

On the other hand, this does not mean that its mechanisms cannot be involved in the processes of memory impairment in different neuropsychiatric diseases. The studies carried out are only an initial stage in the research of anterograde amnesia mechanisms. However, the obtained facts inspire some hope in searching for ways of memory loss pharmacological correction and its recovery.

We should especially note that the anterograde amnesia described in this work is an amnesia type that occurs after memory impairment. In particular, we have previously shown that impairment of the consolidation or reconsolidation of conditioned food aversion memory in snails by a serotonin receptor antagonist induced amnesia, during which anterograde amnesia did not develop – repeated training led to the formation of food aversion [19]. Thus, with the same form of training, long-term memory impairment by different amnestic agents causes different amnesia types, which differ in the ability to form memory during repeated training. In this regard, it will be interesting to compare the role of DNA methylation in the mechanisms of maintenance of different amnesia types and memory recovery mechanisms.

Declarations

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Author contributions

Conceptualization: N.V.P., S.S.V.; Methodology, Solntseva S.V., Nikitin V.P., Kozyrev S.A., Nikitin P.V.; Investigation, Solntseva S.V., Nikitin V.P., Kozyrev S.A., P.V.N.; Writing – Original Draft, Nikitin V.P., Nikitin P.V.; Writing – Review & Editing, Nikitin V.P., Solntseva S.V., Kozyrev S.A., Nikitin P.V.; Funding Acquisition, Nikitin V.P.; Resources, Nikitin V.P., Nikitin P.V.; Supervision, Nikitin V.P.

Competing Interests

The authors declare no competing interests.

Data Availability Statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

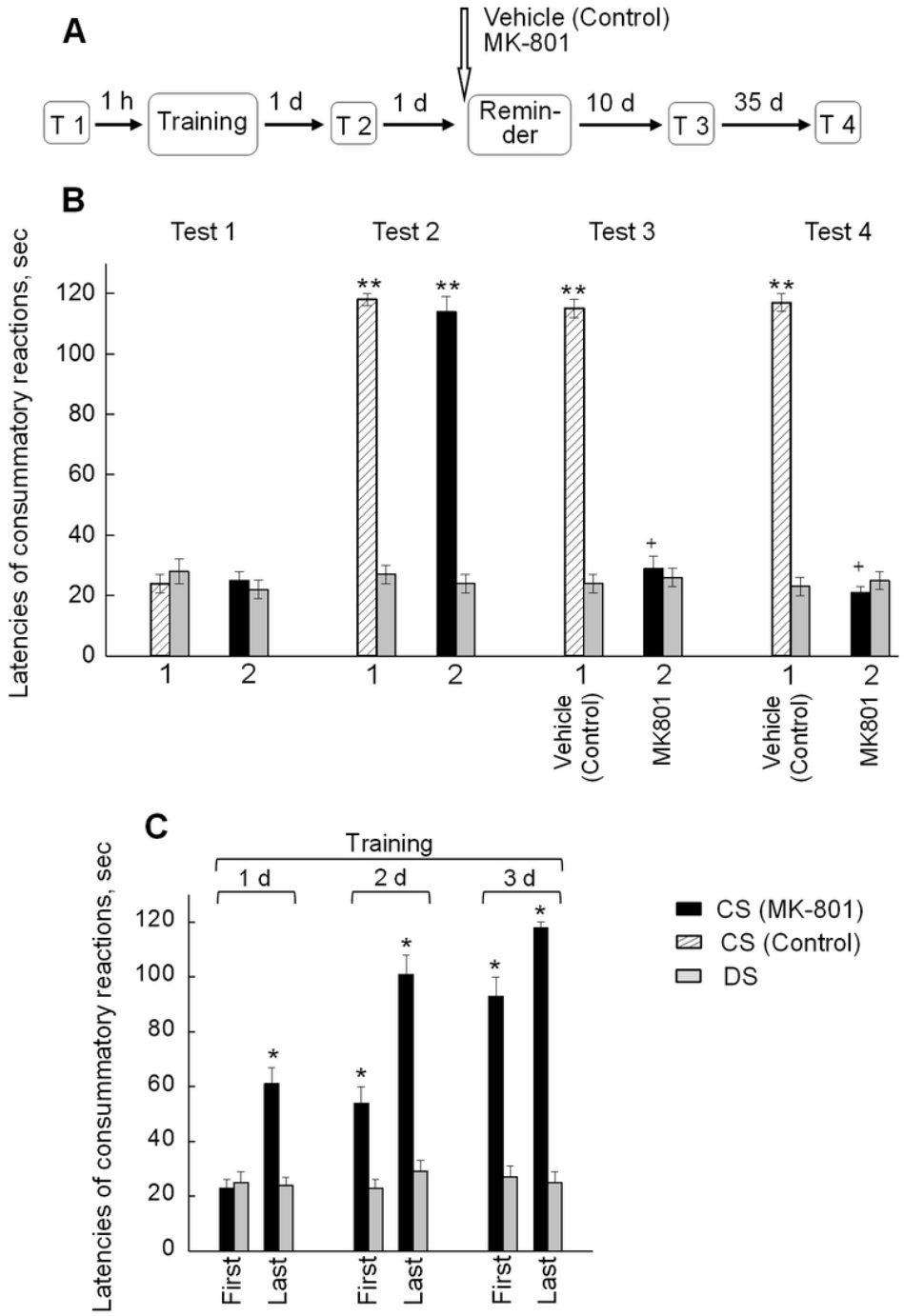

Figure 1

Impairment of memory reconsolidation by the antagonist of NMDA glutamate receptors MK- 801 two days following training for conditioned food aversion caused the development of amnesia in snails, which persisted for more than 45 days. A – experimental scheme. h, hour; d, day. The arrow – time of solutions injection. B – latencies of consummatory reactions. Test 1 (T1) – basic reactions, test 2 (T2) – responses one day after training. Test 3 (T3) and Test 4 (T4) – reactions 10 and 45 days after the solutions+reminder administration. The graphs below: 1 – reactions to food stimuli of control snails that

received vehicle injections before reminder, 2 – the reactions of snails injected before reminder with MK-801. Shaded bars – responses to the conditioned stimulus (CS) of control snails receiving vehicle + reminder; dark bars – reactions to the CS, administrated with MK-801+reminder, grey bars – responses to the differentiating stimulus (DS), not combined with an unconditioned stimulus (US). Vertical – latencies of consummatory reactions to food stimuli, in seconds. ** – $p < 0.0001$, CS vs. DS; + – $p < 0.0001$, concerning the responses to CS among control snails who received vehicle injections. C. Graphs of changes in responses to food stimuli during three days of initial training. In each of the three training days, the latencies of consummatory reactions to the first and last CS and DS presentation on the day of training were shown. * - $P < 0.05$, CS vs. DS. For each of the three days of second training, the latencies of responses to CS were increasing and were longer than those to the DS and CS on the previous day. Thus, at the initial training in animals, there is short-term and long-term memory formation.

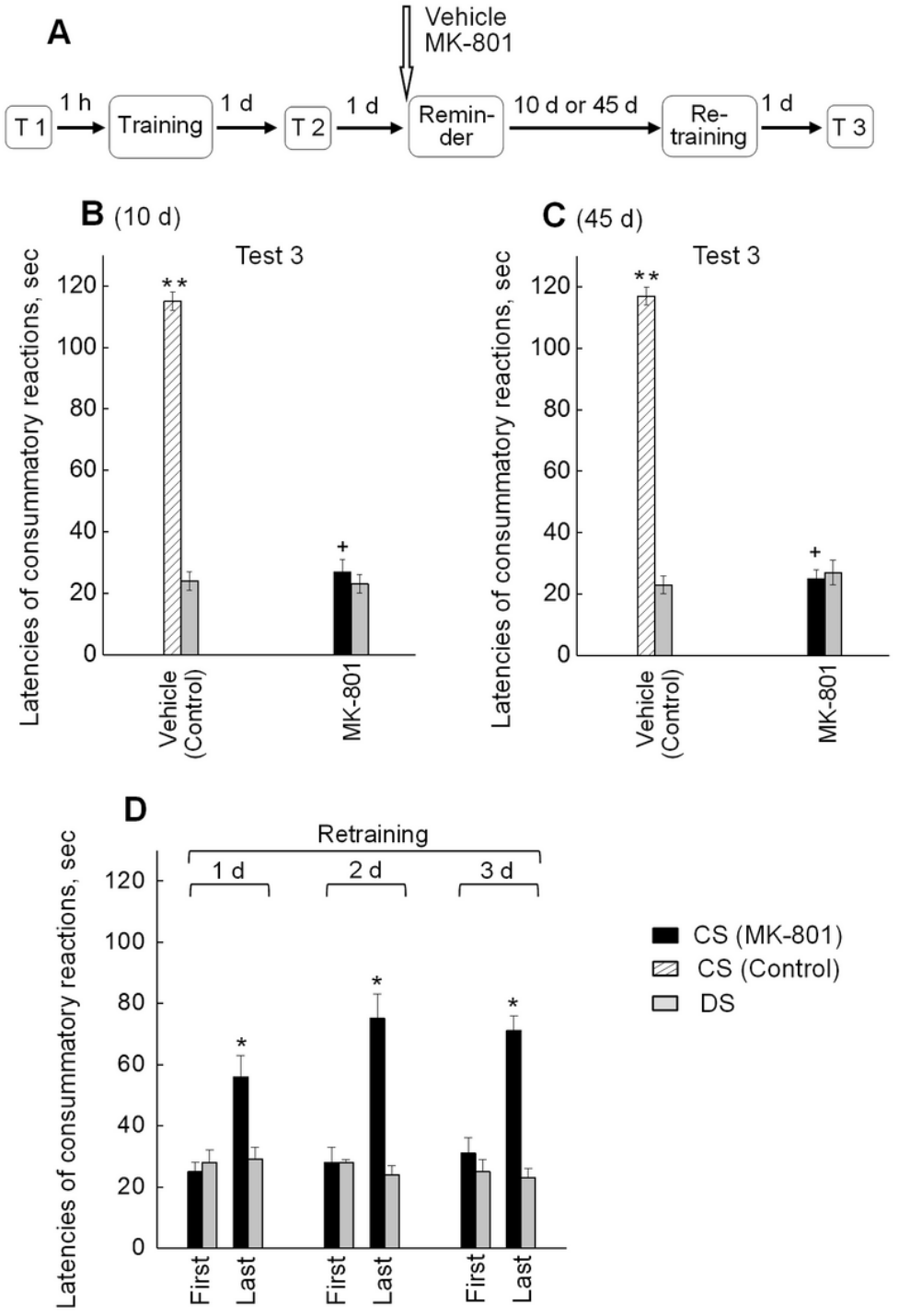

Figure 2

During second training 10 (B) or 45 (C) days after the induction of amnesia caused by administration of MK-801+reminder, the ability of short-term memory formation was saved in animals, while long-term memory was not formed. A. Experiment scheme. B and C – animal responses to food stimuli one day after repeated training. D. Graphs of changes in responses to food stimuli during repeated training ten days after amnesia induction. Each of the three days of the second training shows the latencies of consummatory reactions to the first and last presentation of CS and DS on the day of training. The

remaining symbols are the same as in Fig. 1. For each of the three days of second training, the latencies of responses to CS were increasing and were longer than those to the DS. That is, short-term memory was formed in animals on the day of training. However, long-term memory was not formed – upon presentation of the CS for each following day of training, the latencies of reactions to CS and DS did not differ.

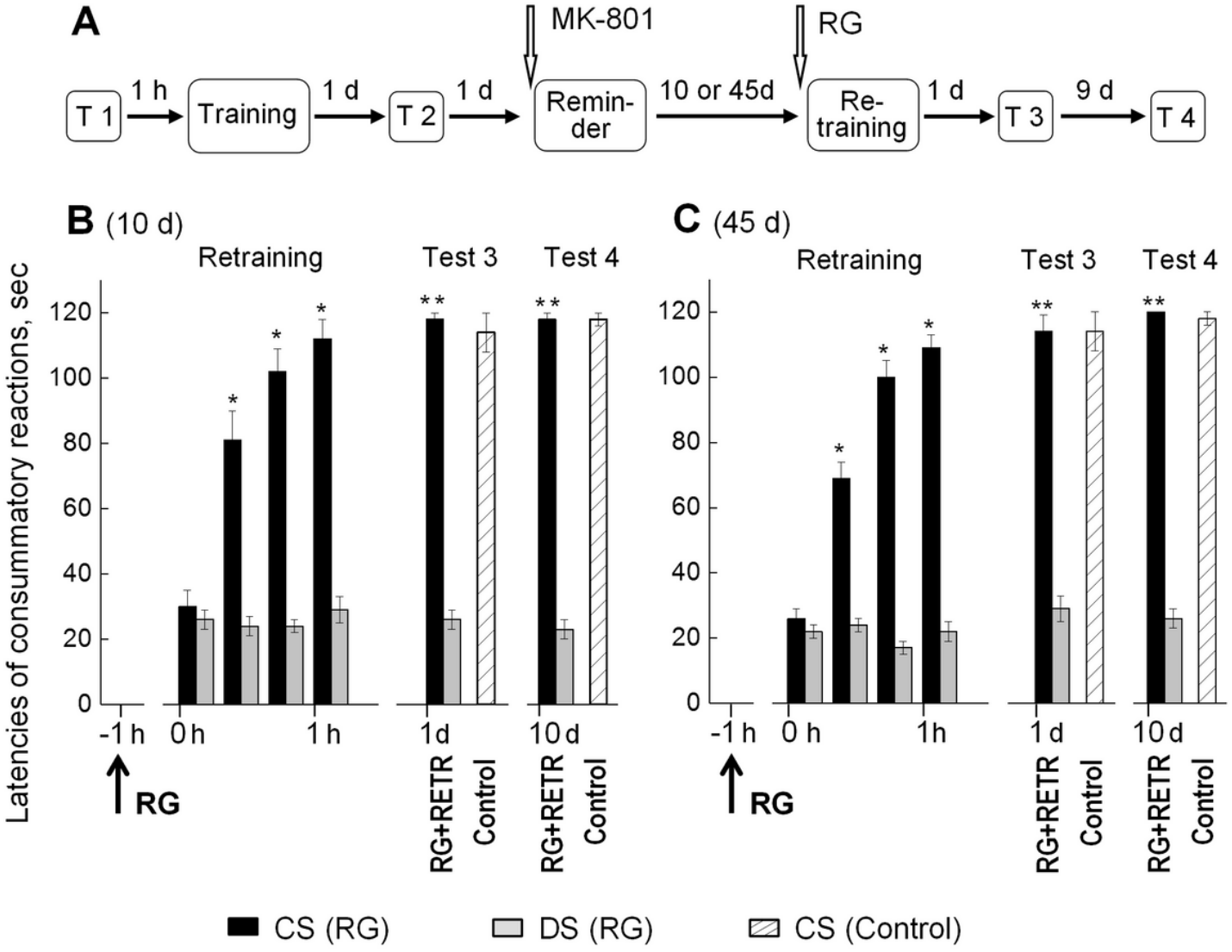

Figure 3

10 or 45 days after amnesia induction, administration of the DNMT inhibitor RG108 before repeated training led to the rapid formation of long-term memory. A. Scheme of the experiment. B and C – effects of RG108 administration before repeated training 10 (B) or 45 (C) days after amnesia induction (MK-801+reminder). Repeated training – reactions to CS and DS during 4 CS+US pairing presentations). Test 3 and Test 4 – testing 1 and 10 days after training. Figure captions: h – hours; d – days; RG is RG108; RETR – repeated training. The remaining symbols are the same as in Fig. 1. After the RG108 administration, a rapid memory formation was found within one day, which persisted for at least ten days.

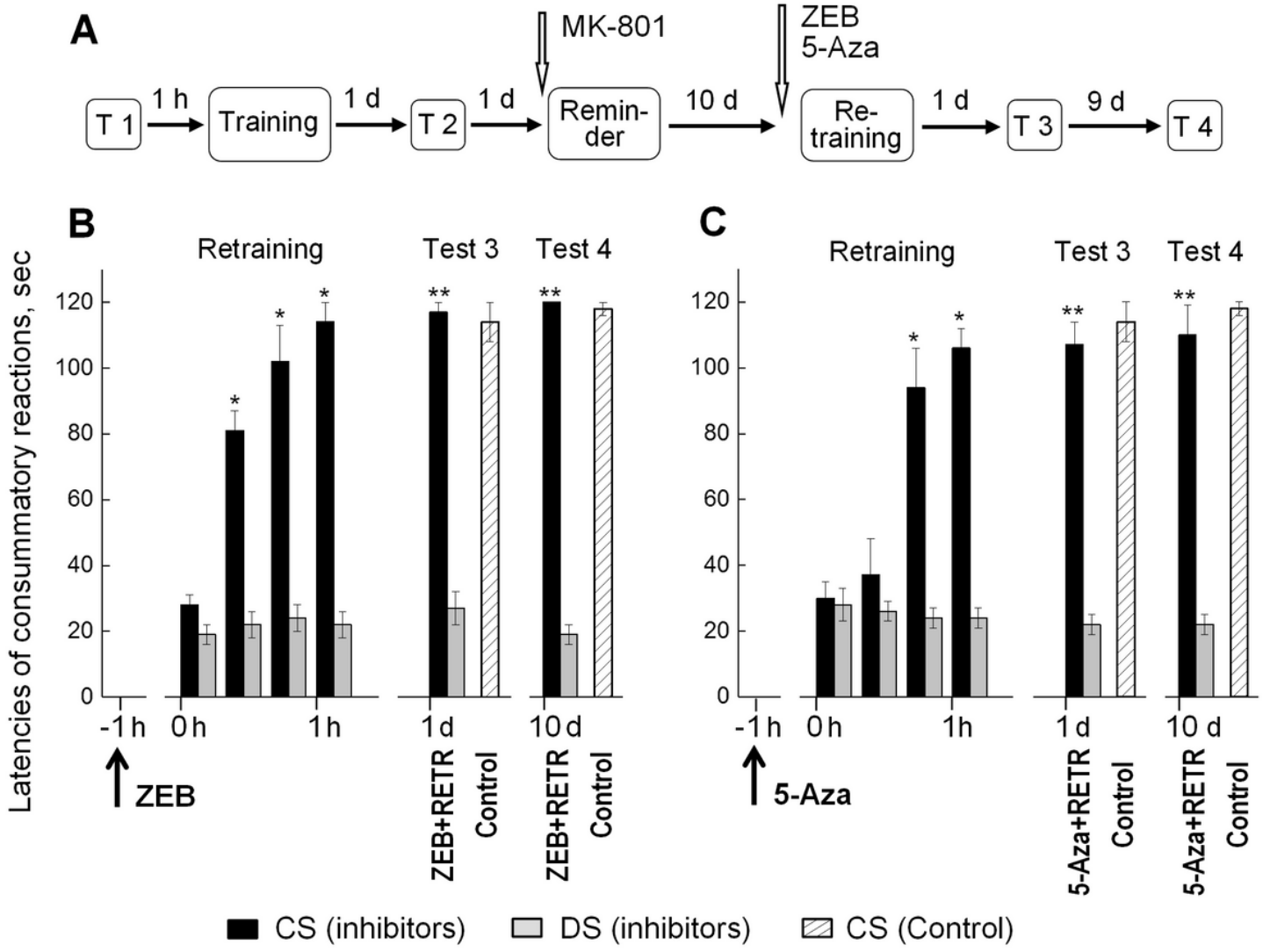

Figure 4

Dose-dependence of the RG108 effects on the long-term memory formation. B and C – effect of RG108 at doses of 6 mg/kg and 1 mg/kg, respectively, on long-term memory formation during repeated training. The symbols are the same as in Fig. 1 and 3. Administration of RG108 at a dose of 6 mg/kg before repeated treating induces the expression of aversive responses to both CS and DS. The aversive reactions to DS persist one day after training and disappear after ten days. RG108 administration at a 1 mg/kg dose before repeated training did not lead to long-term memory formation.

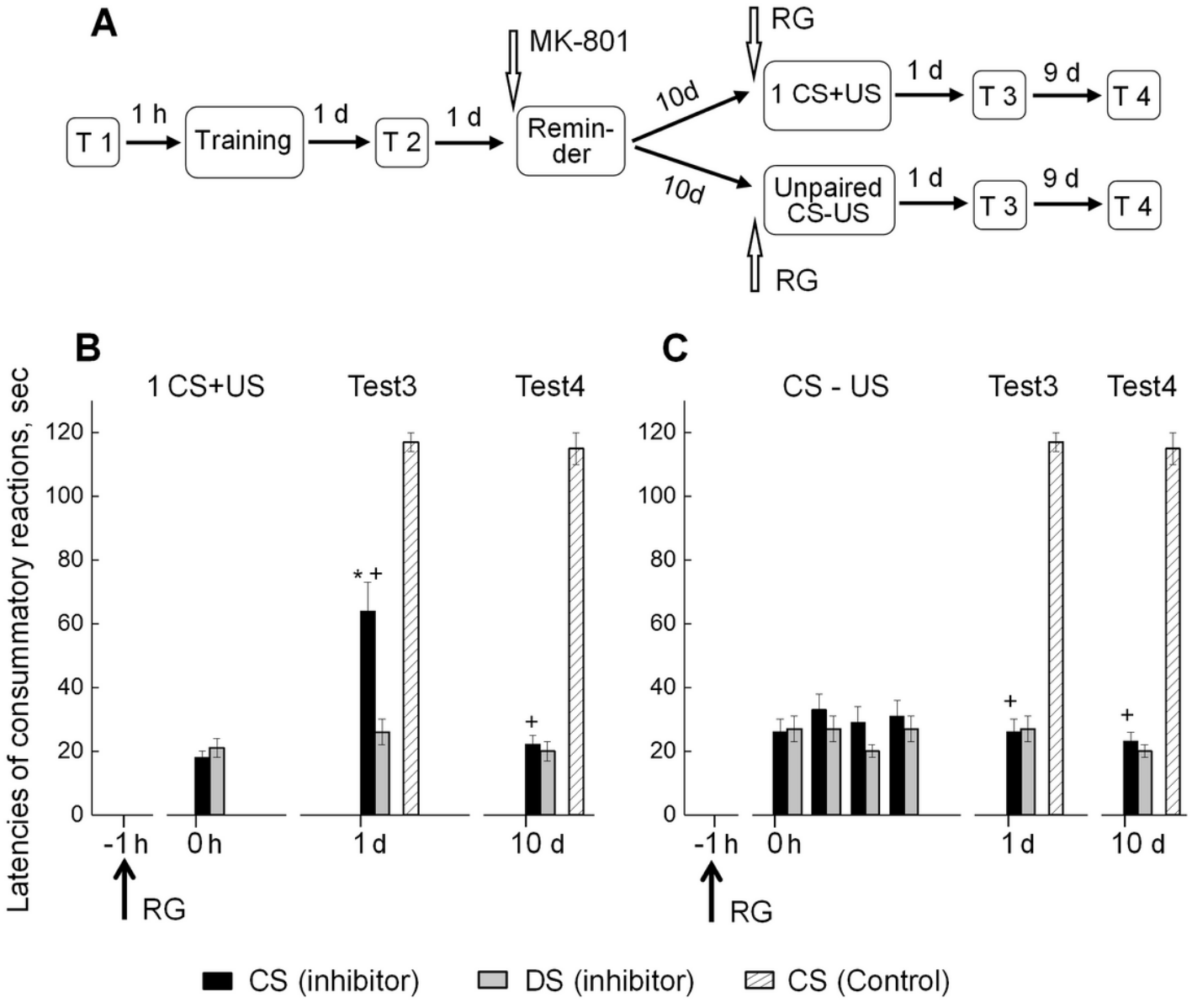

Figure 5

Determination of the “time window” of the effectiveness of RG108 concerning repeated training. The symbols are the same as in Fig. 1 and 3. Injections of RG108 15 min or four h after repeated training resulted in the formation of long-term memory.

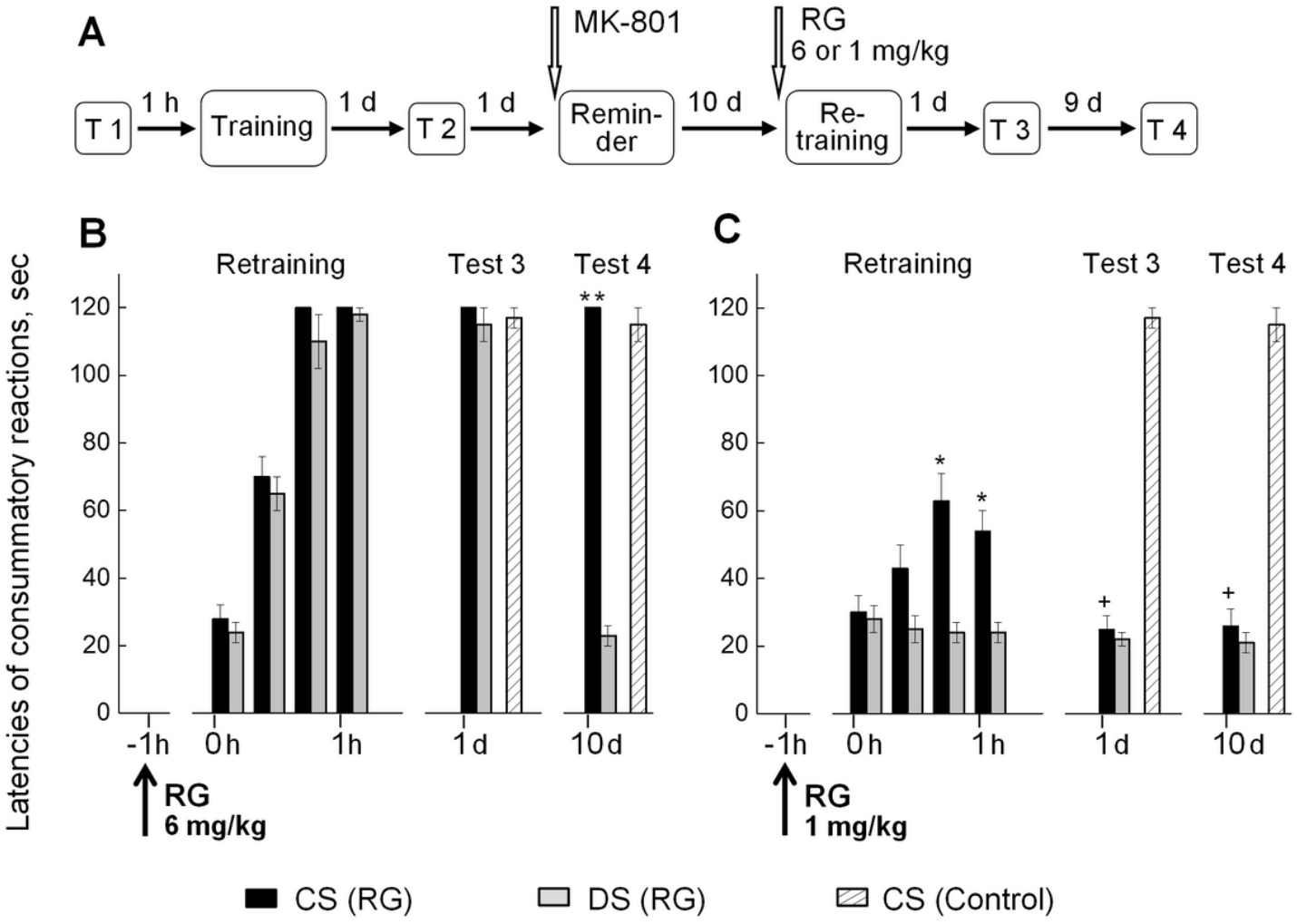

Figure 6

Ten days after amnesia induction, administration of ZEB (zebularine) or 5-AZA (5-Aza-2'-deoxycytidine) before repeated training led to the rapid formation of long-term memory. The symbols are the same as in Fig. 2 and 3. Thus, DNMT inhibitors with different mechanisms of action had a similar effect on the formation of long-term memory during repeated training.

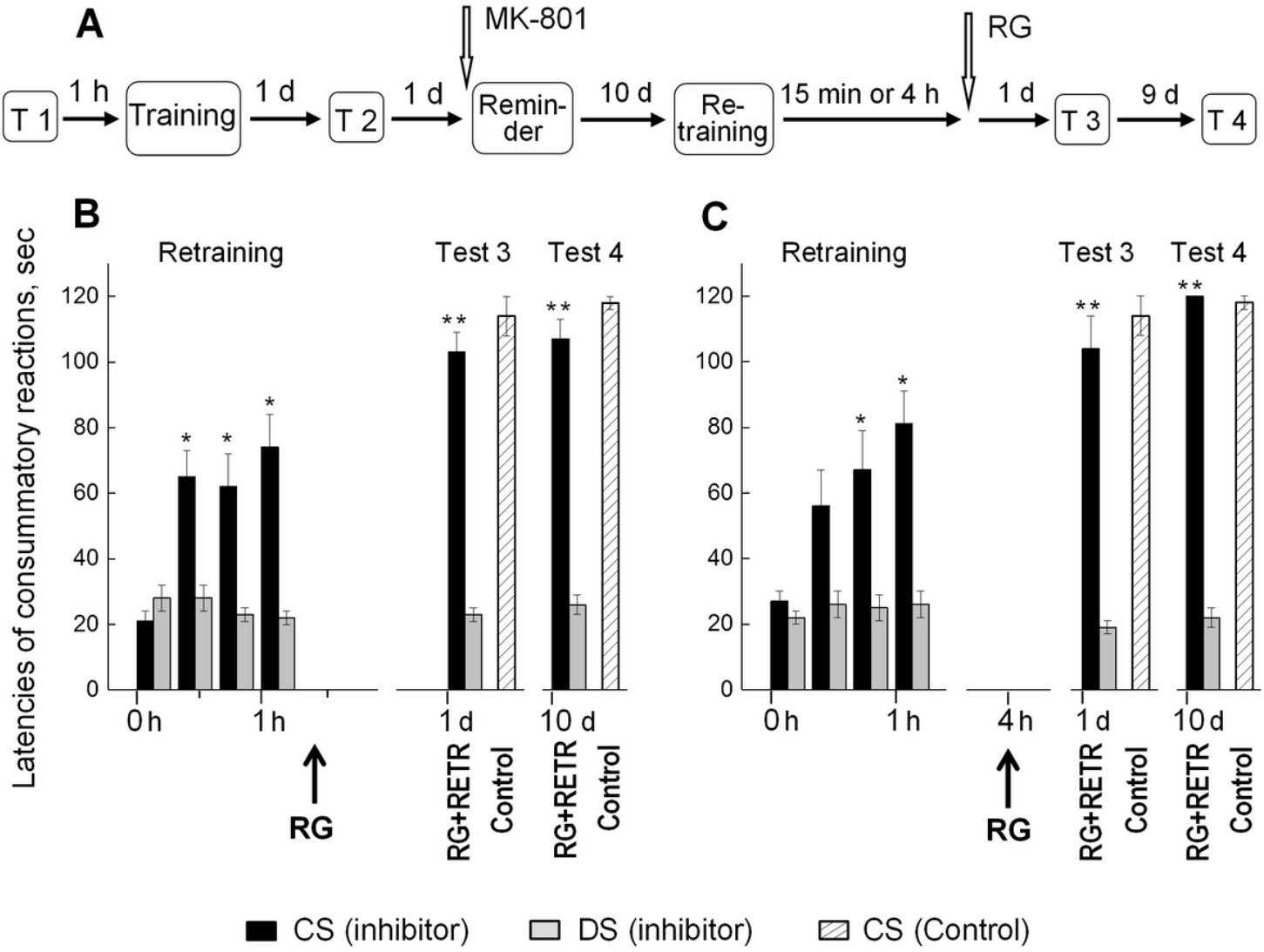

Figure 7

Long-term memory formation dependence on protein and RNA synthesis upon administration of RG108 + repeated training. A. CYH — cycloheximide, ACD — actinomycin D. B — paired injections of RG108 and

CYH 1 hour before repeated training. C and D – CYH injections 15 minutes or 4 hours, respectively, after the administration of RG108 + repeated treating. E – paired injections of RG108 and ACD 1 hour before repeated training. F and G – injections of ACD 15 minutes or 4 hours, respectively, after the administration of RG108 + repeated treating. The symbols are the same as in Fig. 2 and 3. The protein synthesis inhibitor completely suppressed the formation of long-term memory induced by RG108 upon injections of the inhibitor at two-time intervals – before and 15 min after training. At the same time, the administration of RNA synthesis inhibitor before training was not effective, whereas its injections 15 min or four h after training suppressed the formation of long-term memory. Interestingly, both inhibitors did not affect the formation of short-term memory – an increase in the latencies of responses to CS during repeated training.

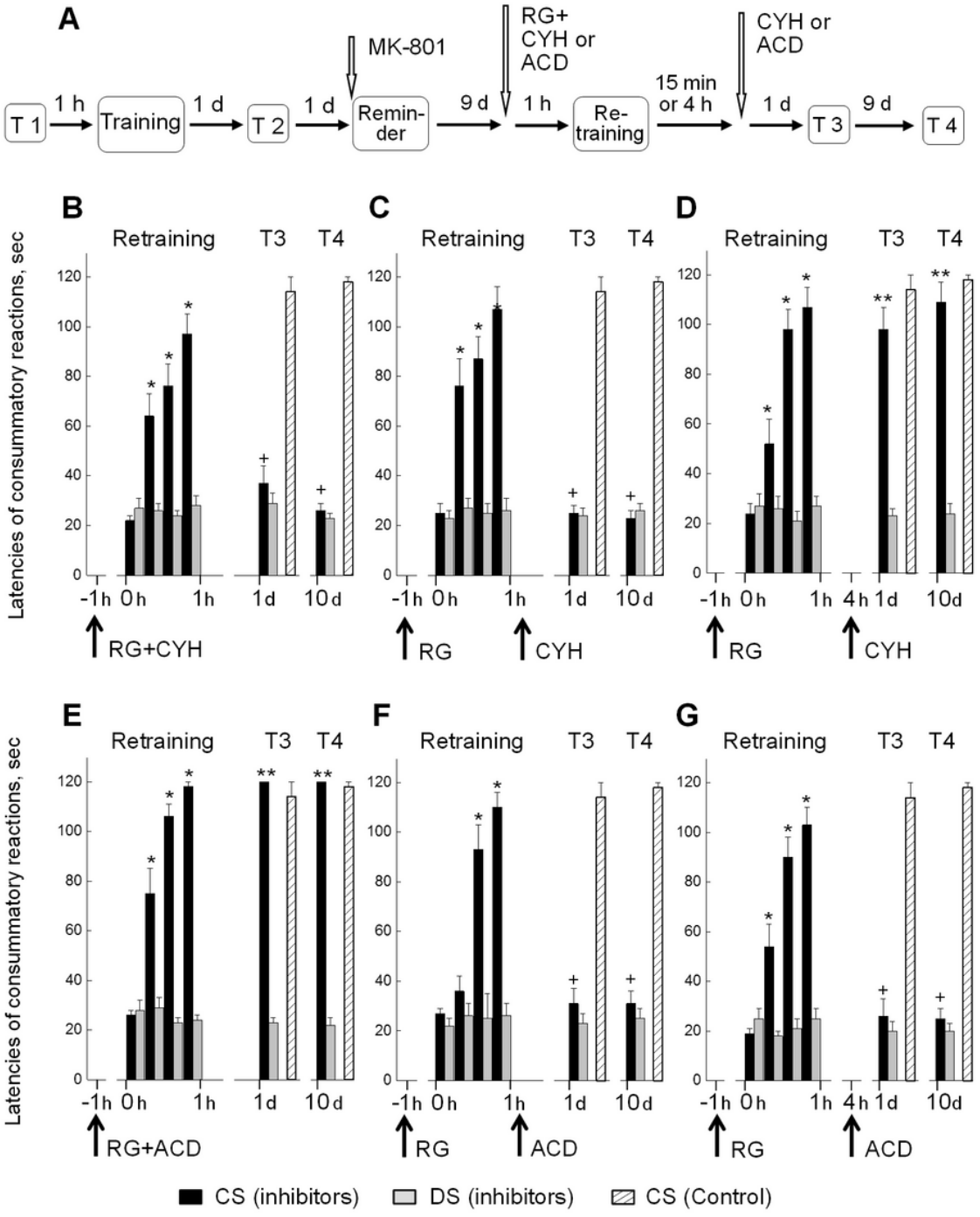

Figure 8

Effects of unpaired presentations of CS and US, as well as single CS+US pairing after RG108 injections. C. Injections of RG108 and a single CS+US pairing caused a slight increase in the latencies of responses to CS after 24 hours; after ten days, the latencies of reactions to the CS and DS did not differ. C. Administration of four unpaired presentations of CS and US after RG108 injections did not reveal an increase in the latent periods of responses to food stimuli. The symbols are the same as in Fig. 2 and 3.

Thus, for long-term memory formation, more than one CS+US pairing is required. Injections of a DNMT inhibitor before the unpaired presentation of CS and US did not lead to long-term memory formation.