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Nitric oxide is necessary for labilization of a consolidated context memory during reconsolidation in terrestrial snails

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Abstract

Nitric oxide (NO) is known to be involved in associative memory formation. We investigated the influence of blocking NO function on the reconsolidation of context memory in terrestrial snails (*Helix lucorum* L.). After a 10 day session of electric shocks in one context only, context memory in snails was observed in test sessions as the significant difference of amplitudes of withdrawal responses to tactile stimuli in two different contexts. After a 1 day rest, a session of 'reminding' was performed, preceded by injection in different groups of the snails with either vehicle or combination of the protein synthesis blocker anisomycin (ANI) with one of the following drugs: the NO scavenger carboxy-PTIO, the NO-synthase inhibitors *N*-omega-nitro-L-arginin, nitroindazole and NG-nitro-L-arginine methyl ester hydrochloride, or the NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine. Testing the context memory at different time intervals after the reminder under ANI injection showed that the context memory was impaired at 24 h and later, whereas the reminder under combined injection of ANI and each of the NO-synthase inhibitors used or the NO scavenger showed no impairment of long-term context memory. Injection of the NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine with or without reminder had no effect on context memory. The results obtained demonstrated that NO is necessary for labilization of a consolidated context memory.

Introduction

The concept of reconsolidation assumes that newly acquired memories are not consolidated once and for all (Nader & Hardt, 2009). Several reports have shown that, after the presentation of a specific reminder, reactivated old memories become labile and again susceptible to amnesic agents. Such vulnerability diminishes with the progress of time and implies a restabilization phase, usually referred to as reconsolidation (Sara, 2000; Nader, 2003).

One of the most interesting properties of memory is that a stable consolidated memory can be disturbed by the same factors (e.g. protein synthesis blockade) that can impair newly formed memories shortly after acquisition, if applied along with 'reminder' cues representing a part of the learning situation (Misanin *et al.*, 1968; Nader *et al.*, 2000; Sara, 2000; Anokhin *et al.*, 2002).

Context-specific learning and memory have been shown in many invertebrates (Colwill *et al.*, 1988; Haney & Lukowiak, 2001). The context memory was described in detail in terrestrial snails (Balaban, 1993, 2002; Balaban & Bravarenko, 1993). A reactivation of a consolidated memory resulting in the reconsolidation phenomenon was also shown in invertebrates (Sekiguchi *et al.*, 1997; Pedreira *et al.*, 2002; Sangha *et al.*, 2003; Eisenhardt & Menzel, 2007; Stollhoff *et al.*, 2008; Kaczer *et al.*, 2011). Recently, a reconsolidation-like process was demonstrated in neural networks consisting of co-cultured *Aplysia* neurons at the level of single sensory-to-motor neuron synapses (Cai *et al.*, 2012; Lee *et al.*, 2012).

Much evidence has accumulated to show that nitric oxide (NO) is an important neurotransmitter and neuromodulator in both vertebrates and invertebrates (Jacklet, 1997; Muller, 1997; Fedele & Raiteri, 1999; Katzoff *et al.*, 2002; Eisenhardt & Menzel, 2007). An increasing number of studies also indicate that the NO–cyclic guanosine monophosphate (cGMP) pathway is involved in associative memory formation (Hawkins *et al.*, 1998; Rose, 2000; Schweighofer & Ferriol, 2000; Kemenes *et al.*, 2002; Matsumoto *et al.*, 2013). In the terrestrial snail *Helix*, it was shown (Gainutdinova *et al.*, 2005) that the protein synthesis blocker anisomycin (ANI) impairs the context memory if injected immediately after a 'reminding' session.

Nitric oxide is synthesized as needed by NO synthase (NOS) and does not react with receptors but locally diffuses in synapses and adjacent cells. Instead of reversible interactions with targets, NO forms covalent linkages to a multiplicity of targets, which may be enzymes, such as guanylate cyclase, or other protein or non-protein targets (Calabrese *et al.*, 2009). One effect of NO on nerve cell activity is the direct *S*-nitrosylation of proteins (the covalent attachment

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of a nitrogen monoxide group to the thiol side-chain of cysteine), which changes the physiological functions of existing proteins, inhibiting their normal role in physiological functions including memory. It has been shown that *S*-nitrosylation is involved in plasticity (Tooker *et al.*, 2013), mediating the activity-dependent plasticity of retinal bipolar cell output. Simultaneously, the influence of NO via the cGMP pathway activates intracellular signaling cascades and triggers increased synthesis of proteins, including those participating in memory maintenance (for review of dual NO effects see Calabrese *et al.*, 2009). Here we suggest that the local and selective increase of NO concentration in neurons activated during memory reactivation may be the reason for memory labilization making a reconsolidation process necessary.

In the present study, we tested the hypothesis of NO involvement in memory labilization during the reconsolidation process using a reminding procedure under different conditions in the terrestrial snail *Helix*.

Materials and methods

Subjects

Adult snails (*Helix lucorum* L.) (Crimea population) weighing 20–30 g were used in our experiments. The snails were kept in an active state at least 2 weeks before the experiment in a wet environment and were fed regularly with carrots. At 2 days before the training session, the snails were deprived of food. Each snail was used in only one series of experiments. The scores of 119 animals that survived the training and testing procedures, and were in good health at least 1 week after the end of the experiment were used for statistical evaluation. The experimental procedures were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, and the protocol was approved by the Ethical Committee of the Institute of Higher Nervous Activity and Neurophysiology of the Russian Academy of Sciences.

Apparatus and analysis of behavior

In the experimental set-up (Context 1), the snail was tethered by its shell in a manner allowing it to crawl on a ball that rotated freely in a water solution containing 0.01% NaCl (Fig. 1). The ball was covered with aluminum foil to complete an electrical circuit between the animal's foot and a carbon electrode placed in the water. Electric shock was delivered using a 1–4 mA, 1 s current through a macroelectrode applied manually to the dorsal surface of the snail's foot, and the second carbon electrode (Fig. 1). Punctate mechanical stimuli were applied with calibrated von Frey hairs, permitting the delivery of pressures ranging from 6 g/mm^2 (estimated as weak) to 68 g/mm^2 (estimated as noxious). After several pilot series, the behavioral response, intensity (25 g/mm^2), and location of tactile stimulation were chosen. The withdrawal of ommathophores (posterior tentacles) in response to tactile stimulation of the rostral part of the skin at 4–5 mm behind the posterior tentacles appeared to be at the level of 10–30% of maximal in normal animals. In pilot experiments, it was shown that responses to such test stimulation were sensitized after noxious stimuli, and this part of the foot skin was chosen as the standard place for tactile stimulation. An investigator, blind to the experimental histories of the animals, applied the tactile stimuli to the snail's skin and video-recorded the tentacle withdrawal. We analysed video-recordings offline using PHYSVIS 1.4 software (Kenyon College), which is available free on the internet. To quantify and average the results, we analysed the distance between the tip and base of the tentacle and scored the withdrawal amplitude as a percentage of the initial length of the tentacle in each trial.

Statistical evaluation of data

Blind testing was performed at different time intervals after injections as shown in the insets of Figs 2 and 4. Comparison between groups was made only for parallel groups of animals in one experimental series. We used the Wilcoxon signed-rank test for comparison of performance of the same group. Significant differences in performance of the same group of animals in two contexts are indicated in all figures.

Reminder

Before training, each snail was exposed for 30 min daily for 2 days to the experimental set-up. The first test session (T) was then performed for all groups (first day, Fig. 2A). After obtaining the pre-training scores, snails of the experimental and control groups received five electrical shocks per day with 20–30 min intervals for

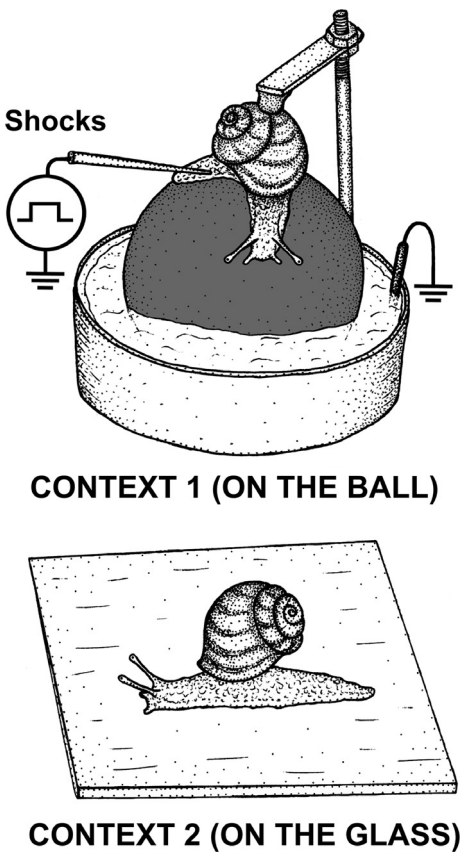

FIG. 1. Scheme of context conditioning in terrestrial snails. In Context 1, the snail was tethered by its shell in a manner allowing it to crawl on a ball that rotated freely in a water bath containing 0.01% NaCl. The ball was covered with aluminum foil to complete an electrical circuit between the animal's foot and a carbon electrode placed in the water. Electric shock was delivered using a 1–4 mA current of 1 s duration through a macroelectrode applied manually to the dorsal surface of the snail's foot. In Context 2, the snails were only tested. Scoring of the tentacle withdrawal amplitude in both contexts was performed using video-recording and a moderate intensity tactile stimulation of the same skin area.

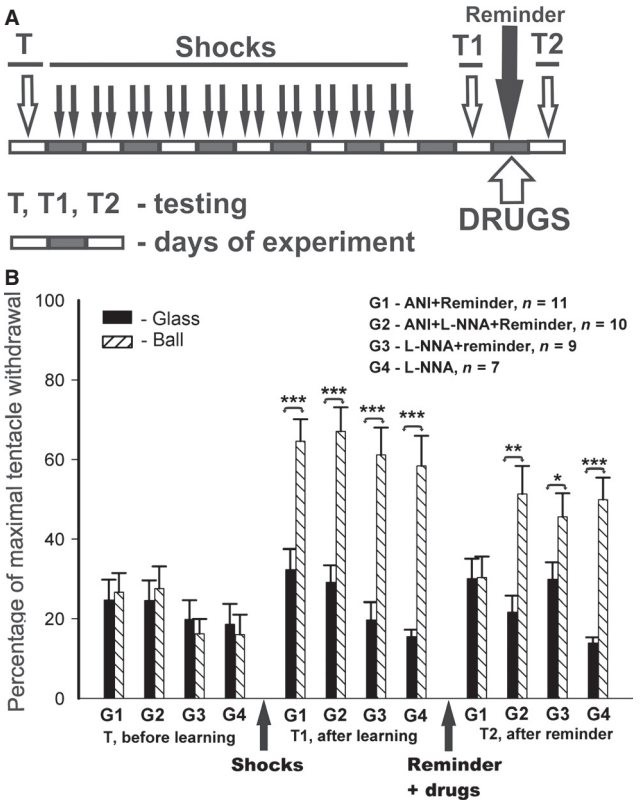

FIG. 2. Reconsolidation is absent under ANI+blockade of NO synthesis with L-NNA. (A) Protocol of a context conditioning experiment with ANI/L-NNA injections at 20 min before the reminder. T, T1, T2, tests for context conditioning, timings are as shown on the scheme. (B) Averaged amplitudes (+SEM) of withdrawal responses in four groups of snails measured in two different contexts, i.e. on the ball (reinforced context) and on the glass. G1, $n = 11$; G2, $n = 10$; G3, $n = 9$; G4, $n = 7$. G1 was injected at 20 min before the reminder with ANI, G2 with the ANI+L-NNA+reminder, G3 with the L-NNA+reminder, whereas G4 was not subjected to reminder, i.e. only L-NNA injection was performed. *Y*-axis, amplitude of tentacle withdrawal as a percentage of the length before the test. Significance of differences in response amplitudes in the two contexts was estimated for each group using a Wilcoxon signed-rank test. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

5–10 days in Context 1. A longer training session was necessary in some series to ensure that the 'reminder' would be effective (details in Gainutdinova *et al.*, 2005). The current was individually chosen for each snail so that a complete withdrawal of the anterior part of the body was observed in response to a shock. No testing was performed during the training session. On the second day after completion of the training session (animals were fed during the rest period), the responsiveness to the same test tactile stimuli (T1, Fig. 2A) was compared in all parallel groups of snails. Blind testing was always performed for each snail in two alternating contexts. The order in which the animals were tested in each context was randomized.

On the day after the second test session (T1), the experimental and control groups of snails were reminded of the training by placing them for 20 min in the same Context 1 where they were shocked (ball, Fig. 1). At 20 min before the reminder, the snails were injected with either ANI (0.4 mg in 0.2 mL of saline plus 0.5 mL of saline to equalize the volume per snail weighing 20–30 g), the NO-synthase inhibitor *N*-omega-nitro-L-arginin (L-NNA) [0.22 mg in 0.5 mL saline (+0.2 mL saline) per snail weighing 20–30 g], or L-NNA+ANI (same doses, total injected volume 0.7 mL). On the second day after a session of 'reminding', the third test session (T2) was performed for all parallel groups in two different contexts. Similar testing protocols and injection procedures were used for all tested drugs.

Drugs and injections

Anisomycin (Sigma) was dissolved in sterile saline, with the addition of an equimolar amount of 3 N HCl and adjustment of the pH of the resulting solution to 7.2 with 3 N NaOH. The water-soluble NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (carboxy-PTIO), and neuronal NOS inhibitors (all from Sigma) NG-nitro-L-arginine (L-NNA) (non-specific NOS inhibitor), NG-nitro-L-arginine methyl ester hydrochloride (L-NAME) (non-specific NOS inhibitor), and 7-nitroindazole (NI, reversible and non-selective NOS inhibitor) were used. The approximate final concentration of carboxy-PTIO in the hemolymph was 250 μM and the approximate final concentrations of the neuronal nitric oxide synthase (nNOS) inhibitors were 50 μM for L-NNA and L-NAME, and 100 μM for 7-nitroindazole. The selected concentrations were effective in our electrophysiological experiments in snails without obvious toxic effects (Korshunova & Balaban, 2014). For calculating the final concentrations in the nervous system, each gram of the snail body weight was scored as 1 mL. Animals were also treated with 50 μM (calculated final concentration) of the NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) (Tocris), which was dissolved in saline just before the injection. A similar concentration of SNAP depolarizes the giant metacerebral neuron of the *Aplysia* cerebral ganglion (Jacklet & Tieman, 2004). This concentration of SNAP produced no obvious effects on animal behavior.

Drugs were prepared in Ringer saline as a stock solution at a concentration that was 28.6-fold greater than required. Because the snails used in these experiments were similar in weight (20 ± 2 g), 0.7 mL of the drug solutions was injected into the hemocoel, thereby achieving a concentration in the animal that was appropriate to the experiment ($0.7 \times 28.6 = 20$ mL).

Intracoelomic injection was performed with a fine needle via a non-sensitive part of the foot skin normally hidden under the shell. During injections, the snails stopped locomotion and lowered the ommatophores, mostly because the shell was fixed by the experimentator, but never showed a generalized withdrawal into the shell.

Results

In a first experimental series we decided to analyse the role of NO in reconsolidation during which a consolidated memory is thought to be labilized and reconsolidated again (Nader *et al.*, 2000; Anokhin *et al.*, 2002; Duvarci & Nader, 2004). We hypothesized that if the NO is involved in memory labilization, then the NOS blocker may prevent the disappearance of memory during reactivation of memory in conditions of protein synthesis blockade. It was shown previously (Gainutdinova *et al.*, 2005) that the protein synthesis blocker ANI impairs the contextual memory in the terrestrial snail *Helix* if injected immediately after or before a 'reminding' session. We decided to inject ANI and the NOS blocker simultaneously, which might preserve the memory labilization.

In this series of experiments, four groups of snails were randomly tested in two different contexts (ball and glass) before the training session (T, see protocol in Fig. 2A). The percentage of maximal withdrawal to tactile stimulation was scored from video-recordings. The snails were then trained (shocked) for 10 days to remember the context in which they were shocked (Context 1, on the ball, Fig. 1)

and tested with a 1 day rest interval for aversive context memory (T1 in Fig. 2A). All four groups were then reminded of the context in which they were shocked (no shocks during reminding) under the drug injections, and tested 24 h later for maintenance of context memory (T2, Fig. 2A). Group 1 (G1) was injected with ANI at 20 min before the reminder, Group 2 (G2) with ANI+L-NNA at 20 min before the reminder, Group 3 (G3) with L-NNA at 20 min before the reminder, and Group 4 (G4) with L-NNA only without any reminder.

Prior to the training session, the behavioral responses in two contexts did not differ significantly in all groups ('T, before learning' in Fig. 2B). On the second day after a 10 day session of electric shocks in Context 1, the context conditioning was observed as a highly significant difference of behavioral response amplitudes in two contexts in all groups ('T1, after learning' in Fig. 2B, $P < 0.001$ for all groups, Wilcoxon signed-rank test, $z = 3.9$ for G1, $z = 3.7$ for G2, $z = 3.7$ for G3, $z = 3.6$ for G4). On the day following testing of context memory, a session of 'reminding' (no shocks, just 20 min in Context 1) was performed under drug injections. Next day testing of long-term context memory demonstrated that ANI injections and reminder ('T2, after reminder' in Fig. 2B, G1, no significant difference in amplitudes of tentacle withdrawal responses in two contexts) impaired the context conditioning, as was shown previously with much larger groups of snails and all necessary controls (Gainutdinova *et al.*, 2005), whereas under injection of ANI+L-NNA (Fig. 2B, G2, $P < 0.01$, Wilcoxon signed-rank test, $z = 2.8$), no impairment of the context memory was observed. This result demonstrated that, in the conditions when a new memory cannot be formed (protein synthesis is blocked), the 'old' memory is maintained after the reminder only if NOS is blocked. The presence of memory after the reminder presentation combined with an ANI and NOS blocker injection presumes that the reconsolidation process did not happen.

Responses in snails from G3 (reminder+L-NNA) still demonstrated the existence of a significant difference ('T2, after reminder', Fig. 2B, $P < 0.05$, Wilcoxon signed-rank test, $z = 2.1$ for G3) between two contexts. Injection of L-NNA without the reminder also did not show any effect on the memory ('T2, after reminder', Fig. 2B, $P < 0.001$, Wilcoxon signed-rank test, $z = 3.4$ for G4).

Taken together, these data demonstrate that the NO blockade allows the existing memory to be revealed in the conditions of memory reactivation under protein synthesis blockade when new memory cannot be formed. Considering the fact that, normally, the reminder under ANI completely abolishes the memory, the hypothesis that NO participates in labilization of existing memory appears justified.

Using a similar protocol, we tested whether the observed behavioral effects of preserving memory after reminder under blockade of protein synthesis and simultaneous blockade of NO synthesis still persist in conditions of binding the NO molecules with the potent NO scavenger carboxy-PTIO. In this series of experiments we again used a group of snails that were injected with ANI only as a control for reconsolidation, and again we observed the disappearance of context memory after reminder under ANI (Fig. 3, G1, after reminder), but no changes in memory were observed when carboxy-PTIO was injected simultaneously with ANI (Fig. 3, after reminder, $P < 0.001$, Wilcoxon signed-rank test, $z = 3.4$ for G2). Injection of carboxy-PTIO alone before the reminder did not impair the memory (Fig. 3, after reminder, $P < 0.05$, Wilcoxon signed-rank test, $z = 2.1$ for G3).

To better understand the time-course of changes in behavior during reconsolidation, we performed a similar series of experiments

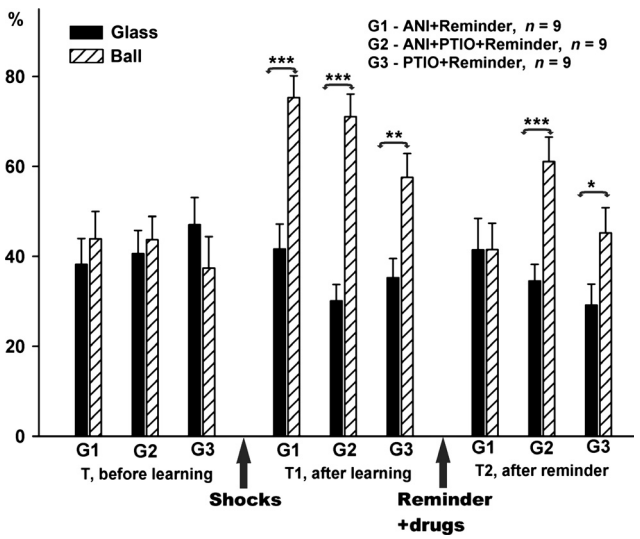

FIG. 3. Reconsolidation is absent under ANI+blockade of NO functioning with the NO scavenger carboxy-PTIO. Protocol of a context conditioning experiment with ANI/PTIO injections is similar to Fig. 2A. Plot shows averaged amplitudes (+SEM) of withdrawal responses in three groups of snails measured in two different contexts, i.e. on the ball (reinforced context) and on the glass. G1 ($n = 9$) was injected at 20 min before the reminder with ANI, G2 ($n = 9$) with ANI+PTIO+reminder, and G3 ($n = 9$) with PTIO+reminder only. Significance of differences in response amplitudes in two contexts was estimated for each group using a Wilcoxon signed-rank test. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

but tested behavioral performance at two additional time-points (4 and 72 h after the reminder; see protocol in Fig. 4A). It was also essential to test other types of NO-synthase inhibitors, and we used two drugs in addition to L-NNA, i.e. L-NAME hydrochloride (non-specific inhibitor) and 7-nitroindazole (reversible and non-selective NOS inhibitor). The results of this complex experiment, involving six groups of snails trained and tested simultaneously to avoid seasonal differences in behavior, are shown in Fig. 4B–D. Initially, each group consisted of eight animals, but we discarded the scores of animals that failed to meet our survival criterion of at least 1 week after the end of experiments. As expected, after training, the animals in all groups showed highly significant ($P < 0.001$ in all groups, Wilcoxon signed-rank test) differences of responses in two contexts. It is interesting to note that, under ANI, the context memory was still present at 4 h after the reminder ($P < 0.001$ at the 4 h time-point, Wilcoxon signed-rank test, z = 3.1, Fig. 4B, 'Anisomycin'), whereas 24 and 72 h later the difference in two contexts in the same animals was not significant. The saline-injected animals showed highly significant differences at all testing points ($P < 0.001$, Fig. 4B, 'Saline'). Administration of L-NAME and 7-nitroindazole rescued the memory in conditions of reminder under ANI (Fig. 4C, $P < 0.01$ at the 24 h time-point, Wilcoxon signed-rank test, z = 2.2; Fig. 4D, $P < 0.05$ at the 24 h time-point, Wilcoxon signed-rank test, z = 1.9), supporting an NO requirement for manifestation of the reconsolidation process. Administration of these NO-synthase inhibitors by itself, similar to what was shown for L-NNA, had no significant effects on the memory (Fig. 4C,D, left panels).

We tested the effect of administration of the potent NO donor SNAP using the same protocol (Fig. 5). No significant differences were observed between the groups of animals injected with saline or SNAP before the reminder (Fig. 5, compare G1 and G2), whereas the animals injected with ANI (Fig. 5, G3) demonstrated a complete

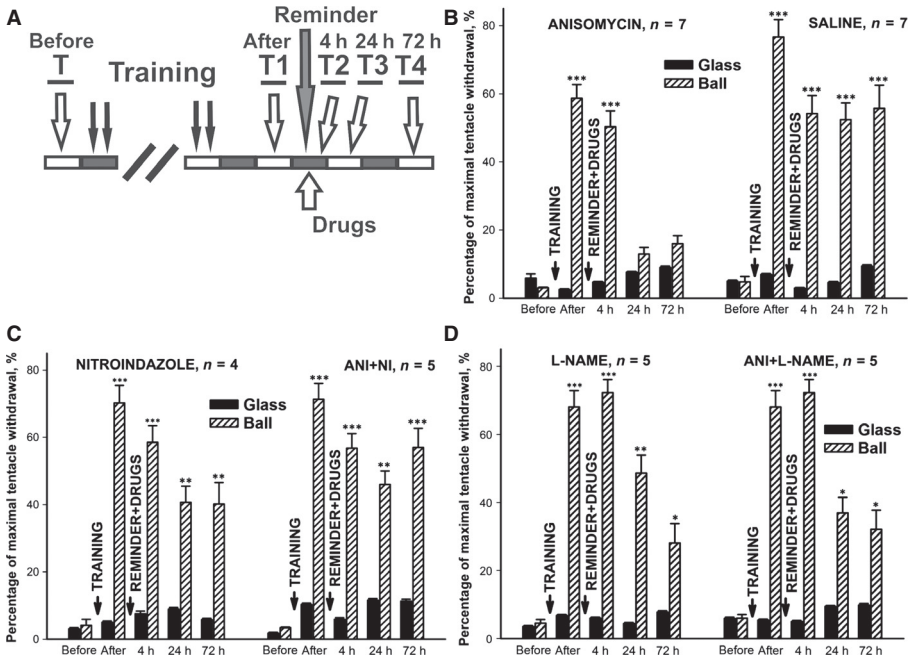

FIG. 4. Reconsolidation is absent under ANI+blockade of NO synthesis with the NOS inhibitors L-NAME or 7-nitroindazole tested at time-points 4, 24, and 72 h after the reminder. (A) Protocol of a context conditioning experiment with ANI/drug injections at 20 min before the reminder. T, T1, T2, T3, T4, tests for context conditioning at different time-points. (B–D) Averaged amplitudes (+SEM) of withdrawal responses in six groups of snails measured randomly in two different contexts, i.e. on the ball (reinforced context) and on the glass. In all cases, memory is present at 4 h after the reminder, but disappears under ANI when tested 24 and 72 h later (B), whereas under ANI+7-nitroindazole+reminder (C) or ANI+L-NAME+reminder (D) the memory is not impaired. *Y*-axis, amplitude of tentacle withdrawal as a percentage of the length before the test. Significance of differences in response amplitudes in two contexts was estimated for each group using Wilcoxon signed-rank test. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

loss of memory the next day after the reminder, as expected. The idea of this experiment was to validate whether an excessive concentration of NO affects learning, but the results obtained suggest that changes in NO concentrations must be very local and addressed to certain neurons of the network in order to change the behavior.

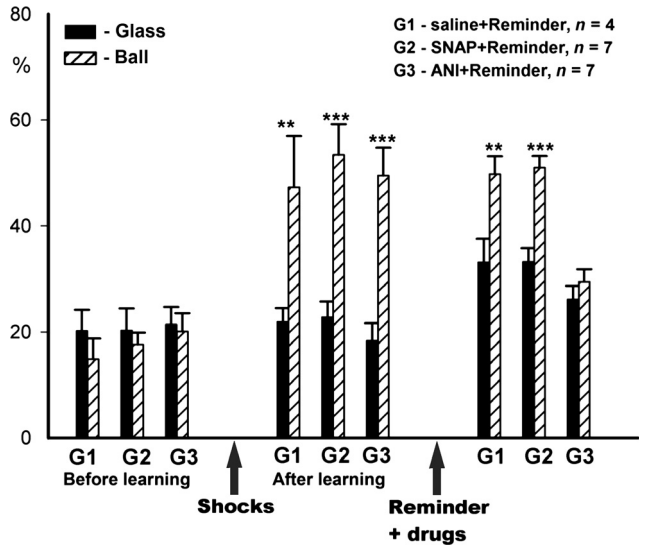

FIG. 5. Memory is not impaired under the NO donor SNAP. Protocol of a context conditioning experiment with ANI/SNAPinjections is similar to Fig. 2. Averaged amplitudes (+SEM) of withdrawal responses in three groups of snails measured in two different contexts, i.e. on the ball (reinforced context) and on the glass. G1 (n = 4) was injected at 20 min before the reminder with saline, G2 (n = 7) with SNAP+reminder, and G3 (n = 7) with ANI+reminder. Significance of differences in response amplitudes in two contexts was estimated for each group using Wilcoxon signed-rank test. $**P < 0.01$ and $***P < 0.001$.

Discussion

In the present study, we exploited the reconsolidation hypothesis that states that a consolidated memory, when reactivated, enters a changeable (labile) state that requires *de-novo* protein synthesis for new consolidation or reconsolidation of memory, and the existence of this labile state is proved by the disappearance of memory when protein synthesis is blocked during a reminder of the experimental situation (Nader *et al.*, 2000; Anokhin *et al.*, 2002; Duvarci & Nader, 2004). We hypothesized that the proteins involved in memory maintenance are changed by some agent during the labile state elicited by memory reactivation. For instance, the PkMZeta molecules, shown to be involved in memory maintenance and localized at post-synaptic densities, may be the target (Hernández *et al.*, 2014). We have analysed the literature about existing in synapses agents, the concentration of which is increased during strong activation very locally and can impair proteins. The only strong candidate was NO, which was shown to be present in post-synaptic densities in the spines of pyramidal cells (Hardingham *et al.*, 2013). It is known that NO acts via the direct *S*-nitrosylation of proteins, the covalent attachment of a nitrogen monoxide group to the thiol side-chain of cysteine, which changes the physiological functions of existing proteins, changing their role in physiological functions including synaptic plasticity (Selvakumar *et al.*, 2013; Tooker *et al.*, 2013). Only neurons that are strongly activated during memory reactivation can locally produce NO in large concentrations, and can change (via fast diffusion of NO to pre-synapses) their own synaptic

inputs (Hardingham *et al.*, 2013). Therefore, we started to investigate the role of NO in reconsolidation.

In our previous experiments (Balaban *et al.*, 2011), we tried to answer the question of whether NO is necessary for learning in snails, and whether NO participates in a situation of relearning to an alternative behavior. At first, we trained the snails to perceive a certain context as aversive for food intake, and then retrained them to shift their perception to view the same context as positive. If the animals were injected each day during training sessions with the NOS blocker, they demonstrated no difference in responses in the two contexts, indicating that NO is involved in the development of new memory. The saline-injected group in these experiments demonstrated perfect memory to context, and perfect relearning (Balaban *et al.*, 2011). In electrophysiological experiments, the participation of NO in long-term facilitation also was shown in the terrestrial snail (Korshunova & Balaban, 2014).

The reconsolidation phenomenon was shown in terrestrial snails in our earlier experiments (Gainutdinova *et al.*, 2005). In the present experiments, we used the same experimental paradigm and found that, in the ANI-injected group, the animals demonstrated disappearance of memory (compare the results of G1 snails in Figs 2B and 3, ANI data in Fig. 4B) after the reminder in the presence of ANI. Note that all experiments were performed with long (especially for snails) periods of training and rest days to account for long-lasting changes in behavior (Figs 2–5).

Recently, a review of the current understanding of the cytotoxic vs. cytoprotective effects of NO in the central nervous system was published (Calabrese *et al.*, 2009), highlighting the 'Janus-faced properties of this small molecule'. In this review it is described that signaling by reactive nitrogen species is carried out mainly by targeted modifications of critical cysteine residues in proteins, including *S*-nitrosylation and *S*-oxidation, as well as by lipid nitration.

Multiple roles of NO in the nervous system are stressed in a review concerning the NO regulation of transcription factors (Contestabile, 2008). The author states that, in addition to regulating the proliferation, survival and differentiation of neurons, NO is also involved in synaptic activity, neural plasticity and memory formation. The long-lasting effects of NO, a simple and unstable molecule, occur through the regulation of transcription factors and modulation of gene expression. cAMP-response-element-binding protein is an important transcription factor that regulates the expression of several genes involved in survival and neuroprotection as well as in synaptic plasticity and memory formation. It is described that NO promotes the survival and differentiation of neural cells, activating, through cGMP signaling, cAMP-response-element-binding phosphorylation-dependent transcriptional activity, and simultaneously promoting the *S*-nitrosylation of nuclear proteins that favor cAMP-response-element-binding binding to its promoters on target genes (Contestabile, 2008). Using the anti-NOS-2 RNA in the central nervous system of the pond snail *Lymnaea* it was shown that establishing memories occurs through the regulation of NO signaling at the synapse (Korneev *et al.*, 2013).

The overall impression derived from the literature on NO is that this molecule is everywhere and participates in everything. Here, we tried to focus only on events elicited by strong activation of specific cells involved in the network underlying the reactivation of memory and leading to the local release of NO.

Nitric oxide and formation of memory

A role of NO in memory formation was repeatedly described in all animals studied (Jacklet, 1997; Muller, 1997; Hawkins *et al.*, 1998; Fedele & Raiteri, 1999; Rose, 2000; Schweighofer & Ferriol, 2000; Katzoff *et al.*, 2002, 2006; Antonov *et al.*, 2007). Itzhak & Anderson (2007) stated that, in the absence of nNOS activity, particularly during the reconsolidation phase, long-term memory of cocaine-associated context is extinguished. It is surprising that this memory was developed in NOS knockout mice at all, because Tanda *et al.* (2009) clearly showed that spatial memory in NOS knockout mice is impaired. The mechanisms of consolidation and reconsolidation are implied to be similar. In our recently published experiments (Korshunova & Balaban, 2014), it is shown that daily injection of NOS blocker during 5 days of training completely prevents the formation of long-term memory in snails using 'negative' reinforcement (electric shocks in a given context), whereas application of the NOS blocker after the development of long-term changes does not influence memory. Some contradictions between the results of Itzhak & Anderson (2007) and our data may be caused by different paradigms of learning, involving quite different neurochemical systems (positive or negative reinforcing systems).

In gastropods, NO was shown to be involved in several forms of learning *in vivo* and synaptic plasticity *in vitro*, and during learning in a simplified preparation of the *Aplysia* siphon-withdrawal reflex (Antonov *et al.*, 2007). It was shown that NO makes an important contribution during conditioning and acts directly in both the sensory and motor neurons to affect different processes of facilitation at the synapses between them. In addition, the results of this study suggest that NO does not come from either the sensory or motor neurons but rather comes from another source, perhaps neighboring interneurons (Antonov *et al.*, 2007), which is an essential conclusion for the interpretation of possible NO effects.

In a behavioral analysis (Kemenes *et al.*, 2002) using one-trial appetitive associative conditioning of the snail *Lymnaea stagnalis*, it was shown that there is an obligatory requirement for the NO–cGMP signaling pathway in the formation of long-term memory, and this requirement lasts for a critical period of 5 h after training. This represents the first demonstration that associative memory formation after single-trial appetitive classical conditioning is dependent on an intact NO–cGMP signaling pathway.

In *Helix*, it was shown (Teyke, 1996) that blocking the NOS prior to conditioning significantly impaired their food-finding ability. Food-conditioned snails, after inhibition of NOS, still remained able to locate the conditioned food. These important results indicate that the acquisition of memory in the terrestrial snail depends on NO, whereas memory recall and olfactory orientation are less or not at all dependent on NO.

In experiments in *Aplysia* it was shown that the NO signaling during training plays a critical role in the formation of multiple memory processes (Katzoff *et al.*, 2002). In these experiments, a possibility of rescuing memory formation by NO donors was shown. It is essential to note that L-NAME had little or no effect on feeding behavior *per se* or on most aspects of the animals' behavior while they were being trained (Katzoff *et al.*, 2002).

Nitric oxide and labilization of memory

In the published literature we have found only one article in which the NO blocker was used simultaneously with other pharmacological substances during analysis of learning and memory (Wass *et al.*, 2006). It is of interest that the NOS inhibitor L-NAME in these experiments reversed the phencyclidine-induced disruption of acquisition learning, thus suggesting the necessity of NO for the disruption of memory.

The question is still open of whether the labilized memory during reconsolidation is erased or only the retrieval is blocked, due to quite different published data and different interpretations (Rose, 2000; Anokhin *et al.*, 2002; Sara & Hars, 2006). Some evidence suggests that the extinction of memory is a consequence of new inhibitory learning (Archbold *et al.*, 2010) but not memory erasure or labilization. However, it is demonstrated that the amnesia induced by blockade of reconsolidation does not show any spontaneous recovery in many experiments (Duvarci & Nader, 2004).

The potential mechanism of how NO rescues the ANI effect on memory can be assumed on the basis of our results and literature data. It is known that NO molecules generate dual effects of changing protein properties via *S*-nitrosylation, and trigger protein synthesis (Calabrese *et al.*, 2009). In the presence of ANI the second mechanism cannot be implemented, and thus it is rather the inhibition of labilization of the reactivated consolidated memory that is affected by the NO blockers.

In this study, our results advance the idea that NO is necessary for labilization of the existing memory, and provides confirming evidence that NO participates in development of new memory.

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Abbreviations

ANI, anisomycin; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt; cGMP, cyclic guanosine monophosphate; G1, group 1; G2, group 2; G3, group 3; G4, group 4; L-NAME, NG-nitro-L-arginine methyl ester hydrochloride; L-NNA, *N*-omega-nitro-L-arginin; NI, 7-nitroindazole; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine.

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