Stimulation of the cAMP system by the nitric oxide-cGMP system underlying the formation of long-term memory in an insect

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Abstract

The nitric oxide (NO)-cGMP signaling system and cAMP system play critical roles in formation of multiple-trial induced, protein synthesis-dependent long-term memory (LTM) in many vertebrates and invertebrates. The relationship between the NO-cGMP system and cAMP system, however, remains controversial. In honey bees, the two systems have been suggested to converge on protein kinase A (PKA), based on the finding *in vitro* that cGMP activates PKA when sub-optimal dose of cAMP is present. In crickets, however, we have suggested that NO-cGMP pathway operates on PKA via activation of adenylyl cyclase and production of cAMP for LTM formation. To resolve this issue, we compared the effect of multiple-trial conditioning against the effect of an externally applied cGMP analog for LTM formation in crickets, in the presence of sub-optimal dose of cAMP analog and in condition in which adenylyl cyclase was inhibited. The obtained results suggest that an externally applied cGMP analog activates PKA when sub-optimal dose of cAMP analog is present, as is suggested in honey bees, but cGMP produced by multiple-trial conditioning cannot activate PKA even when sub-optimal dose of cAMP analog is present, thus indicating that cGMP produced by multiple-trial conditioning is not accessible to PKA. We conclude that the NO-cGMP system stimulates the cAMP system for LTM formation. We propose that LTM is formed by an interplay of two classes of neurons, namely, NO-producing neurons regulating LTM formation and NO-receptive neurons that are more directly involved in formation of long-term synaptic plasticity underlying LTM formation.

Main text

Recent studies have suggested that many of the molecular mechanisms underlying learning and memory are conserved among vertebrates and invertebrates, the most convincing evidence for which has been obtained by the study of the mechanisms of the formation of LTM [7]. LTM is defined as a protein synthesis-dependent phase of memory lasting for day to a lifetime. It is usually formed by multiple pairing trials but not by a single trial. It has been demonstrated that the cAMP signaling system plays critical roles in producing LTM, or long-term synaptic plasticity considered to underlie LTM formation, in mammals [1], insects [6, 19] and mollusks [2]. In all of these animals, production of cAMP stimulates PKA, and this activates the transcription factor CREB (cAMP element-binding protein). Activation of CREB leads to a protein synthesis-dependent long-term synaptic plasticity that underlies LTM formation [7, 20].

The NO-cGMP system also plays critical roles in the formation of LTM in mammals [9], honey bees [15, 16], crickets [14] and mollusks [8]. NO is a membrane-permeable intercellular signaling molecule produced by NO synthase (NOS). NO diffuses into neighboring cells and stimulates soluble guanylyl cyclase, and produced cGMP plays various physiological roles [5], including induction of LTM in many animals.

In crickets, we have reported that the NO-cGMP system and cAMP system play major roles in formation of associative olfactory LTM in studies using a differential conditioning procedure [14]. We found that: (1) multiple-trial conditioning leads to LTM formation, which is measured as 1-day retention, but single-trial conditioning leads to medium-term memory (MTM), which is measured as 30-min retention, (2) injection of inhibitors of key enzymes of the NO-cGMP system or cAMP system, such as the NOS inhibitor L-NAME, the soluble guanylyl cyclase inhibitor ODQ, the adenylyl cyclase inhibitor 2', 5'-dideoxyadenosine (DDA) or the PKA inhibitor KT5720, prior to multiple-trial conditioning fully impaired LTM formation but had no effect on MTM formation, and (3) injection of an activator of the NO-cGMP system or cAMP

system, such as 8Br-cGMP or DB-cAMP, prior to single-trial conditioning led to the induction of LTM. The simplest model to account for these and some other findings [14] is depicted in Fig. 1, with slight modifications to explain the rationale of the present experiments (see below).

Whether the NO-cGMP system and cAMP system are arranged in series or in parallel, however, remains controversial. In honey bees, it has been suggested that prolonged activation of PKA during multiple-trial conditioning induces LTM [16], and an *in vitro* study using PKA holoenzyme purified from the brain led to the conclusion that PKA could be activated by cGMP in the presence of a low concentration of cAMP [10] and thus the NO-cGMP pathway and the cAMP pathway are thought to converge on PKA to induce LTM. If cGMP produced by multiple-trial conditioning indeed activates PKA to lead to LTM formation in the presence of a low concentration of cAMP, the NO-cGMP pathway and cAMP pathway should act in parallel, and PKA should serve as a molecular convergence site to integrate signals of the two systems and to trigger biochemical cascades for LTM formation. In crickets, on the other hand, we found that the effects of an externally applied cGMP analog to facilitate LTM formation was fully blocked by co-injecting an adenylyl cyclase inhibitor DDA [14], thus suggesting that cGMP does not activate PKA to lead to LTM formation (see Fig. 1). This observation, however, does not rule out the possibility that cGMP directly activates PKA when sub-optimal dose of cAMP is present and thus, the possibility that NO-cGMP system and cAMP system are arranged in parallel, as well as in serial, has not be ruled out, as is depicted in Fig. 1. Whether the NO-cGMP system and cAMP system are arranged in series or parallel is not a trivial question, because it provides critical information to understand the basic design of neural systems underlying LTM formation, as we will discuss later.

The aim of this study was to resolve this controversy. We performed a set of experiments to study whether cGMP activates PKA in the presence of sub-optimal dose of cAMP during actual LTM formation in crickets. The results of our study suggest that

(1) an externally applied cGMP analog can activate PKA when sub-optimal dose of a cAMP analog is present, but (2) cGMP produced by multiple-trial conditioning cannot activate PKA even when a low concentration of a cAMP analog is present, thus indicating that cGMP produced by multiple-trial conditioning is not accessible to PKA.

Adult male crickets, *Gryllus bimaculatus,* at 1-2 weeks after the imaginal molt were used. They were reared in a 12 h:12 h light:dark cycle at 27±2°C and were fed a diet of insect pellets and water *ad libitum*. Three days before the start of the experiment, crickets were individually placed in 100-ml glass beakers and fed a diet of insect pellets *ad libitum* but deprived of drinking water to enhance their motivation to search for water.

We used a classical conditioning and operant testing procedure [11, 12, 14]. In short, individual animals received single- or multiple-trial (four-trial) elemental appetitive conditioning. For single-trial appetitive conditioning, peppermint odor was paired with water reward. For multiple-trial conditioning, four sets of appetitive conditioning trials were performed with an inter-trial interval of 5 min. Hypodermic syringes of 1 ml each were used for conditioning. A small filter paper was attached to the needle of the syringe. The syringe was filled with water, and the filter paper was soaked with peppermint essence (Mikoya Kosho, Tokyo, Japan). For odor presentation, the filter paper was placed within 1 cm of the cricket's head. At 2 sec after the onset of odor presentation, a drop of water was given to the mouth of the cricket for 2 sec.

Odor preference tests were performed a few hours before the onset of conditioning and at 30 min or 1 day after the end of conditioning. Animals were placed in a test apparatus and allowed to choose between reward-associated odor (peppermint) and control odor (vanilla), as described previously [11]. In short, on the floor of the “test chamber” of the apparatus, there were two circular holes that connected the chamber with two of three sources of odor. Each odor source consisted of a cylindrical plastic container containing a filter paper soaked with 3 µl solution of vanilla essence (Kyoritsu Shokuhin, Tokyo, Japan) or peppermint essence, covered with a fine gauze net. The

three containers were mounted on a rotatable holder. Two odor sources could be located simultaneously just below the holes at the “offer position” by rotating the holder.

Before the preference test, an animal was transferred from the beaker to the “waiting chamber” of the apparatus and left for 4 min to become accustomed to the surroundings, and then the door to the test chamber was opened. The test started when the animal entered the test chamber. Two minutes later, the relative positions of the vanilla and peppermint sources were changed by rotating the container holder. An odor source was considered to have been visited when the animal probed the net top with its mouth. The time spent for visiting each odor source was measured cumulatively. The preference test lasted for 4 min. If the total time of visits of an animal to either source was less than 10 sec, we considered that the animal was less motivated to visit odor sources, possibly due to a poor physical condition, and the data were rejected. At the end of the training, the sliding door was opened and the animal was gently pushed into the waiting chamber, and then the animal was transferred to a beaker. After completing the test session, animals were provided a diet of insect pellets *ad libitum*.

For the study of the effect of drugs, animals were injected with 3 µl cricket saline [13] containing drugs into the hemolymph of the head using a 10-µl microsyringe at 20 min before training. Rp-8Br-cAMPS was purchased from Calbiochem (Tokyo, Japan) and all other drugs were purchased from sources described previously [14]. ODQ and KT5720 were dissolved in cricket saline containing 0.1% DMSO, and all other drugs were dissolved in cricket saline.

For statistical evaluation of odor preferences of animals, relative odor preference of each animal was measured using the preference index (PI) for rewarded odor (peppermint) (%), defined as $t_P/(t_P+t_V)x100$, where t_P is the time spent exploring the peppermint source and t_V is the time spent exploring the vanilla source [14]. Because crickets prefer vanilla over peppermint, the PI before conditioning is below 50% in most groups [11, 14]. Wilcoxon's (WCX) test was used to compare odor preferences in different tests of a given animal group. The Mann-Whitney *U* (M-W) test was used to

compare odor preferences of different two groups. For multiple comparisons, Holm method was used to adjust the significance level. *P* values of <0.05 were considered statistically significant.

The basic rationale of the present experiments is depicted in Fig. 1. In this study, we compared the effect of multiple-trial conditioning for producing LTM against the effect of an externally applied cGMP analog, in the presence of sub-optimal dose of cAMP analog and in condition in which adenylyl cyclase was inhibited. This was to clarify whether cGMP, produced by multiple-trial conditioning or externally applied, can directly activate PKA when sub-optimal dose of cAMP analog was present. Before performing this experiment, we needed preparatory experiments to establish adequate conditioning procedure and also adequate doses of cGMP analog, cAMP analog and adenylyl cyclase inhibitor.

In this study we used an elemental appetitive conditioning procedure, not the differential conditioning procedure we used in our previous study [14]. This change was to simplify the procedure and thus to facilitate detailed analysis of biochemical processes underlying LTM formation. We, therefore, carried out preparatory experiment to confirm that our previous findings that the NO-cGMP system, cAMP system and protein synthesis participate in the formation of LTM by differential conditioning [14] are applicable to LTM formed by elemental appetitive conditioning (Supplemental Fig. S1). We found that injection of soluble guanylyl cyclase inhibitor (ODQ), adenylyl cyclase inhibitor (2', 5'-dideoxyadenosine: DDA), PKA inhibitors (KT5720, Rp-8Br-cAMPS) or protein synthesis inhibitor (cyclohexmide: CHX) at 20 min prior to multiple-trial appetitive conditioning completely impairs the formation of 1-day retention (Fig. S1Bc-g) but not the formation of 30-min memory (for details, see Fig. S1Ac-g). Thus we conclude that the NO-cGMP system, cAMP system and protein synthesis participate in LTM formation, but not of MTM formation, by multiple-trial appetitive conditioning.

Next, we performed another preparatory experiment to determine adequate doses

of drugs used in main experiments to examine whether injection of a cGMP analog (8Br-cGMP) prior to single-trial conditioning can stimulate PKA to lead to the formation LTM when a low concentration of a cAMP analog (DB-cAMP) is also co-injected. In this experiment, the adenylyl cyclase inhibitor DDA needed to be co-injected, because we have shown that an increase of cGMP concentration leads to an activation of adenylyl cyclase, and this in turn leads to an increase of cAMP concentration and activation of PKA [14], as is depicted in Fig. 1. We thus performed experiments to determine adequate doses of 8Br-cGMP, DDA and DB-cAMP (Fig. S2).

For the 8Br-cGMP, we found that injection of 3 μl of saline containing 200 μM or 1 mM 8Br-cGMP at 20 min prior to single-trial conditioning yielded a saturated level of LTM (Fig. S2c, d), while injection of 3 μl of saline containing 20 μM 8Br-cGMP yielded only a low level of LTM (Fig. S2b). Thus, we conclude that injection of 3 μl of saline containing 200 μM 8Br-cGMP prior to single-trial conditioning is suitable to achieve a saturated level of LTM and a full activation of PKA. For the DDA, we found that LTM formed by injection of 3 μl of saline containing 200 μM, 1 mM or even 5 mM 8Br-cGMP prior to single-trial conditioning is fully impaired by co-injection of 1 mM DDA (Fig. S2e-f). We thus used 1 mM DDA in subsequent experiments. For the DB-cAMP, we studied the effects of injection of 3 μl of saline containing 20, 50, 100, 200 or 1000 μM DB-cAMP and 1 mM DDA before single-trial conditioning on LTM formation (Fig. S3a-e) and found that a low level of LTM is achieved by 50 or 100 μM DB-cAMP and 1 mM DDA. We thus conclude that injection of 3 μl of saline containing 50 μM DB-cAMP and 1 mM DDA is suitable to emulate a sub-optimal dose of cAMP.

On the basis of the results of preparatory experiments, we carried out a main experiment examining whether an externally applied cGMP analog can activate PKA to induce LTM in the presence of a low concentration of a cAMP analog, and we found that an externally applied cGMP analog can induce LTM. A group of animals was injected with 3 μl of saline containing 200 μM 8Br-cGMP, 50 μM DB-cAMP and 1 mM DDA before single-trial conditioning. The group exhibited a significant level of 1-day

retention (Fig. 2c; $p<0.05$, WCX test adjusted by Holm method). The level of 1-day retention in this group was significantly greater than that in the group injected with 3 μl saline containing 50 μM DB-cAMP and 1 mM DDA (but not 200 μM 8Br-cGMP) before single-trial conditioning (Fig. 2a; $p<0.05$, M-W test adjusted by Holm method) and did not significantly differ from that in the group injected with 3 μl saline containing 200 μM DB-cAMP and 1 mM DDA (but not 200 μM 8Br-cGMP) before single-trial conditioning (Fig. 2b; $p>0.05$, M-W test adjusted by Holm method), thus demonstrating an enhancement of LTM formation by 8br-cGMP. We also found that the enhancement of LTM formation by 8Br-cGMP is mediated by activation of PKA, because the enhancement of formation of LTM by 8Br-cGMP was fully inhibited by further co-injecting the PKA inhibitor KT5720: A group of animals injected with 3 μl of saline containing 200 μM 8Br-cGMP, 50 μM DB-cAMP, 1 mM DDA and 200 μM KT5720 exhibited no significant level of 1-day retention (Fig. 2e, $p<0.05$, WCX test). Thus, externally applied 8Br-cGMP can activate PKA to lead to LTM formation when sub-optimal dose of cAMP is present.

Finally, we performed an experiment to determine whether cGMP produced by multiple-trial conditioning can activate PKA to lead to LTM formation in the presence of sub-optimal dose of cAMP, and the results suggested that cGMP produced by multiple-trial conditioning cannot activate PKA. One group of animals was injected with 3 μl of saline containing 50 μM DB-cAMP and 1 mM DDA before multiple-trial conditioning (Fig. 2d). If cGMP produced by multiple conditioning stimulates PKA (as depicted by the dotted line in Fig. 1), this group should exhibit a higher level of LTM than that of the group injected with the same solution before single-trial conditioning (Fig. 2a). However, the level of 1-day retention of the former group (Fig. 2d) did not significantly differ from that of the latter group (Fig. 2a; $p>0.05$, M-W test adjusted by Holm method).

The results of our study suggest that an externally applied cGMP analog can activate PKA in the presence sub-optimal dose of a cAMP analog, in agreement with the

previous reports by Leboulle and Müller [10] in honey bees. However, our results also suggest that cGMP produced by multiple-trial conditioning cannot activate PKA even in the presence of sub-optimal dose of a cAMP analog. The simplest and the most likely explanation of this finding is that cGMP production and PKA activation occur in different subcellular compartments in the same population of neurons. Alternatively, cGMP production and PKA activation may occur in different population of neurons. In this case, however, elongated subcellular and cellular processes needs to be assumed between activation of calmodulin and activation of adenylyl cyclase. By any reasons, cGMP produced by multiple-trial conditioning is not accessible to PKA. Therefore, we conclude that the NO-cGMP pathway and cAMP pathway are arranged in series in the biochemical cascade for the formation of LTM; that is, the NO-cGMP pathway stimulates the cAMP pathway to lead to LTM formation.

On the basis of the finding that the NO-cGMP pathway and cAMP pathway are arranged in series, not in parallel, in the signaling cascades for LTM formation, we propose that LTM is formed by an interplay of two classes of neurons, namely, NO-generating neurons that play regulatory roles in formation of LTM and NO-receptive neurons that are more directly involved in formation of long-term synaptic plasticity underlying LTM formation. Our next steps are to identify these NO-generating and NO-receptive neurons in the cricket brain and also to confirm that production of cGMP and activation of cAMP system occur in the same population of neurons. We have shown that mRNA for *NOS* gene and that for *SGCβ* gene, the latter codes β subunit of soluble guanylyl cyclase, are densely distributed in outer Kenyon cells and inner Kenyon cells of the mushroom body (a higher-order olfactory center as well as multisensory association center), respectively, in addition to some neurons around the antennal lobe (primary olfactory center) [18, unpublished results]. Similarly, in the mushroom body of locusts, axons of Kenyon cells have been reported to form tubular NO-generating zones that surround central cores of NO-receptive Kenyon cell axons [17]. Roles of NO-generating and NO-receptive neurons in the mushroom body

and the antennal lobe in LTM formation need to be determined.

Another important subject is to clarify which receptors or ion channels are responsible for the entry of calcium ions in NO-generating cells, to lead to activation of calmodulin and then that of NO synthase. Notable findings related to this subject are that stimulation of nicotinic acetylcholine receptors follows NO production in honey bees [3] and moths [21] and that antagonists of nicotinic acetylcholine receptors impair formation of LTM, but not MTM, in honey bees [4]. Studies on receptors or ion channels upstream of NO production are promising to elucidate why multiple, spaced conditioning triggers NO production and hence LTM formation, but single conditioning or multiple, massed conditioning does not [11].

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Figure legends

Fig. 1. A scheme to explain the experimental design. A model proposed to account for the results of our studies on biochemical pathways for LTM formation by associative olfactory conditioning in crickets [14] is modified to explain the basic rationale of the present experiments. In the figure, the points of pharmacological manipulation (1-4) in experiments to study whether or not cGMP activates PKA (dotted line with a question mark) are indicated. Single-trial conditioning induces only short-term synaptic plasticity that underlies amnesic treatment-sensitive STM and amnesic-treatment resistant MTM [11]. Multiple-trial conditioning activates the NO-cGMP system, and this in turn activates the adenylyl cyclase (AC)-PKA-cAMP responsive element-binding protein (CREB) system via the cyclic nucleotide-gated (CNG) channel and calcium-calmodulin (CAM) system, resulting in protein synthesis that is necessary to achieve long-term plasticity of synaptic connection (a column of gray triangles) upon another neurons that underlies LTM. On the upper right, the drugs (1-4) used to inhibit (marked by X) or activate (marked by upper arrows) the elements of the biochemical systems are indicated. Arg: arginine, NOS: NO synthase, sGC: soluble guanylyl cyclase.

Fig. 2. Experiments to determine whether cGMP produced by multiple-trial conditioning can activate PKA to lead to LTM formation in the presence of sub-optimal dose of cAMP. Four groups of animals were injected with 3 μl of saline containing 50 μM DB-cAMP and 1 mM DDA (a), 200 μM DB-cAMP and 1 mM DDA (b), or 200 μM 8Br-cGMP, 50 μM DB-cAMP and 1 mM DDA without (c) or with (e) co-injection of 200 μM KT5720 at 20 min prior to single-trial conditioning. Another group of animals was injected with 3 μl of saline containing 50 μM DB-cAMP and 1 mM DDA at 20 min prior to multiple-trial conditioning (d). The preference indexes (PIs) measured before conditioning (white bars) and at 1 day after conditioning (hatched bars in a, b, d, shaded bar in c) are shown as means + S.E. The results of statistical comparisons within each group (WCX test) or between groups (M-W test), adjusted by Holm method, are shown

as asterisks. (NS) $p>0.05$, (*) $p<0.05$. The number of animals is shown at each data point.

Supplementary Figure List

Fig. S1. Participation of the NO-cGMP system, cAMP system and protein synthesis in LTM formation by multiple-trial elementary appetitive conditioning. Effects of injection of the soluble guanylyl cyclase inhibitor ODQ, the adenylyl cyclase inhibitor inhibitor DDA, the PKA inhibitor KT5720, another PKA inhibitor Rp-8Br-cAMPS and the protein synthesis inhibitor cycloheximide (CHX) prior to multiple-trial appetitive conditioning on 30 min retention (MTM) (**A**) or 1 day retention (LTM) (**B**) were studied. Two groups of animals were subjected to four-trial conditioning (intact; a) and another ten groups of animals were each injected with 3 μl of saline (saline; b) or saline containing 200 μM ODQ (c), 1 mM DDA (d), 200 μM KT5720 (e), 200 μM Rp-8Br-cAMPS (f) or 10 mM CHX (g) at 20 min prior to four-trial conditioning. Their odor preferences were tested before conditioning (white bars) and at 30-min (shaded bars in **A**) or 1-day after conditioning (shaded bars in **B**). The results of statistical comparisons are shown as means + S.E. Significant differences of the PIs are indicated by asterisks (WCX test). (NS) $p>0.05$, (**) $p<0.01$, (***) $p<0.001$. The number of animals is shown at each data point. The estimated final concentration of the drugs after diffusion was 0.7, 3.5, 0.7, 0.7 and 35 μM, respectively, calculated from injected volume and the approximate body weight of 850 mg. The timing of injection and the concentrations of the drugs, except for Rp-8Br-cAMPS, were determined on the basis of our previous study (see ref. 14 of the main text). The concentration of Rp-8Br-cAMPS was determined by observation that injection of 3 μl of saline containing 200 μM of Rp-8Br-cAMPS after multiple-trial appetitive conditioning fully impaired 1 day retention (Fig. S1Af), but injection of saline containing 20 μM of Rp-8Br-cAMPS had no significant effect (data not shown).

All groups of animals tested at 30 min after conditioning exhibited significant levels of retention: the preferences for the reward-associated odor were significantly greater than those before conditioning in all groups (Fig. S1Aa-g; $p<0.001$ in Fig. 1SAa-e, g,

$p<0.01$ in Fig. S1Af, WCX test). Thus, these drugs had no effects on the formation of 30-min memory, which matches MTM (see ref. 11 of the main text).

For 1-day retention, both the intact group and saline-injected group exhibited significant levels of retention (Fig. S1Ba, b). In contrast, all drug-injected groups exhibited complete impairments of 1-day retention (Fig. S1Bc-g): The preference for the reward-associated odor 1 day after conditioning did not significantly differ from that before conditioning ($p>0.05$, WCX test). Because ODQ and KT5720 were dissolved in saline containing 0.1% DMSO, we also tested the effect of injection of 3 μl of saline containing 0.1% DMSO at 20 min prior to multiple-trial conditioning in another group of animals, and we found that the level of 1-day retention was as high as that of the saline-injected group (data not shown). Thus we conclude that the NO-cGMP system, cAMP system and protein synthesis participate in formation of LTM by multiple-trial appetitive conditioning.

Fig. S2. Effects of injection of different doses of 8Br-cGMP with or without co-injection of the adenylyl cyclase inhibitor DDA prior to single-trial conditioning on LTM formation. Four groups of animals were each injected with 3 μl of saline (a) or saline containing 20 μM (b), 200 μM (c) or 1 mM (d) 8Br-cGMP at 20 min prior to single-trial conditioning. Another three groups of animals were each injected with 3 μl saline containing 200 μM (e), 1 mM (f) or 5 mM (g) 8Br-cGMP and 1 mM DDA at 20 min prior to single-trial conditioning. The preference indexes (PIs) measured before conditioning (white bars) and at 1 day after conditioning (hatched bars) are shown as means + S.E. The results of statistical comparisons within each group (WCX test) or between groups (M-W test) adjusted by Holm method are shown as asterisks. (NS) $p>0.05$, (*) $p<0.05$. The number of animals is shown at each data point.

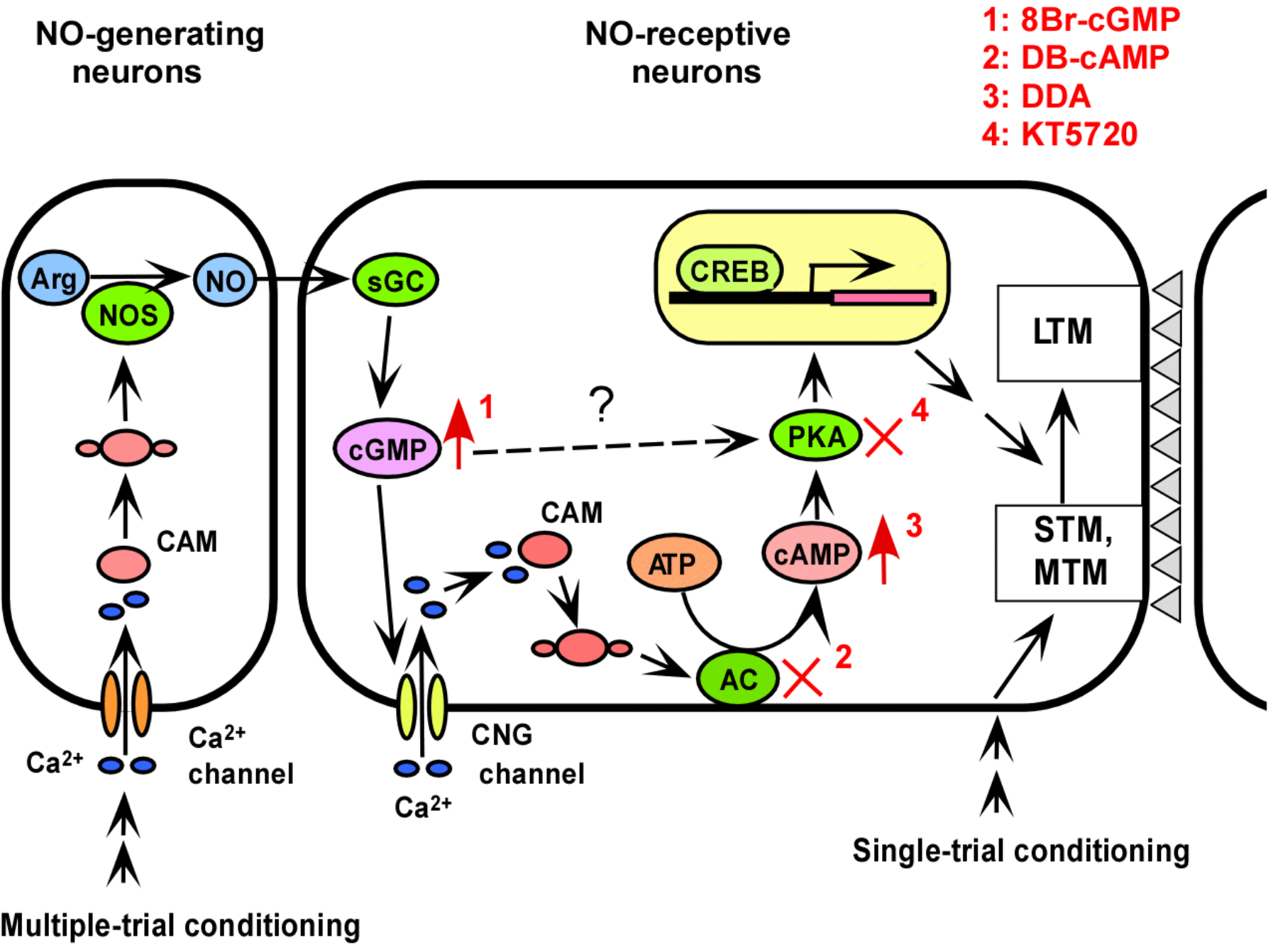
A saline-injected control group exhibited no significant level of 1-day retention, namely, the odor preference did not significantly differ from that before conditioning (Fig. S2a, $p>0.05$, WCX test). The group injected with 20 μM 8Br-cGMP (20 μM

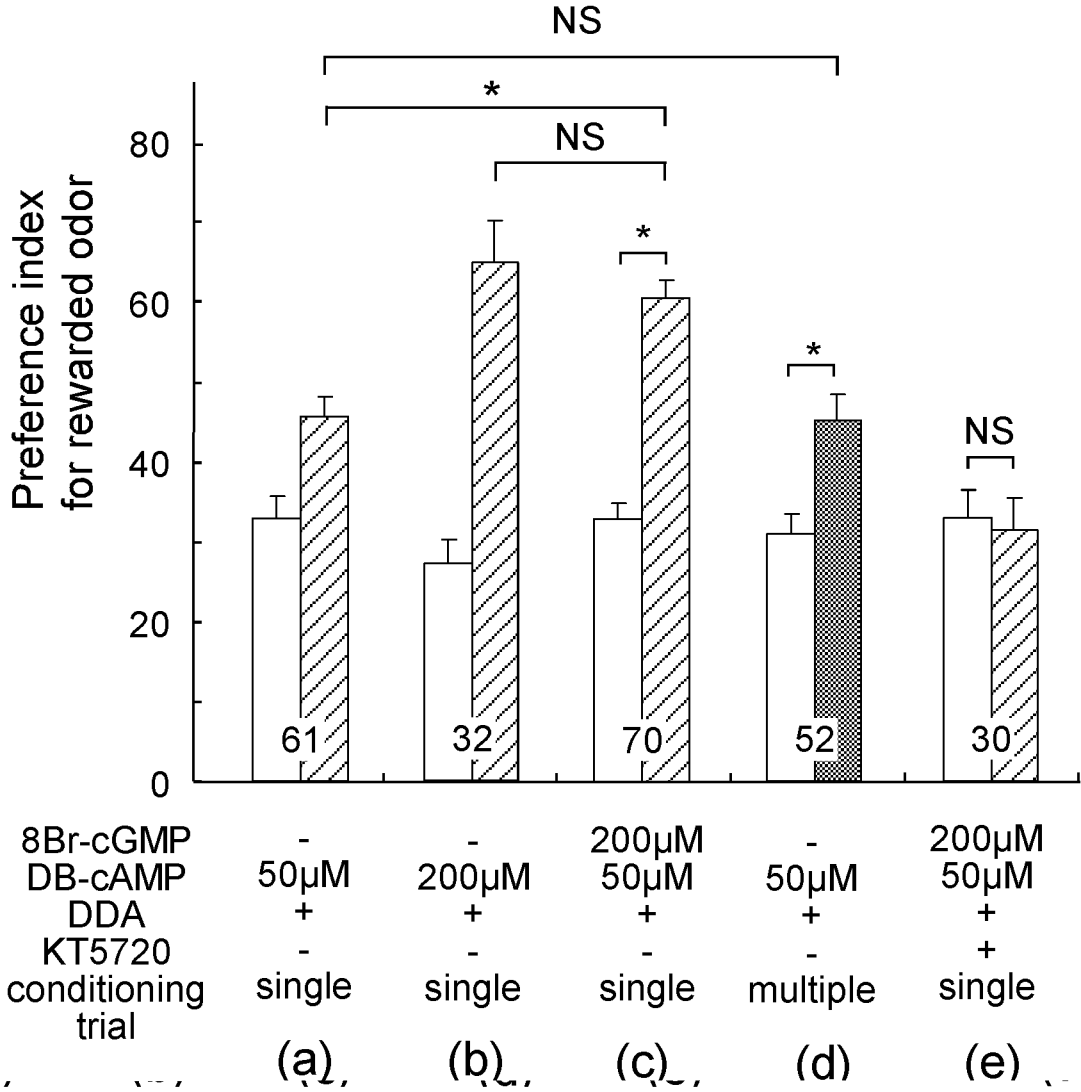
group) exhibited a significant level of LTM: The preference for the rewarded odor was significantly greater than that before conditioning (Fig. S2b, $p<0.05$, WCX test adjusted by Holm method), but the retention level of the 20 μM group was significantly less than that of the 200 μM group or 1 mM group (Fig. S2c, d; $p<0.05$ to 200 μM group or 1 mM group, M-W test adjusted by Holm method). The level of 1-day retention of the 200 μM group did not significantly differ from that of the 1 mM group or the group subjected to multiple-trial conditioning (see Fig. S1Bb, $p>0.05$, M-W test adjusted by Holm method), thus indicating that the effect of 8Br-cAMP to induce LTM is saturated. We thus conclude that injection of 3 μl of saline containing 200 μM 8Br-cGMP prior to single-trial conditioning can achieve a saturated level of LTM, thus indicating that full activation of PKA is achieved.

Groups of animals injected with 3 μl of saline containing 1 mM DDA and 200 μM, 1 mM or 5 mM 8Br-cGMP prior to single-trial conditioning (Fig. S2e-g) exhibited no significant level of 1-day retention ($p>0.05$, WCX test). Previously, we observed that DDA of 1-log order of magnitude lower concentration only partially impaired LTM induced by injection of 8Br-cGMP prior to single-trial conditioning (see ref. 14 of the main text). We conclude that injection of 3 μl of 1 mM DDA is adequate to achieve complete impairment of LTM induced by 8Br-cGMP.

Fig. S3. Effects of injection of different doses of DB-cAMP and DDA before single-trial conditioning on LTM formation. Five groups of animals were each injected with 3 μl of saline containing 20 μM (a), 50 μM (b), 100 μM (c), 200 μM (d) or 1 mM (e) 8Br-cGMP and 1 mM DDA at 20 min prior to single-trial conditioning. The preference indexes (PIs) measured before conditioning (white bars) and at 1 day after conditioning (hatched bars) are shown as means + S.E. The results of statistical comparisons within each group (WCX test) or between groups (M-W test) followed by Holm method are shown as asterisks. (NS) $p>0.05$, (*) $p<0.05$. The number of animals is shown at each data point.

The group injected with 20 μM DB-cAMP and 1 mM DDA (20 μM group) exhibited no significant level of 1-day retention (Fig. S3a; $p>0.05$, WCX test). The 50 or 100 μM groups exhibited significantly higher levels of 1-day retention (Fig. S3b, c; $p<0.05$, WCX test followed by Holm method), but the retention level of the 50 μM group was significantly lower than that of the 1 mM group (Fig. S3b, e; $p<0.05$, M-W test adjusted by Holm method). The level of 1-day retention of the 200 μM group did not significantly differ from that of the 1 mM group (Fig. S3d, e; $p>0.05$, M-W test adjusted by Holm method) or the group subjected to multiple-trial conditioning (Fig. 1SBb, ($p>0.05$, M-W test) thus indicating a saturation of the LTM-inducing effect of DB-cAMP. Thus, we conclude that co-injection of 3 μl of saline containing 50 μM DB-cAMP and 1 mM DDA is adequate to mimic sub-optimal dose of cAMP to achieve partial activation of PKA and a low level of LTM.


NO-generating neurons
NO-receptive neurons
1: 8Br-cGMP
2: DB-cAMP
3: DDA
4: KT5720
Arg
NOS
NO
sGC
CREB
LTM
cGMP
1
?
PKA
4
CAM
ATP
cAMP
3
STM, MTM
AC
2
Ca2+
Ca2+ channel
CNG channel
Ca2+
Single-trial conditioning
Multiple-trial conditioning


NS
*
NS
*
*
NS
Preference index for rewarded odor
80
60
40
20
0
61
32
70
52
30
8Br-cGMP
DB-cAMP
DDA
KT5720
conditioning trial
-
50μM
+
-
single
-
200μM
+
-
single
200μM
50μM
+
-
single
-
50μM
+
-
multiple
200μM
50μM
+
+
single
(a)
(b)
(c)
(d)
(e)