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| Citation | Neuroscience Letters, 2013(541), 4-8 https://doi.org/10.1016/j.neulet.2013.01.010 |
| Issue Date | 2013-04-29 |
| Doc URL | http://hdl.handle.net/2115/52903 |
| Type | article (author version) |
| File Information | Mizunami (revised).pdf |



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Participation of NO signaling in formation of long-term memory in salivary conditioning of the cockroach

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3959 words, 14 text pages, 3 figures

Keywords: olfactory learning; long-term memory; nitric oxide synthase; protein synthesis inhibitor; salivary conditioning; insect.

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Abstract

The molecular and neural basis of protein synthesis-dependent long-term memory (LTM) has been the subject of extensive studies in vertebrates and invertebrates. In crickets and honey bees, it has been demonstrated that nitric oxide (NO) signaling plays critical roles in LTM formation, but no experimental system appropriate for electrophysiological study of neural mechanisms by which production of NO leads to LTM formation has been established in insects. We have reported that cockroaches, as do dogs and humans, exhibit conditioning of salivation, i.e., they exhibit an increased level of salivation in response to an odor paired with sucrose reward. Salivary conditioning can be monitored by activity changes of salivary neurons in rigidly immobilized animals and thus is useful for the study of brain mechanisms of learning and memory. In the present study, we found that injection of cycloheximide, a protein synthesis inhibitor, into the hemolymph before multiple conditioning trials impairs formation of 1-day memory, but not that of 30-min memory. This indicates that formation of 1-day memory requires protein synthesis but that of earlier memory does not. We also found that injection of L-NAME, an inhibitor of NO synthase, before multiple conditioning impairs formation of 1-day memory but not that of 30-min memory. We thus conclude that NO signaling participates in the formation of protein synthesis-dependent LTM but not that of earlier memory in salivary conditioning. Salivary conditioning in cockroaches should become a pertinent system for the study of neural mechanisms by which activation of NO synthase leads to LTM formation.

(250 words)

1. Introduction

The molecular and neuronal basis of protein synthesis-dependent long-term memory (LTM) has been the subject of extensive studies in vertebrates and invertebrates [7]. LTM is defined as a protein synthesis-dependent phase of memory lasting from one day to a lifetime. It is usually formed by multiple pairing trials but not by a single trial. Many studies have suggested that LTM storage is accomplished by enduring changes in synaptic strength that require transcription and translation of genes [7]. This is often achieved by activation of cAMP signaling and resulting phosphorylation of the transcription factor cAMP responsive element-binding protein (CREB) [3,7].

It has been reported that NO-cGMP signaling plays critical roles in the formation of LTM in some species of insects, namely, honey bees [1,18,20] and crickets [12,13], as in mammals [9] and mollusks [8,25]. Surprisingly, although molecular mechanisms of LTM formation have been studied in great detail in the fruit-fly *Drosophila* [2], there are no reports suggesting participation of NO in LTM formation in this species. NO is a membrane-permeable intercellular signaling molecule produced by NO synthase. NO diffuses into neighboring neurons and stimulates soluble guanylyl cyclase, which produces cGMP. In crickets, we pharmacologically studied signaling cascades underlying LTM formation and concluded that the NO-cGMP signaling stimulates the cAMP signaling, namely, the NO-cGMP signaling activates adenylyl cyclase and leads to cAMP production, via activation of CNG (cyclic nucleotide-gated) channel and calcium-calmodulin signaling, for LTM formation [12,13,26]. However, little is known about the neural circuitry mechanisms by which NO production leads to LTM formation in insects. An appropriate experimental system needs to be established for allowing electrophysiological and optophysiological studies on neural mechanisms by which NO production leads to LTM formation.

We have reported that cockroaches, as do dogs [24] and humans [4], exhibit salivary conditioning, namely, they exhibit an increased level of salivation in response to an odor paired with sucrose reward [27,28]. This conditioning can be monitored by changes in

activities of salivary neurons of the subesophageal ganglion, the axons of which run along the salivary duct and innervate salivary glands [27,29]. The activity of salivary neurons can be monitored in rigidly immobilized animals and thus this conditioning system is suitable for the study of brain mechanisms of learning and memory. Indeed, we have suggested, based on the results of a study using local brain microinjection of mecamylamine (an antagonist of a type of acetylcholine receptor), that the mushroom body (multisensory association center of the insect brain) is the site of convergence of olfactory CS and sucrose US in salivary conditioning [30].

In the present study, we first investigated whether formation of 1-day memory after salivary conditioning of cockroaches is impaired by administration of cycloheximide, a protein synthesis inhibitor. Next, we studied whether formation of this memory phase is impaired by L-NAME, an antagonist of NO synthase.

2. Materials and methods

Adult male cockroaches *Periplaneta americana*, maintained in a colony under a light-dark cycle of 12h : 12h at 28 ± 2 C, were used. More than 1 week prior to the training, 10-20 cockroaches were isolated from their colony and placed in a plastic container. Drinking water and sugar-free yeast extract were provided *ad libitum*. The preparation for extracellular recordings from salivary neurons was described previously [10,27]. In short, individual animals were immobilized ventral side up on a paraffin plate. The restrained cockroaches were kept in darkness for 1-2 hours at room temperature. A small incision was made in the ventral region of the neck cuticle to expose the salivary duct. The salivary duct nerve, which contains axons of two salivary neurons, runs along the surface of the salivary duct. Thus the salivary duct nerve, together with the salivary duct, was hooked on a pair of tungsten electrodes. The activity of salivary neurons was amplified with a differential AC amplifier and stored on DAT tape. The number of spikes was counted using LabVIEW software (National Instruments, Tokyo). For the study of effects of drugs, animals were injected with 3 μ l of

cockroach saline [27] containing drugs into the hemolymph of the head. All drugs used in this study were purchased from Sigma.

Immobilized animals were subjected to a differential conditioning procedure in which one of a pair of odors (peppermint and vanilla odors) applied to an antenna (conditioned stimulus, CS+) was paired with sucrose solution applied to the mouth (unconditioned stimulus, US) and the other odor was presented alone (control stimulus, CS-) (Fig. 1). Delivery of odor to an antenna was achieved by the use of a continuous airflow system described previously [21]. The air around the preparation was continuously sucked out of the experimental room by an exhaust system. For delivery of US, the mouth was gently touched with a bamboo skewer soaked in 500 mM sucrose solution. To evaluate the conditioning effect, responses of salivary neurons to odors were tested. Details of the conditioning trials and the retention test are given in the legends of Fig. 1.

Salivary neurons exhibit spontaneous spike activities [10,27-30]. Responses of salivary neurons to peppermint or vanilla odor (R) were measured as relative increase of the spike frequency from the spontaneous level, i.e., $(R_s - R_o)/R_o$, where R_s or R_o is the summed spike frequency of two salivary neurons during a 2-s period of odor stimulation or that during a 2-s period before the onset of odor stimulation, respectively [10]. It is zero when there is no change of spike frequency by odor stimulation. Responses of salivary neurons to vanilla odor and peppermint odor did not differ before conditioning [10,27,29,30]. In all experiments, animals were divided into two groups: one group in which peppermint odor was used as CS+ and vanilla odor was used as CS- and one group that received conditioning with the opposite stimulus contingency. Since we observed no statistical difference in the conditioning effect between these two groups [10,27,29,30], data from these two groups were pooled. The effect of conditioning was evaluated by comparing responses to CS+ and CS- using the Wilcoxon's test [10].

3. Results

First, we studied whether the formation of 1-day memory after conditioning requires protein synthesis. Cockroaches in three groups were immobilized on stages and each cockroach received an injection of 3 μ l of saline or saline containing 2.5 mM or 25 mM of cycloheximide into the hemolymph at 30 min prior to 5 sets of differential conditioning. At one day after conditioning, the animals were dissected for recording of the activities of salivary neurons, and then responses of salivary neurons to CS+ and CS- were tested (Fig. 1A). The group injected with saline exhibited significantly greater responses of salivary neurons to CS+ than to CS- (Fig. 2A, statistical results shown in legends), indicating that 1-day memory was formed. In contrast, the group injected with saline containing 25 mM cycloheximide exhibited no significantly different responses to CS+ than to CS- (Fig. 2A), indicating impairment of the formation of 1-day memory. The group injected with saline containing 2.5 mM cycloheximide exhibited significantly greater responses to CS+ than to CS-, indicating formation of 1-day memory (Fig. 2A). Therefore, the effect of cycloheximide was dose-dependent. We conclude that 1-day memory can be characterized as protein synthesis-dependent LTM.

To determine whether the effect of cycloheximide is specific to long-term (1-day) memory formation, we studied the effect of cycloheximide on formation of 30-min memory. Cockroaches were immobilized on stages and dissected for recording of the activities of salivary neurons. Then they were injected with 3 μ l saline or saline containing 25 mM cycloheximide at 30 min prior to conditioning. A retention test was performed at 30 min after completion of conditioning trials (Fig. 1B). The group injected with 25 mM cycloheximide exhibited significantly greater responses of salivary neurons to CS+ than to CS- (Fig. 2B), as did the group injected with saline solution (Fig. 2B), indicating that formation of earlier (30 min) memory does not require protein synthesis. This observation also indicates that cycloheximide does not impair discrimination of odors.

Next, we studied the effect of L-NAME, an NO synthase inhibitor, on formation of 1-day memory. Three groups of cockroaches were immobilized on stages and each cockroach

received an injection of 3 μ l saline containing 50 μ M, 500 μ M or 5 mM L-NAME. Another group received injection of 3 μ l saline containing 5 mM D-NAME, an inactive isomer. Thirty min later, each group received five sets of differential conditioning trials. The next day, animals were each dissected for recording of the activities of salivary neurons and were then subjected to a retention test. The group injected with 500 μ M or 5 mM L-NAME exhibited no significantly different responses to CS+ than to CS- (Fig. 3A), indicating impairment of LTM formation. In contrast, the group injected with 5 mM D-NAME exhibited significantly greater responses of salivary neurons to CS+ than to CS- (Fig. 3A), indicating the formation of LTM. The group injected with 50 μ M L-NAME exhibited significantly greater responses to CS+ than to CS- (Fig. 3A), indicating that the effect of L-NAME is dose-dependent.

Finally, we studied the effect of L-NAME on 30-min memory to determine whether the effect of L-NAME is specific to LTM formation. Two groups of cockroaches were immobilized on stages and were dissected for recording of the activities of salivary neurons. Then each group was injected with saline containing 5 mM L-NAME or 5 mM D-NAME at 30 min prior to conditioning. Retention was tested at 30 min after completion of the conditioning. Both groups exhibited significantly greater responses of salivary neurons to CS+ than to CS- (Fig. 3B), indicating that L-NAME does not impair formation of 30-min memory. We thus conclude that L-NAME specifically impairs LTM formation.

4. Discussion

In this study we found that the formation of 1-day memory requires protein synthesis in olfactory conditioning of salivation, monitored by activity changes in salivary neurons, in cockroaches. This was in contrast to that the formation of 30-min memory does not require protein synthesis. To our knowledge, this study is the first to demonstrate that the general notion that memory after multiple conditioning trials consists of two phases: protein synthesis-independent earlier phase and protein synthesis-dependent later phase [1,3] is applicable to conditioning of autonomic function in insects. The time course of the

development of the later phase differs among insects [3,11,14]: the protein synthesis-dependent LTM develops at ~8 hours after conditioning in crickets [11] but 3 or 4 days are needed for it to fully develop in honey bees [14] and fruit-flies [3]. The exact time course of the development of LTM in salivary conditioning in cockroaches needs to be characterized. Secondly, we found that activation of NO synthase is required for formation of 1-day memory but not that of 30-min memory in salivary conditioning in cockroaches. Participation of NO signaling in formation of LTM, but not in formation of earlier memory, has been reported in honey bees [18,20] and crickets [12,13]. In the fruit-fly *Drosophila*, however, there is no report suggesting participation of NO in LTM formation, despite enormous efforts to clarify molecular and neuronal mechanisms of LTM formation [2]. Therefore, we need to pay attention to the possibility that molecular mechanisms of LTM formation differ in different insect species. In crickets, our pharmacological studies suggested that the NO-cGMP signaling is upstream of the cAMP signaling, namely, activation of NO-cGMP signaling activates cAMP signaling, via activation of CNG channel and calcium-calmodulin signaling, for LTM formation [12,13]. It needs to be clarified whether this is applicable to salivary conditioning in cockroaches. In addition, the time window in which activation of NO synthase is required for LTM formation needs to be investigated.

A notable feature of NO-cGMP signaling is that NO can diffuse in the brain over tens of millimeters and can act as a volume transmitter within memory-forming neural circuits [23], and we plan to investigate how this signaling mechanism contributes to LTM formation using the cockroach salivary conditioning assay as a model system. In honey bees, it has been shown that the antennal lobe (primary olfactory center) and the mushroom body (multisensory association center) participate in olfactory learning [5,14,15] and that some neurons in both brain areas exhibit NOS activities [19]. It has been shown that local uncaging of cGMP (but not NO) in the antennal lobe facilitates LTM formation in honey bees [20]. In cockroaches, we obtained evidence suggesting that the mushroom body is the site where olfactory CS and sucrose US converge for salivary conditioning [30]. The mushroom body of

cockroaches is a highly organized neuropil, in which the intrinsic neurons (Kenyon cells) are organized into discrete structural subunits [16,17]. Notably, high levels of NOS activities have been observed in class II Kenyon cells of the mushroom body, the axons of which occupy γ areas of the lobes of the mushroom body, but not in class I Kenyon cells, the axons of which occupy the rest of the lobes [22]. One of our next subjects is to study whether local uncaging of NO in the mushroom body can stimulate LTM formation, using the cockroach salivary conditioning assay.

In conclusion, our results demonstrate participation of NO signaling in the formation of protein synthesis-dependent LTM in salivary conditioning of cockroaches. Salivary conditioning in rigidly immobilized cockroaches should become a pertinent model system for studying neural mechanisms by which production of NO leads to LTM formation.

Acknowledgements

This study was supported by grants from the Japan Society for the Promotion of Science to Y. M. and the Ministry of Education, Science, Culture, Sports and Technology of Japan to M. M.

References

- [1] M. Dacher, M. Gauthier, Involvement of NO-synthase and nicotinic receptors in learning in the honey bee, *Physiology & Behavior* 95 (2008) 200-207.
- [2] R.L. Davis, Olfactory memory formation in *Drosophila*: from molecular to systems neuroscience, *Annual Reviews in Neuroscience* 28 (2005) 275-302.
- [3] J. DeZazzo, T. Tully, Dissection of memory formation: from behavioral pharmacology to molecular genetics. *Trends in Neuroscience* 5 (1995) 212-218.
- [4] B.W. Feather, F.C. Delse, M.R. Bryson, Human salivary conditioning: effect of unconditioned-stimulus intensity, *Journal of Experimental Psychology* 74 (1967) 389-392.
- [5] M. Giurfa, Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well, *Journal of Comparative Physiology Part A* 193 (2007) 801-824.
- [6] M. Heisenberg, Mushroom body memoir: from maps to models, *Nature Reviews Neuroscience* 4 (2003) 266-275.
- [7] E.R. Kandel, The molecular biology of memory storage: a dialogue between genes and synapses, *Science* 294 (2001) 1030-1038.
- [8] I. Kemenes, G. Kemenes, R.J. Andrew, P.R. Benjamin, M. O'Shea, Critical time-window for NO-cGMP-dependent long-term memory formation after one-trial appetitive conditioning, *Journal of Neuroscience* 22 (2002) 1414-1425.
- [9] K.M. Kendrick, R. Guevara-Guzman, J. Zorrilla, M.R. Hinton, K.D. Broad, M. Mimmack, S. Ohkura, Formation of olfactory memories mediated by nitric oxide, *Nature* 388 (1997) 670-674.
- [10] C.S. Matsumoto, Y. Matsumoto, H. Watanabe, H. Nishino H, M Mizunami, Context-dependent olfactory learning monitored by activities of salivary neurons in cockroaches, *Neurobiology of Learning and Memory* 97 (2012) 30-36.
- [11] Y. Matsumoto, S. Noji, M. Mizunami, Time course of protein synthesis-dependent phase of olfactory memory in the cricket *Gryllus bimaculatus*, *Zoological Science* 20 (2003) 409-416.

- [12] Y. Matsumoto, S. Unoki, H. Aonuma, M. Mizunami, Critical role of nitric oxide-cGMP cascade in the formation of cAMP-dependent long-term memory, *Learning & Memory* 13 (2006) 35-44.
- [13] Y. Matsumoto, A. Hatano, S. Unoki, M. Mizunami, Stimulation of the cAMP system by the nitric oxide-cGMP system underlying the formation of long-term memory in an insect, *Neuroscience Letters* 467 (2009) 81-85.
- [14] R. Menzel, Memory dynamics in the honeybee, *Journal of Comparative Physiology Part A* 185 (1999) 323-340.
- [15] R. Menzel R, The honeybee as a model for understanding the basis of cognition, *Nature Reviews Neuroscience* 13(2012) 758-768.
- [16] M. Mizunami, M. Iwasaki, R. Okada R, M Nishikawa, Topography of four classes of Kenyon cells in the mushroom bodies of the cockroach, *Journal of Comparative Neurology* 399 (1998) 162-175.
- [17] M. Mizunami, M. Iwasaki, R. Okada, M. Nishikawa, Topography of modular subunits in the mushroom bodies of the cockroach, *Journal of Comparative Neurology* 399 (1998) 153-161.
- [18] U. Müller, Inhibition of nitric oxide synthase impairs a distinct form of long-term memory in the honeybee, *Apis mellifera*, *Neuron* 16 (1996) 541-549.
- [19] U. Müller, The nitric oxide system in insects, *Progress in Neurobiology* 51 (1997) 363-381.
- [20] U. Müller, Prolonged activation of cAMP-dependent protein kinase during conditioning induces long-term memory in honeybees, *Neuron* 27 (2000) 159-168.
- [21] H. Nishino, S. Yamashita, Y. Yoshiyuki, M. Nishikawa, F. Yokohari, M. Mizunami, Projection neurons originating from thermo- and hygrosensory glomeruli in the antennal lobe of the cockroach, *Journal of Comparative Neurology* 455 (2003) 40-55.
- [22] S.R. Ott, M.R. Elphick, Nitric oxide synthase histochemistry in insect nervous systems: methanol/formal in fixation reveals the neuroarchitecture of formaldehyde-sensitive

- NADPH diaphorase in the cockroach *Periplaneta americana*, *Journal of Comparative Neurology* 448 (2002) 165-185.
- [23] S.R. Ott, A. Philippides, M.R. Elphick, M. O'Shea, Enhanced fidelity of diffusive nitric oxide signaling by the spatial segregation of source and target neurons in the memory centre of an insect brain, *European Journal of Neuroscience* 25 (2007) 181-190.
- [24] I.P. Pavlov, *Conditioned Reflexes: An Investigation of the Physiological Activity of the Cerebral Cortex*, In G. V. Anrep (Trans.). Oxford: Oxford Univ. Press (1927).
- [25] M. Ribeiro, V.A. Straub, M. Schofield, J. Picot, P.R. Benjamin, M. O'Shea, S.A. Korneev, Characterization of NO-sensitive guanylyl cyclase: expression in an identified interneuron involved in NO-cGMP-dependent memory formation, *European Journal of Neuroscience* 28 (2008) 1157–1165.
- [26] T. Takahashi, A. Hamada, K. Miyawaki, Y. Matsumoto, T. Mito, S. Noji, M. Mizunami, Systemic RNA interference for the study of learning and memory in an insect, *Journal of Neuroscience Methods* 179 (2009) 9-15.
- [27] H. Watanabe, M. Mizunami, Classical conditioning of activities of salivary neurones in the cockroach, *Journal of Experimental Biology* 209 (2006) 766-799.
- [28] H. Watanabe, M. Mizunami, Pavlov's cockroach: classical conditioning of salivation in an insect, *PLoS ONE* 6 (2007) e529.
- [29] H. Watanabe, C. Sato, T. Kuramochi, H. Nishino, M. Mizunami, Salivary conditioning with antennal gustatory unconditioned stimulus in an insect, *Neurobiology of Learning and Memory* 90 (2008) 245-254.
- [30] H. Watanabe, C.S. Matsumoto, H. Nishino, M. Mizunami M, Critical roles of mecamylamine-sensitive mushroom body neurons in insect olfactory learning, *Neurobiology of Learning and Memory* 95 (2011) 1-13.

Figure legends

Fig. 1. Experimental procedure. (A) The procedure for the study of the effect of drug injection on formation of one-day memory. Cockroaches were first immobilized on stages and then subjected to drug injection before receiving five sets of differential conditioning trials. One day after conditioning, they were dissected for recording of the activities of salivary neurons and then subjected to a retention test. (B) The procedure for the study of the effect of drug injection on formation of 30-min memory. Immobilized cockroaches were dissected for recording of the activities of salivary neurons. Then they received drug injection at 30 min prior to conditioning. A retention test was performed at 30 min after conditioning. For a set of differential conditioning trials, 4-sec presentation of one of a pair of odors (peppermint and vanilla odors) (CS+) was paired with 4-sec presentation of 500 mM sucrose solution (pairing trial) to the mouth (US; open squares), which was initiated at 2 sec after the onset of CS+ presentation, and the other odor (CS-) was presented alone (unpairing trial). The pairing trial and the unpairing trial were alternately presented with the interval of 2 min. In testing sessions, responses of salivary neurons to vanilla and peppermint odors, presented 3-5 times in a pseudorandom order, were measured. The durations of odor stimulations were 2 s and the intervals between stimulations were >25 s.

Fig. 2. Effects of cycloheximide on formation of 1-day memory after olfactory conditioning of salivation, monitored as activity changes of salivary neurons, in cockroaches. (A) Three groups of cockroaches were each subjected to injection of 3 μ l of saline or saline containing 2.5 mM or 25 mM cycloheximide at 30 min prior to conditioning. Responses of salivary neurons to conditioned odor (CS+) and control odor (CS-) were measured one day (~24 hours) after conditioning. (B) Two groups of cockroaches were each subjected to injection of saline or saline containing 25 mM cycloheximide at 30 min prior to conditioning. Responses of salivary neurons to odors were measured at 30 min after conditioning. The responses to CS+ and CS- are shown as box plots. The line in the box is the median and the box represents

the 25-75 percentiles. Whiskers extend to extreme values as long as they are within a range of $1.5 \times$ box length. The results of statistical comparisons are shown by asterisks (Wilcoxon's test, $**P < 0.01$; $*P < 0.05$; NS $P > 0.05$). The number (n) indicates the number of animals used for data analysis.

Fig. 3. Effects of L-NAME on formation of 1-day memory. (A) Four groups of cockroaches were each injected with 3 μ l of saline containing 5 mM D-NAME or 50 μ M, 500 μ M or 5 mM L-NAME at 30 min prior to conditioning. Responses of salivary neurons to conditioned odor (CS+) and control odor (CS-) were measured one day (~24 hours) after conditioning. (B) Two groups of cockroaches were each injected with saline containing 5 mM D-NAME or 5 mM L-NAME at 30 min prior to conditioning. Responses of salivary neurons to CS+ and CS- were measured at 30 min after conditioning. The responses are shown as box plots. The results of statistical comparisons are shown by asterisks (Wilcoxon's test, $**P < 0.01$; $*P < 0.05$; NS $P > 0.05$). The number (n) indicates the number of animals used for data analysis.

Fig.1

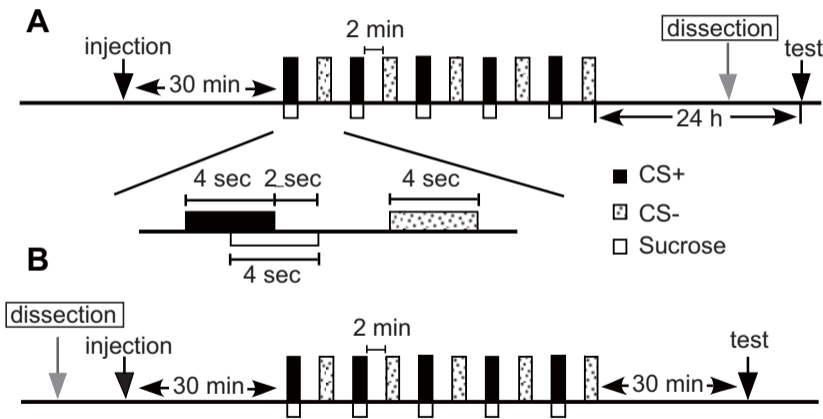


Fig.1

Fig. 2

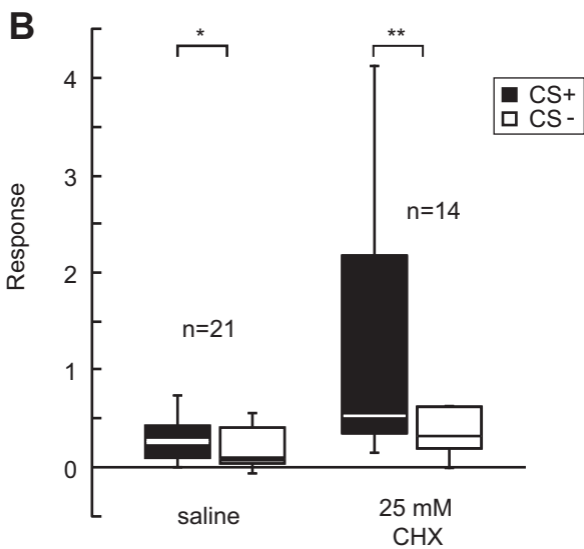
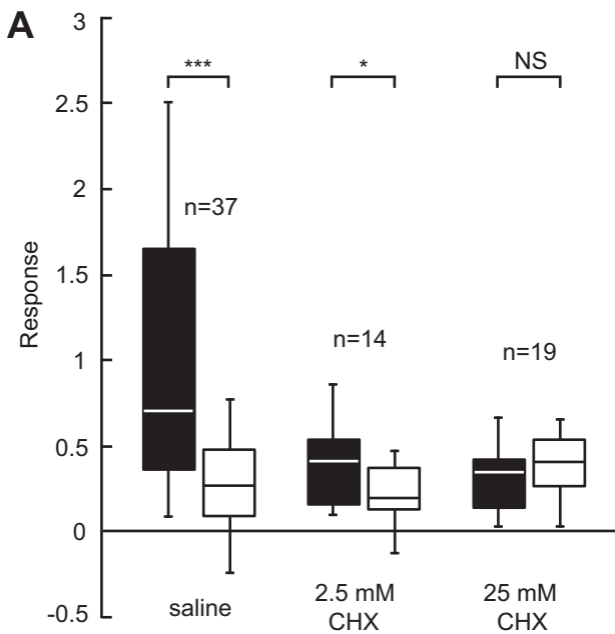


Fig. 3

