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Influence of mRNA and protein synthesis inhibitors on the long-term memory acquisition of classically conditioned earthworms

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Abstract

We investigated the process of memory consolidation following classical conditioning of earthworms. Earthworms were conditioned in paired trials by a weak vibration as a conditioned stimulus (CS), and by light as an unconditioned stimulus (US). The occurrence of a shrinking response upon exposure to the CS increased steadily with the number of paired training trials. When the training procedure was changed by increasing the intertrial interval (ITI), it was found that only those worms trained with a 68 s ITI exhibited long-term memory retention for at least 24 h. The influence of mRNA synthesis inhibition by actinomycin-D or of protein synthesis by anisomycin on memory consolidation was also examined. Induction of the long-term memory was blocked when either of these two compounds was injected into the body cavity of the worm within 25 min of conditioning with the 68 s ITI. These results demonstrate that the long-term memory is dependent upon protein synthesis in response to the upregulation of new transcription messengers.

Keywords: Memory consolidation; Shirinkage response; Associative learning; Anisomycin; Retention of memory; Actinomycin-D; Ventaral nerve cord

1. Introduction

In vertebrates and invertebrates, memory following learning is not a unitary process (Dudai, 1989), but involves at least two processes which we typically refer to as short-term memory and long-term memory acquisition. Short-term memory persists for only a few minutes while long-term memory persists for anything from hours to days, weeks or even years. It has been suggested that long-term memory is dependent upon mRNA and protein synthesis whereas short-term memory is not (DeZazzo & Tully, 1995). In studies on *Hermissenda* (Crow, Siddiqi, & Dash, 1997; Epstein, Child, Kuzirian, & Alkon, 2003) and *Lymnaea* (Sangha, Scheibenstock, McComb, & Lukowiak, 2003), it was shown that either a translation inhibitor anisomycin or a transcription inhibitor actinomycin-D could be used to block long-term memory. It is also indicated that protein synthesis after training induces morphological changes of synapses in *Aplysia* (Bailey, Montarolo, Chen, Kandel, & Schacher, 1992) and chicks (Rose, 2000). In mammalian brain, it was examined that certain forms of long-term memory is dependent on protein or RNA synthesis (Frey, Krug, Reymann, & Matthies, 1988; Nguyen, Abel, & Kandel, 1994).

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The simple nervous system of the earthworm shows both associative and non-associative learning patterns (Bullock, 1945; Roberts, 1966; Yerkes, 1912). In earlier work (Peeke, Herz, & Wyers, 1965; Ratner & Miller, 1959; Ray Jr., 1968), earthworms were classically conditioned with weak vibration as the conditioned stimulus (CS) and light as the unconditioned stimulus (US). Worms subjected to paired presentations of the CS and US showed a heightened shrinkage response to vibration than did worms receiving either random presentation of these stimuli or no stimuli at all. Both the CS and US are processed in the main nervous system of earthworms, the ventral nerve cord (VNC), which is composed of segmental ganglia containing sensory and motor neurons and interneurons, and three giant fibers connecting each segmental ganglion (Günther, 1973; Mill, 1982). In earthworms, the brain ganglion is not used for learning and memory because removal of the first five segments of worms does not affect T-maze task capabilities (Yerkes, 1912). Furthermore, each segmental ganglion which processes the stimuli has a simple structure (Mill, 1982). These results indicate that the function of memory formation is distributed over the segmental ganglia. We, therefore, can extract many specimens for electrophysiological or immunohistochemical experiments from one individual.

As will be shown, earthworms can be conditioned successfully in a high percentage of cases and various parameters, including interstimulus interval, intertrial interval, body length, temperature, and amount of training, that affect worm's acquisition degree of memory (Herz, Peeke, & Wyers, 1964; Peeke et al., 1965, Peeke, Herz, & Wyers, 1967; Ratner & Miller, 1959). However, these previous works have shown no physiological approach. From stated above, the earthworm ventral nervous system is a good model for the study of cellular and synaptic plasticity associated with memory acquisition of classical conditioning, and it is thought that earthworms may be the animal located phylogenetically at the lower limit which can induce associative learning. However, there is no research which investigate the existence of long-term memory and network based understandings of memory consolidation in earthworms.

In this study, we proposed to develop a reliable experimental protocol for induction of long-term memory in earthworms, and determine the effectiveness of mRNA and protein synthesis inhibitors at various times after the completion of training bouts.

2. Materials and methods

2.1. Materials

Earthworms, *Eisenia fetida*, were purchased from a local fishing trader, and kept in a box filled with moist soil prior to experiments. We used only adult earthworms whose clitellums were clearly visible and whose body weights were greater than 300 mg.

2.2. Training apparatus

Worms were placed individually in training chambers (Fig. 1A) which consisted of clear plastic tubes (100 mm inner diameter; 15 mm height) fixed on an acrylic stage supported with springs. An 8 s vibration was used as the conditioned stimulus (maximum acceleration: 5.7 m/s^2, frequency: 180 Hz) which was provided by three flat vibration motors (F203C, Shicoh Engineering, Japan) attached to the stage. A 3 s exposure to light was used as the unconditioned stimulus. The light source was a flood lamp (PRF500WD, Iwasaki Electric, Japan) installed 30 cm above training chambers. We confirmed that the intensity of the light stimulus was sufficient to elicit an avoidance reflex (shrinking response). The timings for applying these stimuli were controlled by an analog I/O board (NI-DAQ 6711, National Instruments, USA) and software (LabVIEW5.1, National Instruments). The apparatus was placed in a dark box, and training chambers were kept moist between behavioral experiments. Earthworm behavior was monitored with an IR-CCD camera (C2400-79, Hamamatsu Photonics, Japan) positioned over the experimental apparatus and recorded using an S-VHS video recorder (NV-SV1, Panasonic, Japan).

2.3. Training procedures

Three training procedures were used: paired conditioning, random conditioning, and naïve. After a 10 min acclimation period in the training chambers, all worms received three sessions of training. In the paired conditioning group, one session consisted of 10 CS-US pairings with a fixed intertrial interval (ITI, 28, 38, 68 or 98 s). The US duration overlapped the last 3 s of the CS period. In the random group, worms received the same number of CS and US presentations, but the ITI was varied randomly from 28 ± 10 s. In the naïve group, worms received only the conditioned stimulus during the test session, but no stimuli during the training session.

2.4. Behavioral tests

To evaluate the shrinking response induced by CS alone, 8 s vibrations (test stimulus, TS) were applied three times in each test session (Fig. 1B). Shrinkage responses were evaluated as the number of body shrinkages elicited in each test session. Because each response was assigned a score of "0" (no response) or "1" (shrinkage response) to each TS, each test session resulted in a score of 0, 1, 2, or 3. In behavioral tests, all behavioral observations were performed "blind." The experimenter did not know the training procedure which worms received.

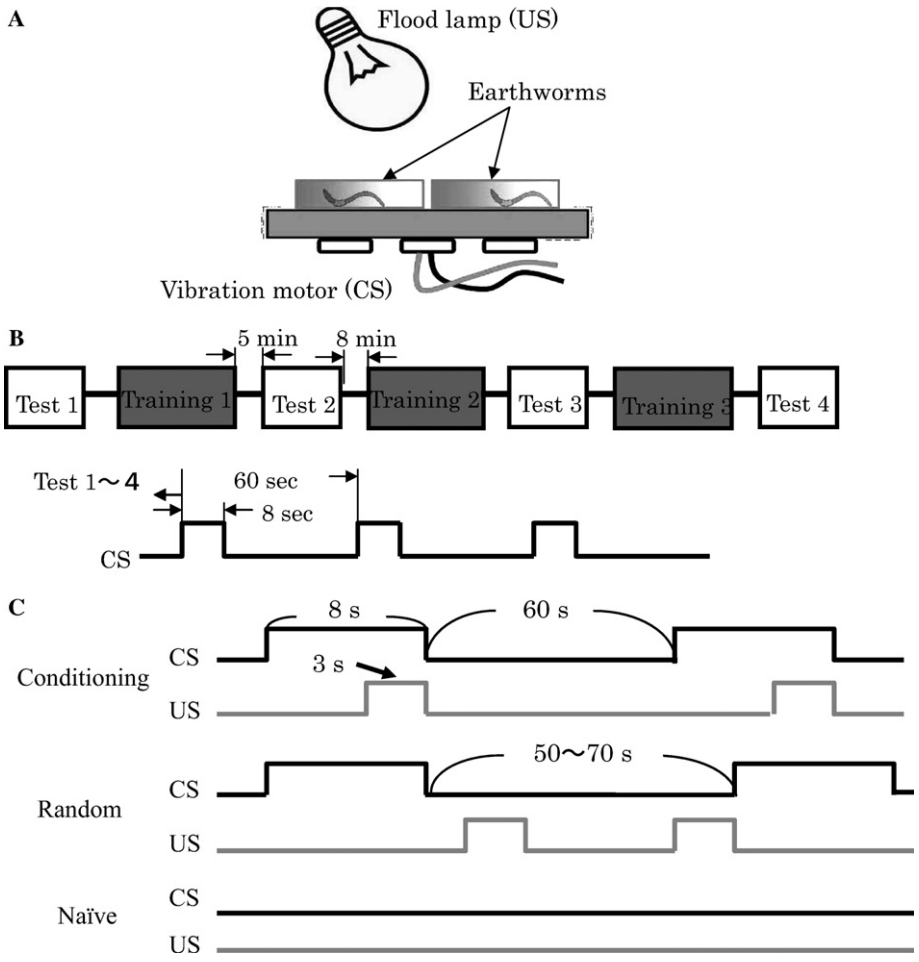

Fig. 1. Experimental set-up and protocol for the classical conditioning of earthworms. (A) The training apparatus was placed in a darkened room and four worms were trained simultaneously. The weak vibration (CS) was applied via three vibration motors, and light stimulation (US) was applied with a flood lamp. (B) Each training session consisted of 10 paired or unpaired stimuli with fixed ITI. A CS was applied three times at 60 s interval in each test session which was performed 1 min before and 5 min after the end of each training session. (C) The three procedures of training sessions.

2.5. Inhibition of mRNA and protein synthesis

After three sessions of training and tests, worms were anesthetized with 10% ethanol for 10 min and then 0.1 ml of actinomycin-D or anisomycin (Nacalai Tesque, Japan) was injected into the body cavity; 20 μg/ml of inhibitor was dissolved in normal earthworm saline (125.5 mM NaCl, 2.5 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10.0 mM $NaHCO_3$, and 10.0 mM Tris-buffer, pH 7.4). The volume and concentration of the inhibitor was based on previous studies on *Hermissenda* (Crow et al., 1997; Epstein et al., 2003) and *Lymnaea* (Sangha et al., 2003). As a control experiment, worms were injected with the same volume of saline (without drugs) 15 min after the third training session.

2.6. Statistical analysis

In Figs. 2–4, statistical analysis was performed using *t* statistics. Differences were considered to be significant if $p < .05$. In Figs. 5 and 6, we performed repeated measures one-way ANOVA. If the ANOVA was significant ($p < .05$), Dunnett's comparison procedure was performed to show which groups were significantly different from the control group. Differences were considered to be significant if $p < .05$.

3. Results

3.1. Classical conditioning of earthworms

Shrinkage responses and scores induced by the CS in each group are shown in Figs. 2A and B. Prior to the first training session, worms did not respond to CS alone. Results shown for the conditioning group are those for the 28 s ITI, where the score increased during the three training sessions, particularly with respect to tests 3 and 4 ($N = 25$, average and *SE*, $p < .001$). In contrast, worms in the random and naïve groups did not show such an

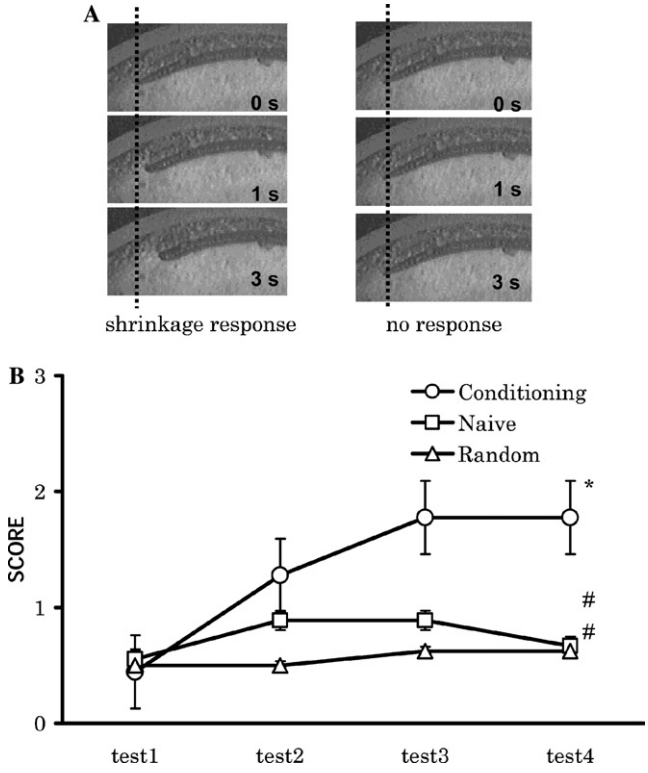

Fig. 2. Effects of classical conditioning on earthworm's behavior. (A) After the conditioning training using a 28 s ITI, worms showed a shrinkage response just after application of the CS. (B) Average score for each test for the three training groups.

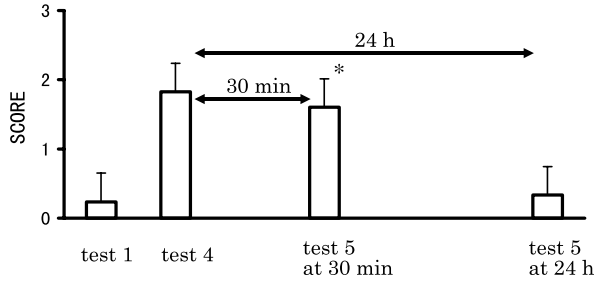

Fig. 3. Memory retention time in the conditioning group using a 28 s ITI protocol. Worms received an additional test stimuli (test 5) 3 min or 24 h after test 4.

increase in score during three sessions ($N = 26$, 12, respectively). These results show that earthworms could be classically conditioned with paired training with the 28 s ITI.

3.2. Retention of memory

To examine the retention time of this memory response, an additional test session (test 5) was applied 30 min after the end of test 4. Here, test 5 scores were found to be significantly greater than test 1 scores for the 28 s ITI (Fig. 3 $*p < .005$ compared with test 1 of conditioning group). When the interval between the training periods (test 4) and test 5 was extended to

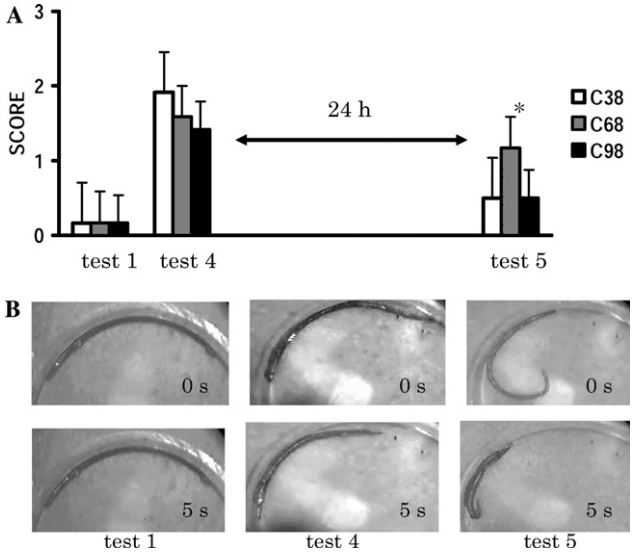

Fig. 4. (A) Effects of ITI on memory retention. Scores for worms trained with 38, 68 or 98 s ITI (C38, C68, and C98, respectively). (B) Worm's shrinkage response to TS. In test 1, worms of C68 group showed no response for CS. But, in tests 4 and 5, they show the shrinkage response at a higher percentage of case.

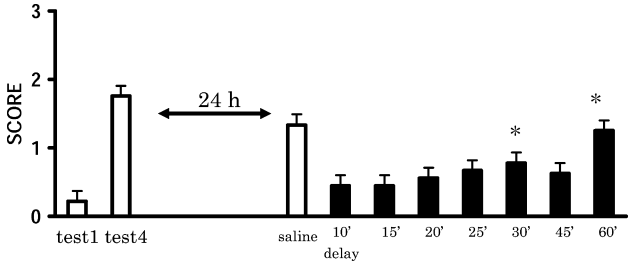

Fig. 5. Influence of actinomycin-D on long-term memory. Test 4 scores were significantly higher than test 1 scores ($p < .05$). $*p < .05$: only the saline group and the 30, 60 min delay group showed evidence of long-term memory retention, with scores at test 5 being significantly greater than those of test 1. All worms were tested again at 24 h later. Scores of the saline group were significantly greater than those of the 10, 15, 20, 25, and 45 min delay groups ($p < .05$), but not significantly different from the 30 and 60 min delay group.

24 h, there were no differences between the averages of test 1 and test 5 scores ($*p < .005$ compared with test 1 of conditioning group). These results indicate that conditioning training with the 28 s ITI (C28) resulted in short-term memory lasting for at least 30 min, but failed to induce long-term memory lasting 24 h or more.

In many other species, it is known that paired training with longer ITIs results in the formation of longer term memory (Rescorla, 1988), On this basis we increased the ITI for the conditioning group from 28 to 38, 68, and 98 s (C38, C68, and C98, respectively) in an attempt to induce long-term memory.

Worms were trained in the same manner except for the ITI employed, and received an additional test at 24 h

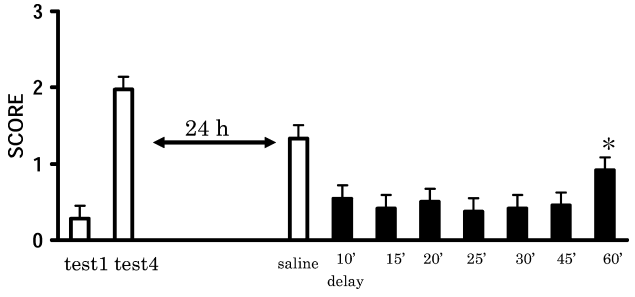

Fig. 6. Influence of anisomycin on long-term memory retention. Scores for the saline group are the same as that shown in Fig. 5. *$p < .05$: all worms were tested again 24 h after the first training session, with scores for the saline group being significantly greater than those for all groups injected with inhibitor ($p < .05$). Only the saline group and the 60 min delay groups showed evidence of long-term memory retention, with test 5 scores being significantly greater than test 1 scores ($p < .05$). In all groups, test 4 scores were significantly greater than those of test 1 ($p < .05$).

after the end of first training period. Scores at test 1, test 4, and test 5 are shown in Fig. 4A. When the ITI was 68 s, test 5 scores were significantly greater than test 1 scores ($N = 12$, $p < .005$). However, there were no significant differences between the scores of test 1 and test 5 in the C38 and C98 groups ($N = 12$, $p > .05$). The worms of C38 and C98 groups showed 30 min memory retention (data not shown) but did not show long-term memory lasting for 24 h (Fig. 4A).

These experiments, therefore, demonstrated that the C68 training procedure was moderate and effective for consolidation of the memory for the classical conditioning in earthworms.

3.3. Influence of an mRNA synthesis inhibitor on memory retention

To determine the time window for the effect of an mRNA inhibitor on memory retention, actinomycin-D was injected into the body cavity at 10, 15, 20, 25, 30, 45, or 60 min after test 4 in C68 conditioned animals and memory retention was then tested 24 h later. Only those worms injected with inhibitor at 30 and 60 min after test 4 showed increased shrinking response scores at test 5 compared to test 1 (Fig. 5). Given that control worms injected with normal saline showed an increased response at test 5, these results reveal that early injection of actinomycin-D blocks the induction of long-term memory.

Furthermore, between-groups comparisons at test 5 showed that actinomycin-D injected groups did not demonstrate long-term memory ($F_{(116,7)} = 6.18669$; $p < .001$). The score of the normal saline group was significantly greater than that of 10, 15, 20, 25, or 45 min delay group ($p < .05$), and was not significantly different from the score at 30, or 60 min delay group ($p > .05$).

3.4. Influence of protein synthesis inhibitor on the memory

In the same manner as for the actinomycin-D experiments, worms were injected anisomycin at 10, 15, 20, 25, 30, 45, or 60 min after test 4. Result of memory retention tests 24 h later are shown in Fig. 6, and appear similar to those obtained for actinomycin-D. Within-group comparisons revealed that long-term memory formation only occurred in the group given anisomycin 60 min post-conditioning ($F_{(160,7)} = 2.61574$; $p < .05$). However, in contrast to the results for actinomycin-D experiments, test 5 scores of the normal saline group were significantly greater than those for all the anisomycin-injected groups after the training ($p < .05$). This shows that the entire process needed for memory consolidation is not complete and the protein synthesis takes place within the first 60 min after conditioning.

4. Discussion

In the present study, we have succeeded in inducing long-term memory in earthworms by classical conditioning and also found that mRNA synthesis is essential for long-term memory consolidation. In previous studies (Peeke et al., 1967; Ratner & Miller, 1959; Ray Jr., 1968), it has been reported that earthworms can be classically conditioned with vibration and light stimulus pairings, however, no studies to date have investigated the time-dependence of this learning process. To induce long-term memory, we changed the training procedure employed by increasing the ITI from 28 to 38, 68, or 98 s. In several other species such as *Aplysia* (Botzer, Markovich, & Susswein, 1998), moth (Fan, Anderson, & Hansson, 1997), honeybee (Menzel, Manz, Menzel, & Greggers, 2001; Sandoz, Roger, & Pham-Delegue, 1995), and nematode (Beck & Rankin, 1997), it has been shown that long ITI or spaced training could produce longer memory than short ITI or massed training procedures. Interestingly, in our experiments, worms trained with a 68 s ITI showed evidence of long-term memory, whereas the C98 group did not in spite of being trained with the longest ITI. Because worms of the C98 group showed short-term memory lasting for 30 min (data not shown), it is considered that there is a mechanism facilitating short-term memory formation when the ITI is 98 s. But, it is unknown why they failed to consolidate the long-term memory.

The long-term memory could be blocked when either a transcription blocker (actinomycin-D) or a translation blocker (anisomycin) was injected into the body cavity of the worm within 25 min of completion of the conditioning training. In studies on *Lymnaea*, it was shown that the injection of anisomycin prevents the establishment of both intermediate- (lasting 3 h) and long-term memory (lasting over 6 h), whereas injection of

actinomycin-D prevents only the establishment of long-term memory (Sangha et al., 2003; Scheibenstock, Krygier, Haque, Syed, & Lukowiak, 2002). Moreover, in *Hermissenda*, both anisomycin and actinomycin-D blocked the formation of long-term memory (Crow et al., 1997; Epstein et al., 2003). These results therefore suggest that despite species differences, the induction of long-term memory results from a common mechanism that is dependent on mRNA and protein synthesis. In *Lymnaea*, the hypothesis that there is at least two stages in long-term memory formation is supported (Sangha et al., 2003). However, our result is not enough to support the hypothesis in earthworms. If the hypothesis can be applied also in earthworms, it is considered that the second stage of long-term memory formation may occur later than other species.

In several species, intermediate-term memory was also reported. It has been shown that intermediate-term memory requires new protein synthesis as well as long-term memory, but it does not require new mRNA synthesis (Sangha et al., 2003; Sutton, Masters, Bagnall, & Carew, 2001). In our results, earthworms require both protein and RNA synthesis within 60 min after training, so, we believe that it is "long-term memory" and not "intermediate-term memory." There is a possibility that earthworms also have a certain form of intermediate-term memory, but it is not supported in our results. To research and evaluate earthworm's intermediate memory further, it may be suitable to reduce the number of training sessions or parings of stimuli.

From our previous study, it was demonstrated that of the earthworm can be stained in an activity dependent manner with a fluorescent dye, FM1-43 (Shimizu et al., 1999). So, simultaneous observation of animal's behavior and fluorescent image enable us to identify the location of long-term memory formation by classical conditioning. Our previous work also reported that the earthworm *Eisenia fetida* produces nitric oxide (NO) as a neuromodulator, and basal NO production from the neuron is relatively high compared with other animal nervous systems (Kitamura, Naganoma, Horita, Ogawa, & Oka, 2001). And, it is reported that NO is necessary for long-term memory consolidation in several species (Katzoff, Ben-Gedalya, & Susswein, 2002; Kemenes, Kemenes, Andrew, Benjamin, & O'Shea, 2002; Muller, 1996). Therefore, earthworms may be an advantageous preparation for investigating the relation between NO and learning.

Acknowledgments

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