Neurobiology of Learning and Memory 141 (2017) 189–198

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Weak involvement of octopamine in aversive taste learning in a snail

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ARTICLE INFO

Article history:
Received 28 January 2017
Revised 20 April 2017
Accepted 23 April 2017
Available online 24 April 2017

Keywords:
Conditioned taste aversion
Food deprivation
Long-term memory
Lymnaea
Octopamine

ABSTRACT

The pond snail *Lymnaea stagnalis* is capable of learning taste aversion by pairing presentations of a sucrose solution and an electric shock and consolidating it into long-term memory (LTM), which is referred to as conditioned taste aversion (CTA). We asked here if the neurotransmitter octopamine is involved in CTA. We first determined the levels of octopamine and its catabolites in the central nervous system (CNS) of snails with varying degrees of food deprivation, because CTA grades are correlated with degrees of food deprivation. We next manipulated the octopamine signaling using both an agonist and an antagonist of octopamine receptors and correlated their respective effects with CTA grades. We found that snails with the least amount of food-deprivation obtained the best CTA grade and had low levels of octopamine; whereas the most severely food-deprived snails did not form CTA and had the highest CNS octopamine levels. In modestly food-deprived snails, octopamine application increased the basal level of feeding response to a sucrose solution, and it did not obstruct CTA formation. Application of phentolamine, an octopamine receptor antagonist, to the most severely food-deprived snails decreased the basal level of feeding elicited by sucrose, but it did not enhance CTA formation. We conclude that octopamine involvement in CTA formation in *Lymnaea* is at best weak, and that the changes in CNS octopamine content are an epiphenomenon.

1. Introduction

Octopamine, discovered by Vittorio Erspamer in octopus (Erspamer, 1948), acts as a neurotransmitter in invertebrates. It plays a role in mediating various behaviors including flying, egg-laying, jumping, aggression as well as sleep in insects, spiders and crustaceans (David & Coulon, 1985; Kerkut, 1973; Roeder, 1999, 2005; Sakura & Aonuma, 2013). In insects, octopamine plays a major role in learning and memory (Farooqui, 2007; Giurfa, 2006; Kim, Lee, Lim, & Han, 2013). For example, octopamine can substitute for the appetitive (reward) reinforcement used in olfactory conditioning in insects (Mizunami & Matsumoto, 2010). Blocking octopaminergic signaling abolishes appetitive learning, and optogenetic activation of octopaminergic neurons triggers appetitive learning in *Drosophila* larvae (Schroll et al., 2006).

In molluscs, the studies of octopamine have been pursued using the pond snail *Lymnaea stagnalis* by Vehovszky and her colleagues (Elliott & Vehovszky, 2000; Pitt, Vehovszky, Szabó, & Elliott, 2004; Vehovszky & Elliott, 2000, 2001, 2002; Vehovszky, Hiripi, & Elliott, 2000; Vehovszky, Szabó, & Elliott, 2005; Vehovszky, Szucs, Szabó, Pitt, Vehovszky, Szabó, & Elliott, 2004). These data show that octopamine plays a role in mediating feeding behavior. Kemenes et al. extended those findings showing that octopamine may play a role in the formation of long-term memory (LTM) in a form of aversive food conditioning in *Lymnaea* (Kemenes, O'Shea, & Benjamin, 2011).

Here we examined the involvement of octopamine in conditioned taste aversion (CTA) in *Lymnaea* (Ito, Kobayashi, Kojima, Sadamoto, & Hatakeyama, 1999; Ito, Kojima, Lukowiak, & Sakakibara, 2013; Yamanaka et al., 1999). In our CTA training, snails receive a sucrose solution as the conditioned stimulus (CS)

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http://dx.doi.org/10.1016/j.nlm.2017.04.010
1074-7427/

and an electric shock as the unconditioned stimulus (US) (Ito, Yamagishi, Takigami, et al., 2015; Ito, Yamagishi, Hatakeyama, et al., 2015; Takigami, Sunada, Lukowiak, & Sakakibara, 2013). Recently we examined the role played by dopamine in CTA (Aonuma et al., 2016). In those studies, food-deprived animals of different durations were used (1–5 days) and it was found that the best CTA occurred with snails experiencing a single day of food deprivation. In those snails, the grades assigned to the snails based on their response to the CS were the best whilst the levels of dopamine in the central nervous system (CNS) were the lowest. The opposite was found in snails receiving the longest period of food deprivation, the grades were the poorest and the dopamine levels were the highest. Thus, there was a negative correlation between grades and CNS dopamine levels. In the Kemenes et al. study referred to above, they found that dopamine played a key role in appetitive food conditioning and thought it acted as a reward transmitter (Kemenes, O'Shea, & Benjamin, 2011). As we showed previously (Aonuma et al., 2016), high dopamine levels were correlated with poorer CTA. Octopamine is also thought to be a reward transmitter (Mizunami, Hamanaka, & Nishino, 2015), and thus we hypothesized that CNS levels of octopamine should change in a similar manner as did CNS dopamine.

2. Materials and methods

2.1. CTA training procedure

Specimens of *Lymnaea stagnalis* (Linnaeus, 1758) with an 18–23 mm shell length obtained from our snail-rearing facility (original stocks from Vrije Universiteit Amsterdam) were used in the present study. The method of rearing snails and the procedure of CTA training were as described previously (Azami et al., 2006; Hatakeyama et al., 2006; Sugai et al., 2006, 2007). Briefly, all snails were first given a pretest. We counted by visual inspection the number of feeding responses (i.e., bites) elicited by the CS (10 mM sucrose solution, 15 s) in the 1-min period after presentation of the CS. Following to the pretest, the snails were subjected to the conditioning and control procedures. In the CTA training procedure, we paired the CS with the US (high-voltage electric shock, 3 s). The CS was rinsed out by distilled water and then followed by the US. The US period was set as 15 s, because following the 3-s electric shock a 12-s recovery time was needed for the body to re-emerge from the shells. The inter-trial interval was 10 min. Snails received 20 paired presentations of the CS-US. Controls included a backward-conditioning (US-CS) group and a naive group to validate associative learning. For the naive control group, only distilled water (DW) was applied to the lips instead of the CS and US. The number of snails used was 20 for each group. In the post-test sessions, snails were again challenged with the CS, and the number of bites was recorded in the 1-min interval in distilled water after a 15-s application of the CS. The post-tests were performed 10 min, 1 h, 1 day and 1 week after training. In all experiments, after the 10-min post-test, snails were allowed *ad libitum* access to food. All tests were performed blindly. The behavioral experiments were performed in the morning, because it has been shown that the learning grades are better in the morning than at other times (Wagatsuma et al., 2004).

2.2. Effects of the length of food deprivation and application of octopamine agonist and antagonist on CTA

We used 4 cohorts of snails for the CTA training: (A) Snails that were in the act of eating just before the commencement of the CTA training procedure (i.e., Eating snails); (B) Snails that had been given *ad libitum* access to food but were not eating just prior to the CTA training procedure (called Day -1 snails); (C) Snails that had been food-deprived for 1 day (i.e., modestly hungry snails, called Day 1 snails). That is, Day -1 snails could eat freely before the start of CTA training, whereas Day 1 snails experienced food deprivation for 1 day before the training; and (D) Snails that had been food-deprived for 5 days (i.e., severely hungry snails, called Day 5 snails) (Mita, Yamagishi, Fujito, Lukowiak, & Ito, 2014; Mita, Okuta, et al., 2014). An octopamine receptor agonist, octopamine hydrochloride, was purchased from Sigma-Aldrich (St. Louis, MO, USA), and an antagonist, phentolamine hydrochloride, was from LKT Laboratories (St. Paul, MN, USA). Phentolamine has been widely used as an antagonist of octopamine receptors (Miyamae et al., 2010; Roeder, 1990). We applied these chemicals by transdermal application, that is, we dissolved them in DW and immersed the snails in these solutions for 24 h before training (Miyamae et al., 2010). The washout time for these chemicals was 1 h. The suppression ratio of feeding response after CTA training was calculated as follows: the mean value of number of bites elicited by the CS in the 10-min post-test was divided by that in the pretest, and then we expressed these values as a percentage (Aonuma et al., 2016).

2.3. Measurement of octopamine content in the CNS

The measurement method was as described previously (Aonuma & Watanabe, 2012; Aonuma et al., 2016). Briefly, each CNS dissected out from snails was homogenized in 50 μl of ice-cold 0.1 M perchloric acid containing 5 ng of *N*-ω-methyl-5-hydroxytryptamine oxalate (NMET; Sigma-Aldrich) as an internal standard. After centrifugation of the homogenate [0 °C, 21,500*g*, (15,000 rpm), 30 min], 40 μl of supernatant was collected. Octopamine, tyramine and *N*-acetyloctopamine (NacOA) in the single CNS were measured using high-performance liquid chromatography with electrochemical detection (HPLC-ECD; EICOM, Kyoto, Japan). Tyramine is the precursor of octopamine, and NacOA is the catabolite of octopamine (Aonuma & Watanabe, 2012). The number of single CNSs used was 10 for each group. All the CNS ganglia were used. The mobile phase containing 0.18 M chloroacetic acid and 16 μM disodium EDTA was adjusted to pH 3.6 with NaOH. Sodium-1-octanesulfonate at 1.85 mM as an ion-pair reagent and CH_3CN at 8.40% (v/v) as an organic modifier were added to the mobile phase solution. The chromatographs were acquired using the computer program PowerChrom (eDAQ Pty, Denistone East, NSW, Australia). The supernatants of samples were injected directly onto the HPLC column. The snails used for the measurement of octopamine were independent from those used for behavioral experiments to avoid the effects of sucrose application on the octopamine contents.

2.4. Data analysis

Data are expressed as the mean ± s.e.m. Differences in the behavioral changes were tested using one-way ANOVA and the appropriate post hoc Tukey test, or Student's *t*-test or Welch's *t*-test. Significance was $P < 0.05$. Differences in the octopamine contents were tested using one-way ANOVA and post hoc Scheffé test ($P < 0.05$) because the number of samples in each group was different.

3. Results

3.1. CTA grades

As described in Methods, we used 4 separate cohorts of snails for CTA training that were termed: (A) Eating snails, (B) Day -1