Increase in cyclic AMP concentration in a cerebral giant interneuron mimics part of a memory trace for conditioned taste aversion of the pond snail

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Conditioned taste aversion (CTA) can be classically conditioned in the pond snail *Lymnaea stagnalis* and subsequently be consolidated into long-term memory (LTM). The neural trace that subserves CTA-LTM can be summarized as follows: A polysynaptic inhibitory postsynaptic potential recorded in the neuron 1 medial (N1M) cell in the conditioned snails as a result of activation of the cerebral giant cell (CGC) is larger and lasts longer than that in control snails. The N1M cell is ultimately activated by the CGC via the neuron 3 tonic (N3t) cell. That is, the inhibitory monosynaptic inputs from the N3t cell to the N1M cell are facilitated. The N1M and N3t cells are the members of feeding central pattern generator, whereas the CGC is a multimodal interneuron thought to play a key role in feeding behavior. Here we examined the involvement of a second messenger, cAMP, in the establishment of the memory trace. We injected cAMP into the CGC and monitored the potentials of the B3 motor neuron activated by the CGC. B3 activity is used as an index for the synaptic inputs from the N3t cell to the N1M cell. We found that the B3 potentials were transiently enlarged. Thus, when the cAMP concentration is increased in the CGC by taste aversion training, cAMP-induced changes may play a key role in the establishment of a memory trace in the N3t cell.

The pond snail *Lymnaea stagnalis* has the ability to learn and remember to avoid specific tastes. This phenomenon is known as conditioned taste aversion (CTA)[1–3]. To produce CTA, an appetitive stimulus (*e.g.*, sucrose) is used as the conditioned stimulus (CS). Application of the CS to the lips increases the feeding response (*i.e.*, the number of bites) in snails. An aversive stimulus (*e.g.*, KCl) is used as the unconditioned stimulus (US). Application of the US to the snails induces a withdrawal response into the shell, resulting in inhibiting feeding behavior. In the taste aversion-training procedure, the CS is paired with the US. After repeated temporal contingent presentations of the CS and US, the CS no longer elicits a feeding response, and this taste aversion persists for more than a month[1].

The neural mechanisms underlying CTA in *Lymnaea* have been examined[4–7]. The cerebral giant cells (CGCs) act as a pair of multimodal interneurons that play key roles in the mediation of learning and memory of feeding behaviors[8–12]. We showed that both the CS and US used in taste aversion-training alter the activity of the CGCs[13–15]. Based on the results from Paul Benjamin's laboratory[16], we focused our experiments on a polysynaptic inhibitory postsynaptic potential (IPSP) recorded in the neuron 1 medial (N1M) cell by activation of the CGC via the neuron 3 tonic (N3t) cell. This IPSP was larger and lasted longer in the taste aversion-trained snails than that in the control snails[13]. These data are

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consistent with the hypothesis that an enhanced IPSP to the N1M cell underlies the suppression of feeding response in CTA of *Lymnaea*[17].

To determine whether the enhancement of this IPSP to the N1M cell in the taste-aversion trained snails is the result of a change in properties of the CGC, the electrical properties of the CGC were compared between the taste aversion-trained snails and the control snails[13]. No significant differences were found in the resting membrane potential, the input resistance, the half width of spontaneous action potential, the half width of after-hyperpolarization of spontaneous action potential, and the threshold for an action potential. We therefore examined the changes in the postsynaptic N1M cell. No significant differences were either found in the resting potential between the taste aversion-trained snails and the control snails. These results suggested to us that CTA of *Lymnaea* is the result of a memory trace in the N3t cell. This neuron receives an excitatory monosynaptic input (*i.e.*, EPSP) from the CGC and connects to the N1M cell by means of a monosynaptic IPSP.

We have monitored N3t activity following the establishment of CTA[18]. However, the N3t cell is too small to be able to consistently record its activity via standard sharp electrode recording techniques. Thus, the synaptic inputs from the CGC to the N3t cell and those from the N3t cell to the N1M cell are inferred by monitoring the EPSPs recorded in the large B1 and B3 motor neurons, respectively (Fig. 1)[18,19]. The spontaneous EPSPs recorded in the B3 motor neuron were significantly enlarged in taste-aversion trained snails compared to control snails[18]. These data suggested that, after

taste aversion training, the monosynaptic inputs from the N3t cell to its follower neurons, including the N1M cell, are facilitated. That is, a neural correlate of CTA in *Lymnaea* is an increase in neurotransmitter release from the N3t cell. Thus, in taste aversion-trained snails the CS causes the N3t cell to suppresses activity in the N1M cell and suppresses the feeding central pattern generator (CPG).

In general, the molecular mechanisms underlying long-term memory (LTM) consolidation are thought to be mediated by cAMP signaling cascades across phyla in the animal kingdom[20,21]. It is thought that cAMP activation mediates LTM mainly by activating the cAMP-sensitive protein kinase A (PKA) that can then phosphorylate various downstream kinases and transcription factors required for LTM. Although we have no direct data showing that the cAMP concentration is increased in the CGC by the taste-aversion training procedure, our pervious results have led us to hypothesize that if cAMP is increased in the CGC[22,23], such cAMP cascades might result in a change in activity that resembles the changes seen following CTA training. That is, cAMP cascades may result in predictable changes not only in the CGC-N3t pathway (*i.e.*, the B1 activity) but also in the N3t-N1M pathway (*i.e.*, the B3 activity). In the present study, we show that causing an increase in cAMP concentration in the CGC activates the N3t-N1M pathway as evidenced by alterations in the synaptic input to the B3 motor neuron.

Materials and Methods

CNS preparations

The pond snails *Lymnaea stagnalis* with a 15–25 mm shell (young adults)[24], originally supplied from Vrije Universiteit Amsterdam, were maintained in dechlorinated tapwater (*i.e.*, pond water) under a 12 : 12 light-dark cycle at 20°C and fed *ad libitum* on a kind of turnip leaf *Brassica rapa var. peruviridis* (*Komatsuna* [in Japanese]) and a spiral shell food (Nisso, Saitama, Japan) every other day. Snails were anesthetized with 25% Listerine® before dissection[13]. The isolated central nervous system (CNS) was immersed in *Lymnaea* saline and pinned in a Sylgard®-lined dish for electrophysical recording. *Lymnaea* saline contained: 50 mM NaCl, 1.6 mM KCl, 2.0 mM $MgCl_2$, 3.5 mM $CaCl_2$, 10 mM HEPES (pH 7.9)[25].

Intracellular recording

The CGC, the B1 motor neuron and the B3 motor neuron were impaled with glass microelectrodes filled with 2 M potassium acetate giving tip resistances of 20–50 MΩ. A train of *ca.* 10 spikes in the CGC, which was produced by a current injection (1.1–1.5 nA) for 1 s, evoked a single large compound EPSP both in the B1 motor neuron and the B3 motor neuron (electric stimulator: SEN-7203, Nihon Kohden, Tokyo, Japan; intracellular recording amplifier: MEZ-8300, Nihon Kohden; AD converter: DIGIDATA 1322A, Axon Instruments, Foster City, CA, USA). We used the neurons
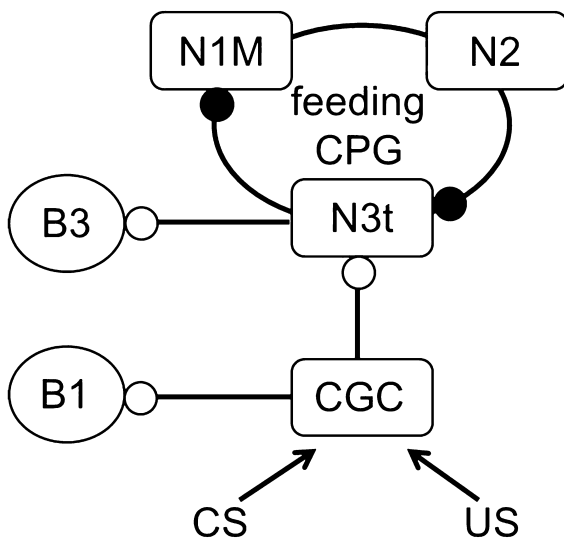

Figure 1 Schematic presentation of the neural circuitry underlying taste aversion training. Presentation of the CS and the US both independently elicit activity in the CGCs. Rectangles and circles indicate interneurons and motor neurons, respectively. At synapses, open circles and closed circles indicate excitatory monosynaptic inputs and inhibitory monosynaptic inputs, respectively. The N1M, N2 and N3t cells form part of the feeding CPG.

located in the ipsilateral side. Cyclic AMP (Sigma-Aldrich, St. Louis, MO, USA), which was filled in a glass micro-electrode (80–120 MΩ) as a 200 mM solution dissolved in 20 mM Tris buffer (pH 7.5), was injected into the CGC by passing hyperpolarizing current pulses (50 ms on, 50 ms off) of 4 nA for 20 min. The injection period was decided by conferring with the work of *Aplysia*[26]. Before and after the injection of hyperpolarizing current into the CGC, the EPSP recorded in the B1 and B3 motor neurons is not changed by activation of the CGC[22,23]. The data were recorded in the same preparation at the following 4 time points after cAMP injection: 1, 3 and 6 h. The data at 0 h were recorded before cAMP injection. For estimation of EPSP changes recorded in the B1 and B3 motor neurons, the area of EPSP was cal-culated. That is, the unit for Figures 2B and 3B is arbitrary.

Statistics

The data are expressed as the mean±SEM. Significant differences at *P*<0.05 between 2 groups were examined by Student paired *t*-test. Significant differences at *P*<0.05 among 3 groups were examined by two-way repeated mea-sure ANOVA and post hoc Scheffé test.

Results and Discussion

Recent studies on appetitive conditioning of feeding behavior in *Lymnaea* have elucidated three points[19]. (A) Tonic inhibition in the feeding network is provided by the N3t cell. This interneuron makes a monosynaptic inhibitory connection (IPSP) to the N1M cell. (B) There is a reduction in N3t spiking after appetitive conditioning, and this reduc-tion in N3t firing inversely correlates with an increase in the conditioned fictive feeding response. (C) Computer simula-tion of N3t-N1M interactions suggests that changes in N3t firing are sufficient to explain the increase in the fictive feed-ing activity produced by appetitive conditioning. These data showed that appetitive conditioning of feeding behavior in *Lymnaea* occurs due to the combined effects of reduced tonic inhibition and enhanced excitatory synaptic connections between the CS pathway and feeding command neurons.

These afore mentioned findings led us to hypothesized that 'taste aversion learning' would occur via a mechanism that was the inverse of the mechanism proposed for 'appeti-tive conditioning'. That is, there would be an increase in N3t spiking after conditioning, and this increase in N3t firing would inversely correlate with a reduction in the condi-tioned fictive feeding response. However, as described in the first section, because the N3t cell is too small to consis-tently record from using standard sharp electrode recording techniques, we inferred the synaptic inputs from the CGC to the N3t cell and those from the N3t cell to the N1M cell by monitoring the EPSPs recorded in the large B1 and B3 motor neurons, respectively (Fig. 1)[18].

Both the CS and the US elicit activity in the CGC during taste aversion training[14,15]. However, we have no data show-
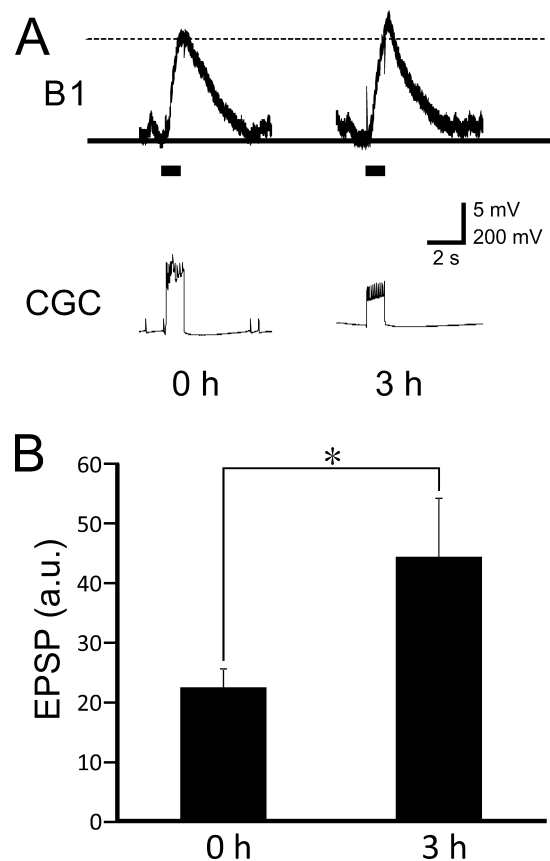

Figure 2 EPSP recorded in the B1 motor neurons by depolariza-tion of the CGC after injection of cAMP into the CGC. (A) The EPSP recorded in the B1 motor neurons can be used as a monitor for the changes in the CGC-N3t synaptic connection. The bars indicate the duration of depolarization of the CGC. The data were recorded at 0 h (before injection of cAMP into the CGC) and 3 h after injection of cAMP into the CGC and were identical to the previous ones[22]. (B) The enlargement of B1 EPSPs is shown as the summarized data (**P*<0.05, Student paired *t*-test). *y*-axis shows the size of the EPSP that was cal-culated from the area of the EPSP. The data are expressed as the mean±SEM. The number of data was 11 each for 0 h and 3 h that were obtained from 5 snails.

ing that the cAMP concentration is increased in the CGC as a result of taste aversion training, because no method is applicable to single-cell cAMP measurement. Previous stud-ies, however, showed that if the cAMP concentration was increased in the CGC by injection of cAMP, the EPSP recorded in the B1 motor neuron as a result of depolariza-tion of the CGC was enlarged[22,23]. Here, we replicated those previous findings (Fig. 2A). That is, the EPSP recorded in the B1 motor neuron was enlarged after injection of cAMP into the CGC (Fig. 2B). This enlargement of EPSP in the B1 motor neuron is associated with the increase in the amount of serotonin release from the CGC[22,27]. We consider that this enlargement of EPSP in the B1 motor neuron is equivalent to the facilitation of the synaptic inputs from the CGC to the N3t cell.

Our most important finding in the present study is that

when the cAMP concentration was increased in the CGC, the EPSP recorded in the B3 motor neuron after depolarization of the CGC was significantly larger (Fig. 3). This enlargement occurred transiently 3 h after injection of cAMP into the CGC, thus the synaptic inputs from the N3t cell to the N1M cell were facilitated transiently. To our knowledge, there is no monosynaptic connection between the CGC and the B3 motor neuron[28]. Therefore, the injection of cAMP into the CGC whereas directly enhancing synaptic connections made by the CGC indirectly alters connections made by neurons downstream of the CGC (*i.e.*, interneurons in the feeding CPG including the N3t cell and the synaptic connections between the N3t cell to the N1M cell).

Previous studies have shown that in the CNS isolated from the taste-aversion trained snails the EPSPs recorded in the B1 motor neuron as a result of activation of the CGC were identical to those in the control snails, whereas the spontaneous EPSPs recorded in the B3 motor neuron (*i.e.*, no stimulation for the CGC) were significantly enlarged[18]. Our present data showed that a cAMP injection into the CGC increased the B3 activity, *i.e.*, the N3t activity. We therefore conclude that an increase in cAMP concentration in the CGC mimics part of the memory trace in the feeding neural network for CTA in *Lymnaea*. We still have an issue to be addressed. After taste aversion training, only the B3 motor neuron was activated (*i.e.*, N3t cell was activated) but the B1 motor neuron was not[18]. This issue should be considered in the near future because the function of second messengers is not so simple.

A further issue to be addressed is how the transient enhancement of synaptic activity in the N3t-N1M pathway can be stabilized into a persistent LTM. That is, an increase in cAMP in the CGC solely is not sufficient to explain the long-lasting enhancement of synaptic activity of the N3t-N1M pathway following successful CTA-LTM in *Lymnaea*. A possible key molecule that is necessary for the establishment of the long-lasting neuronal memory trace is 'insulin'. Previous studies have shown that molluscan insulin-related peptides (MIPs) were up-regulated in snails exhibiting CTA[29]. Recently, when we applied MIPs to the isolated CNS, we observed a long-term change in synaptic enhancement of the synaptic connection between the CGC and the B1 motor neuron[30,31].

We further examined whether the observed changes in synaptic plasticity were the result of pre- and/or postsynaptic alterations using the paired pulse procedure. The paired pulse ratio was unaltered following insulin application, suggesting that insulin's effects on synaptic plasticity are mediated postsynaptically in the B1 motor neuron[32]. Thus, it was suggested that both the postsynaptic changes due to insulin's actions and the presynaptic plasticity by cAMP cascades are needed for the neural correlate for LTM[33].

The molecular cascades following to cAMP in the CGC are thought to include PKA, cAMP-responsive element binding protein (CREB) and CCAAT/enhancement binding pro-
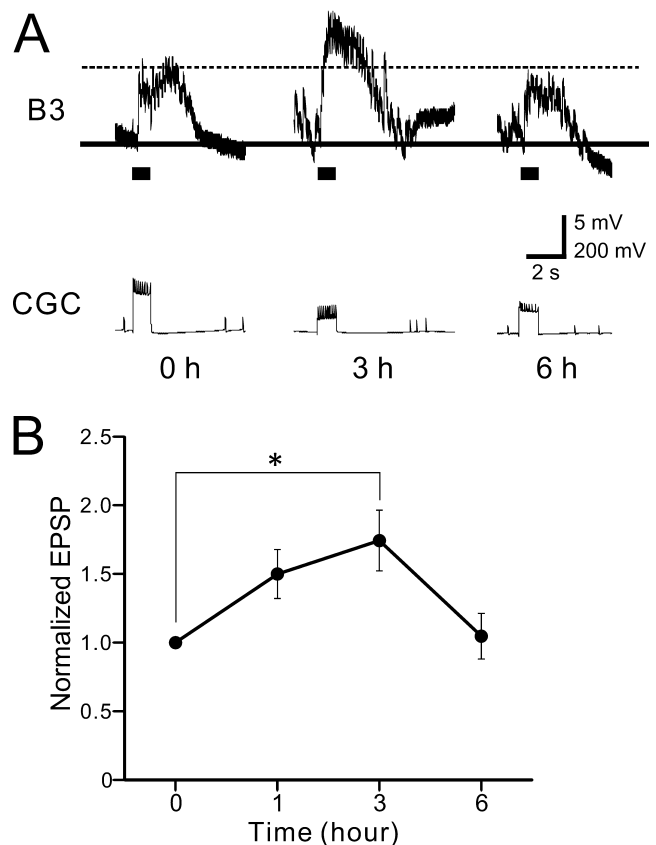

Figure 3   EPSP in the B3 motor neurons by depolarization of the CGC after injection of cAMP into the CGC. Changes in the EPSP in the B3 motor neurons can be used as a monitor for changes in the N3t-N1M synaptic connection. The data were recorded at 0 h (before injection of cAMP into the CGC) and 1, 3 and 6 h after injection of cAMP into the CGC. (A) The B3 EPSPs were transiently enlarged 3 h after cAMP injection, and returned to the basal level at 6 h. The bars indicate the duration of depolarization of the CGC. (B) The enlargement of B3 EPSPs is shown as the summarized data (*$P<0.05$, two-way repeated measure ANOVA and post hoc Scheffé test). *y*-axis shows the normalized EPSP that was calculated from the area of the EPSP. The data are expressed as the mean±SEM. The number of data was 21 each for 0 h, 1 h and 3 h that were obtained from 7 snails. The number of data was 12 from 4 snails for 6 h.

tein (C/EBP)[22,23,34–39]. In particular, we note that the mRNA copy number of CREB repressor (CREB2) is tens to hundreds in a single CGC, whereas that of CREB activator (CREB1) was below the detection limits of the assay. These results suggested that the CREB cascade is regulated by an excess amount of CREB2 in the CGC[38].

Conclusion

In the present study, we showed that when the cAMP concentration is increased in the CGC by taste aversion training for *Lymnaea*, cAMP-induced changes may play a key role in the establishment of a memory trace in the N3t cell. Although we focused our attention on only cAMP as a second messenger, we also have to keep in mind that alter-

ations in the level of calcium occur in the CGCs following taste aversion training. We have observed an increase in calcium in the CGC with pairing of the CS and US (Ito *et al.*, unpublished data). A rise in intracellular calcium concentration is most likely also important in learning and memory for feeding behaviors in *Lymnaea*[40].

Acknowledgments

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