Associative Learning in the Pond Snail, Lymnaea stagnalis

Authors: Ito, Etsuro, Kobayashi, Suguru, Kojima, Satoshi, Sadamoto, Hisayo, and Hatakeyama, Dai

Source: Zoological Science, 16(5) : 711-723

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.16.711

ZOOLOGICAL SCIENCE **16**: 711–723 (1999)

[REVIEW]

Associative Learning in the Pond Snail, *Lymnaea stagnalis*

Etsuro Ito*, Suguru Kobayashi, Satoshi Kojima, Hisayo Sadamoto and Dai Hatakeyama

Laboratory of Animal Behavior and Intelligence, Division of Biological Sciences, Graduate School of Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan

INTRODUCTION

Dudai (1990) described that an ideal subject for studying the cellular and molecular bases of learning should, probably, have ten large nerve cells, ten genes, a generation time of 1 week, and the ability to play a cello and recite Shakespeare (note that this sentence originated with W. G. Quinn). Nobody knows such organisms, but gastropod molluscs are such compromises. Gastropod molluscs are excellent experimental preparations that not only neurobiologists but also psychologists are using to help understand the basic mechanisms underlying learning and memory. They have relatively simple central nervous systems (CNSs) with large, identifiable neurons. The neurons are accessible for detailed electrophysiological, biophysical, biochemical, and molecular studies. Such a small brain in invertebrate was recently termed "microbrain" by Mizunami *et al*. (1999). To date, many scientists have analyzed various neural mechanisms on production of complex, long-lasting behavioral changes of gastropod molluscs including *Aplysia*, *Hermissenda*, *Limax*, and so on (Alkon *et al*., 1993; Bailey and Kandel, 1993; Frank and Greenberg, 1994; Gelperin *et al*., 1996; Abel and Kandel, 1998; Alkon *et al*., 1998; Matzel *et al*., 1998; Silva *et al*., 1998; Ito *et al*., 1999; Kimura *et al*., 1999). Associative learning in the pond snail, *Lymnaea stagnalis* (Fig. 1), can be also used as an important system for investigating the neurobiology of learning and memory. It includes classical and operant conditioning, for example, using feeding behavior (Alexander *et al*., 1982; Audesirk *et al*., 1982; Kemenes and Benjamin, 1989a, b, 1994; Whelan and McCrohan, 1996; Kemenes *et al*., 1997; Staras *et al*., 1998, 1999a, b), respiratory behavior (Lukowiak *et al*., 1996, 1998; Hermann and Bulloch, 1998; Spencer *et al*., 1999), withdrawal behavior (Sakakibara *et al*., 1998), and the isolated brain (Veprintsev and Rosanov, 1967). We here review our recent findings for associative learning, particularly conditioned taste aversion (CTA), in *L. stagnalis*.

Fig. 1. The pond snail, *Lymnaea stagnalis*.

* Corresponding author: Tel. +81-11-706-2615;
FAX. +81-11-706-4448.
E-mail. eito@sci.hokudai.ac.jp.

CONDITIONING BY CHEMOSENSORY STIMULI

First, we considered that it was necessary to determine whether, in *L. stagnalis* for the same behavior such as feeding, aversive and appetitive conditioning yield different strengths and periods of either acquisition or retention. To this end, we examined the effects of various chemosensory and physical stimuli on feeding and avoidance behavioral responses (Kojima *et al*., 1996). Then, using these findings, we constructed classical-conditioning paradigms with aversive and appetitive stimuli (Kojima *et al*., 1995, 1996). In the aversive conditioning paradigm, an appetitive stimulus (sucrose, conditioned stimulus: CS), which increased the feeding response, was paired with an aversive stimulus (KCl, unconditioned stimulus: UCS), which inhibited feeding behavior. Upon presentation of KCl, the aversive conditioning, which is generally called conditioned taste aversion (CTA), was acquired quickly and persisted for up to a month (Fig. 2A, B). On the other hand, the appetitive conditioning paradigm paired a neutral stimulus (vibration, CS) with an appetitive stimulus (sucrose, UCS). This conditioning took longer to acquire and persisted

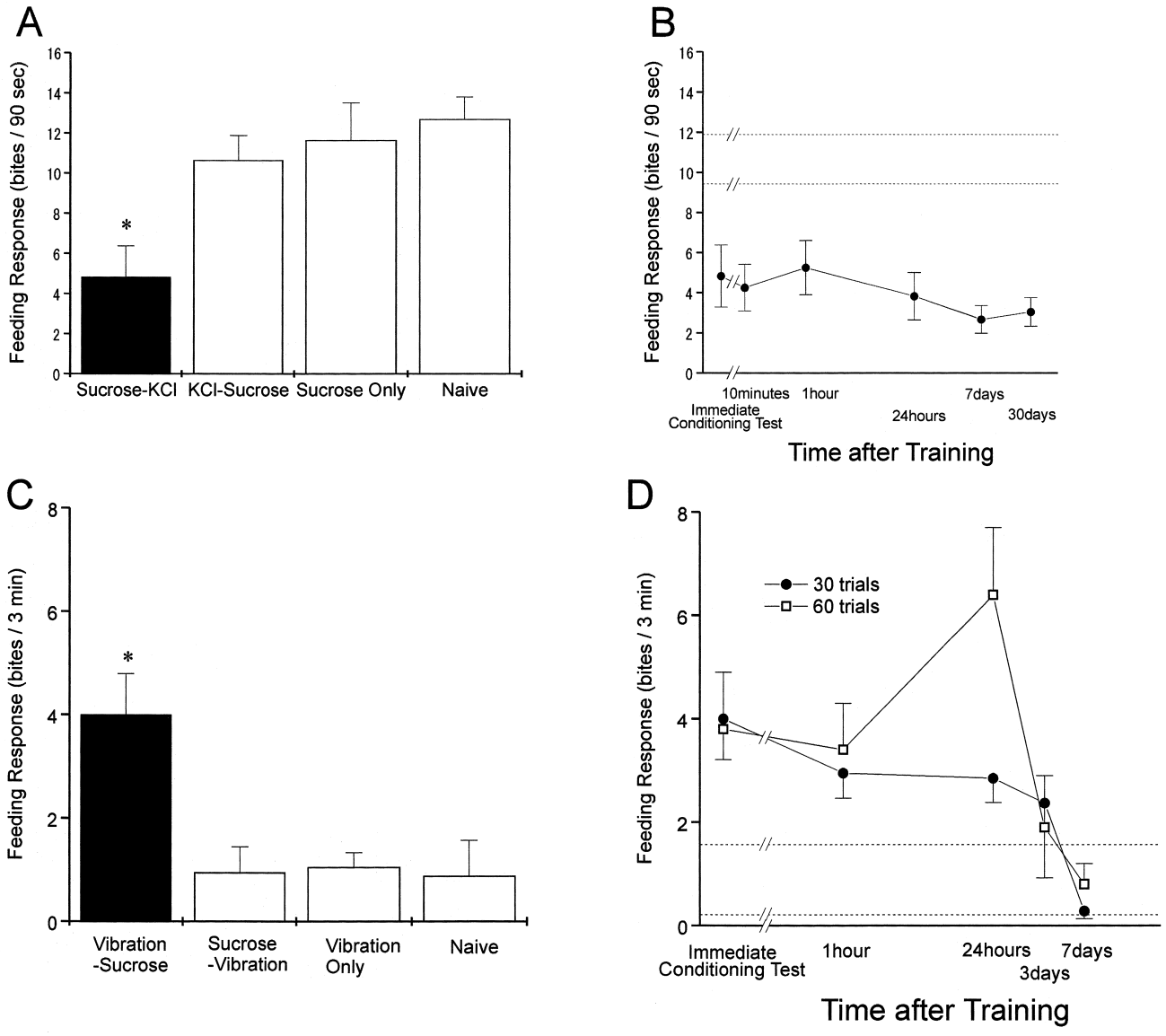

Fig. 2. Classical conditioning. The feeding responses evoked by the conditioned stimulus (sucrose or vibration) were counted. See Kojima *et al.* (1996) for the details. (A, B) Feeding response and time dependence of retention in the aversive (sucrose-KCl) conditioning, *i.e.* conditioned taste aversion. (C, D) Feeding response and time dependence of retention in the appetitive (vibration-sucrose) conditioning. The areas between the two dashed lines in (B and D) show the means±SEM of feeding responses in the control snails at the immediate conditioning tests. The time in the abscissas of (B and D) is expressed in a logarithmic scale. All data are means±SEM. * indicates $p<0.01$.

for a shorter period of time than the CTA (Fig. 2C, D).

Second, we demonstrated a sensory preconditioning in *L. stagnalis* (Kojima *et al.*, 1998). An appetitive sucrose solution (CS1) and weak vibration (CS2) were first associated, and then the CS2 and an aversive KCl solution (UCS) were done. To build the conditioning, two different training procedures, massed and spaced, were examined. It is well known in psychology that spaced training, interposing a rest interval between the multiple training sessions, produces stronger and longer-lasting memory than massed training, which has the same number of training sessions with no rest interval (Hintzman, 1974). After the both training, the sensory preconditioning was built: significantly fewer feeding response to the CS1 became elicited; slower latency to the first bite to the CS1 was induced (Fig. 3). However, no significant differences on the memory retention between these training procedures were found in the sensory preconditioning, possible because this conditioning included a neutral conditioning (CS1-CS2) (see Kojima *et al.*, 1996 for the details).

Third, an operant conditioning that *L. stagnalis* suppressed its naturally occurring behavior of escape from a water tank was examined by using a negative reinforcement (*i.e.* an aversive KCl stimulus) prepared outside the tank (Fig. 4A, Kobayashi *et al.*, 1998). During the training period, the number of escapes from a tank was strongly suppressed. One of behavioral factors for this suppression was confirmed as the elongation of latency to the first escape after training. The effects on the memory retention were examined in the massed

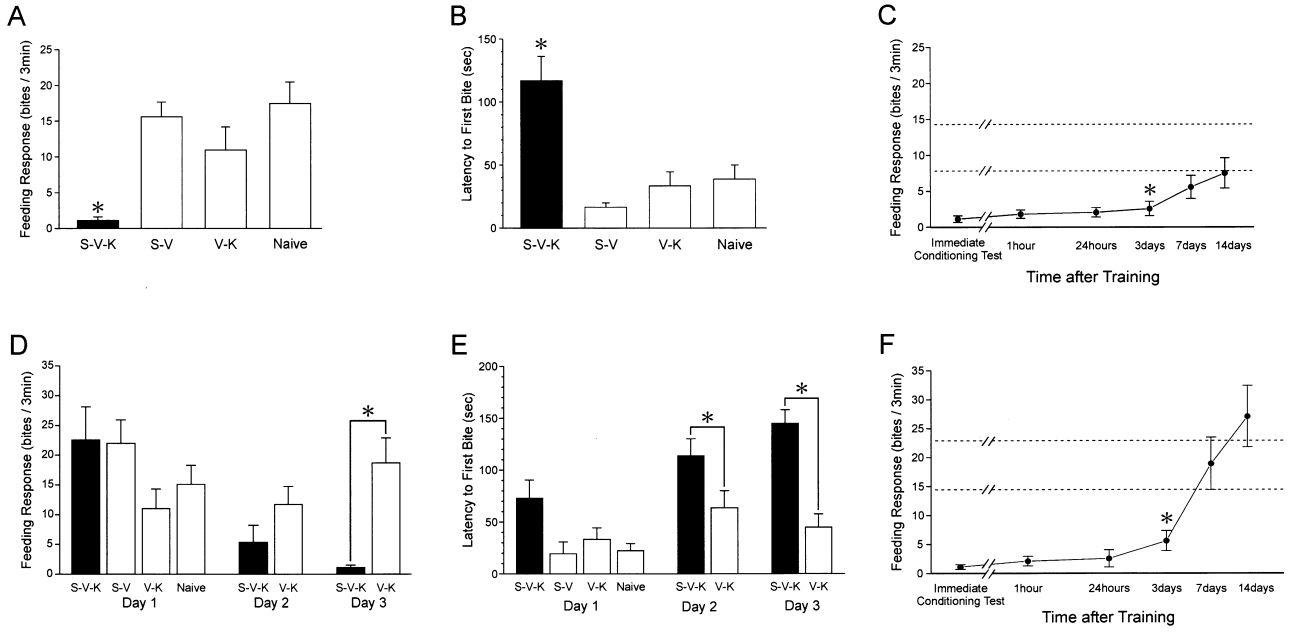

Fig. 3. Sensory preconditioning. The feeding responses and the latencies to the first bites evoked by the first conditioned stimulus (sucrose) were measured. See Kojima *et al.* (1998) for the details. (A–C) Massed training. (D–F) Spaced training. S, V and K are sucrose (CS1), vibration (CS2), and KCl (UCS) stimulus training, respectively. The areas between the two dashed lines in (C and F) show the means±SEM of feeding responses in the control snails at the immediate conditioning tests. The time in the abscissas of (C and F) is expressed in a logarithmic scale. All data are means±SEM. * indicates $p<0.05$.

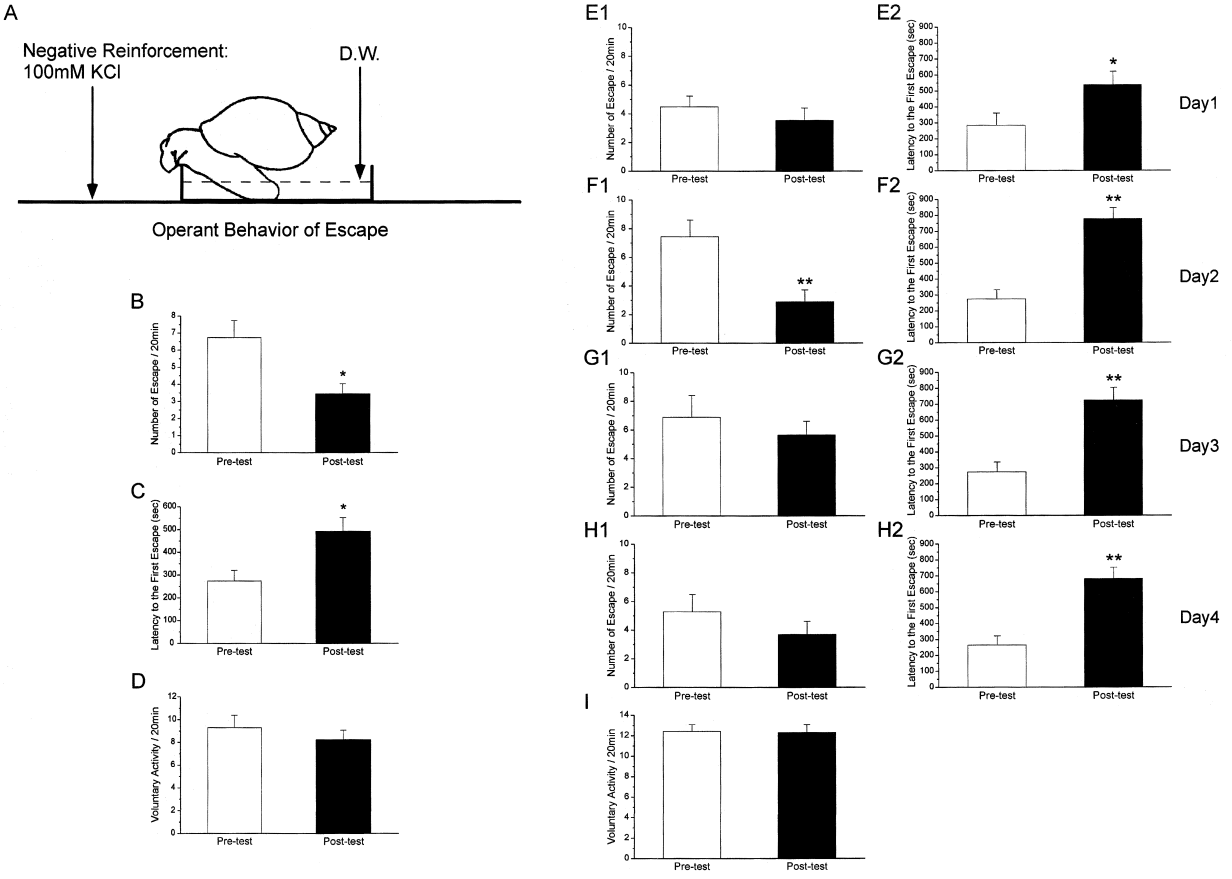

Fig. 4. Operant conditioning. See Kobayashi *et al.* (1998) for the details. (A) Schematic presentation of training apparatus for the operant conditioning. (B–D) Massed training. (E–I) Spaced training. The voluntary activity shown in (I) was measured from the conditioning snails in the pre-test at Day 1 and in the post-test at Day 4. All data are means±SEM. * and ** indicate $p<0.05$ and $p<0.005$, respectively.

and spaced training procedures. The memory retention by the massed training was observed within 20 min after training (Fig. 4B–D). By the spaced training, the learning acquisition was found to be stronger, which was observed as the slower latency to the first escape, than by the massed training, but the longer-lasting memory retention, which had been expected first, was not formed (Fig. 4E1–I). These results suggest that once *L. stagnalis* recognize the external environment is safe after training, they may extinguish their memory of the past situation quickly, resulting in no or very little difference in the memory retention by two different training procedures in this operant conditioning. Compared with the behavior in classical conditioning (Kojima *et al.*, 1995, 1996, 1998) and its neural mechanism described in the next sections (Kojima *et al.*, 1997, Nakamura *et al.*, 1999a, b, c), these findings may help to address not only the neural basis of operant conditioning but also the relation between the classical and operant conditioning.

CELLULAR MECHANISM OF CONDITIONED TASTE AVERSION

Based on the behavioral experiments for the CTA in *L. stagnalis* (Kojima *et al.*, 1995, 1996), we proposed a neuromodulatory model (Fig. 5A). When the CS (sucrose) is followed by the UCS (KCl) in the training session, the association of the CS and UCS potentiates an inhibitory pathway, resulting in suppression of the feeding response to the CS. Taking account of the underlying neural circuits so far reported (Benjamin and Elliott, 1989; Ferguson and Benjamin, 1991; Syed and Winlow, 1991; Elliott and Kemenes, 1992, McCrohan and Kyriakides, 1992; Inoue *et al.*, 1996a, b; Yeoman *et al.*, 1996), our model further proposed that sensory neuron(s) sensitive to the appetitive CS excite the feeding central pattern generator (CPG) in the CS pathway to induce the feeding response, and that sensory neuron(s) for the aversive UCS excite interneurons in the UCS pathway, resulting in the withdrawal response (Fig. 5B). The cerebral giant cells (CGCs) exert weak excitatory monosynaptic and strong inhibitory polysynaptic influences upon the neuron 1 medial (N1M) cells in the CPG, and the repetitive firing of the CGCs results in inhibitory influence on the N1M cells (Yeoman *et al.*, 1996). Thus, it was presumed that the inhibitory influence of the CGCs upon the N1M cells may be potentiated in the conditioned snails to suppress the feeding response. To test the validity of this model, we examined the differences in synaptic inputs to

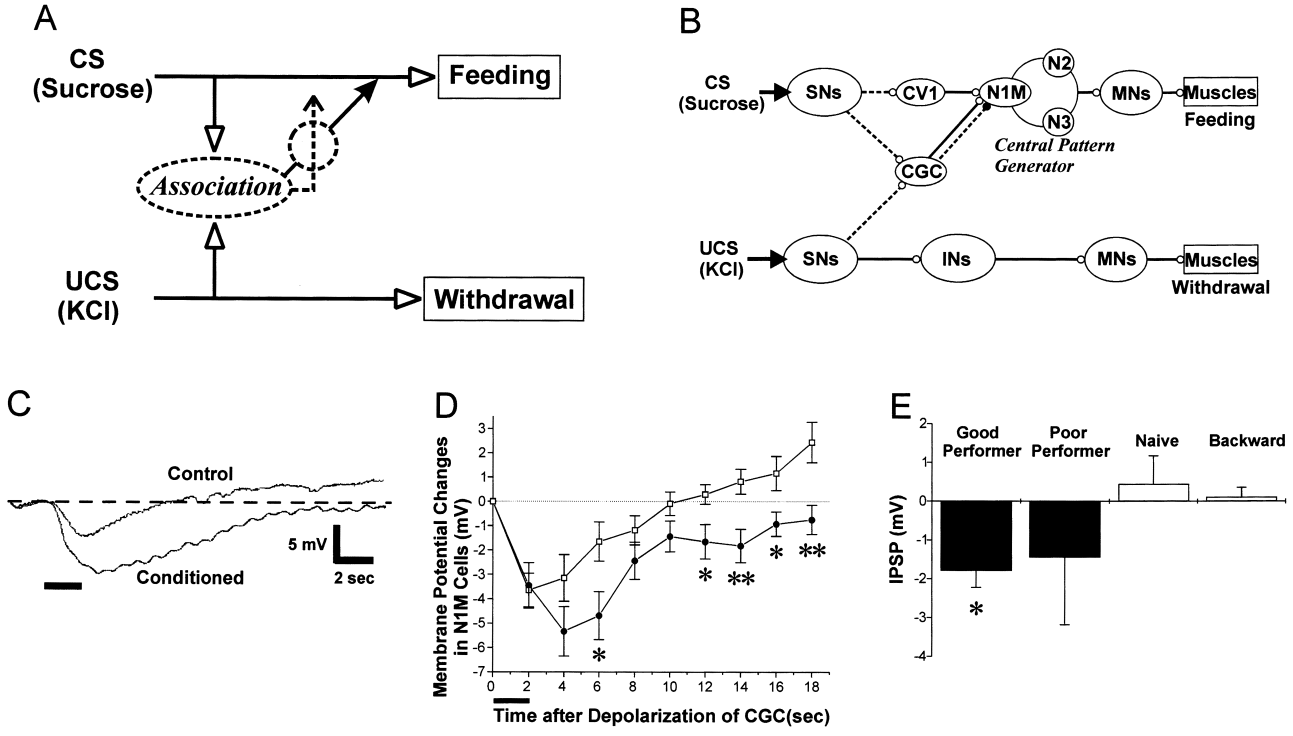

Fig. 5. Cellular mechanism of conditioned taste aversion. See Kojima *et al.* (1997) for the details. (A, B) Neuromodulatory model. A white and a black arrow or circle indicate an excitatory and an inhibitory influence or synapse, respectively. A dashed line part in (A) shows a variability of transmission strength. A solid and a dashed line in (B) indicate a monosynaptic and a polysynaptic projection, respectively. CV1: cerebral ventral 1 cell; CGC cerebral giant cell; N1M: neuron 1 medial cell; SN: sensory neuron; IN: interneuron; MN: motoneuron. (C–E) Enhancement of IPSP to the feeding central pattern generator. Typical IPSPs (C) and their summarized data (D) in the N1M cells, which were induced by depolarizing current injection into the CGCs during a bar, were recorded in the conditioned (filled circles) and control (open circles) snails. In (E), we defined the conditioned snails, which bit less than twice for 90 sec by the conditioned stimulus (sucrose), as the good performers, and compared the IPSPs at 12 sec after the onset of CGC depolarization. Backward indicates backward-conditioning control. The data are means±SEM. * and ** indicate $p<0.05$ and $p<0.01$, respectively.

the N1M cells by stimulating the CGCs in the conditioned and control snails (Kojima *et al*., 1997), and further studied whether or not the association of the chemosensory inputs (sucrose and KCl) occurs in the CGCs (Nakamura *et al*., 1999a, b).

Inhibitory postsynaptic potential (IPSP) which was evoked in the N1M cell by activation of the CGC was larger and lasted longer in the conditioned snail than that in the control snail (Fig. 5C–E). The electrical properties of the cell bodies of CGCs and the responses of the CGCs to the chemosensory inputs were not changed during the CTA. These results, together with the previous report indicating the existence of excitatory projection from the N1M cells to the buccal motoneurons 1 (B1 cells) involved in feeding behavior (Kemenes and Elliott, 1994), suggested that enhanced IPSP in the N1M cells may underlie the suppression of feeding responses in the CTA of *L. stagnalis*.

The neural circuits from the chemosensory neurons receiving the CS and UCS have not yet been demonstrated. Generally, as compared to the motoneuronal system, the chemosensory neuronal system has been poorly understood as regards its role in feeding behavior of gastropod molluscs (Kemenes *et al*., 1986). Even in *L. stagnalis*, only six types of endings of primary neurons are known (Zylstra, 1972). These neurons have been morphologically classified as sensory neurons in the epithelia of lips, tentacles, and dorsal surface of head (Zylstra, 1972; Zaitseva and Bocharova, 1981). Neither the secondary chemosensory neurons nor the interneurons directly following the primary ones have been identified. Therefore, to understand the cellular mechanism of the CTA, the neural pathways which transmit taste signals to the feeding CPG in *L. stagnalis* should be elucidated clearly.

We thus targeted to clarify pathways for the feeding responses between the chemosensory neurons and the regulatory neurons, such as the CGCs. To achieve this goal, the lip and tentacle nerves of *L. stagnalis*, were characterized using histological techniques (Nakamura *et al*., 1999a). Anatomical drawings showed the detailed distributions of the superior lip, median lip, and tentacle nerves in the lip and tentacle; particularly it was found that the mouth is mainly innervated by the superior lip nerve. The neurons in the CNS by backfilling of the superior lip nerve and/or the median lip nerve with fluorescence dyes made some clusters, whereas those stained from the tentacle nerve made other clusters. These stained neurons were not part in the CPG or its regulatory neurons for feeding. These results, therefore, suggested that the superior lip nerve may be employed as a principal factor in the chemosensory transduction from the mouth, and that no direct projections from the CPG or its regulatory neurons for feeding to the lip and tentacle nerves.

Furthermore, the lip and tentacle nerves of *L. stagnalis*, were characterized using electrophysiological techniques (Nakamura *et al*., 1999b). When the activity of those nerves was induced in lip-tentacle preparations, aversive taste (KCl) signals were transmitted through all the lip and tentacle nerves, but appetitive (sucrose) signals could be recorded only through the superior lip nerve. In the CNS immersed in high Mg^{2+}-high Ca^{2+} saline, electrical stimuli applied to any of the nerves failed to induce action potentials in the CGCs, implying that the signals are polysynaptically transmitted to the CGCs (Fig. 5B). Intracellular recordings revealed that the CGCs in semi-intact preparations received both appetitive and aversive taste signals not only through the superior lip nerve but also through the median lip nerve (Fig. 6). In addition, an osphradium was ruled out as a candidate for appetitive reception. The present results, together with our preceding data arrived at by the histochemical analyses (Nakamura *et al*., 1999a), indicated that the appetitive taste transduction responsible for generating the feeding responses is performed through the superior lip nerve with some contribution of the median lip nerve. The data showing that the CGCs can receive various taste signals suggested that they may play a crucial role in feeding behavior as demonstrated in the study of CTA (Fig. 5B).

On the other hand, serotonin (5-HT) has been emphasized of neurotransmitters in the CNS of *L. stagnalis*, because 5-HT is known to play an important role to activate the feeding motor pattern (Yeoman *et al*., 1994, 1996; Kemenes *et al*., 1997). The CGCs are known to be serotonergic (Boer *et al*., 1984; Tuersley and McCrohan, 1988; Yeoman *et al*., 1996). Although these previous studies in immunohistochemistry let us gain deep insights into the neurobiology of *L. stagnalis*, all the results were obtained by two-dimensional (2-D) observation according to conventional methods, leading some defects that a 3-D reconstruction of immunoreactive neurons in the CNS was ambiguous and that discrimination of individual small neurons from cell clusters was difficult. Therefore, we examined 3-D arrangement of 5-HT-like immunoreactive neurons in the CNS of *L. stagnalis*, by a combination of immunohistochemistry and confocal laser scanning microscopy (Hatakeyama and Ito, 1999a). In addition to the confirmation of previously identified serotonergic neurons, some important new findings were obtained (see Hatakeyama and Ito, 1999a for the details). These results could produce the exactly 3-D maps for 5-HT-like immunoreactive neurons in the CNS, and will help the further analyses of neural networks employing 5-HT.

Recently, γ-aminobutyric acid (GABA) has been noted. Injection of GABA into the haemocoel of intact *L. stagnalis* resulted in the modulation of feeding activity (Romanova *et al*., 1996). Such injection also immediately evoked the rhythmic movements of radula, including opening the mouth and three-phase cyclic radula biting: protraction, rasping, and swallowing. Therefore, we examined the distribution of GABA-like immunoreactive neurons in the CNS of *L. stagnalis* using a 3-D analysis by immunohistochemistry combined with confocal laser scanning microscopy (Hatakeyama and Ito, 1999b). Our results showed that GABA is ubiquitously located in the CNS, particularly that the paired GABA-like immunoreactive neurons in the buccal ganglia resemble a pair of buccal motoneurons 19 (B19 cells) of *Helisoma trivolvis* (Kater, 1974; Richmond *et al*., 1991). To our knowledge, this is the first description of such neurons in *L. stagnalis*. Functional studies demonstrated that application of GABA to the B19 cells in

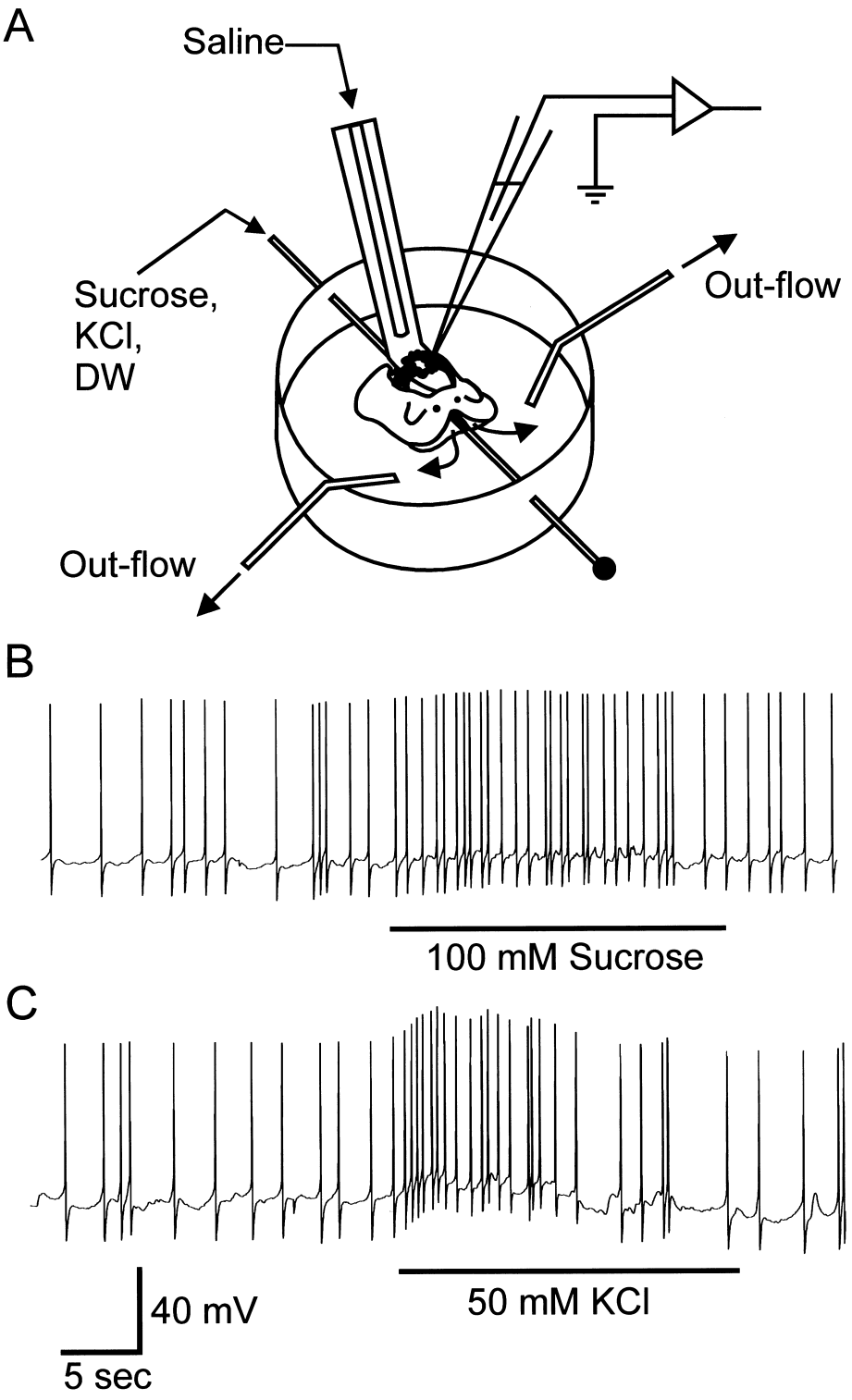

Fig. 6. Intracellular recordings of the CGCs in response to appetitive and aversive chemical stimuli applied to the lips in semi-intact preparations. See Nakamura *et al.* (1999b) for the details. (A) Schematic presentation of the semi-intact preparation. (B, C) Responses of the CGCs to sucrose and KCl which are used for the acquisition of conditioned taste aversion.

H. trivolvis stimulated the rhythmic patterned activity, which resembled fictive feeding (Richmond *et al.*, 1994). Taken together, these results emphasized the need for further analysis of the functional role of GABA-like immunoreactive neurons in *L. stagnalis* (corresponding to the B19 cells of *H. trivolvis*) and their influence of the activity of feeding motor pattern.

DEVELOPMENTAL STUDY OF CONDITIONED TASTE AVERSION

The relationships between development and learning were strongly emphasized in gastropod molluscs, particularly in *Aplysia californica* by Carew and his colleagues (Marcus *et al.*, 1994; Nolen and Carew, 1994; Marcus and Carew, 1998), because neurobiologists have long speculated that growing processes involved in the development of CNS may persist in the adult where they could subserve learning and memory. As a first step to study the relationships between the development and learning in the CNS of *L. stagnalis*, we examined

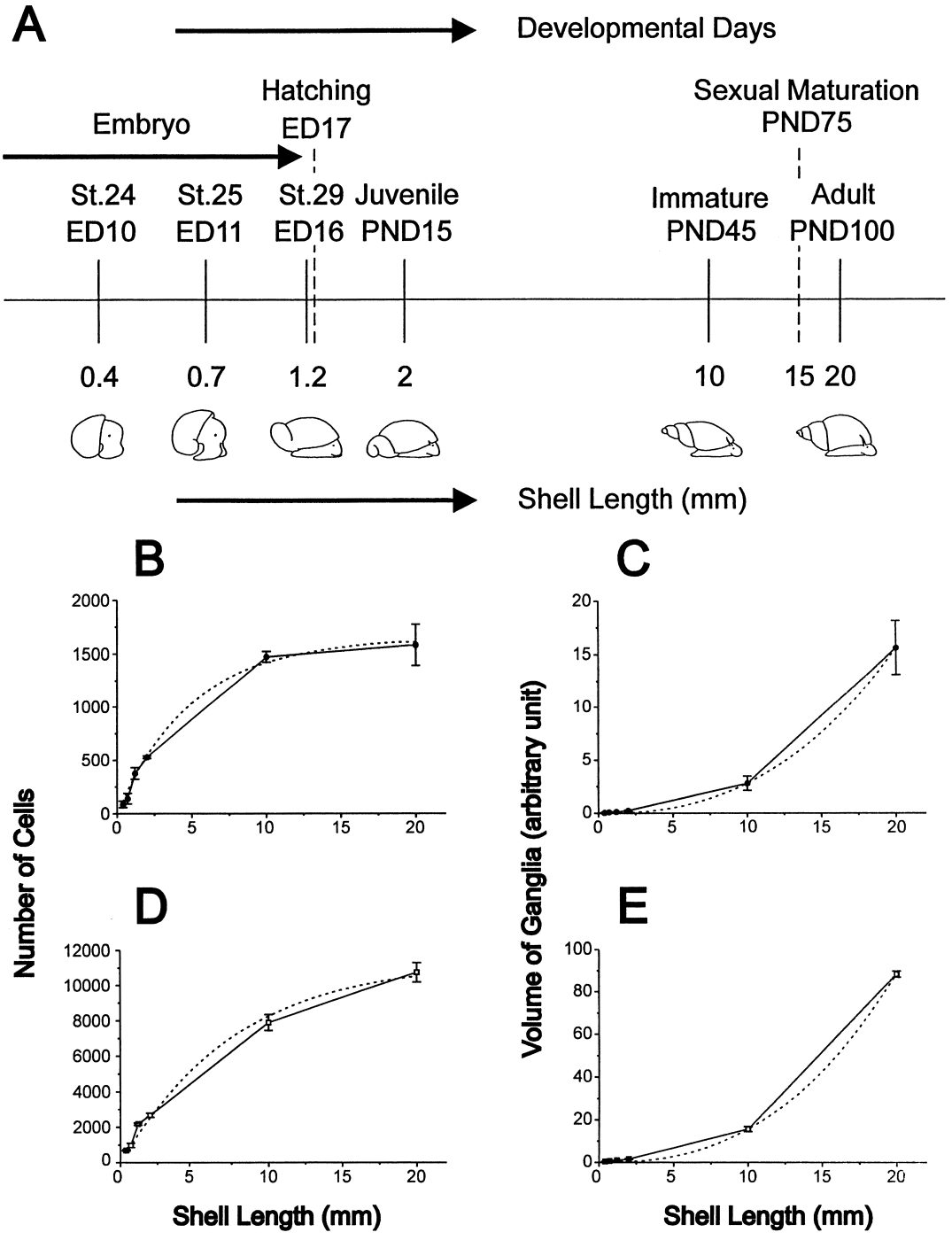

Fig. 7. Developmental changes in conditioned taste aversion. See Yamanaka *et al.* (1999) and Sadamoto *et al.* (1999) for the details. (A) Outline of developing snails kept at 20°C. The shell length is expressed in a logarithmic scale. ED and PND indicate embryonic days and postnatal days, respectively. St.: embryonic stage according to Meshcheryakov's criteria (1990). (B–E) Increase in numbers of cells and that in volumes of the buccal (B, C) and cerebral (D, E) ganglia. The shell lengths of 0.4 mm, 0.7 mm, 1.2 mm, 2 mm, 10 mm, and 20 mm correspond to St. 24, St. 25, St. 29 embryos, juvenile snails, immature snails, and adult snails, respectively.

developmental changes in the acquisition and retention of CTA (Yamanaka *et al.*, 1999). We found that *L. stagnalis* developed ability of a CTA as a long-term memory through three critical stages (see Fig. 7A for the outline of developing snails). Embryos in veliconcha (stage 25) started to respond to appetitive sucrose at the first critical stage. This response was in good agreement with morphological observations (Meshcheryakov, 1990) that embryos at this developmental stage seemed to be physically ready to eat. However, they could not associate this appetitive stimulus (CS) with an aversive stimulus of KCl (UCS). At the second critical stage, embryos just before hatching (stage 29) acquired the CTA, but the conditioned response did not persist. Through this stage, they may acquire learning ability to safely seek out food in an external environment. At the third critical stage, immature snails with a 10 mm shell could use a long-term memory to maintain the conditioned response. This memory persisted for at least a month, showing that now they are able to maintain a long-term memory so that they can safely eat a variety of food when they cover wide territory to search for a mate. The findings indicated that the development of learning ability in snails, which secures acquisition of better survival ability, is coincident with the major changes in their life cycle.

Next, to provide the anatomical substrate upon which the CTA is superposed during the development, we examined the number of cells and the volumes of the buccal and cerebral ganglia in *L. stagnalis* at the critical developmental stages described above (Sadamoto *et al.*, 1999). The buccal and cerebral ganglia include the majority of neurons involved in the CTA. We found that the numbers of cells in these ganglia are almost saturated in the immature snails, but the volumes of these ganglia still increase from the immature snails to the adults (Fig. 7B-E). These results suggested that most of the cells indispensable to the CTA emerge at the immature stage, but that individual cells in the ganglia continue to enlarge even in adulthood. Furthermore, a pair of key neurons (CGCs) for the CTA were found to mature at the immature stage. This study provided the anatomical substrate upon the long-term CTA, by which snails can eat safe food in a wide territory. Furthermore, we studied the synthesis and storage of 5-HT in the CGCs, using *L. stagnalis* at the above critical developmental stages. There was a positive correlation between the acquisition of CTA and the first appearance of 5-HT immunoreactivity in the CGCs at the embryonic stage 29 (Yamanaka *et al.*, unpublished data). These results, therefore, indicated that the development of a pair of key neurons (CGCs) for the CTA stimulates the developmental changes in the learning ability.

PKA-DEPENDENT REGULATION OF SYNAPTIC ENHANCEMENT

As described repeatedly, the CGCs play a crucial role in the regulation of CTA in *L. stagnalis*. However, the mechanisms of signal transduction from the CGCs to the follower buccal interneurons and motoneurons are not clear. To our knowledge, only the work by McCrohan and Gillette (1988) suggested that cyclic AMP (cAMP) is a candidate of the second messenger in the CGCs by demonstrating the appearance of Na^+ current by the cAMP injection. We thus examined whether cAMP-dependent protein kinase (PKA) contributes to enhancement of a monosynaptic connection between the presynaptic CGCs and the postsynaptic B1 cells (Nakamura *et al.*, 1999c). Injection of cAMP into the CGCs or inhibition of phosphodiesterase by isobutylmethylxanthine in the CGCs increased the amplitude of excitatory postsynaptic potential (EPSP) in the B1 cells, whereas no changes were detected in the electrical properties of the CGCs (Fig. 8A–C). The synaptic enhancement in the B1 cells was completely blocked by inhibition of PKA in the CGCs but did not require a *de novo* protein synthesis due to a PKA phosphorylation (Fig. 8D, E). The increase in the EPSP amplitude of B1 cells was associated with the increase in the amount of 5-HT release from the CGCs (Fig. 8F, G). These results thus provided the physiological evidence of the direct regulation of a synaptic enhancement by PKA in the CNS of *L. stagnalis*, indicating the completely different mechanism from that in the well-studied siphon- and gill-withdrawal reflex in *A. californica* (Abel and Kandel, 1998).

INVOLVEMENT OF NITRIC OXIDE

The function of nitric oxide (NO), particularly a specific role in feeding behavior, has been started to be examined (Elofsson *et al.*, 1993; Moroz *et al.*, 1993, 1994a, b, 1995, 1999; Elphick *et al.*, 1994, 1995; Martinez, 1995; Korneev *et al.*, 1998; Serfözö *et al.*, 1998). To examine whether NO-generative neurons are included in the central circuitry for generation of feeding pattern in *L. stagnalis*, two staining techniques for NADPH diaphorase and 5-HT were applied for its CNS (Sadamoto *et al.*, 1998). The former technique is known to show localization of NO synthase (NOS); the latter is well employed as a marker for the feeding circuitry because 5-HT is a main transmitter in it (Kemenes, 1997). In the buccal ganglion, B2 cells were found to be a pair of putative NO-generative neurons (Fig. 9A). These motoneurons are not involved directly in the coordination of feeding pattern (Fig. 9B) but are activated simultaneously with feeding to control the oesophageal and gut tissues for digestion. Taking account of the diffusion effects of NO, the NO released from the B2 cells, when the feeding is started, is considered to sufficiently modulate the feeding circuitry. In the cerebral ganglion, the superior lip, median lip, and tentacle nerves included both putative NO-generative fibers and serotonergic fibers. These fibers were not identical, but the NO released in the nerves may activate the serotonergic fibers, resulting in the influence upon the initiation of feeding. Therefore, our findings clearly showed that NO is not involved in transmission within the central circuitry for feeding, but suggested that NO can crucially affect feeding behavior, such as initiation and modulation of the feeding pattern.

To demonstrate that NO is generated in the CNS accom-

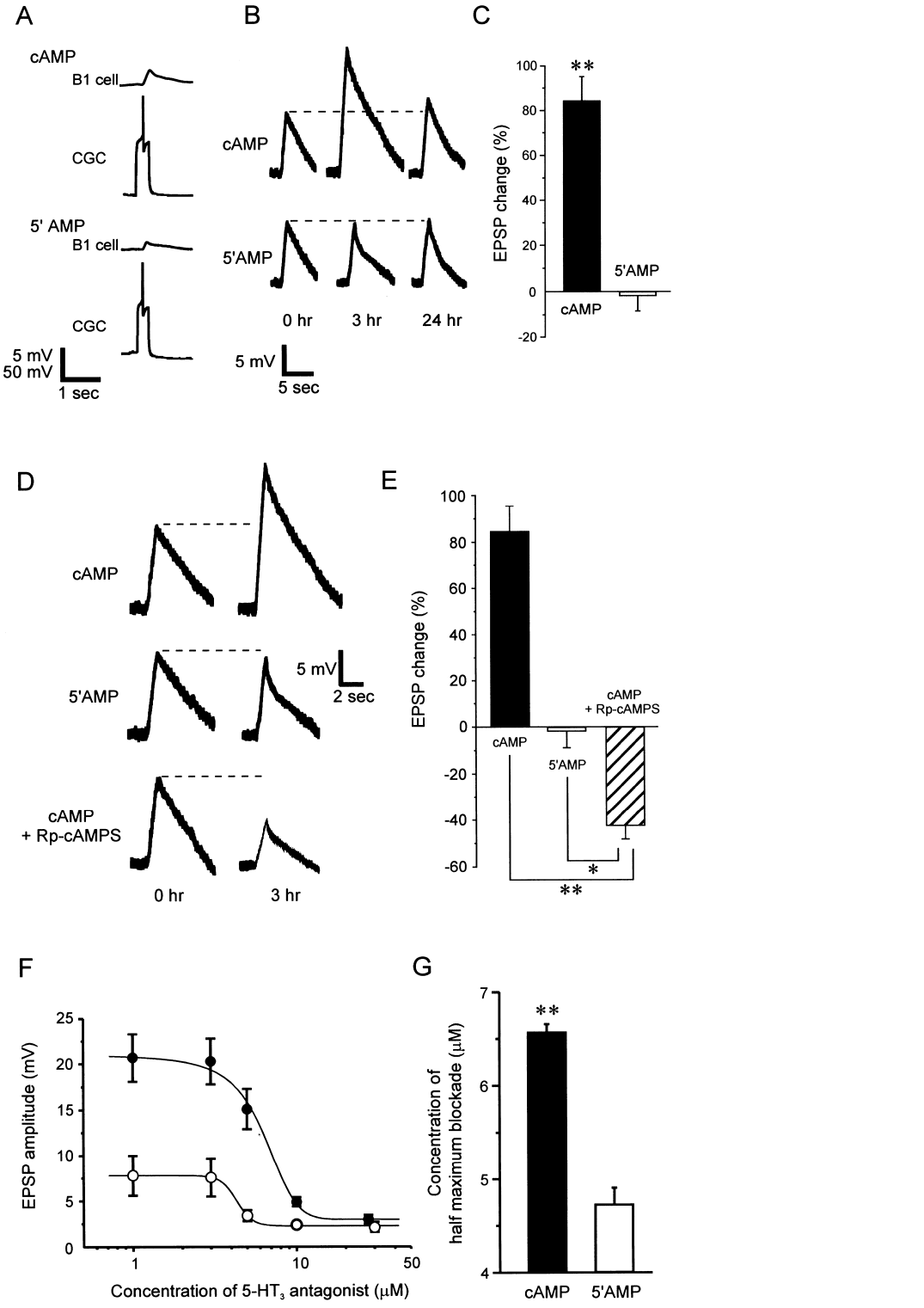

Fig. 8. PKA-dependent regulation of synaptic enhancement between the CGCs and the follower B1 motoneurons. See Nakamura *et al.* (1999c) for the details. (A–C) Synaptic enhancement by injection of cAMP into the CGCs. Single EPSPs recorded in the B1 cells 3 hr after the onset of injections are shown in (A), and compound EPSPs are shown in (B). The changes in the amplitudes of compound EPSPs recorded in the B1 cells 3 hr after the onset of injections are summarized in (C). (D, E) Blockade of the increase in EPSP amplitude in the B1 cells by PKA-inhibitor (Rp-cAMPS) application on the cerebral ganglia including the CGCs. The changes in the EPSP amplitudes recorded in the B1 cells 3 hr after the onset of injections are summarized in (E). (F, G) Dose-response of the EPSP amplitude in the B1 cells to 5-HT_3 antagonist. The EPSP amplitudes shown in (F) were recorded in the B1 cells 3 hr after the onset of injections of cAMP (filled circles) and 5'AMP (open circles), when the 5-HT_3 antagonist was applied on the whole CNS. The concentrations of 5-HT_3 antagonist for the half maximum blockade are summarized in (G). All data are means±SEM. * and ** indicate $p<0.01$ and $p<0.001$, respectively.

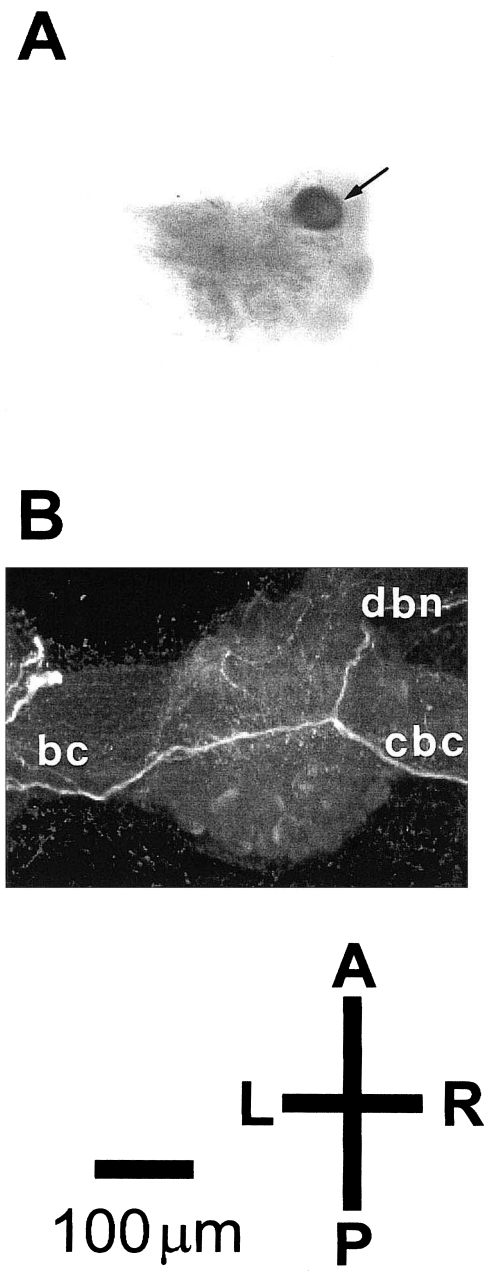

Fig. 9. Histochemistry to reveal the relation between NO-generative neurons and feeding circuitry in the buccal ganglia. See Sadamoto *et al.* (1998) for the details. (A) NADPH-diaphorase staining in the right buccal ganglion. An arrow points the B2 motoneuron. (B) 5-HT immunohistochemistry showing the feeding circuitry. bc: buccal commissure; dbn: dorsobuccal nerve; cbc: cerebrobuccal connective.

panied with feeding behavior, we measured the increase in NO concentration at the buccal ganglia in semi-intact preparations of *L. stagnalis* using an NO specific electrode, when the lips of these preparations were stimulated by sucrose (Kobayashi *et al.*, unpublished data). The NO concentration at the buccal ganglia was significantly increased by an application of sucrose to the lips. The rhythmic NO response well corresponded to the rhythmic bursting (fictive feeding response) of the B2 cells. These data provided the first direct evidence that NO is actually generated in the CNS of *L. stagnalis* and involved in a specific behavior such as feeding.

IMAGING BY NEW MICROSCOPES

New microscopes have been recently developed to image morphology and function of multiple sites in tissues or small cellular elements that are inaccessible by conventional light and electron microscopy and electrophysiological techniques. In the neurobiology of learning and memory, one of the most striking findings is that long-term memory involves morphological changes in neurons (Bailey and Kandel, 1993). To observe the fine structures of neurons isolated from the CNS of *L. stagnalis*, we thus attempted to detect fine 3-D structures of living neuronal terminals with at least 40-nm vertical information, and succeeded in observing the real-time dynamics of their terminals, such as attraction and repulsion with each other, using an atomic force microscope (AFM) for a liquid environment (Nagayama *et al.*, 1996, 1997; see Shao *et al.* for the detail of AFM). The time-dependent fine dynamics of the neurons obtained here is beyond the resolution power of an optical microscope, and actually it has never been accomplished with an electron microscope that requires of fixing and staining.

More recently, we began to image the changes in membrane potential by using an optical recording technique (see Kojima *et al.*, 1999 for the detail of our optical recording system) as well as the those in intracellular Ca^{2+} concentration (Alkon *et al.*, 1992; Collin *et al.*, 1992; Ito *et al.*, 1994). The electrical responses in the cerebral ganglion and the median lip nerve of *L. stagnalis* were measured, when a short current pulse was delivered to the median lip nerve, which transmits chemosensory signals to the cerebral ganglion (Kojima *et al.*, unpublished data). We detected a composite depolarizing response in the cerebral ganglion, which consisted of a sharp depolarizing response corresponding to a compound action potential and a following slow depolarizing response. After pharmacological analyses, the slow depolarizing response was found to originate from glial cells. Our results thus provided the first evidence for neuron-independent signals in glial cells of gastropod molluscs and implied the contribution of the glial signaling to the chemosensory processing in the CNS of *L. stagnalis*.

CONCLUDING REMARKS

The studies of CTA in *L. stagnalis* provide us with insight into the richness of cellular correlates of associative learning behavior in the relatively simple CNS, or microbrain. The next critical step in our understanding of the neural mechanisms of associative learning in *L. stagnalis* is to proceed studies at molecular level. In *A. californica*, it has been possible to identify some of the genes and gene products that contribute importantly to induction of the long-lasting neural and behavioral changes underlying the long-term memory (Abel and

Kandel, 1998). Unfortunately, the molecular analyses involved in the associative learning of *L. stagnalis* are future work. We just started the molecular cloning for genes concerned with the CTA (Sadamoto *et al*., unpublished data). We really hope that many molecular biologists take part in studying the molecular mechanisms of associative learning in *L. stagnalis* and that gene-knockout snails will be produced in near future.

ACKNOWLEDGMENTS

We thank the Editor-in-Chief of this journal, Professor Norio Suzuki, for recommending us to write this review. The pond snails, *L. stagnalis*, were originally gifted by Professor Wijnand P.M. Geraerts at Vrije Universiteit in Amsterdam, the Netherlands. We have learned a lot about the cellular mechanisms of feeding behavior of *L. stagnalis* from a series of excellent studies by Professor Paul R. Benjamin and his colleagues at University of Sussex in East Sussex, UK. This work was partly supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan to E.I. and a grant from Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists to S.K.

REFERENCES

Abel T, Kandel E (1998) Positive and negative regulatory mechanisms that mediate long-term memory storage. Brain Res Rev 26: 360–378

Alexander JE, Jr, Audesirk TE, Audesirk GJ (1982) Rapid, nonaversive conditioning in a freshwater gastropod. II. Effects of temporal relationships on learning. Behav Neural Biol 36: 391–402

Alkon DL, Sánchez-Andrés J-V, Ito E, Oka K, Yoshioka T, Collin C (1992) Long-term transformation of an inhibitory into an excitatory GABAergic synaptic response. Proc Natl Acad Sci USA 89: 11862-11866

Alkon DL, Collin C, Ito E, Lee C-J, Nelson TJ, Oka K, Sakakibra M, Schreurs BG, Yoshioka T (1993) Molecular and biophysics steps in the storage of associative memory. In "Molecular basis of ion channels and receptors involved in nerve excitation, synaptic transmission and muscle contraction" Ann NY Acad Sci Vol 707, Ed by H Higashida, T Yoshioka, K Mikoshiba, The New York Academy of Sciences, New York, pp 500–504

Alkon DL, Nelson TJ, Zhao W, Cavallaro S (1998) Time domains of neuronal Ca^{2+} signaling and associative memory: steps through a calexcitin, ryanodine receptor, K^+ channel cascade. Trends Neurosci 21: 529–537

Audesirk TE, Alexander JE, Jr, Audesirk GJ, Moyer CM (1982) Rapid, nonaversive conditioning in a freshwater gastropod. I. Effects of age and motivation. Behav Neural Biol 36: 379–390

Bailey CH, Kandel ER (1993) Structural changes accompanying memory storage. Annu Rev Physiol 55: 397–426

Benjamin PR, Elliott CJH (1989) Snail feeding oscillator: the central pattern generator and its control by modulatory interneurons. In "Neuronal and Cellular Oscillators" Ed by JW Jacklet, Marcel Dekker, New York, pp 173–214

Boer HH, Schot LP, Steinbusch HW, Montagne C, Reichelt D (1984) Co-existence of immunoreactivity to anti-dopamine, anti-serotonin, anti-vasotocin in cerebral giant neuron of pond snail *Lymnaea stagnalis*. Cell Tissue Res 238: 411–412

Collin C, Ito E, Oka K, Yoshioka T, Sánchez-Andrés J-V, Matzel LD, Alkon DL (1992) The role of calcium in prolonged modification of a GABAergic synapse. J Physiol (Paris) 86: 139–145

Dudai Y(1990) The Neurobiology of Memory. Concepts, Findings, Trends. Oxford University Press, Oxford, p 49

Elliott CJH, Kemenes G (1992) Cholinergic interneurons in the feeding system of the pond snail *Lymnaea stagnalis*. II. N1 interneurons make cholinergic synapses with feeding motoneurons. Phil Trans R Soc Lond B 336: 167–180

Elofsson R, Carlberg M, Moroz L, Nezlin L, Sakharov D (1993) Is nitric oxide (NO) produced by invertebrate neurons? Neuro Report 4: 279–282

Elphick MR, Riveros-Moreno V, Moncada S, O'Shea M (1994) Identification of nitrergic neurons in invertebrates. In "The Biology of Nitric Oxide: Physiology and Clinical Aspects Vol 3" Ed by S Moncada, M Feelisch, R Busse, EA Higgs, Portland, London, pp 377–381

Elphick MR, Kemenes G, Staras K, O'Shea M (1995) Behavioral role for nitric oxide in chemosensory activation of feeding in a mollusc. J Neurosci 15: 7653–7664

Ferguson GP, Benjamin PR (1991) The whole-body withdrawal response of *Lymnaea stagnalis*. I. Identification of central motoneurones and muscles. J Exp Biol 158: 63–95

Frank DA, Greenberg ME (1994) CREB: A mediator of long-term memory from mollusks to mammals. Cell 79: 5–8

Gelperin A, Kleinfeld D, Denk W, Cooke IRC (1996) Oscillations and gaseous oxides in invertebrate olfaction. J Neurobiol 30: 110–122

Hatakeyama D, Ito E (1999a) Three-dimensional reconstruction and mapping of serotonin-like immunoreactive neurons in the central nervous system of the pond snail, *Lymnaea stagnails*, with the confocal laser scanning microscope. Bioimages 7: 1–12

Hatakeyama D, Ito E (1999b) Distribution and developmental changes in GABA-like immunoreactive neurons in the central nervous system of pond snail, *Lymnaea stagnalis*. J Comp Neurol in press

Hermann PM, Bulloch AGM (1998) Developmental plasticity of respiratory behavior in *Lymnaea*. Behav Neurosci 112: 656–667

Hintzman DL (1974) Theoretical implications of the spacing effect. In "Theories in Cognitive Psychology, The Loyola Symposium" Ed by RL Solso, Lawrence Erlbaum Associates, Hillsdale, pp 77–99

Inoue T, Takasaki M, Lukowiak K, Syed NI (1996a) Identification of a putative mechanosensory neuron in *Lymnaea*: Characterization of its synaptic and functional connections with the whole-body withdrawal interneuron. J Neurophysiol 76: 3230–3238

Inoue T, Takasaki M, Lukowiak K, Syed NI (1996b) Inhibition of the respiratory pattern-generating neurons by an identified whole-body withdrawal interneuron of *Lymnaea stagnalis*. J Exp Biol 199: 1887–1898

Ito E, Oka K, Collin C, Schreurs BG, Sakakibara M, Alkon DL (1994) Intracellular calcium signals are enhanced for days after Pavlovian conditioning. J Neurochem 62: 1337–1344

Ito I, Kimura T, Suzuki H, Sekiguchi T, Ito E (1999) Effects of electrical stimulation of the tentacular digits of a slug upon the frequency of electrical oscillations in the procerebral lobe. Brain Res 815: 121–125

Kater SB (1974) Feeding in *Helisoma trivolvis*: The morphological bases of a fixed action pattern. Am Zool 14: 1017–1036

Kemenes G, Elliott CJH, Benjamin PR (1986) Chemical and tactile inputs to the *Lymnaea* feeding system: Effects on behaviour and neural circuitry. J Exp Biol 122: 113–137

Kemenes G, Benjamin PR (1989a) Appetitive learning in snails shows characteristics of conditioning in vertebrates. Brain Res 489: 163–166

Kemenes G, Benjamin PR (1989b) Goal-tracking behavior in the snail, *Lymnaea stagnalis*. Behav Neural Biol 52: 260–270

Kemenes G, Benjamin PR (1994) Training in a novel environment improves the appetitive learning performance of the snail, *Lymnaea stagnalis*. Behav Neural Biol 61: 139–149

Kemenes G, Elliott CJH (1994) Analysis of the feeding motor pattern in the pond snail, *Lymnaea stagnalis*: Photoinactivation of axonally stained pattern-generating interneurons. J Neurosci 14: 153–166

Kemenes G (1997) *In vivo* neuropharmacological and *in vitro* laser ablation techniques as tools in the analysis of neuronal circuits

underlying behavior in a molluscan model system. Gen Pharmacol 29: 7–15
Kemenes G, Staras K, Benjamin PR (1997) In vitro appetitive classical conditioning of the feeding response in the pond snail *Lymnaea stagnalis*. J Neurophysiol 78: 2351–2362
Kimura T, Iwama A, Sekiguchi T (1999) Contributions of superior and inferior tentacles to learned food-avoidance behavior in *Limax marginatus*. Zool Sci 16: 595–602
Kobayashi S, Kojima S, Yamanaka M, Sadamoto H, Nakamura H, Fujito Y, Kawai R, Sakakibara M, Ito E (1998) Operant conditioning of escape behavior in the pond snail, *Lymnaea stagnalis*. Zool Sci 15: 683–690
Kojima S, Yamanaka M, Nagayama S, Fujito Y, Ito E (1995) Neuromodulation models for associative learnings with central pattern generator in the pond snail, *Lymnaea stagnalis*. In "Nervous Systems and Behaviour" Ed by M Burrows, T Matheson, PL Newland, H Schuppe, Georg Thieme Verlag, Stuttgart, p 208
Kojima S, Yamanaka M, Fujito Y, Ito E (1996) Differential neuroethological effects of aversive and appetitive reinforcing stimuli on associative learning in *Lymnaea stagnalis*. Zool Sci 13: 803–812
Kojima S, Nakamura H, Nagayama S, Fujito Y, Ito E (1997) Enhancement of an inhibitory input to the feeding central pattern generator in *Lymnaea stagnalis* during conditioned taste-aversion learning. Neurosci Lett 230: 179–182
Kojima S, Kobayashi S, Yamanaka M, Sadamoto H, Nakamura H, Fujito Y, Kawai R, Sakakibara M, Ito E (1998) Sensory preconditioning for feeding response in the pond snail, *Lymnaea stagnalis*. Brain Res 808: 113–115
Kojima S, Nakamura T, Nidaira T, Nakamura K, Ooashi H, Ito E, Watase K, Tanaka K, Wada K, Kudo Y, Miyakawa H (1999) Optical detection of synaptically induced glutamate transport in hippocampal slices. J Neurosci 19: 2580–2588
Korneev SA, Piper MR, Picot J, Phillips R, Korneeva EI, O'Shea M (1998) Molecular characterization of NOS in a mollusc: Expression in a giant modulatory neuron. J Neurobiol 35: 65–76
Lukowiak K, Ringseis E, Spencer G, Wilderring W, Syed N (1996) Operant conditioning of aerial respiratory behaviour in *Lymnaea stagnalis*. J Exp Biol 199: 683–691
Lukowiak K, Cotter R, Westly J, Ringseis E, Spencer G, Syed N (1998) Long-term memory of an operantly conditioned respiratory behaviour pattern in *Lymnaea stagnalis*. J Exp Biol 201: 877–882
Marcus EA, Emptage NJ, Marois R, Carew TJ (1994) A comparison of the mechanistic relationships between development and learning in *Aplysia*. Prog Brain Res 100: 179–188
Marcus EA, Carew TJ (1998) Developmental emergence of different forms of neuromodulation in *Aplysia* sensory neurons. Proc Natl Acad Sci USA 95: 4726–4731
Martinez A (1995) Nitric oxide synthase in invertebrates. Histochem J 27: 770–776
Matzel LD, Talk AC, Muzzio IA, Rogerts RF (1998) Ubiquitous molecular substrates for associative learning and activity-dependent neuronal facilitation. Rev Neurosci 9: 129–167
McCrohan CR, Gillette R (1988) Cyclic AMP-stimulated sodium current in identified feeding neurons of *Lymnaea stagnalis*. Brain Res 438: 115–123
McCrohan CR, Kyriakides MA (1992) Motor programme selection and the control of feeding in the snail. In "Neurobiology of Motor Programme Selection, New Approaches to the Study of Behavioural Choice" Ed by J Kien, CR McCrohan, W Winlow, Pergamon Press, Oxford, pp 37–51
Meshcheryakov VN (1990) The common pond snail *Lymnaea stagnalis*. In "Animal Species for Developmental Studies. Vol. 1. Invertebrates" Ed by TA Dettlaff, SG Vassetzky, Plenum Publishing, New York, pp 69–132
Mizunami M, Yokohari F, Takahata M (1999) Exploration of the adaptive design of the arthropod "microbrain". Zool Sci 16: 703–709
Moroz LL, Park J-H, Winlow W (1993) Nitric oxide activates buccal motor patterns in *Lymnaea stagnalis*. NeuroReport 4: 643–646
Moroz LL, Bulloch AGM, Lukowiak K, Syed NI (1994a) Putative NO-synthesizing neurons of *Lymnaea in vivo* and *in vitro*. Netherlands J Zool 44: 535–549
Moroz LL, Winlow W, Turner RW, Bulloch AGM, Lukowiak K, Syed NI (1994b) Nitric oxide synthase-immunoreactive cells in the CNS and periphery of *Lymnaea*. NeuroReport 5: 1277–1280
Moroz LL, Radbourne S, Winlow W (1995) The use of NO-sensitive microelectrode for direct detection of nitric oxide (NO) production in molluscs. Acta Biol Hung 46: 155–167
Moroz LL, Gillette R, Sweedler J (1999) Single-cell analysis of nitrergic neurons in simple neurons systems. J Exp Biol 202: 333–341
Nagayama S, Morimoto M, Kawabata K, Fujito Y, Ogura S, Abe K, Ushiki T, Ito E (1996) AFM observation of three-dimensional fine structural changes in living neurons. Bioimages 4: 111–116
Nagayama S, Tojima T, Morimoto M, Sasaki S, Kawabata K, Ushiki T, Abe K, Ito E (1997) Practical scan speed in atomic force microscopy for live neurons in a physiological solution. Jpn J Appl Phys 36: 3877–3880
Nakamura H, Ito I, Kojima S, Fujito Y, Suzuki H, Ito E (1999a) Histological characterization of lip and tentacle nerves in *Lymnaea stagnalis*. Neurosci Res 33: 127–136
Nakamura H, Kojima S, Kobayashi S, Ito I, Fujito Y, Suzuki H, Ito E (1999b) Physiological characterization of lip and tentacle nerves in *Lymnaea stagnalis*. Neurosci Res 33: 291–298
Nakamura H, Kobayashi S, Kojima S, Urano A, Ito E (1999c) PKA-dependent regulation of synaptic enhancement between a buccal motor neuron and its regulatory interneuron in *Lymnaea stagnalis*. Zool Sci 16: 387–394
Nolen TG, Carew TJ (1994) Ontogeny of serotonin-immunoreactive neurons in juvenile *Aplysia californica*: Implications for the development of learning. Behav Neural Biol 61: 282–295
Richmond JE, Bulloch AGM, Bauce L, Lukowiak K (1991) Evidence for the presence, synthesis, immunoreactivity, and uptake of GABA in the nervous system of the snail *Helisoma trivolvis*. J Comp Neurol 307: 131–143
Richmond JE, Murphy AD, Lukowiak K, Bulloch AGM (1994) GABA regulates the buccal motor output of *Helisoma* by two pharmacologically distinct actions. J Comp Physiol A 174: 593–600
Romanova EV, Rubakhin SS, S -Rózsa K (1996) Behavioral changes induced by GABA-receptor agonists in *Lymnaea stagnalis* L. Gen Pharmacol 27: 1067–1071
Sadamoto H, Hatakeyama D, Kojima S, Fujito Y, Ito E (1998) Histochemical study on the relation between NO-generative neurons and central circuitry for feeding in the pond snail, *Lymnaea stagnalis*. Neurosci Res 32: 57–63
Sadamoto H, Yamanaka M, Hatakeyama D, Nakamura H, Kojima S, Yamashita M, Ito E (1999) Developmental study of anatomical substrate for conditioned taste aversion in *Lymnaea stagnalis*. Zool Sci in press
Sakakibara M, Kawai R, Kobayashi S, Horikoshi T (1998) Associative learning of visual and vestibular stimuli in *Lymnaea*. Neurobiol Learn Mem 69: 1–12
Serfözö Z, Elekes K, Varga V (1998) NADPH-diaphorase activity in the nervous system of the embryonic and juvenile pond snail, *Lymnaea stagnalis*. Cell Tissue Res 292: 579–586
Shao Z, Yang J, Somlyo AP (1995) Biological atomic force microscopy: From microns to nanometers and beyond. Annu Rev Cell Dev Biol 11: 241–265
Silva AJ, Kogan JH, Frankland PW, Kida S (1998) CREB and memory. Annu Rev Neurosci 21: 127–148
Spencer GE, Syed NI, Lukowiak K (1999) Neural changes after operant conditioning of the aerial respiratory behavior in *Lymnaea stagnalis*. J Neurosci 19: 1836–1843
Staras K, Kemenes G, Benjamin PR (1998) Neurophysiological cor-

relates of unconditioned and conditioned feeding behavior in the pond snail *Lymnaea stagnalis*. J Neurophysiol 79: 3030–3040

Staras K, Kemenes G, Benjamin PR (1999a) Cellular traces of behavioral classical conditioning can be recorded at several specific sites in a simple nervous system. J Neurosci 19: 347–357

Staras K, Kemenes G, Benjamin PR (1999b) Electrophysiological and Behavioral analysis of lip touch as a component of the food stimulus in the snail *Lymnaea*. J Neurophysiol 81: 1261–1273

Syed NI, Winlow W (1991) Coordination of locomotor and cardiorespiratory networks of *Lymnaea stagnalis* by a pair of identified interneurones. J Exp Biol 158: 37–62

Tuersley MD, McCrohan CR (1988) Serotonergic modulation of patterned motor output in *Lymnaea stagnalis*. J Exp Biol 135: 473–486

Veprintsev BN, Rosanov SI (1967) Learning of isolated ganglia of the mollusc *Lymnaea stagnalis*. In "Symposium on Neurobiology of Invertebrates 1967", pp 413–421

Whelan HA, McCrohan CR (1996) Food-related conditioning and neuronal correlates in the freshwater snail *Lymnaea stagnalis*. J Moll Stud 62: 483–494

Yamanaka M, Sadamoto H, Hatakeyama D, Nakamura H, Kojima S, Kimura T, Yamashita M, Urano A, Ito E (1999) Developmental changes in conditioned taste aversion in *Lymnaea stagnalis*. Zool Sci 16: 9–16

Yeoman MS, Pieneman AW, Ferguson GP, Ter Maat A, Benjamin PR (1994) Modulatory role for the serotonergic cerebral giant cells in the feeding system of the snail, *Lymnaea stagnalis*. I. Fine wire recording in the intact animal and pharmacology. J Neurophysiol 72: 1357–1371

Yeoman MS, Brierley MJ, Benjamin PR (1996) Central pattern generator interneurons are targets for the modulatory serotonergic cerebral giant cells in the feeding system. J Neurophysiol 75: 11–25

Zaitseva OV, Bocharova LS (1981) Sensory cells in the head skin of pond snails. Fine structure of sensory endings. Cell Tissue Res 220: 797-807

Zylstra U (1972) Distribution and ultrastructure of epidermal sensory cells in the freshwater snails *Lymnaea stagnalis* and *Biomphalaria pfeifferi*. Neth J Zool 22: 283–298

(Received August 19, 1999 ; invited article)