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Developmental Study of Anatomical Substrate for Conditioned Taste Aversion in *Lymnaea stagnalis*

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ABSTRACT—The pond snail, *Lymnaea stagnalis*, is a useful preparation for analyzing the commonality between development and learning. To promote this analysis, the anatomical substrate should be provided upon which learning is superposed during development. Because we previously demonstrated that *L. stagnalis* change their ability of conditioned taste aversion (CTA) as a long-term memory from veliconcha embryos to immatures, we examined in the present study the numbers of cells and the volume of the buccal and cerebral ganglia in the snails at the critical developmental stages. 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 immatures, but the volumes of these ganglia still increase from the immatures to the adults. 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, the key neuron for the CTA was found to mature at the immature stage. The present study provided the anatomical substrate upon the long-term CTA, by which snails can eat safe food in a wide territory.

INTRODUCTION

A simple central nervous system (CNS) in the pond snail, *Lymnaea stagnalis*, is highly advantageous for analyzing fundamental principles of neural development (Cumin, 1972; Morrill, 1982; Meshcheryakov, 1990; Moffett, 1995). To promote analyses of different neural functions to understand various behaviors in *L. stagnalis*, the developmental changes in immunoreactivity in the CNS were studied for neurotransmitters such as 5HT, dopamine, octopamine (Croll and Chiasson, 1989; Marois and Croll, 1992; Voronezhskaya and Elekes, 1993; Elekes *et al*., 1996; Croll *et al*., 1999; Voronezhskaya *et al*., 1999) and neuropeptides like FMRFamide (Croll and Voronezhskaya, 1995; Voronezhskaya and Elekes, 1996, 1997). The expression of nitric oxide synthase (NOS), which putatively indicates NO-generative neurons, was also examined in the developing snails (Serfözö *et al*., 1998).

Relationships between a neural circuit for feeding and a number of identified neurons in *L. stagnalis* can now be examined owing to the series of studies by Benjamin and colleagues (*e.g.* Staras *et al*., 1998). Deep insights were also gained into pathways from the peripheral tissues to the CNS by examining with histological and physiological techniques how the lip and tentacle nerves transmit taste signals (Nakamura *et al*., 1999a, b) . These academic conditions resulted in stimulating to investigate the behavioral and cellular mechanisms of appetitive and aversive classical conditioning (Kojima *et al*., 1996, 1998; Staras *et al*., 1999). In particular, the aversive conditioning called as conditioned taste aversion (CTA) was well examined, indicating that a specific interneuron (cerebral giant cell: CGC) regulating the central pattern generator for feeding rhythm plays an important role to acquire and maintain the CTA (Kojima *et al*., 1997).

More recently, we started to combine a developmental strategy with behavioral and cellular approaches to analyze the emergence and assembly of the CTA in *L. stagnalis* (Yamanaka *et al*., 1999). We found that *L. stagnalis* developed their ability of the CTA as a long-term memory through three critical stages (Fig. 1). Embryos in veliconcha (stage 25) started to respond to appetitive sucrose at the first critical stage. However, they could not associate this appetitive stimulus, or conditioned stimulus, with an aversive stimulus of KCl, or unconditioned stimulus. At the second critical stage, embryos (stage 29) just before hatching acquired the CTA, but the conditioned response did not persist. At the third critical stage, immatures with a 10 mm shell could use a long-term memory to maintain the conditioned response. These findings indicate that the development of learning ability in

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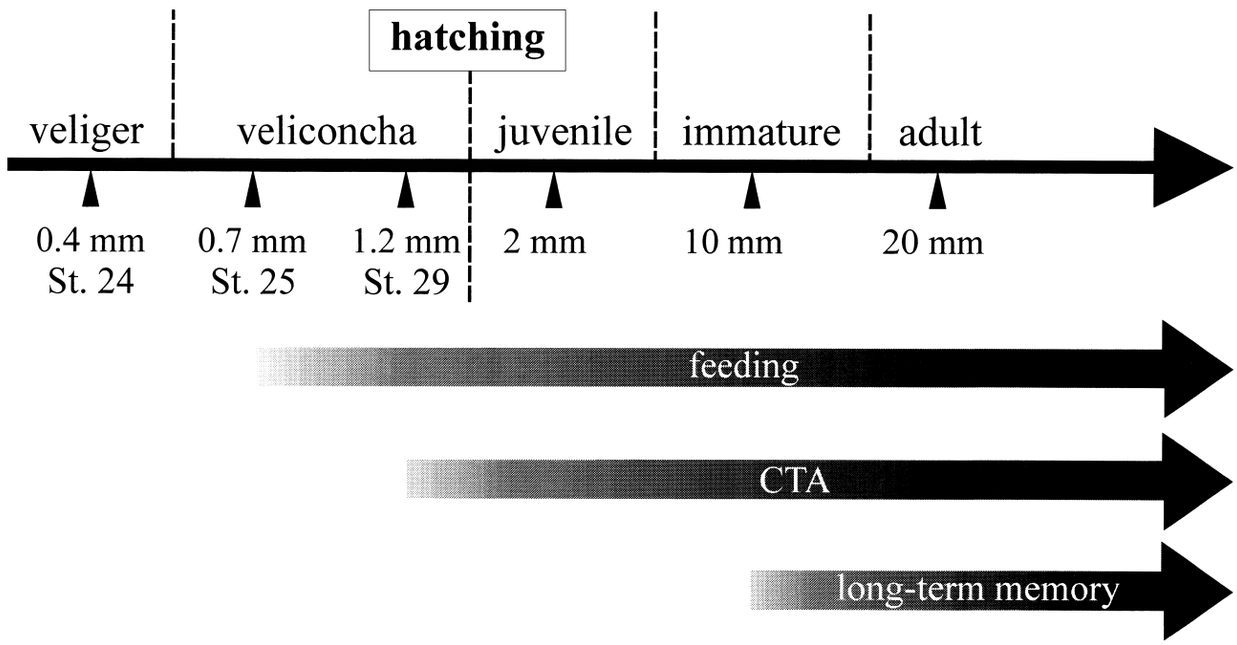

Fig. 1. Outline of the development of *L. stagnalis*. The numerals expressed in mm are the shell lengths. St.: embryonic stage according to Meshcheryakov's criteria (1990). The buccal ganglia are connected by their commissure at St. 24; the cerebral ganglia connected at St. 22. Feeding response starts at St. 25. Conditioned taste aversion (CTA) is acquired from St. 29. A long-term memory is used to maintain the CTA from immatures.

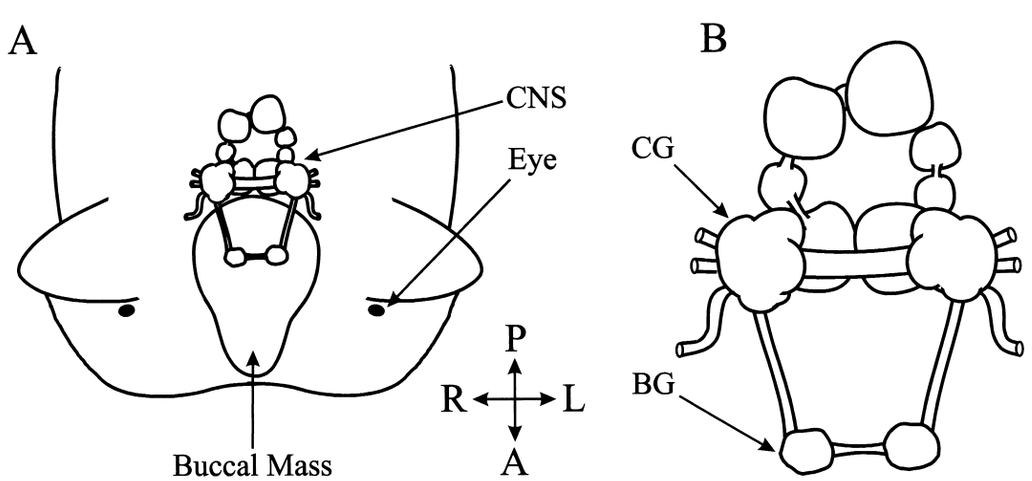

Fig. 2. Diagrams of the CNS. (A) Dorsal view of the head. (B) Dorsal view of the isolated CNS. The CNS consists of 11 ganglia. BG: buccal ganglia, CG: cerebral ganglia. A: anterior, P: posterior, R: right, L: left.

snails, which secure the better survival ability, is coincident with the major changes in their life cycle.

In the present study, to provide the anatomical substrate upon which the CTA is superposed, we examined the numbers of cells and the volume of the buccal and cerebral ganglia in developing snails, because these two ganglia include the majority of neurons involved in the CTA (Fig. 2). We found that the numbers of cells in these ganglia were almost saturated in the immatures. This result suggested that most of the cells indispensable to the feeding and CTA emerge at the immature stage.

MATERIALS AND METHODS

Snails

Locally-reared pond snails, *Lymnaea stagnalis*, were fed with lettuce and turtle food (Tetra ReptoMin, TetraWerke, Mell, Germany), and were maintained on a 12:12 light-dark cycle at 20°C. We used embryos of developmental stages 24–29, juveniles with a 2 mm shell, immatures with a 10 mm shell, and adults with a 20 mm shell (see Fig. 1 to see outlines of the developing snails). The embryonic stages

were morphologically classified using Meshcheryakov's criteria (Meshcheryakov, 1990). The detailed definition for embryos, juveniles, and immatures was shown in our previous work (Yamanaka *et al.*, 1999).

Azan staining

For fixation, embryos, juveniles and immatures were prepared as whole-animal preparations, while adults were handled as isolated CNSs. They were fixed in 4% paraformaldehyde in phosphate buffer or Bouin's solution, embedded in paraffin, and sectioned at a thickness of 5 μm. The two fixatives gave essentially the same result. We followed a standard protocol of azan staining method (*e.g.* McManus and Mowry, 1960). We estimated the relative volumes of the buccal and cerebral ganglia by weighing the camera-lucida drawings of the stained sections. Thus the unit was arbitrary. The numbers of cells in these ganglia could be determined by counting the dark-red stained nuclei. The number of snails was 3 each, except for the adults (n=2).

Lucifer yellow staining

The CNSs were isolated from juveniles, immatures, and adults, and pinned in a recording dish under HEPES-buffered saline (in mM: NaCl 24.0, KCl 2.0, $CaCl_2$ 4.0, $MgCl_2$ 2.0, NaH_2PO_4 0.1, glucose 0.3, HEPES 35.4, pH 7.9 adjusted by NaOH) at room temperature. Their outer sheath of connective tissue was removed. The CNSs were then treated with 1 mg/ml protease (type XXIV, Sigma, St. Louis, MO) for 3 min for juveniles and 15 min for others, and washed thoroughly. Glass electrodes with a resistance of 25 MΩ in 2 M KAc solution were filled with 5% Lucifer yellow CH (Molecular Probes, Eugene, OR) in 0.1 M LiCl solution. After penetration of a target cell under a light microscope (a transmission type or a stereoscopic type), current pulses of –5 nA lasting 500 msec, repeated at 1 Hz, were used for the injection for 15 min for juveniles and 2 hr for others. The preparations were fixed with 10% formaldehyde in DW, dehydrated by ethanol, and cleared in methyl salicylate. They were then observed with a confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA; 512×512 pixels/section, 10 μm thick optical section). The number of snails was 3 each.

Statistical analyses

Data were expressed as means±SEM. Plots in Fig. 5 were fitted by suitable curves with the software program 'Origin' (ver. 5.0J, Microcal Software, Northampton, MA).

RESULTS

Reconstruction of buccal and cerebral ganglia

We three-dimensionally reconstructed the buccal and cerebral ganglia from the azan-stained sections (Figs. 3 and 4). Meshcheryakov (1990) described that the buccal ganglia are being laid down at stage 23, and that they are connected by a buccal commissure at stage 24. Therefore, we decided to analyze the stage 24 CNS as the first preparation. The cerebral ganglia are connected by their cerebral commissure at stage 22.

The azan staining shown in Fig. 3 revealed the following points. Neuropile (blue) was surrounded with somata (red) in the ganglia. Even though the commissure connects two buccal ganglia at stage 24, the neuropile was not seen well in the azan-stained sections. The cerebral commissure was observed clearly at this stage (Fig. 3A). Accompanied with development, the neuropile was found to occupy the major parts of the buccal and cerebral ganglia. As a result, the commissures lengthened and the left and right ganglia appeared to spread apart.

The geometrical relationship between the oesophagus and the buccal mass was found to be changed accompanied with development (Fig. 4). For this reason, the cerebral ganglia appeared to be located at the more anterior side than the buccal ganglia between the stage 24 and the stage 29 embryos (Fig. 4A–C). After juveniles, the cerebral ganglia occurred in at the more posterior side than the buccal ganglia (Fig. 4D).

Cell number and ganglion volume

Figure 5 shows the increase in the numbers of cells and that in the volumes of the buccal and cerebral ganglia from embryonic stage 24 to adulthood. Both the number and volume were examined in pairs of ganglia. The increase curves in Fig. 5A, C and those in Fig. 5B, D were considered to be saturation curves and parabolas, respectively. Therefore, the number of cells was expressed as a saturation curve of $y=a[1-\exp(-bx)]$, and the volume was expressed as a parabola of $y=cx^d$. Here y is the number of cells or the volume of ganglion, and x is the shell length in mm. The parameters a and b represent the final number of cells in the adults and the degree of increase in the number of cells, respectively. The parameter d represents the change in size of the ganglion against the change in the shell length of developing snails. The parameter c will be discussed later.

We obtained the following values for these parameters. For the number of cells, $a=1.65\times10^3$ and $b=2.00\times10^{-1}$ for the buccal ganglia; $a=11.5\times10^3$ and $b=1.28\times10^{-1}$ for the cerebral ganglia. For the volume, $c=9.46\times10^{-3}$ and $d=2.47$ for the buccal ganglia; $c=50.9\times10^{-3}$ and $d=2.49$ for the cerebral ganglia.

Shape of cerebral giant cell

Lucifer yellow staining revealed that the shapes of CGCs in the juveniles (Fig. 6A) , the immatures (Fig. 6B) and adults (Fig. 6C) were very similar, but these in the juveniles were different in size. The ipsilateral lengths of the axons of CGCs were estimated as about 240 μm, 1000 μm, and 1600 μm in the juveniles, immatures, and adults, respectively. The diameters of CGC somata were about 27 μm, 73 μm, and 95 μm in the juveniles, immatures, and adults, respectively. This estimation for the axon lengths and the diameters of CGC somata was supported by the data for neurons showing 5HT-like immunoreactivity (Yamanaka *et al.*, unpublished observation). Our another experiment using an electrophysiological technique showed that the right and left CGCs electrically coupled in the juveniles and immatures (data not shown) as well as in the adults (McCrohan and Benjamin, 1980b). The coupling between the pair of CGCs may already occur prior to the stage 29 in which the snails can acquire the CTA ability. Embryo CGCs could not be stained with Lucifer yellow, nor electrophysiologically recorded, because of technical limitation. Electrically coupled cells can behave as a single neural unit (McCrohan and Benjamin, 1980a).

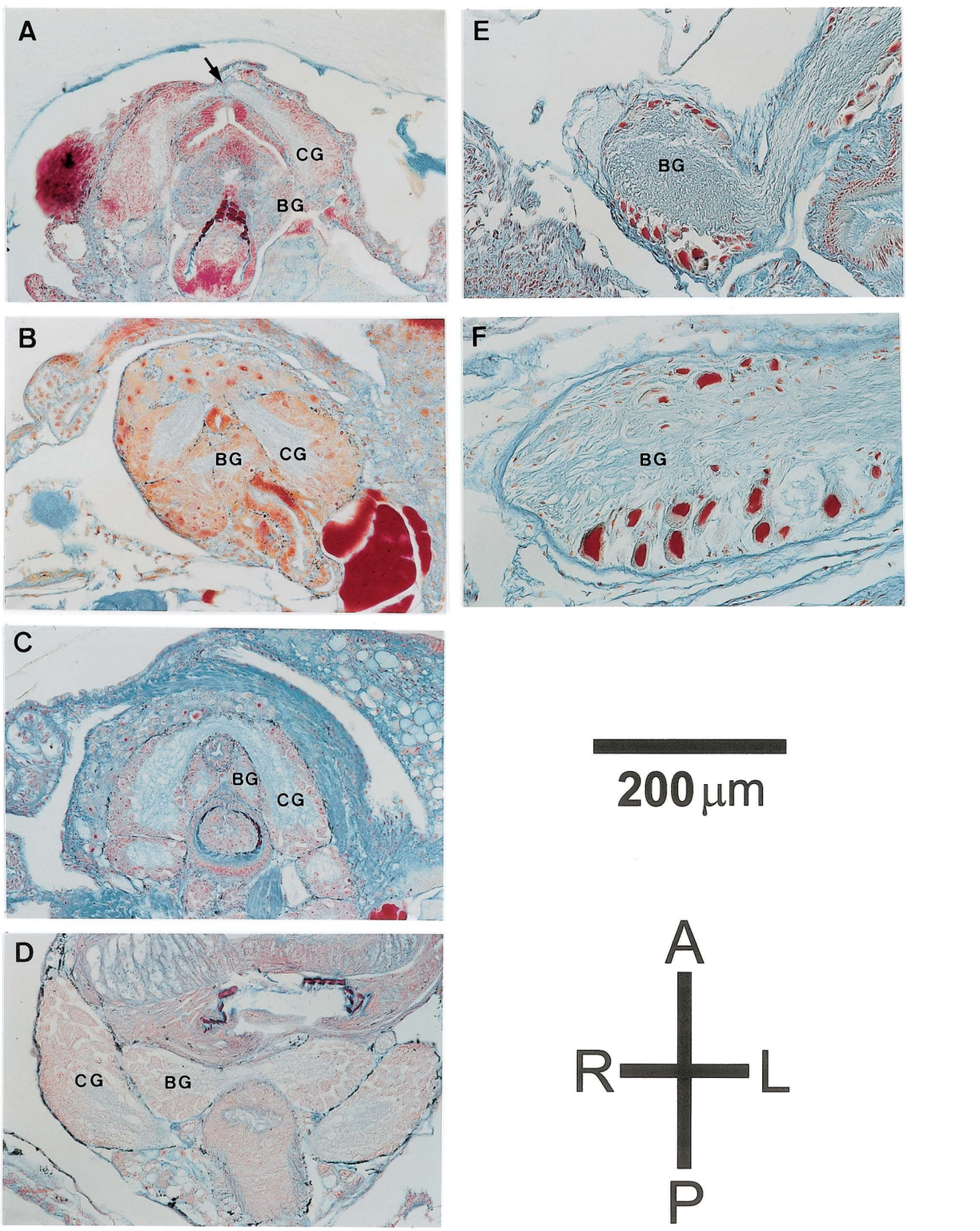

Fig. 3. Azan-stained horizontal sections of the buccal (BG) and cerebral ganglia (CG) in a St. 24 embryo (A), a St. 25 embryo (B), a St. 29 embryo (C), a juvenile (D), an immature (E), and an adult (F). All the pictures were viewed from the ventral side. The nuclei were stained dark red; the cytoplasm was light red; the neuropile was blue.
Cerebral commissure is pointed by an arrow in (A).

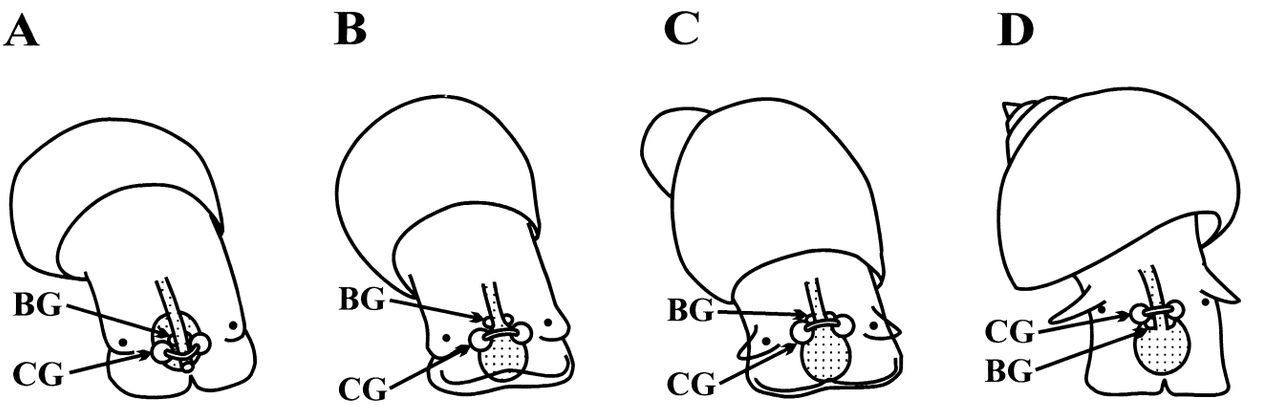

Fig. 4. Schematic drawings of the buccal and cerebral ganglia in the developmental stages. (A) St. 24, (B) St. 25, (C) St. 29, (D) adult. BG: buccal ganglia, CG: cerebral ganglia.

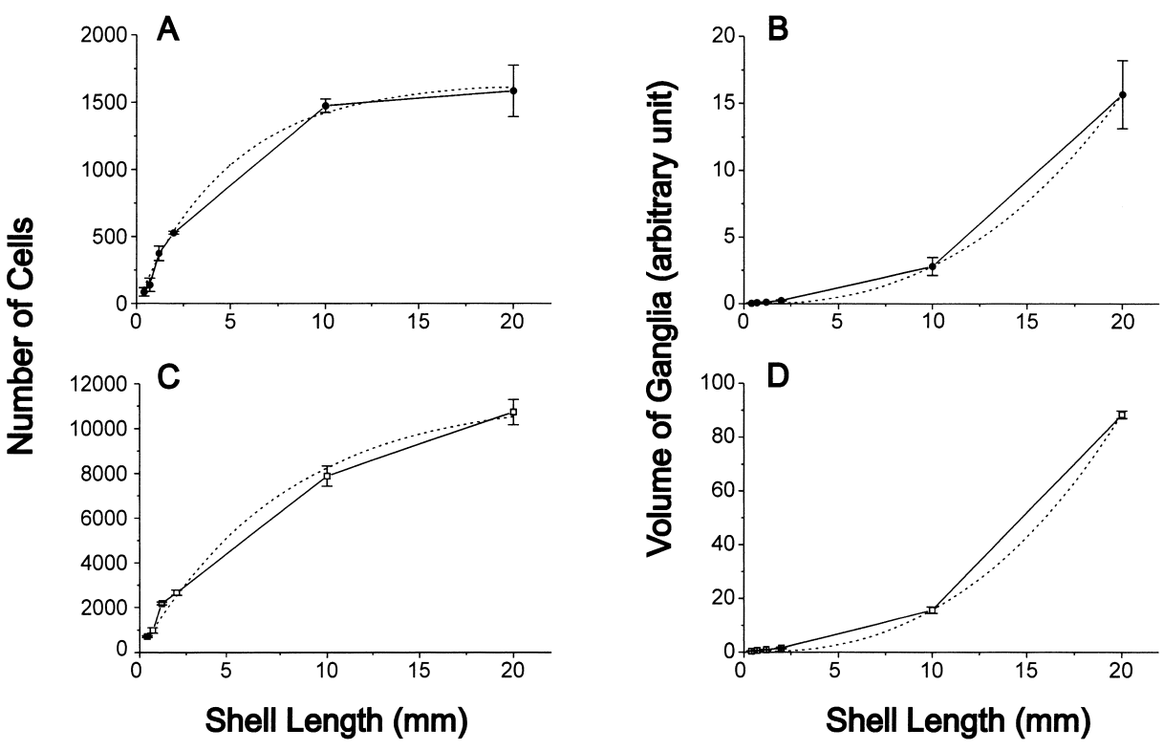

Fig. 5. Increase in the numbers of cells and that in the volumes of the buccal and cerebral ganglia. The shell lengths of 0.4 mm, 0.7 mm, 1.2 mm, 2 mm, 10 mm, and 20 mm correspond to St. 24, St. 25, St. 29 embryos, juveniles, immatures, and adults, respectively. (A) Number of cells in a pair of buccal ganglia. (B) Volume of a pair of buccal ganglia. (C) Number of cells in a pair of cerebral ganglia. (D) Volume of a pair of cerebral ganglia. Dotted curves are suitable saturation curves and parabolas. Note that the unit for the volume is arbitrary (see text).

DISCUSSION

Development of nervous system

The values *a* obtained by curve fitting in Fig. 5A, C indicated that the final number of cells in a pair of buccal ganglia in adulthood is about 1650, and that in a pair of cerebral ganglia is about 11500. The fact that the value *b* for the buccal ganglia was a little larger than that for the cerebral ganglia indicated that the cells in the buccal ganglia emerge earlier than those in the cerebral ganglia (Fig. 5A, C). This is reasonable because motoneurons are located in the buccal ganglia, and interneurons mainly located in the cerebral ganglia. This also corresponds with the behavioral data in which embryos acquired the ability of feeding earlier than the ability of learning (Yamanaka *et al*., 1999). The values *d* for the buccal and cerebral ganglia were almost same. However, the value *c* for the cerebral ganglia was much larger than that for the buccal ganglia. The ratio between these values *c* means the ratio

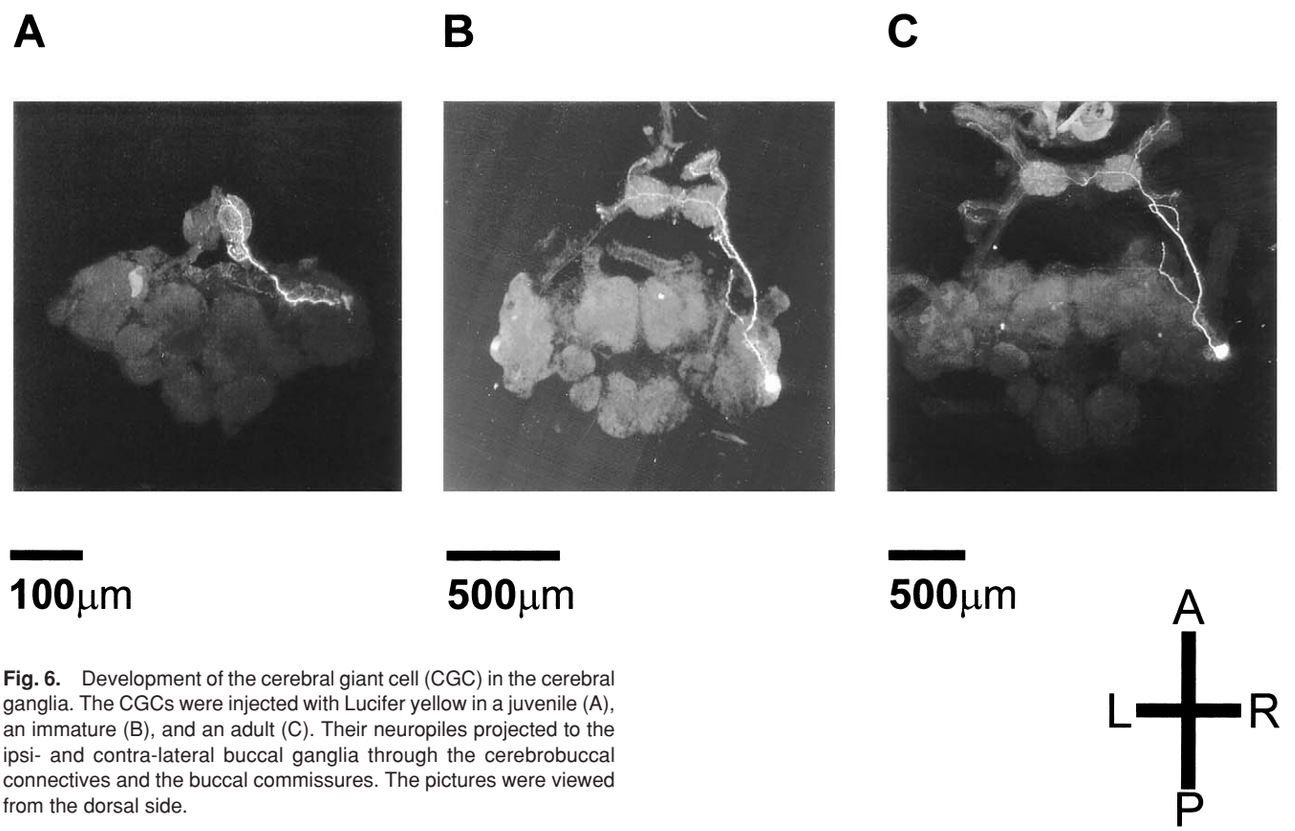

Fig. 6. Development of the cerebral giant cell (CGC) in the cerebral ganglia. The CGCs were injected with Lucifer yellow in a juvenile (A), an immature (B), and an adult (C). Their neuropiles projected to the ipsi- and contra-lateral buccal ganglia through the cerebrobuccal connectives and the buccal commissures. The pictures were viewed from the dorsal side.

between the volumes of the buccal and cerebral ganglia. That is, the volume of the cerebral ganglia is 5.4 times larger than that of the buccal ganglia independent of development.

As seen from Fig. 5A, C, the cells in the buccal and cerebral ganglia increased very much during embryonic stages and juveniles. Most of the cells emerged in the immatures. However, the volume of the buccal and cerebral ganglia still increased from the immatures to adults (Fig. 5B, D). These results suggested that most of the cells indispensable to the feeding and CTA have already emerged at the immatures, but that individual cells in the CNS continue to enlarge even in the adults. Cash and Carew (1988) examined the quantitative analysis of the development of CNS in *Aplysia*, and reported the significant increase in the cell number at a specific developmental stage of juvenile. They proposed that the increasing of interneurons and the maturation of the nervous system may occur in this developmental stage.

Neurotransmitter and key neuron in feeding circuit

In *L. stagnalis*, 5HT acts crucially in the neural circuit controlling feeding rhythm (Kemenes, 1997). The main 5HT-containing neurons involved in the feeding circuit are a pair of CGCs (Sadamoto *et al.*, 1998; Hatakeyama and Ito, 1999), and are known to have synaptic and modulatory actions on neurons in the buccal ganglia (Staras *et al.*, 1998; Nakamura *et al.*, 1999*c*). As described in Introduction, Kojima *et al.* (1997) demonstrated that these CGCs have an important key to form the CTA. In our preliminary work, the 5HT immunoreactivity in the CGCs appears first at stage 29, suggesting that there is a positive correlation between the acquisition of CTA and the role of 5HT (Yamanaka *et al.*, unpublished observation). This also supports the positive correlation between the acquisition of CTA and the functional commencement of CGCs.

Figure 6 showed that the CGCs in the immatures already grow in the almost same shape and size as in the adults. This result indicated that the CGCs grow faster than other cells in the buccal and cerebral ganglia, and thus the maturation of CGCs in the immature stage may be required for a long-term memory of CTA. This faster growth of CGCs is supported by another histological experiment. Croll and Chiasson (1989) compared the postembryonic developments of CGCs and other 5HT-like immunoreactive neurons, and found that the CGCs grow faster than other cells, particularly than large ones.

Development and CTA

The embryos (stage 24 to 29) showed very low levels of voluntary activity (Yamanaka *et al.*, 1999), even though the same peristaltic movement seen in the juveniles and adults was observed. Since Meshcheryakov (1990) described that the foot showed spontaneous movements for the first time at stage 23, we can suppose that the basic network for this foot movement has been already formed in the stage 24 embryos tested here. In any case, they do not need to move widely, because the embryos exist only in the egg.

Our previous result that the feeding response was initiated at embryonic stage 25 (Yamanaka *et al*., 1999) is in good agreement with morphological observations by Meshcheryakov (1990), that is, "The radular cavity is connected to the oral cavity. The jaw has formed completely. Salivary glands have been laid down as diverticula of the oral cavity walls." This description suggests that stage 25 embryos are physically ready to eat as is shown in our CTA experiments.

The last two critical stages (stage 29 embryos and immatures), the formation of CTA and its long-term memory, appear to correspond well to the life cycle of snails (Yamanaka *et al*., 1999). The CTA was acquired just before hatching (stage 29 embryos), and its long-term memory began to be formed just before sexual maturation (immatures). The stage 29 embryos may become prepared to safely seek out food in an external environment by acquiring learning ability. After hatching, the voluntary activity in snails increased to accompany their somatic development with sexual maturation. The immatures just before sexual maturation showed strong voluntary activity, suggesting that snails must become 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.

Relationships between other behaviors and development

Lukowiak and colleagues demonstrated behavioral and cellular changes that occurred during operant conditioning of the aerial respiratory behavior in *L. stagnalis* (Spencer *et al*., 1999). The developmental study of this respiratory behavior was also examined (Hermann and Bulloch, 1998). Rearing snails from eggs to adulthood while preventing lung respiration showed that *L. stagnalis* can develop and survive without pulmonary respiration. Such a developmental plasticity is a very useful model system for understanding the mechanisms of the operant conditioning of respiratory behavior.

In addition, the withdrawal response in *L. stagnalis* was also utilized in some learning, for example visual and vestibular associative learning (Sakakibara *et al*., 1998) and operantly escape conditioning (Kobayashi *et al*., 1998). The major ganglia involved in the withdrawal response are the pedal ganglia, and the cellular mechanisms for this response in the pedal ganglia have been partially disclosed (Inoue *et al*., 1996a, b). If the cell number and volume in the pedal ganglia are examined, the anatomical substrate for the learning concerning the withdrawal response will be prepared.

Conclusions

The pond snail, *Lymnaea stagnalis*, proved to be a useful preparation for analyzing the development of learning and memory on both behavioral and cellular levels. An important issue in this analysis concerns the anatomical substrate upon which learning is superposed during development. We examined the numbers of cells and the volumes of the buccal and cerebral ganglia from embryonic stage 24 to adulthood. The present findings suggested that most of the cells indispensable to the feeding and CTA in *L. stagnalis* emerge at the immature stage, in which the snails can use a long-term memory for the CTA, but that individual cells in the CNS continue to enlarge even in adulthood. Furthermore, a pair of key neurons for the CTA, cerebral giant cells, mature at the immature stage. The present study provided the anatomical substrate upon the long-term CTA, by which the snails that are still sexually immature can eat safe food in a wide territory.

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