Cerebral CBM1 Neuron Contributes to Synaptic Modulation Appearing During Rejection of Seaweed in Aplysia kurodai

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Narusuye, Kenji, and Tatsumi Nagahama. Cerebral CBM1 neuron contributes to synaptic modulation appearing during rejection of seaweed in Aplysia kurodai. J Neurophysiol 88: 2778–2795, 2002; 10.1152/jn.00757.2001. The Japanese species Aplysia kurodai feeds well on Ulva but rejects Gelidium with distinctive rhythmic patterned movements of the jaws and radula. We have previously shown that the patterned jaw movements during the rejection of Gelidium might be caused by long-lasting suppression of the monosynaptic transmission from the multiaction MA neurons to the jaw-closing (JC) motor neurons in the buccal ganglia and that the modulation might be directly produced by some cerebral neurons. In the present paper, we have identified a pair of catecholaminergic neurons (CBM1) in bilateral cerebral M clusters. The CBM1, probably equivalent to CBE-1 in A. californica, simultaneously produced monosynaptic excitatory postsynaptic potentials (EPSPs) in the MA and JC neurons. Firing of the CBM1 reduced the size of the inhibitory post synaptic currents (IPSCs) in the JC neuron, evoked by the MA spikes, for >100 s. Moreover, the application of dopamine mimicked the CBM1 modulatory effects and pretreatment with a D1 antagonist, SCH23390, blocked the modulatory effects induced by dopamine. It could also largely block the modulatory effects induced by the CBM1 firing. These results suggest that the CBM1 may directly modulate the synaptic transmission by releasing dopamine. Moreover, we explored the CBM1 spike activity induced by taste stimulation of the animal lips with seaweed extracts by the use of calcium imaging. The calcium-sensitive dye, Calcium Green-1, was iontophoretically loaded into a cell body of the CBM1 using a microelectrode. Application of either Ulva or Gelidium extract to the lips increased the fluorescence intensity, but the Gelidium extract always induced a larger change in fluorescence compared with the Ulva extract, although the solution used induced the maximum spike responses of the CBM1 for each of the seaweed extracts. When the firing frequency of the CBM1 activity after taste stimulation was estimated, the Gelidium extract induced a spike activity of ~30 spikes/s while the Ulva extract induced an activity of ~20 spikes/s, consistent with the effective firing frequency (>25 spikes/s) for the synaptic modulation. These results suggest that the CBM1 may be one of the cerebral neurons contributing to the modulation of the basic feeding circuits for rejection induced by the taste of seaweeds such as Gelidium.

I N T R O D U C T I O N

It has been well established that the neural circuitry in the animal brain is flexible and will be reconfigured according to various kinds of behaviors. One of the important general questions in neurobiology is how a specific sensory modality can alter behaviors by reconfiguring a well-defined neural network. The cellular mechanism for the modulation of neural circuits by mechanoafferent sensory inputs has been well studied in both vertebrate and invertebrate central pattern generator (CPG) networks (Grillner et al. 1995; Katz and Frost 1996; Marder and Calabrese 1996; Pearson 1993, 2000; Rosen et al. 2000a,b). However, the way in which specific types of sensory inputs, such as odors or tastes, can modulate the neural network has not yet been elucidated because of the complex processing mechanism.

In food preference behaviors, animals recognize an odor or a taste, decide between liking and aversion often with help of their memory, and sometimes change their preference by learning the taste. After this processing, the animals show multiple behaviors such as ingestion or rejection. Therefore the study of the neural mechanism for food preference can potentially contribute to our understanding of the reconfiguration of neural circuits according to a specific sensory modality. The gastropod mollusks are very useful models for the study of the neuronal basis of such complex behaviors (Kandel 1979). The behaviors have been well studied for odor preference (Audesirk 1975; Croll and Chase 1977, 1980; Peschel et al. 1996; Teyke 1995; Teyke et al. 1992; Willows 1978; Yamada et al. 1992) and taste preference (Chang and Gelperin 1980; Culligan and Gelperin 1983; Kemenes et al. 1986; Kupfermann and Carew 1974). For taste preference, the learning behaviors that associate specific tastes and textures with inedibility have been well studied (Botzer et al. 1998; Schwarz and Susswein 1986; Schwarz et al. 1988; Susswein et al. 1986). The processing neural network has also been well studied for odor preference (Gelperin and Tank 1990; Gelperin et al. 1993; Schütt and Basar 1994). In contrast, the neuronal basis of taste preference has been little studied except for the neural pathway for the chemosensory inputs of the primary food (Bicker et al. 1982; Delaney and Gelperin 1990; Kemenes et al. 1986, 2001; Rosen et al. 1991).

The marine gastropod Aplysia has been reported to show clear food preferences (Audesirk 1975; Carefoot 1967, 1970; Kupfermann and Carew 1974, Nagahama and Shin 1998). In Aplysia californica, the responses of the cerebral neurons to chemical taste stimulation have also been explored (Chiel et al. 1986; Fredman and Jahan-Parwar 1980; Jahan-Parwar 1972; Rosen et al. 1991; Teyke et al. 1990; Weiss et al. 1978, 1986a).
Moreover, the neural mechanisms generating ingestion and rejection have been well studied. In this case, however, rejection is usually induced by tactile stimulation with objects such as polyethylene tubing (Chiel et al. 1986; Church and Lloyd 1994; Hurwitz et al. 1996; Kabotyanski et al. 1998; Kupfermann 1974; Morton and Chiel 1993a,b; Rosen et al. 1991, 2000b; Weiss et al. 1986a). In a Japanese species, A. kurodai, we have recently found that the animals feed well on Ulva but reject Gelidium or Pachydictyon with distinctive rhythmic patterned movements of the jaws and radula (Nagahama and Shin 1998). These responses can be induced not only by the natural seaweeds but also by extract solutions, suggesting that ingestion or rejection could be induced by taste alone. Therefore our system may be appropriate for the study of the neural mechanism generating taste preference behaviors.

We have previously demonstrated that the difference in patterned jaw movements during ingestion and rejection might be caused by a change in the onset time of the firing of the jaw-closing (JC) motor neurons at each depolarizing phase of the rhythmic responses (Nagahama and Shin 1998). During ingestion, delay of the onset time of the JC firing may be produced by the firing of multiaction MA neurons which monosynaptically inhibit the JC motor neurons (Nagahama and Takata 1988–1990). The MA neurons are probably equivalent to the B4/5 in A. californica (Gardner 1971). In an earlier report (Nagahama et al. 1999), we also demonstrated that advance of the onset time of the JC firing during rejection of Gelidium might be caused by transient suppression of the synaptic transmission from the MA neurons to the JC motor neurons. Our results also suggested that some cerebral neurons might directly modulate this synaptic transmission. Therefore in the current experiments we tried to find such cerebral neurons contributing to the reconfiguration of the basic feeding circuits for rejection of Gelidium. The purpose of the present study is to understand how the CPG network generating the firing patterns in the JC motor neurons is modulated by particular neurons activated by the taste of the seaweed.

**METHODS**

**Animals and seaweed**

*A. kurodai*, weighing 30–400 g, were collected from the coast of Awaji Island or Fukui City. Animals were maintained at 14–16°C in aquaria filled with aerated and filtered artificial seawater (ASW). Two species of seaweed, *Ulva pertusa* (*Ulva*) and *Gelidium amansii* (*Gelidium*) were also collected at a location with many animals. These were maintained at room temperature in aquaria filled with ASW or stored below −20°C in a freezer, respectively. *Ulva* was used for animal food and was supplied once every day.

**Preparations**

In the present experiments, the reduced preparation was used as previously described (Nagahama et al. 1999). The animals were anesthetized by injection of isotonic MgCl\textsubscript{2} solution (25% of body weight) into the body cavity and dissected at room temperature. The preparation consisted of the head structures, cerebral ganglia, buccal ganglia, and buccal mass. The head structures, innervated by the upper labial (ULAB), anterior tentacular (AT), and lower labial (LLAB) nerves arising from the cerebral ganglia, included the anterior tentacles, lips, and perioral zone. The cerebral nerves, ULAB, AT, LLAB, and cerebral-buccal connectives (CBC), were left intact and other nerves were severed. The buccal mass was removed from the head structures and then cut into halves along the midline to separate the paired symmetrical buccal musculature innervated by buccal nerves 2 and 3 (n2 and n3). The peripheral buccal nerves, except n2 and n3, were severed. The nomenclature of the buccal nerves followed Gardner (1971). The head structures, cerebral ganglia, buccal ganglia, and paired buccal mass were separately pinned to the silicone elastomer (Sylgard, Dow) surface of each compartment of a Lucite recording chamber, and petroleum jelly (Vaseline) was placed on the partitions between the compartments as previously reported (Nagahama et al. 1999). The head structures were pinned with the ventral surface upward in a way that revealed the inner portion of the lips and the perioral zone. The cerebral ganglia and the buccal ganglia were pinned with the ventral surface upward or caudal surface upward, respectively. The sheath overlaying the cerebral and buccal ganglia was surgically removed. The anterior aorta supplying the tentacle-lip region was cannulated with polyethylene tubing (tip diameter, 50–100 μm) and perfused with fresh ASW (0.5–0.7 ml/min).

**Taste solutions**

For taste stimulation of the lips, solutions of freshly prepared seaweed extract mixed with ASW were used as previously described (Nagahama and Shin 1998; Nagahama et al. 1999). A 20 g sample of fresh seaweed was ground in a mortar and soaked for 30 min in 30 ml (Ulva) or 40 ml (Gelidium) of ASW at room temperature. The mixture was centrifuged (12,000 g; 20 min), and the supernatant was used for the taste stimulation. In the text, they are described as the original extract solutions (solutions with the original concentration). The *Ulva* extract was used for favorite taste that induced the ingestion-like response, whereas the *Gelidium* extract was used for aversive taste that induced the rejection-like response. These original extract solutions were sufficient to induce the stable rhythmic responses, whereas solutions diluted with ASW often failed to induce the rhythmic responses (Nagahama and Shin 1998). In the present experiments, we used two sizes of chambers depending on the size of the preparation, and the free bath sizes for taste stimulation around the head structure were 2–3 and 3–6 ml, respectively. A constant volume (1–2 ml) of seaweed extract solutions for each preparation was gently applied into the bath solution near the lips with a Pasteur pipette to avoid tactile stimulation. In this procedure, the stimulus solutions were gradually diluted by diffusion, and the resultant responses also tended to decrease with time. Therefore we usually compared the initial responses for 5 s just after onset of stimulation. After the end of the recording, the lip region was completely washed with a sufficient amount of fresh ASW by aspirating the overflow and the next chemical stimulation was given 25–30 min later. The two kinds of extract solutions were applied in a random order and more than two trials each were usually performed. In these experiments, we could usually repeat chemical stimulation four to five times in the same preparation.

**Electrophysiology**

Recordings from individual neurons were performed using conventional electrophysiological techniques. For intracellular recording and stimulation, neurons were impaled with glass microelectrodes filled with 2 M potassium acetate. These electrodes were beveled to 5–7 MΩ. For intracellular recording and dye injection for the calcium imaging, glass microelectrodes filled with 4% Calcium Green-1 hexapotassium salt (Molecular Probes) in distilled water (10–20 MΩ) were used. Extracellular recordings of the nerve activity were made with flexible polyethylene suction electrodes (tip diameter, 100–150 μm) placed on the CBC. Similar suction electrodes were used for electrical stimulation of nerve trunks. The JC motor neuron was also voltage-clamped by a two-microelectrode method. Two electrodes were filled with 2 M potassium acetate (voltage recording electrode, 5–7 MΩ; current-passing electrode, 2–4 MΩ). Membrane currents
were read as the voltage drop across a resistor (1 MΩ) interposed in the feedback loop (chassis ground mode). In these experiments, the reversal potential of the inhibitory postsynaptic currents (IPSCs) produced in the JC motor neurons maintained a stable level throughout the experiment.

The composition of ASW used in the experiments was as follows (in mM): 470 NaCl, 11 KCl, 11 CaCl₂, 25 MgCl₂, 25 MgSO₄, and 10 Tris-HCl (pH 7.8–7.9). In some experiments, polysynaptic pathways were suppressed using a 5 × Ca²⁺, 2 × Mg²⁺ solution or a 3 × Ca²⁺, 3 × Mg²⁺ solution, in which the Ca²⁺ and Mg²⁺ concentrations were raised five- and twofold, respectively, or threefold in both by replacement of Na⁺ to maintain the osmotic balance. In the 5 × Ca²⁺, 2 × Mg²⁺ solution, we obtained stable firing of the MA neuron with constant depolarizing current and large IPSCs in the JC motor neurons, but the firing of the CBM1 frequently induced the MA firing resulting from the large EPSPs (see Results). Therefore this solution was incomplete for demonstrating direct effects of the CBM1 on the synaptic modulation. On the other hand, in the 3 × Ca²⁺, 3 × Mg²⁺ solution, the stable firing of the neuron with constant depolarizing current was hard to obtain, but the firing of the CBM1 could not induce the MA firing, suggesting that this solution could completely suppress the polysynaptic pathways. Therefore in the present experiments, we mainly used a 3 × Ca²⁺, 3 × Mg²⁺ solution to demonstrate the direct effect, and subsequently showed that a similar effect of the CBM1 could be obtained even in a 5 × Ca²⁺, 2 × Mg²⁺ solution.

Biocytin backfilling and paraformaldehyde-glutaraldehyde histofluorescence

The locations of the cell bodies of the neurons in the cerebral ganglia that send axons to the buccal ganglia were determined by backfilling the CBC with biocytin following the method of Xin et al. (1999). The cerebral ganglia were dissected from the animal and pinned to the Sylgard bottom of a chamber filled with ASW. The cut end of the CBC attached to the cerebral ganglia was sucked into the polyethylene tubing and the ASW in the tubing was replaced with 3% biocytin (Molecular Probes) in 50 mM NaHCO₃ (pH 8.0). The ganglia were maintained in ASW containing 1 mM probenecid at 4°C for 2 days. After a brief wash in ASW, the ganglia were fixed in 4% paraformaldehyde and 30% sucrose in 0.1 M phosphate-buffered saline (PBS, pH 7.6) at room temperature for 2 h. The ganglia were washed several times in 0.1 M PBS, each time for >30 min at 4°C. The preparation was permeabilized by overnight incubation in 0.1 M PBS containing 2% Triton X-100 at 4°C and then incubated in 50 μg/ml streptavidin rhodamine conjugate (Molecular Probes) in PBS Triton at 4°C for 12–24 h. After washing the ganglia in 0.1 M PBS for 2–3 days, the preparation was cleared in PBS/glycerol (1:6). The ganglia were viewed and photographed with an Olympus fluorescence microscope fitted with a rhodamine filter set (excitation filter BP520–550, dichroic mirror DM565, emission filter BA580IF). To observe catecholamine-containing neurons, the cerebral ganglia were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde, and 30% sucrose in 0.1 M PBS (paraformaldehyde-glutaraldehyde (FaGlu) method) (Furness et al., 1997; Goldstein and Schwartz, 1989) for 2–4 h at room temperature. The preparations were then viewed with the appropriate filter set (excitation filter BP400–440, dichroic mirror DM455, emission filter BA475) under which catecholamine fluorescence appears blue-green. In some preparations, the biocytin backfilling method and the FaGlu method were performed simultaneously. The FaGlu fixative solution was then used instead of the normal one, and the preparations were viewed with different filter sets for rhodamine and catecholamine.

Calcium imaging

When we explored the spike response of the newly identified CBM1 to taste stimulation of the lips with seaweed extract in a single preparation, the application was repeated four to five times after alternate washing with ASW for >2 h. The electrophysiological procedure is very difficult to use for this because a microelectrode cannot be kept in the cell body for a long time. However, we recently demonstrated that the calcium imaging method was very useful for detecting the spike activity of Aplysia neurons (Yoshida et al. 2001). Once a calcium-sensitive dye had been iontophoresically introduced into a cell body of the neuron with a microelectrode containing the dye, we could detect the spike activity of the neuron for a long time. The spike activity simply induced a fluorescence increase in the cell body, in which the slope of the fluorescence increase was almost relative to the spike frequency and the period of the increase almost corresponded to the firing period. We also demonstrated that the fluorescence intensity and the maximum change in the intensity after the spike activity tended to depend on the intracellular dye concentration. However, these differences did not affect the quantitative detection of the spike activity in each cell. Therefore in the present experiments, this method was used for the detection of the CBM1 spike activity to taste stimulation, and after the experiments were complete, the slope and the period of the fluorescence increase were analyzed in detail.

The calcium imaging was performed as described in our earlier report (Yoshida et al. 2001). A glass microelectrode filled with 4% Calcium Green-1 hexapotassium salt solution was impaled into a cell body of the neuron. After identification of the neuron with the electrophysiological procedure, a hyperpolarizing current (5–10 nA) was passed through the membrane for 2–5 min to introduce the dye into the cell body. The recording chamber was mounted on the stage of a fluorescence microscope (Olympus, BX50WI). A Spectra MASTER (Olympus) was used as the light source at 488 nm. The emission fluorescence passed through a dichroic reflector (DM510) and a BA515 barrier filter was detected. The fluorescence image was visualized with a ×10 objective (0.3 NA) and acquired by a cooled CCD camera (Olympix FE250). A sequence of images was stored on the MERLIN imaging system (Olympus), in which each image was usually binned in 3 × 3 blocks to enhance sensitivity, and images of 128 × 128 pixels were acquired at 20 frames/s for 120–150 s. The brightness and contrast levels were adjusted to achieve minimal background fluorescence and sufficient dynamic range for increases in fluorescence. In the data analyses, circular region of interests (ROIs) were defined at the region of a cell body of the CB neuron. A change in fluorescence was expressed as ΔF/F (as a percentage), where F is the average fluorescence intensity at the resting state before stimulation and ΔF is the time-dependent change in fluorescence. In plotting the change in fluorescence against the time course, the representative ΔF/F every 0.5 s was obtained by averaging the ΔF/F over 11 images, including five images before and after the 0.5 s image, to reduce data dispersion owing to apparatus noise. When the initial slope for 5 s was calculated, the representative ΔF/F every 0.25 s was obtained by averaging the ΔF/F over five images before and after the 0.25 s image, and each slope obtained from the increment of ΔF/F for the neighboring representative images was averaged over 5 s. After the calcium imaging experiments, the ganglia were fixed with a FaGlu solution to confirm whether the measured neurons contained catecholamine.

RESULTS

Candidates for cerebral modulatory neurons

Our previous results (Nagahama et al. 1999) suggested that the synaptic transmission from the MA neurons to the JC motor neurons may be selectively suppressed during rejection of Gelidium and that the modulatory action of some cerebral neurons projecting axons to the buccal gan-
glia may directly contribute to the decrement of the synaptic efficacy. In the present experiments, we tried to identify such cerebral modulatory neurons. The locations of the cell bodies of the candidates were determined by backfilling the CBC with biocytin. The cell bodies of *Aplysia* cerebral neurons have been reported to be concentrated into several clusters (Jahan-Parwar and Fredman 1976; Ono and McComas 1980; Rosen et al. 1979). In the present experiments, the cell bodies in the G, M, and E clusters were mostly stained (n = 25). We also obtained mirror images for the results of backfilling either the left or right CBC. A typical result is shown in Fig. 1A. In the ipsilateral G cluster located in the anterior portion of the ganglia, one large cell body (diameter >200 μm) immunoreactive to serotonin (unpublished data) was stained. This neuron may be equivalent to the metacebral cells (MCCs) in *A. californica* (Weiss and Kupfermann 1976; Weiss et al. 1978) on the basis of the location of the cell body and the axonal projection to the CBC and the LLAB nerves. A small cell body of the neuron was also stained on the dorsal side within the same cluster. In the ipsilateral M cluster located at the separation between the ULAB and the AT nerves, four cell bodies were stained on the ventral side and one cell body on the dorsal side (Fig. 1D). In addition, one cell body was also stained on the ventral side in the contralateral M cluster (Fig. 1C). In the ipsilateral E cluster, several cell bodies were stained on both the ventral and dorsal sides. No cell bodies could be stained in the J and K clusters (n = 25) where the cell bodies of some mechanosensory neurons (ICBMs) sending axons to the CBCs are located in *A. californica* (Rosen et al. 1982). In the present experiments, we termed the backfilled cerebral neurons in the G, M, and E clusters CB neurons (cerebral-buccal neurons) except for the large serotonergic neuron. These neurons may be equivalent to the cerebral-to-buccal interneurons (CBIs) in *A. californica* (Hurwitz et al. 1999; Perrins and Weiss 1998; Rosen et al. 1991; Xin et al. 1999). Moreover, the CBIs in the M cluster have been reported to have command-like or modulatory functions (Hurwitz et al. 1999; Rosen et al. 1991, 2000a). Therefore in the present experiments, we addressed the CB neurons in the M cluster (CBM) as the candidates and explored whether either of these CBM neurons has modulatory functions for suppressing the synaptic transmission at the MA-JC connections.

**Cateteholamine-containing neurons**

A pair of catecholaminergic neurons (CBI-1) has been reported in the M cluster of *A. californica* (Rosen et al. 1991). In *A. kurodai*, we explored the location of the cell bodies of the catecholamine-containing CBM neurons by the use of the FaGlu method in combination with the biocytin backfilling (n = 24). Among four cell bodies of the ipsilateral CBM neurons on the ventral side (Fig. 2B), only one cell body in the most anterior portion exhibited catecholamine-like histochemistry (blue-green fluorescence, Fig. 2D), and one cell body of the contralateral CBM also exhibited similarity fluorescence (Fig. 2A and C). Considering the mirror images for backfilling of the left or right CBC, these ipsilateral and contralateral neurons may belong to the same type of neurons, and they may send their axons to the buccal ganglia via the bilateral CBCs. These neurons may be equivalent to the CBI-1 in *A. californica*, and we termed these neurons CBM1s to avoid confusion between the animal species (Fig. 2, E and F). We termed the other CBM neurons...
of the CBm1 was useful for us to identify by the electrophysiological procedure. Intracellular stimulation of the CBm1 by passing a depolarizing current through the membrane simultaneously produced extracellular nerve activity in the bilateral CBCs following the evoked neuronal spikes one for one (Fig. 4A). In addition, we found that firing of the CBm1 produced excitatory postsynaptic potentials (EPSPs) in the MA and JC neurons in the ipsilateral buccal ganglion. Even when the cerebral and buccal ganglia were bathed in a \( 3 \times \text{Ca}^{2+}, 3 \times \text{Mg}^{2+} \) solution, these EPSPs followed the neuronal spikes one for one with constant latency [25.0 ± 0.2 (SE) ms, \( n = 8 \) in Fig. 4B], suggesting a monosynaptic connection with the ipsilateral MA and JC neurons. It was noted that the size of the EPSPs induced in the MA neurons was always larger (~3 times) than that in the JC neurons. Additional certification was often performed after the double-labeling procedure when only electrophysiological experiments were performed. In this procedure, we impaled the cell body of the CBm1 with a microelectrode filled with a rhodamine-lissamine dye. After the electrophysiological experiments, the dye was iontophoretically injected into the cell body and the tissue was subsequently fixed with a FaGlu mixture. As shown in Fig. 3, the positively identified CBm1 showed both rhodamine (red; B) and catecholamine-like (blue-green; C) fluorescence.

**Identification of CBm1**

Figure 3A shows examples of fluorescence images of the CBm1 filled with 5(6)-carboxyfluorescein which was injected iontophoretically into the cell body from a micropipette. The CBm1 sent bifurcated axons to the ipsi- and contralateral CBCs and had many dendritic trees extending toward the central region of the hemiganglion. The structure
Modulatory effects of CBM1 on MA-induced IPSCs

We first explored whether the CBM1 neuron had modulatory effects on the MA-induced IPSPs in the JC motor neurons. In these experiments, the cerebral and buccal ganglia were bathed in a 3 × Ca^{2+}, 3 × Mg^{2+} solution to suppress polysynaptic activity. The JC motor neurons were voltage-clamped to remove the effects of membrane potential changes induced by CBM1 firing on the IPSP sizes. The holding potential was −40 mV because the inhibitory effects of the MA neurons on the JC motor neurons during the rhythmic feeding response usually appeared near the firing threshold of the JC motor neurons. A single spike was repetitively evoked in the MA neuron with a constant depolarizing current every 10 s, and the size of the inhibitory postsynaptic currents (IPSCs) produced in the JC motor neurons was compared before and after firing of the CBM1 neuron for 30 s. A typical result (n = 19) is shown in Fig. 5A. When the CBM1 neuron was fired, the MA neuron largely depolarized, and inward currents were also induced in the JC motor neurons, corresponding to the monosynaptic excitation of these neurons by the CBM1. Some examples of the faster recordings for each individual IPSC in the same experiment are shown in Fig. 6B, together with those for the control experiment in the absence of the CBM1 firing in the same preparation (Fig. 6A). The test immediately prior to the onset of the CBM1 firing was assigned as time 0 and the size of the sampled IPSC relative to that at time 0 was obtained every 10 s. During depolarization induced by the CBM1 firing, MA neuron often evoked more than two spikes (Fig. 5B) and in those cases the first IPSC was sampled. Figure 7 shows time courses of the relative IPSC sizes for two trials of the CBM1 firing (○, 41.1 spikes/s; △, 42.3 spikes/s on average) together with the control data in the same preparation. As previously reported (Nagahama et al. 1999), even in the control the relative IPSC size gradually decreased with time (●). In contrast, after the CBM1 firing, the relative IPSC size rapidly decreased and attained the minimum level (<70%) within 40 s. In comparison with the control data, it was found that this suppressive effect was often prolonged to >100 s. Similar experiments were repeated in nine preparations when the CBM1 was fired at −40 spikes/s for 30 s, and the minimum size of the IPSCs relative to the control value at that time and the minimum-attaining time after onset of the CBM1 firing were averaged over all preparations. In the CBM1 firing at 40.1 ± 1.1 (SE) spikes/s, the average minimum size of the IPSCs was 67.9 ± 2.6% and the average minimum-attaining time was 40.8 ± 3.7 s.

These results suggest that the CBM1 neuron may have a direct modulatory effect on the synaptic transmission at the
MA-JC connections and may largely suppress the inhibitory effects of the MA neurons on the JC motor neurons.

Effects of the firing frequency of the CBM1 on the synaptic modulation

We further explored the effects of the firing frequency of the CBM1 neuron on the change in IPSC size by bathing the preparation in a $3\times\text{Ca}^{2+}, 3\times\text{Mg}^{2+}$ solution. In seven preparations, the effects of the CBM1 firing at low frequency and high frequency for 30 s were compared. Then the strength of the depolarizing current was adjusted for 30 s to evoke the CBM1 firing at 15–25 and 35–45 spikes/s on average in each preparation. A typical result for the two firing frequencies is shown in Fig. 8A. The CBM1 firing at high frequency (44.3 spikes/s on average) induced a large decrease in the IPSC size, whereas the firing at low frequency (19.0 spikes/s) induced a slight decrease. Figure 8B shows the minimum IPSC size averaged over all preparations for the two types of frequencies. The average values were $94.0 \pm 2.1\%$ at $18.9 \pm 1.3$ spikes/s and $68.0 \pm 2.6\%$ at $40.1 \pm 1.1$ spikes/s, and the difference was significant ($P < 0.001$, paired t-test, $n = 7$). In contrast, the

FIG. 6. Modulation of the MA-induced IPSC in the JC motor neuron by spike activity of the CBM1. A time series of the IPSCs in the absence (control, $A$) or presence ($B$) of the CBM1 firing in the same preparation. These data were obtained from the same preparation as shown in Fig. 5.

MA-JC connections and may largely suppress the inhibitory effects of the MA neurons on the JC motor neurons.

FIG. 7. Time courses of the relative sizes of the MA-induced IPSCs in the JC motor neuron before and after firing of the CBM1 (bar). The peak size of the IPSCs was measured every 10 s for 2 trials in the preparation as shown in Fig. 5, $\bigcirc$ and $\triangle$, the results obtained from the 1st and 2nd trials, respectively. The 2nd trial was performed 10 min after the 1st trial.

FIG. 8. Dependence of synaptic modulation on the firing frequency of the CBM1. $A$: time courses of the relative sizes of the MA-induced IPSCs in the JC motor neuron before and after the CBM1 firing (bar) at low and high frequencies (19.0 spikes/s, $\bigcirc$, 44.3 spikes/s, $\triangledown$) in the same preparation. $\bullet$, the control data in the absence of the CBM1 firing. $B$: comparison of the modulatory effects of the CBM1 averaged over all preparations ($n = 7$) for low (18.9 $\pm$ 1.3 spikes/s) and high (40.1 $\pm$ 1.1 spikes/s) firing frequencies. Bars show the SE. $C$: relationship between the average frequency of the CBM1 firing and the minimum IPSC size. These data were obtained from preparations bathed in a $3\times\text{Ca}^{2+}, 3\times\text{Mg}^{2+}$ solution ($\bigcirc$) and a $5\times\text{Ca}^{2+}, 2\times\text{Mg}^{2+}$ solution ($\bullet$).
difference between the control and the minimum IPSC value for the low frequency was less significant (P < 0.05, paired t-test). The average value of the minimum-attaining time was 41.3 ± 2.3 s for the high frequency and 40.0 ± 3.6 s for the low frequency with no significant difference (P > 0.1, paired t-test). These results suggest that the synaptic modulation largely depends on the firing frequency of the CBm1 neuron with the constant minimum-attaining time.

To explore the dependence of the synaptic modulation on the firing frequency of the CBm1 in more detail, the minimum IPSC sizes obtained from all preparations bathed in a 3 × Ca²⁺, 3 × Mg²⁺ solution were plotted against the raw data of the firing frequency in Fig. 8C (C). It appears that the modulatory effect was very small at firing frequencies <25 spikes/s and largely increased above this frequency. This figure also shows data obtained from the preparations bathed in a 5 × Ca²⁺, 2 × Mg²⁺ solution (●). As described in the METHODS, in this solution the CBm1 firing at high frequency frequently induced spike activity of the MA neuron, indicating the incomplete effect of this solution in blocking polysynaptic pathways. Figure 8C shows little difference between the data for the two kinds of solution, suggesting that direct modulatory effects were mainly obtained even in a 5 × Ca²⁺, 2 × Mg²⁺ solution.

Modulatory chemicals released from the CBm1 neuron

In the next step, the chemicals contributing to the synaptic modulation were explored. The results obtained from the FaGlu experiments suggested that the CBm1 contained catecholamines and may release these for the synaptic modulation. The most likely candidate is dopamine, by analogy with the CBI-1 in A. californica (Rosen et al. 1991), and we therefore explored the effects of this chemical on the IPSC size. Bath application of 5 × 10⁻⁵ M dopamine largely reduced the size of the IPSC, mimicking the CBm1 effects, and washing out with the control solution almost recovered the size (Fig. 9A). The average values of the relative minimum size of IPSCs reduced by dopamine was 73.5 ± 1.5% (n = 10). It was found that the modulatory effects of dopamine were obviously induced at concentrations of >10⁻⁵ M.

Several dopamine antagonists were also used to verify the contribution of dopamine. In A. californica, methylergonovine has been reported to affect dopaminergic transmission (Ascher 1972; Nargeot et al. 1999; Teyke et al. 1993). However, we found that it did not have any effect on the dopamine-induced suppression of the IPSC even after methylergonovine in sufficient concentration (5 × 10⁻⁵ M) was applied for 30 min (n = 4). Therefore we further explored specific antagonists for the D1 and D2 dopamine receptors, SCH23390 and sulpiride, previously used in other gastropods (Audesirk 1989; Magoski et al. 1995; Stoof et al. 1985). Pretreatment with a sufficient concentration of sulpiride (5 × 10⁻⁵ M) for 30 min had no effect on the dopamine-induced suppression of the IPSC size (n = 4). In contrast, the dopamine-induced modulation was obviously reduced when the preparation was pretreated with the D1 antagonist SCH23390 (10⁻⁵ M) for 20 min prior to dopamine application (Fig. 9B). The blockage was obviously induced at a concentration of 3 × 10⁻⁶ M, but a solution at a higher concentration was used to induce sufficient effects in these experiments. Figure 9C shows a comparison of the average relative minimum sizes of the IPSCs reduced by dopamine application in the absence or the presence of SCH23390. The average value was 95.3 ± 1.7% for dopamine application with pretreatment of SCH23390 (n = 7), and there was a significant difference from the average value for the application of dopamine only described in the preceding text (P < 0.001, 2-sample t-test). In addition, the difference from the control was low (P < 0.05, 2-sample t-test). These results suggest that the D1 receptors may contribute to the suppression of the IPSC by dopamine.

Moreover, to investigate whether a similar mechanism participates in the synaptic modulation by the CBm1, we explored...
the effect of SCH23390 on the suppression of the IPSC by the CBM1. After the application of $10^{-5}$ M SCH23390 for 20 min, the CBM1 effects were obviously, but not completely, blocked in the same preparation and a typical result is shown in Fig. 10, A–C ($n = 4$). The average values of the relative minimum IPSC size reduced by the CBM1 firing in the absence or the presence of SCH23390 were 68.1 ± 1.6 and 90.8 ± 1.0%, respectively (Fig. 10D), and the difference was significant ($P < 0.005$, paired $t$-test). In this case, there was also a significant difference between the sizes in the control and the pretreatment with SCH23390 ($P < 0.005$, paired $t$-test). These results suggest that the CBM1 may partly release dopamine that acts directly on the D1 receptors in the MA-JC synaptic sites and may suppress the transmission. On the other hand, similar application of $10^{-5}$ M SCH23390 did not affect the sizes of the EPSPs monosynaptically produced in the MA neuron by the CBM1 firing (Fig. 11), suggesting that the CBM1 may use some chemical other than dopamine as a transmitter producing these EPSPs.

Responses of the CBM1 to taste stimulation of the lips with seaweed extracts

In the next step, we explored the response of the CBM1 neuron to taste stimulation of the lips with the seaweed extracts in the reduced preparations. In the present experiments, we found that it was very difficult to detect spike activity of the CBM1 for a long time (≥2 h) during repetitive application (4–5 times) of the extract using the electrophysiological method, and we therefore used the calcium imaging method for this purpose (see METHODS).

After identification of the CBM1 neuron, a calcium-sensitive dye, Calcium Green-1, was iontophoretically introduced into the cell body of the CBM1, and the change in fluorescence in the cell body region was explored when the seaweed extract was applied to the lips. In the present experiments, we used the Ulva and Gelidium extracts (original extract solutions, see METHODS), which can induce the patterned jaw movements for ingestion or rejection when these solutions are applied to the lips.

Fig. 10. Effects of D1 antagonist SCH23390 on the modulatory effects of the CBM1. The suppression of the synaptic transmission between the MA and JC neurons induced by the CBM1 firing for 30 s (A) is obviously reduced in the presence of the SCH23390 (B). C: time courses of the relative sizes of the IPSCs before and after firing of the CBM1 (bar) in the absence (○) and the presence (●) with SCH23390 in the same preparations as shown in A and B. The modulatory effect induced by the CBM1 firing for 30 s (39.7 spikes/s on average) was initially explored by bathing the preparation in a $3 \times 10^{-3}$ Ca$^{2+}$, $3 \times 10^{-3}$ Mg$^{2+}$ solution. The CBM1 was fired for 30 s (41.0 spikes/s on average) again after the preparation was bathed in a solution containing $10^{-5}$ M SCH23390 for 20 min. $D$: comparison of the average relative minimum sizes of the IPSCs induced by the CBM1 firing in the absence (CBM1) or the presence (SCH23390 + CBM1) of pretreatment with SCH23390 ($n = 4$). Bars represent ±SE.

Fig. 11. Effects of SCH23390 on the EPSPs monosynaptically produced in the MA by firing of the CBM1. These EPSPs are not affected by pretreatment with $10^{-5}$ M SCH23390 for 20 min. - - -, correspondence between the CBM1 spikes and the EPSPs produced in the MA.
lips as previously shown (Nagahama and Shin 1998; Nagahama et al. 1999). Figure 12 shows typical results for changes in the pseudocolor images of the CBM 1 cell body after application of the Ulva (A) or Gelidium (B) extract after a control recording for 10 s in the same preparations (n = 13). The time courses of the changes in fluorescence (ΔF/F, see METHODS) averaged over the whole region of the cell body are shown in Fig. 12C. Application of ASW scarcely induced a change in fluorescence. In contrast, just after stimulation with either of the two kinds of seaweed extract, the ΔF/F began to increase and attained the peak values at ~10 s. The peak value for the Gelidium extract was larger than that for the Ulva extract. After recording the fluorescence images for 120–150 s, the lips were completely washed with a sufficient amount of fresh ASW, and the fluorescence intensity usually decreased to the control resting level within 5 min. In the present experiments, the two kinds of taste stimulation were presented in a random order at 25–30 min intervals and more than two trials each were usually performed. Figure 13 shows one example of the time courses of the ΔF/F after alternate application of the Ulva (A) and Gelidium (B) extracts for two trials each. In all preparations, the fluorescence responses to taste stimulation with the same species of seaweed were very similar, while the responses for Gelidium were always larger in comparison with those for Ulva. Moreover the results indicated that the slope of the ΔF/F increase was always larger in taste stimulation with the Gelidium extract compared with the Ulva extract, but the maximums of the ΔF/F were attained at almost the same time.
Considering the relationships between the spike activity and the fluorescence change (see METHODS), application of either kind of extract may induce spike activity of the CBM1 neuron with a similar firing duration, but the neuron may fire at a higher frequency after application of the Gelidium extract compared with the Ulva extract.

Dependence of the fluorescence response on the concentration of the extract solution

In the present study, we initially expected that the cerebral modulatory neurons contributing to rejection would show spike activity only following application of the Gelidium extract. However, the results obtained indicated that the application of the Ulva extract also induced the spike activity of the CBM1 neuron. It was therefore necessary to consider the effects of the concentration of the extract solutions on the CBM1 responses. In our previous study, the concentration of the original extract solutions was determined so as to induce stable rhythmic responses for ingestion or rejection in semi-intact preparations, and dilution of the original extract solutions often failed to induce the rhythmic responses (Nagahama and Shin 1998). However, we have not explored the concentration dependence of the response in detail. Therefore we explored how the concentration dependence, relating to the firing frequency (see METHODS). Figure 14, C and D, shows the average initial slopes for all preparations plotted against the relative concentrations to those of the original Ulva and Gelidium extract solutions. The initial slopes were normalized against the slope for the original extract solution in each preparation. In both kinds of taste stimulation, the relative initial slopes increased at more than 0.02 times the original concentrations and attained the maximum at half the original concentrations. In four of seven preparations, the fluorescence responses following the application of the Ulva extract solutions at twofold the original concentration were also explored. For the Ulva extract solutions at half and twofold the original concentration, the average values of the relative initial slope were 0.97 ± 0.03 and 1.05 ± 0.04, respectively, and there were no significant differences between the initial slopes of the original solution and the solutions with either of the other concentrations (P > 0.1 for half concentration, n = 7; P > 0.1 for twofold concentration, n = 4, paired t-test). In addition, for the Gelidium extract solution at half the original concentration the average value of the relative initial slope was 0.97 ± 0.03, and there was no significant difference from that for the original concentration (P > 0.1, n = 5). These results suggest that the original extract solutions of Ulva and Gelidium may be sufficient to induce the maximum spike response of the CBM1 neuron and the use of more concentrated extract solutions will not increase the responses.

In the other cerebral neurons we further compared the fluorescence responses to taste stimulation with the original extract solutions of Ulva and Gelidium. Several neurons in the M, G, and C clusters responded to the taste stimulation (the response of the G and C clusters was also preliminarily re-
ported in Yoshida and Nagahama 2001). The responses were almost the same for the extract solutions of the two species of seaweed, although a very few neurons showed larger responses following the application of the Ulva extract compared with the Gelidium extract. However, of all the cerebral neurons studied in the present experiments, only the CBM1 neuron obviously showed larger responses to taste stimulation with the Gelidium extract compared with the Ulva extract. Figure 15 shows an example of the fluorescence responses of a CBM2a/b neuron after the application of the Ulva or Gelidium extract for 2 trials each in the same preparation. The extracts were applied to the lips at an arrowhead. From the calcium imaging alone, we could not show the real difference in the firing frequencies of the CBM1 spike activity induced by taste stimulation with the two species of seaweed. To estimate the real firing frequency of the CBM1 spike response to taste stimulation, the CBM1 was fired by repetitive electrical stimulation of the CBC, and the resultant changes in fluorescence were compared with the taste-induced responses of CBM1 to taste stimulation.

**Firing frequency of CBM1 response induced by taste stimulation with seaweed extracts**

From the calcium imaging alone, we could not show the real difference in the firing frequencies of the CBM1 spike activity induced by taste stimulation with the two species of seaweed. To estimate the real firing frequency of the CBM1 spike response to taste stimulation, the CBM1 was fired by repetitive electrical stimulation of the CBC, and the resultant changes in fluorescence were compared with the taste-induced responses of CBM1 to taste stimulation.

**Averaged fluorescence responses of CBM1 to taste stimulation**

The fluorescence responses of the CBM1 to taste stimulation with the original extract solutions of Ulva and Gelidium were explored in 13 preparations. In the present experiments, application of the two kinds of extract solutions produced a similar time course of the change in fluorescence, and we compared only the initial slopes. Figure 16 shows the comparison of the average initial slopes of the responses to taste stimulation with the Ulva and the Gelidium extracts for all preparations. The values were 0.78 ± 0.13%/s for the Ulva extract and 1.42 ± 0.21%/s for the Gelidium extract, and the difference between them was significant (P < 0.001, paired t-test). The average initial slope for ASW application (0.10 ± 0.02%/s, n = 12) is also shown in Fig. 16, and the differences from that for the Ulva extract and the Gelidium extract were significant (P < 0.001 for Ulva, P < 0.001 for Gelidium, 2 sample t-test). These results suggest that the difference between the firing frequencies of the CBM1 spike responses to taste stimulation with the Ulva and Gelidium extracts is clear.

*Fig. 15. Fluorescence responses in the CBM2a/b cell body after application of the Ulva (A) or Gelidium (Geli.) (B) extract for 2 trials each in the same preparation. The extracts were applied to the lips at an arrowhead.*

*Fig. 16. Average initial slopes of the change in fluorescence induced by application of ASW, the Ulva extract or the Gelidium (Geli.) extract. The fluorescence responses to taste stimulation of the 2 species of seaweed were explored in the same preparations and averaged over all preparations (n = 13). In 12 preparations, the responses to ASW application were also averaged. Bars represent ±SE.*
in the same neuron. In these experiments, the strength of each short electrical pulse was adjusted to evoke a single antidromic spike in the CBM1 neuron. Figure 17 shows an example of the change in fluorescence after application of the Ulva and Gelidium extract solutions (A) and electrical stimulation of the CBC (B) in the same CBM1 neuron (n = 8). Electrical stimulation of the ipsilateral CBC for 5 s at various frequencies induced increases in fluorescence depending on the stimulus frequency. The relationship between the stimulus frequency and the initial slope of the change in fluorescence for 5 s is shown in Fig. 17C. The initial slopes were almost proportional to the stimulus frequency, i.e., the firing frequencies of the CBM1. Therefore we could estimate the firing frequency of the CBM1 spikes after taste stimulation by the use of this relationship for the initial slope of the response to taste stimulation in the same preparation, and the obtained frequencies were averaged over all explored preparations. Figure 17D shows a comparison of the average spike frequencies for the two kinds of taste stimulation. The values were 19.7 ± 2.4 spikes/s for the taste of Ulva and 31.8 ± 3.4 spikes/s for the taste of Gelidium and the difference was significant (P < 0.001, n = 8, paired t-test).

Moreover, we explored the CBM1 spike activity induced by the application of the seaweed extracts electrophysiologically, although a pair of responses for the two kinds of extracts could not be compared in the same preparation. Examples of the spike responses to taste stimulation with the two kinds of extract solutions in separate preparations are shown in Fig. 18, A and B. In these experiments, there was an obvious difference between the firing frequencies of the spike activity just after extract application. The spike frequency averaged for 5 s just after the onset of stimulation was calculated and the values for all preparations were averaged. Figure 18C shows a comparison of the average spike frequencies of the CBM1 responses induced by taste stimulation with Ulva and Gelidium. The values were 20.2 ± 1.5 spikes/s for the taste of Ulva (n = 6) and 30.4 ± 1.1 spikes/s for the taste of Gelidium (n = 6) and the difference was significant (P < 0.001, 2-sample t-test). The average spike frequency for ASW application (3.9 ± 0.5 spikes/s, n = 6) is also shown in Fig. 18C, although the value was positive because the CBM1 tended to fire spontaneously even before stimulation. The differences of the spike frequencies between ASW and the Ulva extract application and between ASW and the Gelidium extract application were significant (P < 0.001 for Ulva, P < 0.001 for Gelidium, 2-sample t-test).

These results suggest that taste stimulation with the Gelidium extract may induce the CBM1 firing at ~30 spikes/s sufficient for producing the synaptic modulation at the MA-JC connections while taste stimulation with the Ulva extract may induce the firing at ~20 spikes/s and scarcely produce it.

**DISCUSSION**

In *A. californica* it has been reported that several cell bodies of the command-like neurons and the modulatory neurons locate in the M cluster of the cerebral ganglia (Rosen et al. 1991). Among them the CBI-1 neuron is largely activated by tactile stimulation applied to the lips, tentacles, or radula (Rosen et al. 1991) and may contribute to the rejection of an object such as a piece of polyethylene tubing (Morton and Chiel 1993a; Rosen et al. 2000a). In the present experiments, therefore we focused on the CBI-1 as a candidate for the modulatory neurons for rejection induced by the taste of Ge-
Our previous results for the suppression of the IPSC size by divalent cation solution, a 5<Mg<sup>2+</sup>/H<sub>11003</sub> solution, were often prolonged for 100 s. These results are very similar to those of the CBM1 in A. californica (Nagahama and Takata 1989, 1990). These characteristics of the CBM1 are consistent with those of the CBI-1 in A. californica, suggesting that these neurons are equivalent.

In the present experiments, we first explored the functional role of the CBM1 neuron on the synaptic transmission from the MA neuron to the JC neuron, and secondly the spike responses of the CBM1 to taste stimulation of the lips with the Ulva and Gelidium extracts. Firing of the CBM1 neuron at high-frequency (approximately 40 spikes/s) largely reduced the size of the IPSCs in the JC motor neurons evoked by the MA firing. However, the modulatory effects depended on the firing frequency of the CBM1 spike activity, since the effect was very small at firing frequencies lower than 25 spikes/s and largely increased above this frequency. Compared with the control data, the suppressive effects of the CBM1 neuron were temporary and were often prolonged for >100 s. These results are very similar to our previous results for the suppression of the IPSC size following the application of the Gelidium extract to the lips (Nagahama et al. 1999). In addition, the CBM1 may directly modulate the synaptic transmission at the MA-JC connections since this effect was sustained even when the preparation was bathed in a 3 × Ca<sup>2+</sup>, 3 × Mg<sup>2+</sup> solution to suppress polysynaptic pathways. We also demonstrated that the other high divalent cation solution, a 5 × Ca<sup>2+</sup>, 2 × Mg<sup>2+</sup> solution, can be used to explore the CBM1 direct modulatory effects.

The CBM1 may use dopamine as a modulatory chemical by analogy with the CBI-1 in A. californica (Rosen et al. 1991). We found that application of 5 × 10<sup>-5</sup> M dopamine into the bath solution mimicked the CBM1 effects. Several dopamine antagonists were also used to verify the contribution of dopamine in our systems. In A. californica methylergonovine has been reported to affect dopaminergic transmission (Ascher 1972; Nargeot et al. 1999; Teyke et al. 1993), but it did not have any effect on the dopamine-induced suppression of the IPSC size even in sufficient concentration. We further explored specific antagonists for the D1 and D2 dopamine receptors, SCH23390 and sulpiride, previously used in another gastropod, Lymnaea (Audesirk 1989; Magoski et al. 1995; Stoof et al. 1985). As a result, the modulatory effect of dopamine on the MA-JC synaptic transmission was largely reduced by pretreatment with SCH23390, suggesting that the D1 receptors may contribute to the dopamine modulation. Moreover, we explored the effect of SCH23390 on the synaptic modulation induced by the CBM1 and found that the pretreatment with SCH23390 also obviously reduced the modulatory effects of the CBM1. These results suggest that the CBM1 may release dopamine that directly acts on the D1 receptors in the MA-JC synaptic sites and may suppress the transmission. In the present experiments, we used a sufficient concentration of SCH23390, but the CBM1 effects could not be completely blocked, suggesting that some additional chemicals may also contribute to the modulatory effects. In addition, the CBM1-induced EPSPs in the MA neurons could not be blocked by SCH23390, suggesting that the CBM1 may use some chemicals other than dopamine for the primary synaptic transmission. Further study to identify the modulatory chemicals and the transmitters will be necessary.

In several species of animals, calcium imaging has been used to detect spike activity (Fetch and O’Malley 1995; Lev-Tov and O’Donovan 1995; McClellan et al. 1994; O’Donovan et al. 1993; O’Malley et al. 1996). In the present experiments, we also used calcium imaging to explore the spike responses of the CBM1 induced by taste stimulation of the lips with the seaweed extracts. As demonstrated in our recent report (Yoshida et al. 2001), this method is very useful for detecting spike activity in the small neurons of Aplysia which are unsuitable for microelectrode recordings. The spike responses could be explored repeatedly for a long time once the calcium-sensitive dye was iontophoretically introduced into the cell bodies for a brief time (0.5–5 min/cell). A study of the relationships between the spike activity and the fluorescence change indicated that the slope of the fluorescence increase is
almost relative to the spike frequency and that the period of time the increase lasts almost corresponds to the firing period. Considering these relationships, the present results indicate that the application of either kind of extract induced spike activity of the CBM1 with a similar firing duration, but the neuron fired at higher frequency following the application of the Gelidium extract compared with that of the Ulva extract.

In the present experiments, we used extract solutions for taste stimulation and the CBM1 response may depend on the concentration of the extract solutions. In our previous studies the concentrations of the seaweed extracts for the original extract solutions were determined so as to induce stable rhythmic responses in semi-intact preparations, and dilution of the original solutions often failed to induce the rhythmic responses (see methods) (Nagahama and Shin 1998). When we explored the effects of the concentration of the solutions on the CBM1 responses, we found that the original Ulva and Gelidium extract solutions were sufficient to induce the maximum spike responses for each species of seaweed. These results suggest that a similar spike response will be produced in the CBM1 even if we use more concentrated Ulva extract solutions and that the difference between the responses after taste stimulation with the Ulva and Gelidium extracts will be essential for more concentrated solutions. In addition, the CBM2a/b, probably equivalent to the CBI-2/12 in A. californica, showed similar rhythmic changes in fluorescence in response to the two kinds of original extract solution. In A. californica, the CBI-2/12 neuron has been reported to be a command-like neuron for the basic motor program for feeding (Hurwitz et al. 1999; Rosen et al. 1991, 2000a). The induced rhythmic responses in the CBM2a/b may contribute to the stable rhythmic responses during the ingestion or rejection of seaweed observed in our previous report (Nagahama and Shin 1998). If the original Gelidium extract solution is diluted to adjust the CBM1 activity to that induced by the original Ulva extract, the taste stimulation of Gelidium will lower the firing frequency of the CBM2a/b so as not to produce the rhythmic responses. Moreover, we also found that several other cerebral neurons showed similar spike responses after the application of the two kinds of seaweed extract. These results indicate that the concentrations of the original extract solutions of Ulva and Gelidium are appropriate for the purpose of comparing the CBM1 responses.

To estimate the real firing frequency of the CBM1 spike response to taste stimulation, the CBM1 was fired by repetitive electrical stimulation of the axon, and the resultant changes in fluorescence were compared with the taste-induced responses in the same preparations. We also directly explored the spike responses of the CBM1 to taste stimulation by the use of microelectrodes, although the spike responses for both kinds of tastes could not be obtained from the same preparations. In these experiments, the average firing frequencies for the first 5 s were compared because the stimulus solution was gradually diluted by diffusion into the bath and the response also tended to decrease with time (see methods). In the real feeding behavior, the continuous taste stimulation with the natural seaweed would be expected to induce a stable long-lasting response of the CBM1. The estimation showed that the CBM1 fired at ~20 spikes/s in response to the Ulva extract and at ~30 spikes/s to the Gelidium extract. This conclusion may be consistent with the functional properties of the CBM1, in which the modulatory effects largely increased at a firing frequency of >25 spikes/s. These results strongly suggest that the CBM1 neuron may be one of the modulatory neurons for directly suppressing synaptic transmission at specific MA-JC connection sites during rejection of seaweeds such as Gelidium. Arrows indicate the pathways of information. Small symbols indicate weaker effects and large symbols indicate stronger effects. CPG, central pattern generator; JOM, jaw-opening muscles; JCM, jaw-closing muscles; RM, radula muscles. During ingestion, the onset time of the firing of the JC motor neuron is delayed by inhibitory inputs from the MA neuron, while during rejection the onset time is advanced by suppressive effects of the CBM1 neuron on the inhibitory synaptic transmission.

>FIG. 19. Summary of the modulatory effects of the CBM1 neuron on the synaptic transmission at the MA-JC connection sites during rejection of seaweeds such as Gelidium. Arrows indicate the pathways of information. Small symbols indicate weaker effects and large symbols indicate stronger effects. CPG, central pattern generator; JOM, jaw-opening muscles; JCM, jaw-closing muscles; RM, radula muscles. During ingestion, the onset time of the firing of the JC motor neuron is delayed by inhibitory inputs from the MA neuron, while during rejection the onset time is advanced by suppressive effects of the CBM1 neuron on the inhibitory synaptic transmission. >
In the functional role of the CBM1 neuron, there seems to be a contradiction between the excitatory inputs to the MA neurons and the suppression of the inhibitory effects of the MA neurons. It may be explained by the difference in the stages of these effects, short-term effects and the long-term effects. The MA neurons were usually excited only during the CBM1 firing, whereas the synaptic modulation lasted >100 s after the CBM1 firing. In the experiments using semi-intact preparations, we previously reported that just after stimulation with the seaweed extracts the MA transiently fired at high frequency and then the rhythmic patterned bursts of spikes for ingestion or rejection were induced in the MA and the buccal motor neurons (Nagahama and Takata 1990). Moreover, in freely moving animals, the patterned movements for rejection of Gelidium transiently occurred several seconds after putting the Gelidium on the lips (Nagahama and Shin 1998). The transient CBM1 activity just after the taste stimulation may partly contribute to the initial transient excitation of the MA neurons while the modulatory effect of the CBM1 on the MA-JC connections may contribute to the change in the firing patterns of the JC during the subsequent rhythmic responses. At present, the functional role of the MA firing at high frequency just after taste stimulation is unknown. In the present experiments, we found that the CBM1 also excites the JC motor neurons. These excitatory inputs may compete with the inhibitory inputs from the MA neurons and the firing of the CBM1 may not largely affect the spike activity of the JC motor neurons. Therefore the neural circuits among these three neurons may contribute to a mechanism where the CBM1 excites only the MA activity without changing the JC activity. Further quantitative study for the mechanism controlling the neuronal activity among these neurons is necessary.

In A. californica, it has been reported that the CBI-1 neuron receives monosynaptic excitatory inputs from the mechanosensory ICBM neurons and is activated by tactile stimulation of the lip region (Rosen et al. 1991). Considering that the CBM1 may be equivalent to the CBI-1, the neural pathways originating from the mechanoreception to the CBM1 neuron may be similar to those in A. californica. However, we could not find any cell bodies equivalent to the ICBM neurons in the J and K clusters by backfilling the CBC (Rosen et al. 1982). It is possible that the biocytin backfilling technique was incomplete and did not fill small fine axons adequately. Another possibility is that there may be some discrepancy in the location of the cell bodies of the equivalent neurons in the two species of Aplysia as in the number of the MA neurons in A. kurodai (Nagahama and Takata 1989) and the B4/5 in A. californica (Gardner 1971). It will be necessary to identify the mechanosensory neurons in A. kurodai equivalent to the ICBM. In contrast, the neural pathways originating from the chemoreception to the CBM1 neuron are currently completely unknown. We will further explore the neural mechanism activating the CBM1 neuron after taste stimulation. In addition, the sense of the texture of the natural seaweed may also be caused by the mechanoreception of touch on the lips, and such weak stimulation may also activate the CBM1 to fire at low frequency. We do not currently know how the combination of taste and touch stimulation with natural seaweed activates the CBM1 neuron. Further study for the combined stimulation will be also necessary.

The present results suggest that the modulation of the transmission at the MA-JC synaptic connections may be caused by the CBM1 releasing dopamine from the axon terminals adjacent to the MA-JC synaptic regions. In A. californica, this type of heterosynaptic modulation has been well studied in the CNS and the neuromuscular synapses. One type of modulation concerns learning behavior, such as the sensitization of the gill withdrawal reflex (Castellucci and Kandel 1976; Cohen et al. 1997; Hawkins and Schacher 1989; Mackey et al. 1987; Stopfer and Carew 1996). Another type concerns the modulatory control of the muscle movements at the neuromuscular synapses (Fox and Lloyd 1998; Nagahama et al. 1994; Weiss et al. 1978). Among them, the functional role of the serotoninergic MCC neurons in the cerebral ganglia may be especially in contrast with that of the CBM1 neurons for the feeding behaviors. The MCC neurons receive chemosensory and mechanosensory inputs from the head region including the lips and the MCC activity contributes to the progressive enhancement of the rate and magnitude of the biting responses that characterizes food-induced arousal (Rosen et al. 1982; Weiss et al. 1978, 1986a,b). In some gastropods, the application of dopamine to the CNS has been reported to evoke the buccal motor program thought to represent fictive feeding (Kabotyanski et al. 2000; Kyriakides and McCrohan 1989; Teyke et al. 1993; Trimble and Barker 1984; Wieland and Gelperin 1983). The studies for the dopamine neurotoxin (Kemenes et al. 1990) and for the development of the dopamine-containing neurons (Voronezhskaya et al. 1999) also support the contribution of dopamine to the generation of the feeding motor program. In fact, several dopaminergic neurons have been found in the buccal ganglia and reported to induce the feeding motor program (Kabotyanski et al. 1998; Kemenes et al. 1990; Quinlan et al. 1997; Teyke et al. 1993). In contrast, our present results strongly suggest that a pair of cerebral dopaminergic neurons contribute to the generation of the patterned movements for rejection. These results suggest that two populations of dopaminergic neurons for ingestion and rejection may be present in Aplysia central nervous systems and that the application of dopamine may dominantly activate the neural circuits containing dopaminergic neurons in the buccal ganglia and generate the basic feeding motor patterns. Our present results allow us to propose the hypothesis that the cerebral serotonergic modulatory neurons contribute to the ingestion of the seaweed while the cerebral dopaminergic modulatory neurons contribute to the rejection.

In the crustacean stomatogastric ganglion, exogenous application of dopamine can change the pyloric motor pattern (Anderson and Barker 1981; Eisen and Marder 1984; Flamm and Harris-Warrick 1986), and the cellular mechanisms for the dopaminergic modulation of synaptic transmission, in which activity of the dopaminergic neurons also reduced the IPSP size, have been studied in detail (Ayali et al. 1998; Johnson and Harris-Warrick 1997). A similar mechanism may contribute to the decrement of the IPSP size in our system.

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