Acta Biologica Hungarica 55 (1–4), pp. 201–210 (2004)

SEROTONIN, NITRIC OXIDE AND HISTAMINE ENHANCE THE EXCITABILITY OF NEURON MCC BY DIVERSE MECHANISMS*

J. JACKLET,** J. GRIZZAFFI and D. TIEMAN

Department of Biological Sciences, University at Albany, State University of New York, 1400 Washington Ave., Albany, NY 12222, USA

(Received: August 31, 2003; accepted: December 1, 2003)

Serotonin, nitric oxide (NO) and histamine are neuromodulators used in molluscan nervous systems. We have found that each of them depolarizes and increases the excitability of the serotonergic feeding neural circuit modulator neuron, MCC, of *Aplysia,* but each induces different changes in background ionic currents and uses a different second messenger. Stimulation of neuron C2 in the cerebral ganglion induces a vsEPSP in MCC using NO and histamine. When these neurons are isolated in culture they form synapses that mediate the vsEPSP. The ionic currents induced by these neuromodulators were investigated in isolated cultured MCCs. Histamine reduced a background outward current between –70 and –30 mV that was blocked by cobalt treatment, indicating that it is a calcium activated potassium current. Serotonin reduced a background outward current from –65 mV to –30 mV and enhanced a potassium inward current more negative than –70 mV that was blocked by cesium and barium. This response was mimicked by 8-Br-cAMP. NO donors reduced a cobalt insensitive background outward current between –70 and –30 mV. This response was mimicked by 8-Br-cGMP. These responses show that MCC can produce complex time and state-dependent activity during its modulation of the feeding neural circuit.

Keywords: cAMP – cGMP – guanylyl cyclase – potassium channels – *Aplysia*

INTRODUCTION

The central nervous system of the mollusc *Aplysia* contains many large identifiable neurons that have been used very successfully to identify the neuronal circuits and synaptic mechanisms involved in a variety of behaviors, including feeding [2, 18]. The metacerebral cells (MCCs) are large prominent paired neurons in the cerebral ganglion that provide excitatory modulation of the neuronal circuit for feeding by release of the neurotransmitter serotonin. Each MCC is excited by synaptic input from a sensory neuron C2 during feeding. C2 is a mouth mechanoafferent neuron and a multifunctional modulator in the feeding circuit [2, 18]. It evokes a very slow EPSP (vsEPSP) in MCC [14, 18] that excites MCC. MCC in turn excites the feeding circuit. The vsEPSP is associated with a decrease in membrane conductance, which

* Presented at the 10th ISIN Symposium on Invertebrate Neurobiology, July 1–5, 2003, Tihany, Hungary.

** Corresponding author; e-mail: jwj74@albany.edu

0236-5383/$ 20.00 © 2004 Akadémiai Kiadó, Budapest

appears to be mediated by a decrease in potassium conductance [13, 18]. Nitric oxide (NO) and histamine (HA) are cotransmitter at this synapse [5], and NO stimulates guanylyl cyclase and cGMP production in MCC, decreasing the ionic conductances of the vsEPSP [13]. This synapse may be involved in the NO dependent learning of feeding behavior shown in *Aplysia* [10], and also known in the snail *Lymnaea* [11].

MCC synthesizes serotonin and releases it as a neurotransmitter/neuromodulator at its axon terminals in the buccal ganglion and on buccal muscles. Release of serotonin enhances the effectiveness of synaptic depolarization of buccal muscles and also provide extrinsic modulation of the central neurons that constitute the central pattern generator (CPG), which controls the rate of the rhythmic biting during feeding [15]. Weiss et al. [18] reported that MCC responds to serotonin. We have confirmed that serotonin depolarizes MCC and increases its excitability [9]. Also we have shown that 8-Br-cAMP mimic the effect, suggesting that MCC may contain K, S channels, which are known to contribute to increased excitability and spike broadening in *Aplysia* sensory neurons [1].

In this paper we will review our findings on the membrane currents in MCC that are altered by NO and histamine, and are responsible for the vsEPSP in MCC. In addition, we describe the excitatory responses of MCC to it own transmitter, serotonin, which modulates the feeding neural circuit.

MATERIALS AND METHODS

Aplysia californica (100–150 gm) were supplied by the Aplysia Resource Center, University of Miami, Fl., kept in a sea water aquarium at 18 °C, and fed fresh sea weed (*Gracilaria*). Histamine (HA) (hydrochloric form, Sigma, H-7250) and 8-Br-cAMP (8-bromoadenosine-3'5'-cyclic monophosphate, sodium salt, Sigma B-7880) stocks were aliquoted, frozen to be used later. SNAP (S-nitroso-N-acrtyl-D,L-penicillamine) and serotonin (5-HT) (Sigma H-7752) were made up daily in artificial sea water (ASW).

We used standard neuron culture techniques for *Aplysia* neurons [7, 12]. Ganglia were treated with Sigma protease Type IX at 35EC. The sheath was removed and visually identified C2 and MCC neurons were removed from the ganglion with both sharp and fire-polished micropipettes and transferred to culture dishes. Culture medium [12] consisted of artificial sea water (ASW) and L-15 powder mix (No. 82-5154EA; Gibco, Grand Island, NY) plus glutamine. Neurons were plated in Corning 25,000 culture dishes, previously coated with poly-L-lysine (Sigma, >500,000 MW, p-1524), using a medium composed of 50% modified L-15 medium and 50% filtered (0.45 μm) *Aplysia* hemolymph.

For electrophysiology, the culture dish containing an isolated MCC was placed on a microscope stage. ASW (460 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 48 mM $MgCl_2$, 10 mM HEPES – Sigma, H-3375, pH 7.8) was added to the dish at a flow rate of 2 ml/min and removed by aspiration. We used electrodes (5–8 MΩ) pulled from thin wall glass, filled with 3 M KAc/1 M KCl. Recording were made using an

Axon Instruments, GeneClamp 500B amplifier, in either current or voltage clamp mode. Signals were digitized using Axon Instruments pClamp/Digadata 1200-2 system and analyzed using Clampfit. We recorded neuron activity in current clamp mode to measure the resting membrane potential, the membrane resistance and the action potential characteristics. Neurons were then voltage clamped at –60 mV near the resting potential and voltage clamp protocols were used to generate current-voltage (I–V) curves. We routinely used voltage steps from –100 to –20 mV, 500 to 1000 msec in duration.

RESULTS AND DISCUSSION

The MCCs are paired giant serotonergic neurons in the cerebral ganglion (Fig. 1). Their axons project to the buccal ganglia through the cerebral buccal connectives and branch widely in the buccal ganglion where they modulate many neurons and innervate muscles involved in feeding behavior. MCC synaptic inputs are received from the paired mechano-afferents C2 neurons [2] and the paired C3 neurons [16]. Both the C2 and C3 neurons contain HA [16]. In addition, C2 neurons contain nitric oxide synthase (NOS) and use NO as a cotransmitter with HA [5, 13]. NADPH-diaphorase staining of the cerebral ganglion revealed that many neurons, central fibers and neu-

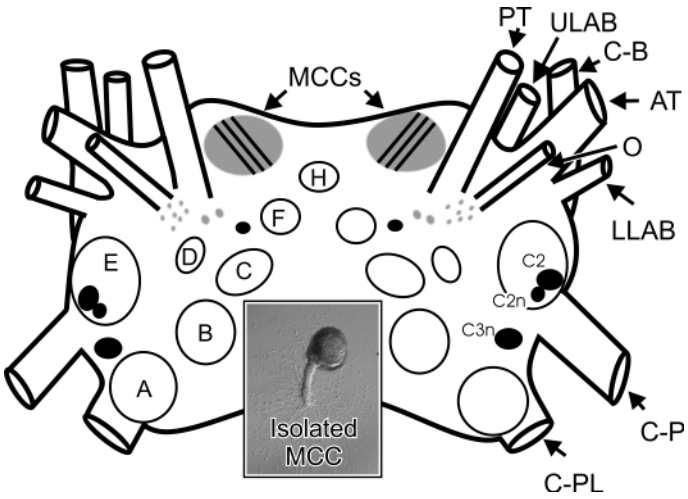

Fig. 1. Cerebral ganglion showing the location of MCCs and the presynaptic neurons C2 and C3. Dorsal view of the cerebral ganglion shows the location of peripheral nerves and connective, and neuron clusters A, B, C, D, E, F and H. Neurons C2 and C2n are in the E cluster. Neuron C3n is on the ventral surface of the ganglion. The MCCs contain 5-HT and cGMP. Neuron C2 and C3 contain histamine and C2, and likely C3, contains NOS. Inset shows an isolated MCC in cell culture. PT – posterior tentacular nerve, ULAB – upper labial nerve, C-B – cerebro-buccal connective, AT – anterior tentacular nerve, O – optic nerve, LLAB – lower labial nerve, C-P – cerebro-pedal connective, C-PL – cerebro-pleural connective

ropil of the ganglion stain, indicating that they contain NOS [6]. C2 neurons, and neurons that are likely the histiminergic C3 neuron, are among the most prominent stained neuron cell bodies.

In the cerebral ganglion, the membrane potential of MCC depolarizes and the membrane conductance decreases in responds to applied HA [13, 14, 18], suggesting that the response is mediated by closure of a potassium channel. MCC responds to NO donors in nearly the same way [5], raising the question of whether the NO and HA mechanisms are different. Evidence from tests of the NO-cGMP pathway [13] show that the mechanisms are different. This finding has been further supported by recent voltage clamp studies of the currents described in this review.

Isolated MCC neurons have been studied in cell culture [7]. They respond vigorously with depolarization to either applied NO or HA, as they do in the ganglion. MCC responds to puff of NO donors delivered by a picopritzer with long lasting, depolarization accompanied by decreased membrane conductance similar to the vsEPSP evoked by stimulation of neuron C2 in the ganglion [13]. Neurons C2 and MCC have also been co-cultured in isolation in preliminary experiments. They form synaptic contacts and C2 stimulation evokes a slow synaptic potential in MCC (Fig. 2) that is like the vsEPSP that is seen when the neurons are in the cerebral ganglion [13]. These C2-MCC neuron pairs have weak electrical coupling as well as slow, long-lasting chemical synaptic coupling.

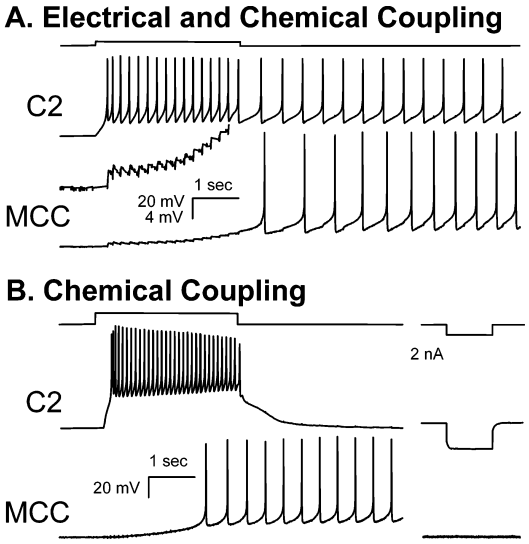

Fig. 2. Recoding from neurons C2 and MCC isolated together in cell culture. Both weak electrical (A) and slow chemical (B) synaptic coupling were observed from C2 to MCC. Direct depolarization of C2 evoked spikes in C2 and corresponding slow synaptic depolarization of MCC and spikes in MCC. A weak electrical component was observed in A but not in B, as shown by the lack of response in MCC when C2 was hyperpolarization. The synaptic input was slow, but powerful and long lasting, as it is in the cerebral ganglion. The contributions of histamine and NO to the responses were not determined

Isolated MCCs in culture have been studied extensively using voltage clamp techniques [8, 9]. HA was applied to MCC to examine the currents responsible for the depolarization and deceased conductance observed in current clamp. Under voltage clamp a steady-state inward current is induced between –60 and –30 mV (Fig. 3A). This inward current appears to be caused by a reduction in background potassium current, in agreement with observation from our current clamp results. Also, Weiss et al. [18] recorded inward current in response to HA from MCC in the cerebral ganglion. They found, as we have (Fig. 3A), that the current in the current-voltage plot did not reverse at the potassium equilibrium potential (–80 mV) as expected. We have tested the HA response in the presence of 10–15 mM cobalt chloride, which reversibly blocks calcium currents and calcium dependent potassium currents in molluscan neurons. Treatment with cobalt chloride blocks the HA response [9], suggesting that HA primarily blocks a calcium dependent potassium current that is normally active at membrane voltages more positive than –60 mV, which is the normal rest-

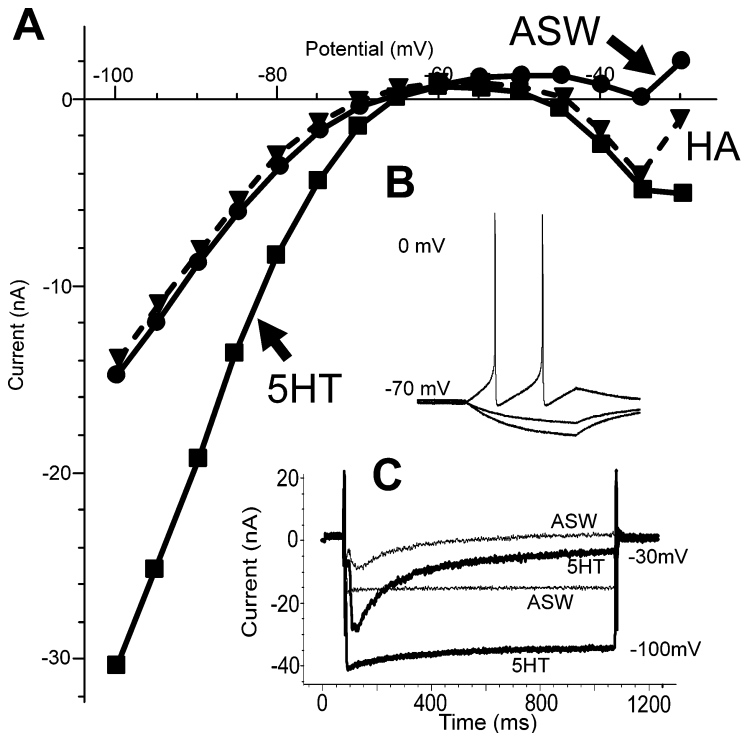

Fig. 3. Currents induced in an isolated MCC by histamine (HA) and serotonin (5-HT). The steady-state I–V curves in A show that HA (20 μM) induces inward current between –60 and –30 mV. 5-HT (30 μM) induces inward current at the same voltage and also between –65 and –100 mV. B shows membrane potential and action potentials evoked in the MCC before voltage clamping. C shows changes in currents induced by 5-HT at 2 voltages, –30 mV and –110 mV. Thin line is the current response in ASW and thick line is the response in 5-HT

ing potential of MCC. The lack of a current reversal at the potassium equilibrium potential may be explained, if the calcium dependent potassium current is not active at the reversal potential and more negative potentials.

Serotonin depolarizes MCC and induces tonic spiking. Under voltage clamp, serotonin induces steady-state inward current between –60 and –30 mV, associated with a decrease in membrane conductance, similar to the current induced by HA (Fig. 3A). Since cobalt does not block the steady state inward current induced by 5-HT in this voltage range, it is not the same current responsible for the calcium dependent background current acted upon by HA. In addition there is an inward shift in steady-state current between –65 and –100 mV, but this current is associated with a increase in membrane conductance. The latter current is blocked by treatment with barium or

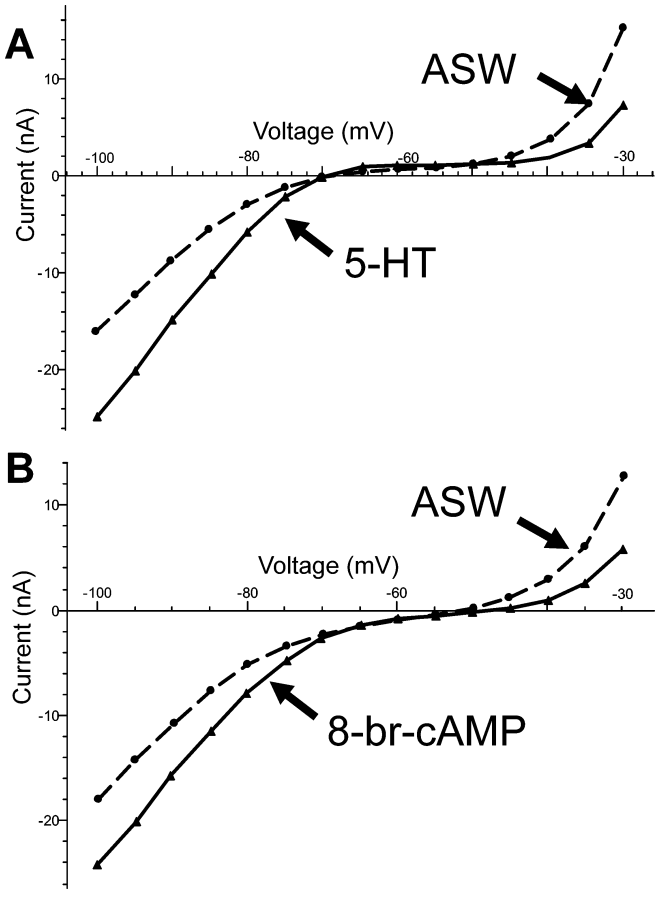

Fig. 4. Currents in an isolated MCC induced by 5-HT and 8-Br-cAMP. A shows steady-state I–V in response to 30 µM 5-HT and B shows response to 40 µM 8-Br-cAMP. The responses are virtually identical, induced inward currents between –50 and –30 mV and between –70 and –100 mV

cesium and therefore it seems to be an increase in potassium current. The inward rectifying potassium current is a candidate because it is known to be active in the voltage range more negative than the potassium equilibrium, and MCC shows a conspicuous increase in membrane conductance (inward rectification) when it is hyperpolarized [17, 18]. In addition to the steady-state currents, serotonin increases a transient calcium current at –30 mV (Fig. 3C) that is blocked by cobalt chloride.

Cyclic AMP mimics the 5-HT response. This was tested by applying the membrane permeant analogue 8-Br-cAMP to MCC. As shown in Fig. 4 inward currents in the –50–30 mV range and the –70 to –100 mV range were increased. This suggest that the inward current in the –60 to –30 range, associated with decreased membrane conductance may be mediated by closure of the serotonin sensitive potassium (K, S) channel, which is well known from studies on *Aplysia* sensory neurons and mediated by increased cAMP [review, 1]. K, S channel closure cannot account for the current induced between –70 and –100 mV, however. This current is likely carried by inward rectifier channel. The result in Fig. 4 indicates that 5-HT enhances the level of cAMP in MCC and cAMP has 2 separate effects on currents. This result is consistent with the observation by others [3] that either the non-specific phospodiesterase inhibitor IBMX or the membrane permeant cAMP analogue 8-benzylthio-cAMP induce an inward current in the range –60 to –30 mV as we have shown in Fig. 4. In voltage clamp experiments [3] both sodium and potassium current appeared to be involved. More experiments are necessary to define the currents involved in the 5-HT induced currents.

The NO donors (SNC, SNAP and SIN-1) depolarize MCC and induce tonic spiking accompanied by decreased membrane conductance [5, 9]. This effect is similar to the effects of HA and 5-HT, but voltage clamp studies show that the membrane mechanisms are different. The SNAP response is shown and compared to the HA response in Fig. 5. HA, as already shown (Fig. 3), enhances an inward shift of current between –60 and –30 mV. SNAP induces inward current over a much broader range (about –80 to –35 mV) and in many cases the current reverses at about –80 mV, the calculated potassium equilibrium potential in *Aplysia* neurons [18]. This suggests that the decrease in membrane conductance could be due to the closure of a background potassium current. This current is not blocked by cobalt, as the HA induced current is. It is not blocked by the usual potassium current blockers, such as barium, cesium and TEA either. These characteristic are similar to currents carried by the K, S channel and the background currents carried by KCNK channels [4].

The membrane permeable cGMP analogue, 8-Br-cGMP at 40 μM, mimics the part of the SNAP response characterized by the inward current between –70 and –35 mV, but does not induce the current reversal shown in Fig. 5. Cyclic GMP is thought to be involved in the NO response because NO elevates cGMP levels in MCC that were detected using a cGMP antiserum [13]. The increased immunostaining was dose dependent and was blocked by 10 μM ODG, a specific soluble guanylyl cyclase (sGC) inhibitor. Thus, it appears that this portion of the NO induced decrease in membrane conductance and membrane depolarization is mediated by the NO-sGC-cGMP pathway.

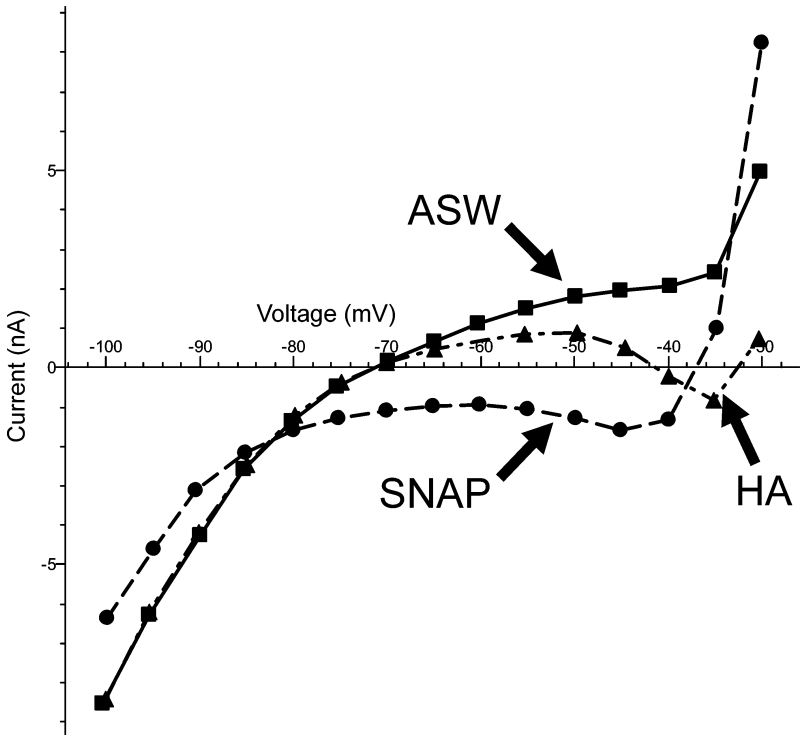

Fig. 5. Currents induced in an isolated MCC by histamine (HA) and the NO donor, SNAP. The steady-state I–V shows that HA (50 µM) induces inward currents between –70 and –30 mV, and SNAP (50 µM) induces inward currents between –80 and –35 mV, and current reversal occurs at –80 mV when current decreases between –80 and –100 mV

SUMMARY

MCC's responses to NO, HA and 5-HT appear to be mediated by three separate pathways involving three different second messenger systems. Their presence suggests that MCC's activity is likely shaped not only by their individual actions but activity that result from interactions between pathways and channels that are well known in *Aplysia* sensory neurons where they result in state and time dependent changes [1]. NO is a water soluble gas that diffuses through membranes and thus spreads widely from the cell where it is produced by NOS activated by calcium influx. Targets include neurons like MCC where it activates GC and cGMP is produced. In MCC this leads to a decrease in a background potassium current. Our experiments suggest that there is also an increase in a persistent background sodium current [9]. Histamine acts through a membrane receptor (preliminary experiments indicate an H1 receptor) that may activate phospholipase C. This leads to a decrease in a calcium activated potassium current, which results in depolarization of the membrane and

an increase in its response to other synaptic inputs. Serotonin (5-HT) appears to act through a membrane receptor coupled to adenylate cyclase and the production of cAMP because 8-Br-cAMP mimics the 5-HT response. Two effects are produced. One appears to be a decrease in potassium current (perhaps IK, S) accompanied by an increase in persistent sodium current. This depolarizes the membrane at and above the resting potential. Another is an increase in a potassium current (likely IK, IR), at potentials more negative than the resting potential. This should lead to opposing effects of depolarization and hyperpolarization, depending upon the membrane potential, as occurs in burster neurons. In fact, 8-benzylthio-cAMP is reported to induce bursting in an otherwise silent MCC [3]. Bursting activity requires sequential depolarizing and hyperpolarizing actions. Our observation that MCC responds to serotonin suggest that MCC's activity could be modulated by 5-HT released in the ganglion by other neurons. Also, if the MCC axonal terminal membranes contain serotonin receptors, the release of serotonin by MCC during its activity could modulate its subsequent transmitter release, via a positive feedback loop.

ACKNOWLEDGEMENTS

Research supported by NIMH MH57746 and SUNY Albany. We thank Jackie White for culturing neurons.

REFERENCES

1. Byrne, J., Kandel, E. (1996) Presynaptic facilitation revisited: state and time dependence. *J. Neurosci. 16,* 425–435.
2. Chiel, H., Weiss, K., Kupfermann, I. (1990) Multiple roles of a histaminergic afferent neuron in the feeding behavior of *Aplysia. Trends Neurosci. 13,* 223–227.
3. Drake, P., Triestman, S. (1981) Mechanisms of action of cyclic nucleotides on a bursting pacemaker and silent neuron in *Aplysia. Brain Res. 218,* 243–254.
4. Goldstein, S., Bockenhauer, D., O'Kelly, I., Zilberberg, N. (2001) Potassium leak channels and the KCNK family of two-P-domain subunits. *Nature Rev. Neurosci. 2,* 175–184.
5. Jacklet, J. (1995) Nitric oxide is used as an orthograde cotransmitter at identified histaminergic synapses. *J. Neurophysiol. 74,* 891–895.
6. Jacklet, J., Gruhn, M. (1994) Nitric oxide as a putative transmitter in *Aplysia:* Neural circuits and membrane effects. *Netherlands J. Zool. 44,* 524–534.
7. Jacklet, J., Koh, H. Y. (2001) Nitric oxide as an orthograde cotransmitter at central synapses of *Aplysia:* Responses of isolated neurons in culture. *Amer. Zool. 41,* 282–291.
8. Jacklet, J., Tieman, D. (2001) Nitric oxide and histamine decrease potassium conductances in Aplysia neurons C4 and M. C. under voltage clamp. *Soc. Neurosci. Abst.* Vol. 27, abstract 31.6.
9. Jacklet, J., Grizzaffi, J., Tieman, D. (2003) Serotonin, nitric oxide and histamine increase excitability in neuron MCC of *Aplysia* by diverse mechanisms. *Soc. Neurosci. Abst.* Vol. 29, abstract.
10. Katzoff, A., Ben-Gedalya, T., Susswein, A. (2002) Nitric oxide is necessary for multiple memory processes after learning that a food is inedible in *Aplysia. J. Neurosci. 22,* 1–14.
11. Kemenes, I., Kemenes, G., Andrew, R., Benjamin, P., O'Shea, M. (2002) Critical time-window for NO-cGMP-dependent long-term memory formation after one-trial appetitive conditioning. *J. Neurosci. 22,* 1414–1425.

12. Kleinfeld, D., Raccuia-Behling, F., Chiel, H. J. (1990) Circuits constructed from identified *Aplysia* neurons exhibit multiple patterns of persistent activity. *Biophys. J. 57,* 697–715.
13. Koh, H. Y., Jacklet, J. (1999) Nitric oxide stimulates cGMP production and mimics synaptic responses in metacerebral neurons of *Aplysia. J. Neurosci. 19,* 3818–3826.
14. McCaman, R. E., Weinreich, D. (1985) Histaminergic synaptic transmission in the cerebral ganglion of *Aplysia. J. Neurophysiol. 53,* 1016–1037.
15. Morgan, P. T., Perrins, R., Lloyd, P. E., Weiss, K. R. (2000) Intrinsic and extrinsic modulation of a single central pattern generating circuit. *J. Neurophysiol. 84,* 1186–1193.
16. Ono, J., McCamen, R. (1980) Identification of additional histaminergic neurons in *Aplysia:* Improvement of single cell isolation techniques for in tandem physiological and chemical studies. *Neuroscience 5,* 835–840.
17. Weiss, K., Kupfermenn, I. (1976) Homology of the giant serotonergic neurons (metacerebral cells) in Aplysia and pulmonate molluscs. *Brain Res. 117,* 33–49.
18. Weiss, K. R., Shapiro, E., Kupfermann, I. (1986) Modulatory synaptic actions of an identified histaminergic neuron on the serotonergic metacerebral cell of *Aplysia. J. Neurosci. 6,* 2393–2402.