Repetition Priming of Motoneuronal Activity in a Small Motor Network: Intercellular and Intracellular Signaling

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The characteristics of central pattern generator (CPG) outputs are subject to extensive modulation. Previous studies of neuromodulation largely focused on immediate actions of neuromodulators, i.e., actions that were exerted at the time when either neuromodulators were present or neuromodulatory inputs to the CPG were active. However, neuromodulatory actions are known to persist when neuromodulators are no longer present. In *Aplysia*, stimulation of cerebral-buccal interneuron-2 (CBI-2), which activates the feeding CPG, produces a repetition priming of motor programs. This priming is reflected in an increase of firing of motoneurons. As CBI-2 contains two neuromodulatory peptides, FCAP (feeding circuit-activating peptide) and CP2 (cerebral peptide 2), we hypothesized that repetition priming may involve persistent peptidergic neuromodulation. We find that these peptides produce priming-like effects, i.e., they increase the firing of radula-opening (B48) and radula-closing (B8) motoneurons during motor programs. Proekt et al. (2004, 2007) showed that repetition priming of neuron B8 is implemented by modulatory inputs that B8 receives from the CPG. In contrast, our current findings indicate that priming of B48 may be implemented by a direct peptidergic modulation of its intrinsic characteristics via a pathway that activates cAMP. We suggest that the direct versus indirect, i.e., CPG-dependent, repetition priming may be related to the type of input that individual motoneurons receive from the CPG. We suggest that in motoneurons that are driven by concurrent excitation-inhibition, repetition priming is indirect as it is preferentially implemented via modulation of the output of CPGs. In contrast, in motoneurons that are driven by alternating excitation-inhibition, direct modulation of motoneurons may be preferentially used.

Introduction

Characteristics of the outputs of vertebrate and invertebrate central pattern generators (CPGs) are sculpted by the presence of neuromodulators (Harris-Warrick and Marder, 1991; Calabrese, 1998; Marder and Thirumalai, 2002; Dickinson, 2006). Importantly, the effects of neuromodulators often persist when they are no longer present. One could therefore hypothesize that modulation of CPG outputs may persist when neuromodulators are no longer present (Marder et al., 1996). Consistent with this idea, repetitive stimulation of peptide-containing neurons (Morgan et al., 2000; Koh et al., 2003) that activate the feeding CPG of *Aplysia* results in repetition priming, i.e., a persistent improvement of the articulation of feeding responses that is expressed as an increase of the firing of specific motoneurons (Proekt and Weiss, 2003; Proekt et al., 2004). This priming could result from a persistent upmodulation of motoneuronal firing or, alternatively, from upmodulation of the synaptic drive that motoneurons receive from CPG elements. Previous work (Jing and Weiss, 2002; Proekt et al., 2004, 2007) indicates that modulation of the synaptic drive from CPG interneurons plays an important role in repetition priming of firing of some, but not all, motoneurons. We analyze

repetition priming of a specific motoneuron and find that its priming involves a direct, i.e., CPG-independent, modulation of its intrinsic properties. We suggest that direct modulation rather than indirect control of synaptic drive to motoneurons may be related to the particular means that CPGs use to drive specific motoneurons.

Here, we study repetition priming of motoneuron B48. B48 increases its firing rate when motor programs are elicited by a repetitive stimulation of the command-like neuron for feeding cerebral-buccal interneuron-2 (CBI-2) (Friedman et al., 2009). Given that CBI-2 contains two neuromodulatory peptides (Morgan et al., 2000; Koh et al., 2003), it is possible that these peptides mediate repetition priming induced by CBI-2 stimulation. We find that, similar to repetitive stimulation of CBI-2 (Friedman et al., 2009), its peptides increase the firing of B48 in motor programs. At the cellular level, CBI-2, feeding circuit-activating peptide (FCAP), and cerebral peptide 2 (CP2) increase the firing of B48 in a cAMP-dependent manner. Importantly, in CBI-2-elicited motor programs cAMP antagonists block repetition priming of B48, whereas cAMP agonists mimic repetition priming. Thus, our data indicate that repetition priming involves direct peptidergic modulation of the intrinsic properties of B48. This is in contrast to repetition priming of B8, which is controlled by CPG interneurons B20 and B40 (Proekt et al., 2007).

Control of B48 and B8 firing by the CPG is representative of two major modes of control that are used in a number of CPGs. B48 receives alternating excitation-inhibition that limits B48 firing to one phase of the program. In contrast, B8 firing is controlled by a balance of concurrent inhibition-excitation that B8

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receives in both phases (Jing and Weiss, 2001, 2002; Sasaki et al., 2009). We suggest that direct modulation of firing may be used in a nondisruptive manner in motoneurons driven by alternating excitation-inhibition, but that the same mechanism may be disruptive in motoneurons driven by a balance of concurrent excitation-inhibition.

Materials and Methods

Animals. Experiments were performed on *Aplysia californica* obtained from Marinus Scientific. *Aplysia* were maintained in circulating artificial sea water (ASW) made from Instant Ocean (Aquarium Systems) at 14–15°C. Animals weighing 150–250 g were anesthetized by injection of approximately half their weight of isotonic $MgCl_2$ (337 mM).

Electrophysiology. Intracellular recordings were made using single-barrel electrodes (6–10 M Ω) filled with an electrolyte containing 2 M K acetate and 100 mM KCl. Intracellular recordings were acquired using either the Axoclamp 2B amplifier (Molecular Devices) or Getting 5A amplifier (Getting Instruments). Extracellular recordings were performed by applying suction electrodes constructed from polyethylene tubing on buccal nerves. Signals were amplified by a CyberAmp 380 (Molecular Devices) and digitized using a Digidata 1200 (Molecular Devices) data acquisition system.

Neuron identification. To identify B48, a semi-intact preparation was used. The cerebral ganglion, buccal ganglia, and the attached buccal mass were dissected out with the cerebral–buccal connectives and buccal nerves intact since they contain the nerves of B48. The buccal mass was separated from the distal digestive system at the buccal–esophageal junction, and a portion of the buccal musculature was cut away to reveal the radula. The cerebral and buccal ganglia were pinned and desheathed in a Sylgard dish with the buccal mass pinned dorsal side down, exposing the radula. The dish was perfused with ASW (in mM: 460 NaCl, 10 KCl, 55 $MgCl_2$, 11 $CaCl_2$, and 10 HEPES buffer, pH 7.6) for 1 h. Motoneuron B48 was identified by its location within the ganglion and the distinct radula-opening movements elicited by its intracellular stimulation (Church and Lloyd, 1994; Evans et al., 1996, 1999; Orekhova et al., 2001; Zhurov et al., 2005).

The cerebral and buccal ganglia were then transferred to a recording chamber with a volume of ~1.5 ml of ASW. The preparations were continuously perfused with ASW at 0.3 ml/min and maintained at 14–17°C for 1 h before beginning experiments. Previously identified neurons CBI-2 and B8 were identified based on location, size, and electrophysiological and morphological characteristics (Rosen et al., 1991; Church and Lloyd, 1994).

Classification of feeding motor programs in *Aplysia*. During the consummatory phase of feeding, *Aplysia* produces behaviors that can be ingestive, intermediate, or egestive (Kupfermann, 1974; Morton and Chiel, 1993a,b; Morgan et al., 2002). In all three types of behavior, the animals first protract and then retract their food-grasping organ the radula. During ingestive behaviors, to pull food in, the radula closes in the retraction phase and opens in the protraction phase (Morton and Chiel, 1993a,b). During egestive behaviors, to push material out, the radula closes during protraction and opens during retraction. Ingestive and egestive behaviors can be distinguished using extracellular recordings obtained *in vivo* by differences in coordination between the activity of the radula-closing motoneuron B8 and the activity of neurons that produce radula protraction and retraction (Morton and Chiel, 1993a,b; Lum et al., 2005; Zhurov et al., 2005).

Neurons that form the CPG for feeding behaviors are located in the buccal ganglion (Susswein and Byrne, 1988; Teyke et al., 1993; Hurwitz and Susswein, 1996; Hurwitz et al., 1997; Kabotyanski et al., 1998; Jing and Weiss, 2001, 2002; Cropper et al., 2004). *In vitro*, this feeding CPG is capable of producing coordinated patterns of activity referred to as “fictive feeding motor programs.” Although not identical, these *in vitro* elicited motor programs share essential characteristics with patterns of activity recorded *in vivo*. Because the phasing of the activity of neuron B8 relative to protraction–retraction can be identified both *in vivo* and *in vitro*, B8 activity is used as a frame of reference for defining different types of motor programs. Specifically, during ingestive motor programs the

radula closer motoneuron B8 is predominantly active in the retraction phase, whereas during egestive motor programs B8 is predominantly active in the protraction phase (Jing and Weiss, 2001, 2002; Morgan et al., 2002; Proekt et al., 2004; Jing et al., 2007; Friedman et al., 2009). Based on previous findings, we define the protraction phase as the period of time during which activity is recorded in the I2 nerve (Hurwitz et al., 1996; Nargeot et al., 1999a,b; Jing and Weiss, 2001, 2002; Morgan et al., 2002; Vilim et al., 2010). The retraction phase is defined as the period of time during which high-frequency activity is recorded in the buccal nerve 2 after the end of activity recorded in the I2 nerve (Morton and Chiel, 1993a,b; Nargeot et al., 1999a; Sasaki et al., 2007; Wu et al., 2007; Sasaki et al., 2008, 2009).

We used a plot of B8 retraction versus B8 protraction phase activity along with the previously described classification scheme to operationally assign motor programs to the ingestive, egestive, or intermediate category (Morgan et al., 2002; Proekt et al., 2004, 2007) (Fig. 1C). The approximate boundaries of the ingestive and egestive clusters are shown as defined previously by the phasing of activity of B8 (Morgan et al., 2002). The intermediate programs are those that are outside the boundaries that define the ingestive and egestive programs. With repeated short interstimulus interval (S-ISI) stimulation of CBI-2, programs migrate from intermediate (Fig. 1C) toward ingestive as manifested by the increase in B8 firing frequency during retraction. Representative recordings of the 1st and the 10th programs elicited in this manner are shown in Figure 1A. Since the radula-opening motoneuron B48 is the focus of the current study, changes in its activity that are associated with the transition of B8 activity-defined intermediate programs to ingestive ones are also illustrated in Figure 1A,D. Firing frequencies of B8 and B48 during repeated S-ISI CBI-2 programs were plotted from data obtained from experiments illustrated in Figure 9. For the purpose of data analysis, activity of individual neurons is expressed in terms of their mean firing frequency within a given phase of the program.

Motor program generation. Motor programs were elicited via stimulation of the command-like neuron CBI-2 (Rosen et al., 1991). CBI-2 is activated when food contacts the mouth area. CBI-2 stimulation can elicit reliable motor programs in both semi-intact preparations and reduced preparations (Jing and Weiss, 2002, 2005; Morgan et al., 2002; Proekt et al., 2004; Sasaki et al., 2008). CBI-2 was stimulated by repetitive depolarizing (8–9 Hz) current pulses, each of which triggered a single action potential. CBI-2 stimulation was manually terminated after the end of the protraction phase, as determined by the termination of activity in the protraction motoneuron-containing I2 nerve (I2N) (Hurwitz et al., 1996, 2000). Two different CBI-2 stimulation paradigms were used. CBI-2 programs were elicited using either an S-ISI of 30 s, in which CBI-2 was stimulated to elicit a program 30 s after the end of retraction of the previous program, or a long interstimulus interval (L-ISI), in which CBI-2 was stimulated to elicit a program 3 min after the end of retraction of the previous program.

Reagents. FCAP (SynPep) (Sweedler et al., 2002) and CP2 (SynPep) (Phares et al., 1996; Vilim et al., 2001) were perfused at 10^{-6} M, a concentration that produces maximum effects (Koh et al., 2003). In all experiments, peptides were perfused at a rate of 0.3 ml/min. For intracellular injections, a 10 mM concentration of 8-bromo-cAMP (8-br-cAMP) was used (Sigma). An 80 mM concentration of the Rp isomer of cAMP (Rp-cAMP) was used for intracellular injections (Sigma). Both 8-br-cAMP and Rp-cAMP were first dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the electrode never exceeded 0.75%. Both 8-br-cAMP and Rp-cAMP were iontophoresed with a single-barrel electrode using 10 ms hyperpolarizing current pulses every 10 s. A 0.2% DMSO solution of buffered distilled water (HEPES 10 mM, pH 7.6) was injected to probe for the potential effects of DMSO. The size of the current pulses was adjusted so that they produced ~10 mV hyperpolarization. A high-divalent ASW was perfused to suppress polysynaptic responses and spontaneous activity during measurements of firing during depolarization and occlusion experiments (in mM: 368 NaCl, 10 KCl, 101 $MgCl_2$, 13.8 $CaCl_2$, and 10 HEPES, pH 7.6) (Trudeau and Castellucci, 1992).

Statistics. Digitized electrophysiological recordings were plotted using CorelDRAW v. 13 (Corel), and data were expressed as the mean $\pm$ SEM.

Two-group statistical comparisons were performed using *t* test statistics. Multigroup data were analyzed using ANOVA, and subsequent comparisons were performed using Bonferroni's multiple-comparison tests. The significance level was set at $p < 0.05$ (** $p < 0.001$; * $p < 0.01$; * $p < 0.05$; n.s., $p > 0.05$). All statistical tests were performed using SigmaPlot (Systat Software).

Results

Peptides and repetition priming

There are two major ways in which activity of the feeding CPG is affected by the command-like neuron CBI-2. First, acting with fast dynamics CBI-2 triggers motor programs. Second, acting with slow dynamics repeated stimulation of CBI-2 modifies these programs so that they evolve from intermediate-type programs to ingestive ones. CBI-2 triggers motor programs using fast cholinergic transmission (Hurwitz et al., 2003). In addition to ACh, two neuropeptides, FCAP and CP2 (Phares et al., 1996; Sweedler et al., 2002), are present in CBI-2 (Morgan et al., 2000; Koh et al., 2003). Given that stimulation paradigms that produce repetition priming have been shown to be favorable for peptide release in *Aplysia* (Whim and Lloyd, 1989, 1990; Cropper et al., 1990; Vilim et al., 1996b, 2000; Brezina et al., 2000b), we reasoned that these peptides may be involved in repetition priming, i.e., an enhancement of motoneuronal firing when motor programs are elicited by repetitive CBI-2 stimulation.

As a first step toward exploring the possible involvement of peptides in the evolution of programs from intermediate to ingestive, we sought to determine whether perfusion of the peptides contained in CBI-2 (CP2 and FCAP) would mimic the effects of repetitive stimulation of CBI-2, i.e., whether it would convert intermediate programs elicited by CBI-2 into ingestive ones. To prevent the motor programs from becoming ingestive, as a result of repetitive stimulation of CBI-2, we stimulated CBI-2 using an L-ISI (3 min). Importantly, consistent with the definition of intermediate programs (see Materials and Methods), under these conditions of stimulation B8 fires at low frequencies during both the retraction and the protraction phase.

Perfusion of FCAP/CP2 dramatically altered the character of motor programs, as exemplified in a recording from a single preparation (Fig. 2, compare A1, A2). Comparison of Figure 2A1 and A2 indicated that in terms of B8 activity, motor programs became ingestive as the firing pattern of B8 changed from one in which B8 fired at a low frequency throughout the retraction and protraction phase, to the pattern in which B8 fired at a high frequency during retraction while maintaining a relatively low firing rate during protraction. Note that this was associated with an increased firing of B48 during protraction. The effects of peptides were reversed with washout.

Quantitative analyses of the aggregate data showed that indeed the changes illustrated in Figure 2A were statistically significant.

Figure 1. Activity of motoneurons B48 and B8 during S-ISI CBI-2-elicited motor programs. **A**, Shown are the 1st and the 10th motor program that were elicited by CBI-2 stimulation. The black bars mark the protraction phase, and the white bars mark the retraction phase. A series of 10 CBI-2 programs were elicited 30 s apart. Repeated CBI-2 stimulation elicited a progressive increase in B48 firing in the protraction phase and an increase in B8 firing in the retraction phase (buildup). **B**, Simplified schematic of the organization and localization of CPG neurons involved in the control of motoneurons B48 and B8. Large and small triangles denote small and large excitatory connections, respectively. Black circles denote inhibitory connections. Zigzags denote electrical coupling. Low-frequency firing is denoted by gray outline. Neurons are shown in the left or right part of the diagram depending on whether they are active in the protraction or the retraction phase, respectively. B8 is shown in both parts of the diagram because it fires in both phases of the motor program. B40 fires at high frequency during the protraction phase, promoting B8 firing in the retraction phase through a slow excitatory input. B20 fires at low frequency (gray outline) during CBI-2-elicited programs. B48 is coupled to a series of protraction-phase interneurons and motoneurons, and fires exclusively in the protraction phase. **C**, Plot of B8 activity during protraction versus B8 activity during retraction illustrates cluster boundaries for ingestive and egestive motor programs. Intermediate programs are motor programs that are between clusters for ingestive and egestive programs. With repeated stimulation of CBI-2, B8 firing frequency increases during retraction, and motor programs migrate toward the ingestive cluster. **D**, Plot of B48 activity during retraction versus B48 activity during protraction. With repeated stimulation of CBI-2, B48 firing frequency increases during protraction, and motor programs migrate toward the ingestive cluster.

Specifically, we performed a one-way ANOVA of B8 firing frequency during protraction phase ($F_{(2,6)} = 3.95$; $p = 0.08$, $n = 4$) and retraction phase ($F_{(2,6)} = 189.64$, $p < 0.001$, $n = 4$). Individual comparisons of firing frequency revealed that after a 10 min perfusion of peptides, retraction-phase firing of B8 was significantly higher compared with controls and washout conditions (control vs FCAP/CP2: $t = 15.88$, $p < 0.001$; FCAP/CP2 vs washout: $t = 17.70$, $p < 0.001$). In contrast, peptides did not affect the frequency of B8 firing during protraction (control vs FCAP/CP2: $t = 2.25$, $p = 0.20$; FCAP/CP2 vs washout: $t = 2.58$, $p = 0.20$). Together, these data indicated that, as defined by B8 activity, perfusion of peptides converted the motor programs to ingestive ones, thus mimicking the effect of repetitive stimulation of CBI-2.

In addition to increasing the retraction-phase firing of B8, repetitive stimulation of CBI-2 increased the frequency of protraction-phase and post-retraction-phase firing of B48 (Friedman et al., 2009). Furthermore, repetitive stimulation of CBI-2 also shortened the latency from the beginning of CBI-2 stimulation to the first B48 action potential. Consistent with the shortening of

Figure 2. Effect of exogenous FCAP and CP2 on L-ISI CBI-2-elicited programs. Black and white bars indicate the protraction and retraction phases, respectively. A series of five CBI-2 programs was elicited every 3 min (L-ISI) (control) that generated intermediate programs. FCAP and CP2 were then superfused during the L-ISI CBI-2-elicited programs (FCAP and CP2). **A1**, Representative recordings of L-ISI CBI-2-elicited intermediate program in which B48 fires weakly in the protraction phase. **A2**, L-ISI CBI-2-elicited program in the presence of FCAP and CP2 in which B48 fired robustly during the protraction phase and B8 fired robustly during the retraction phase. **A3**, L-ISI CBI-2-elicited program after 15 min of washout in which B48 protraction activity and B8 retraction activity were weak. **B**, Aggregate data of the effects of FCAP and CP2 on L-ISI CBI-2-elicited programs on B48 protraction and postretraction firing frequency. **C**, Aggregate data of the effects of FCAP and CP2 on L-ISI CBI-2-elicited programs on B8 protraction and retraction firing frequency. **D**, Aggregate data show that there was a significantly shorter B48 latency and a significantly higher fraction of protraction with B48 activity in the presence of FCAP and CP2.

the latency, the fraction of the protraction phase during which B48 fires also increased. We analyzed the effects FCAP/CP2 on all of these parameters of B48 firing. We performed a one-way ANOVA of B48 firing frequency in the protraction phase and found that there was an overall significant difference between conditions ($F_{(2,6)} = 41.48$, $p < 0.001$, $n = 4$). The differences in the post-retraction-phase firing were also statistically significant ($F_{(2,6)} = 49.52$, $p < 0.001$, $n = 4$). Individual comparisons revealed that there was a significant increase in B48 firing in the presence of FCAP/CP2 compared with control or washout conditions (protraction: control vs FCAP/CP2: $t = 7.23$, $p < 0.001$; FCAP/CP2 vs washout: $t = 8.41$, $p < 0.001$; post-retraction: control vs FCAP/CP2: $t = 9.25$, $p < 0.001$; FCAP/CP2 vs washout: $t = 7.80$, $p < 0.001$). Further analysis of B48 activity during protraction phase revealed that the latency from the onset of CBI-2 stimulation to the first action potential in B48 decreased significantly from 19.6 ± 5.9 to 0.63 ± 0.20 s in the presence of peptides ($t = 4.26$, $p < 0.05$). Consistent with this observation, we found that the fraction of the protraction phase in which B48 was active also

increased significantly ($t = 5.08$, $p < 0.05$) during superfusion of FCAP/CP2.

Together, the data shown in Figure 2 indicated that B48 protraction and B8 retraction activity was upregulated by FCAP/CP2, resulting in an increase of ingestiveness of motor programs. Perfusion of these peptides, FCAP/CP2, reproduces all of the previously reported effects of a repetitive CBI-2 stimulation on B48 and B8 firing frequencies during protraction and retraction phases (Friedman et al., 2009).

B48: effects of peptides and CBI-2 stimulation

In networks that are characterized by a significant degree of interconnectivity, modulation of the intrinsic properties of individual neurons may appear to be the simplest way of selectively regulating the firing frequency of individual neurons. Although it did not have to be so, this mechanism of regulation appeared plausible in the case of neuron B48, which receives its excitatory input and fires during protraction but is inhibited during retraction. Thus, upmodulation of B48 firing by CBI-2 or peptides would not result in a nonspecific firing of B48 during both protraction and retraction phases. In contrast, B8 receives its mixed excitatory-inhibitory input during both protraction and retraction, and fires, albeit to a different degree, in both the protraction and retraction phases in different types of motor programs. Therefore, upmodulation of B8 firing would likely result in an overall increase of B8 activity. This is not what was observed either with repetitive CBI-2 stimulation (Proekt et al., 2004) or with FCAP/CP2 perfusion (Fig. 2). Therefore, it is unlikely that modulation of B8 intrinsic properties would be used to modify the

firing of B8 in response to stimulation of CBI-2 or FCAP/CP2 perfusion.

We thus sought to determine whether both stimulation of CBI-2 and perfusion of FCAP/CP2 differentially altered the number of action potentials elicited by a depolarizing input to B48 and B8. Action potentials were elicited in B48 and B8 using injections of a 3 s constant depolarizing current pulse every 60 s. Current size in individual experiments was selected to generate ~ 10 action potentials under control conditions. The preparations were superfused with a high-divalent ASW to reduce spontaneous activity and polysynaptic effects. We compared the number of action potentials before and after a 10 s stimulation of CBI-2 at 9 Hz. After CBI-2 stimulation, the number of B8 action potentials did not change (Fig. 3B), but the number of B48 action potentials increased from 10.0 ± 1.2 to 23.8 ± 3.5 (Fig. 3A) and then gradually returned to control levels. A one-way repeated-measures ANOVA on the number of action potentials elicited during control, 1 min after CBI-2 stimulation, and after a 25 min recovery showed that for B48 there was an overall significant difference

between these conditions ($F_{(2,8)} = 18.80$, $p < 0.01$, $n = 5$). No such difference was detected for B8 ($F_{(2,8)} = 0.23$, $p = 0.79$, $n = 5$). Individual comparisons revealed that B48 fired at a significantly higher frequency 1 min after CBI-2 stimulation (pre-CBI-2 stimulation vs 1 min post-CBI-2: $t = 5.3$, $p < 0.01$; 1 min post-CBI-2 vs recovery: $t = 5.3$, $p < 0.01$). After CBI-2 stimulation, there was also a significant decrease in the latency to the first B48 action potential elicited during the same size depolarization (from 36.10 ± 1.31 to 93.26 ± 2.76 ms) ($t = 19.12$, $p < 0.001$, $n = 5$). After CBI-2 stimulation, the resting membrane potential became significantly more positive (from -58.88 ± 3.14 to -55.92 ± 2.92 mV) ($t = 3.65$, $p < 0.05$, $n = 5$).

To further characterize the effects of CBI-2 stimulation on B48, we characterized the depolarization required to elicit action potentials before and after CBI-2 stimulation. Two electrodes were used, one to inject a series of increasing depolarizing current pulses (1.0 nA increments; 2 s duration) and a second to record the resulting voltage deflection. Before the injection of the current pulses, the membrane potential of B48 was adjusted to -80 mV. A series of progressively larger current pulses was injected into B48 ($n = 5$). After 10 s of CBI-2 stimulation, an identical series of current pulses produced a significantly larger depolarization (Fig. 3C, depolarization post-CBI-2 is underlined). The 5 nA current pulses after CBI-2 stimulation elicited a larger depolarization (22.68 ± 0.60 vs 18.36 ± 0.71 mV; $t = 5.81$, $p < 0.01$, $n = 5$). The representative traces also illustrated a significant decrease in spike threshold from -43.25 ± 3.73 to -50.61 ± 3.35 mV ($t = 7.40$, $p < 0.01$, $n = 5$) (Fig. 3C). These increases could be expected to enhance B48 firing in motor programs and thus endow the programs with more ingestive-like characteristics.

Next, we sought to determine whether perfusion of FCAP and CP2 mimicked the effects CBI-2 stimulation on B48 and B8 firing as would be predicted if the effects of the stimulation of CBI-2 were to be mediated by the FCAP/CP2 peptides. The stimulation parameters were the same as described above. We found, as illustrated in recordings from a single preparation (Fig. 4A1) and from aggregate data (Fig. 4A2), that the perfusion of FCAP and CP2 increased the number of B48 action potentials elicited during a constant current pulse. Not unexpectedly, we found no differences in the number of B8 action potentials elicited during perfusion of FCAP and CP2 (Fig. 4B).

To determine the overall significance level of differences among the number of B48 action potentials elicited during control, at the end of a 5 min perfusion of FCAP, and after 25 min washout, we performed a one-way repeated-measures ANOVA ($F_{(2,8)} = 24.62$, $p < 0.001$, $n = 5$). Individual comparisons re-

Figure 3. CBI-2 stimulation increases the number of B48 action potentials elicited by the same size current pulse. **A**, Representative recordings of B48 firing before and after 10 s of CBI-2 stimulation. A 3 s current pulse was injected into B48 every 60 s. Aggregate data show a persistent increase in the number of B48 action potentials elicited by the same current injection 1, 5, and 15 min after CBI-2 stimulation. **B**, Representative recordings of B8 firing before and after 10 s of CBI-2 stimulation. A 3 s current pulse was injected into B8 every 60 s. Aggregate data show no significant change in the number of B8 action potentials elicited by the same current injection after CBI-2 stimulation. **C**, CBI-2 stimulation increases the input resistance of B48. B48 membrane potential was held at -80 mV, with a series of 2 s current injections before and 1 min after CBI-2 stimulation (underlined). Note increased depolarization in the after CBI-2 stimulation compared with the same size current injection before stimulation.

vealed that, compared with the control and post-washout period, the number of action potentials elicited in B48 in the presence of FCAP was significantly higher (control vs FCAP: $t = 5.92$, $p < 0.001$; FCAP vs washout: $t = 6.21$, $p < 0.001$; control vs washout: $t = 0.25$, $p = 1.00$). In addition, after the perfusion of FCAP, the latency to the first action potential was significantly shorter (38.92 ± 4.82 vs 155.42 ± 27.53 ms; $t = 4.07$, $p < 0.05$, $n = 5$). In the presence of FCAP, the resting membrane potential became significantly more positive (from -55.40 ± 3.64 to -47.0 ± 2.03 mV; $t = 2.79$, $p < 0.05$, $N = 5$). Using the same paradigm as above, we measured the number of action potentials elicited before CP2 perfusion, in the presence of CP2, and after the washout of CP2. As illustrated in recordings from a single preparation (Fig. 4B1) and the graph of aggregate data (Fig. 4B2), CP2 superfusion also increased the number of B48 action potentials. A one-way repeated-measures ANOVA showed that there was an overall significant difference among the three conditions ($F_{(2,6)} = 20.70$, $p < 0.01$, $n = 4$). Individual comparisons showed a significant increase in the number of B48 action potentials during CP2

Figure 4. FCAP and CP2 increase the number of B48 action potentials, but not the number of B8 action potentials elicited by the same size current pulse. **A**, Representative recordings of B48 firing before and during the presence of FCAP and CP2. A 3 s current pulse was injected into B48 every 60 s. Aggregate data show a significant increase in B48 action potentials elicited by the same current injection in the presence of FCAP and CP2. **B**, Representative recordings of B8 firing before and during the presence of FCAP and CP2. A 3 s current pulse was injected into B8 every 60 s. Aggregate data show no significant change in B8 action potentials elicited by the same current injection in the presence of FCAP and CP2. **C**, FCAP and CP2 increase the input resistance of B48. B48 membrane potential was held at -80 mV with a series of 2 s current injections before and during FCAP and CP2 superfusion (underlined). Note increased depolarization in the presence of peptide compared with the same size current injection.

superfusion compared with control and washout (control vs CP2: $t = 5.75$, $p < 0.01$; CP2 vs washout: $t = 5.38$, $p < 0.01$; control vs washout: $t = 1.34$, $p = 1.00$). In the presence of CP2, the latency to the first action potential was also significantly shorter (38.20 ± 9.60 vs 146.35 ± 9.52 ms: $t = 5.73$, $p < 0.05$, $n = 5$). In the presence of CP2, the resting membrane potential became significantly more positive (from -55.62 ± 6.33 to -48.55 ± 4.60 mV: $t = 3.29$, $p < 0.05$, $n = 5$). Thus, both peptides increased the firing of B48.

Perfusion of either FCAP or CP2 did not alter the number of B8 action potentials elicited, as illustrated in the representative recording (Fig. 4B1) and aggregate data (Fig. 4B2). A one-way repeated-measures ANOVA on the number of action potentials elicited during control, at the end of a 5 min superfusion of FCAP or CP2, and after a 25 min washout showed that, indeed, among the three conditions there were no statistically significant differences in the number of spikes elicited in B8 by constant current injections (FCAP: $F_{(2,8)} = 0.27$, $p = 0.77$, $n = 5$; CP2: $F_{(2,8)} = 0.33$, $p = 0.72$, $n = 5$). Thus, perfusion of either peptide did not alter B8 firing. This lack of modulation of B8 firing was consistent with the previous data showing that B8 firing was controlled by the balance of outputs of premotor neurons that differentially regulate B8 firing during protraction and retraction (Jing et al., 2003; Proekt et al., 2007).

We next sought to determine whether FCAP and CP2 also mimicked the effects of CBI-2 stimulation on the size of depolarization elicited by current injections. Using the same paradigm as in Figure 3C, we examined the alteration in depolarization size during application of FCAP and CP2 (Fig. 4C, underlined), and in all preparations found that they increased the size of the depolarization to the same size current injection compared with the control conditions (representative recording) (Fig. 4C). The 4 nA current pulses in the presence of FCAP/CP2 elicited a significantly larger depolarization (30.42 ± 2.05 vs 27.62 ± 2.26 mV: $t = 9.84$, $p < 0.001$, $n = 5$). The traces also illustrated a significant decrease in spike threshold from -42.29 ± 0.54 to -50.01 ± 0.82 mV ($t = 11.792$, $p < 0.001$, $n = 5$). Thus, both brief stimulation of CBI-2 and peptide perfusion increased the firing and increased the size of the depolarization in B48. Since these experiments were conducted in high-divalent ASW, the results were consistent with the idea that intrinsic properties of B48 may be directly modulated by CBI-2 acting through its peptides, FCAP and CP2.

Because stimulation of both CBI-2 and peptides exerted a similar action on B48, we reasoned that FCAP and CP2 may contribute to the changes in B48 firing observed after CBI-2 stimulation. To explore this possibility, we used an occlusion paradigm (Swensen and Marder, 2000; Koh and Weiss, 2005, 2007) and tested the ability of exogenous peptides to occlude the effects of CBI-2 stimulation on the firing of B48. Again, a high-divalent ASW was used to reduce the threshold for initiation of action potentials, thereby reducing polysynaptic inputs. Current pulses that elicited ~ 10 action potentials in B48 were injected every 60 s.

Perfusion of FCAP and CP2 produced an increase in the number of B48 action potentials elicited by the same size constant current injection (representative recording) (Fig. 5A1). To avoid ceiling effects, in the continued presence of FCAP and CP2, the size of the injected current pulse was reduced to again elicit ~ 10 action potentials, i.e., approximately the same number of action potentials that current pulses elicited before perfusion of FCAP or CP2. In the continued presence of FCAP and CP2, CBI-2 was then stimulated for 10 s, using the same frequency that previously increased the number of action potentials elicited in B48 (Fig. 3A). We found there was no significant increase in B48 firing after CBI-2 stimulation in the presence of FCAP and CP2 ($t = 1.027$, $p = 0.38$, $df = 3$) (Fig. 5A2). Thus, occlusion by FCAP and CP2 of the ability of CBI-2 stimulation to increase the number of B48 action potentials suggested convergent actions of peptides FCAP/CP2 and CBI-2 stimulation.

B48: role of cAMP

If CBI-2 used its peptides to modulate the intrinsic properties of B48, one would expect that the same intracellular signaling pathway would mediate the effects of CBI-2 stimulation and peptide perfusion. To narrow our search, we first sought to determine whether exogenous application of IBMX, a phosphodiesterase inhibitor, increased the CBI-2-elicited enhancement of B48 firing. Action potentials were elicited in B48 using injections of a 3 s constant depolarizing current pulse every 60 s. Current size in individual experiments was selected to generate ~ 10 action potentials under control conditions. We measured the number of B48 action potentials after a 10 s CBI-2 stimulation and found that they were significantly potentiated during perfusion of $100 \mu\text{M}$ IBMX ($t = 4.78$, $p < 0.05$, $n = 4$). Measurements of the time needed for the increased firing to decline by 66% from its peak value after the CBI-2-elicited enhancement in the number of B48 action potentials was significantly longer in the presence of IBMX (control, 5.0 ± 0.58 vs post-CBI-2, 21.3 ± 2.03 m; $t = 6.80$, $p < 0.05$). This suggested that cyclic nucleotides may act to increase B48 firing and may also contribute to regulation of the duration of this enhancement.

Given that previous work showed that the cAMP/protein kinase A (PKA) pathway is an effective means of regulating neuronal firing in *Aplysia*, we investigated the role of this pathway (Klein et al., 1986; Ghirardi et al., 1992; Goldsmith and Abrams, 1992). To examine the possibility that the cAMP/PKA pathway mediated the increased number B48 action potentials, we mea-

Figure 5. Effect of CBI-2 stimulation on B48 firing is occluded in the presence of FCAP and CP2. **A1**, Representative recordings of B48 firing before and after peptide superfusion. Every 60 s, B48 was injected with the same size current pulse for a 3 s duration. After superfusion of FCAP and CP2, the current pulse elicited an increased number of action potentials. **A2**, After the increase in the number of action potentials in B48 in the presence of FCAP and CP2, the size of the current injection is reduced to elicit the same number of control action potentials during the 3 s period. In the presence of FCAP and CP2, there was no change in the number of action potentials 1 min after CBI-2 stimulation. Exogenous FCAP and CP2 occluded the increase in B48 firing after CBI-2 stimulation. **B**, Average number of B48 action potentials and SE are shown for the peptide application and post-CBI-2 stimulation in the presence of peptide.

sured changes in the number of action potentials produced by intracellular injections of 8-br-cAMP, an analog of cAMP. In this paradigm, we used three separate electrodes. The first electrode was used to inject a depolarizing 3 s constant current pulse into B48. Pulse size was adjusted to elicit ~ 10 action potentials every 60 s. Simultaneously, a second electrode recorded the resulting depolarization. After a series of these stimulations, a baseline of firing was established. To increase the level of cAMP in B48, using a third electrode we iontophoretically injected 8-br-cAMP (10 mM) for 15 min. After injection of 8-br-cAMP and removal of the injecting electrode, using the same size current pulses we measured the number of action potentials elicited in B48. We found that intracellular injection of cAMP analog 8-br-cAMP increased the number of action potentials from 10.8 ± 0.6 to 21 ± 4.2 (Fig. 6A) and then gradually returned to control levels. To ensure that the DMSO in which 8-br-cAMP was dissolved did not increase B48 firing, using the same three-electrode paradigm we measured the number of B48 action potentials after a 15 min injection of a 0.20% DMSO solution. We found that DMSO injection alone had no significant effect on B48 firing (control, 9.06 ± 0.24 vs post-DMSO injection, 9.28 ± 0.36 ; $t = 0.55$, $p = 0.61$, $n = 4$).

To analyze the increase in the number of action potentials observed after 8-br-cAMP injection, we performed a one-way repeated-measures ANOVA between the number of B48 action potentials elicited during the control preinjection period, after injection of cAMP, and after a 30 min recovery period (Fig. 6B) ($F_{(2,8)} = 91.81$, $p < 0.001$, $n = 5$). Individual comparison revealed there was a significant increase in the number of action potentials after injection of 8-br-cAMP (preinjection control vs 8-br-cAMP injected, $t = 12.46$, $p < 0.001$; 8-br-cAMP injected vs recovery, $t = 10.84$, $p < 0.001$). Intracellular injections of 8-br-cAMP into B48 mimicked the effects of both FCAP/CP2 and CBI-2 stimulation on the firing of B48, thus implicating the cAMP/PKA pathway as a possible mediator of the FCAP/CP2 and CBI-2 stimulation-induced effects involved in the development of an ingestive program.

Although this demonstrated that cAMP was capable of augmenting the number of B48 action potentials, it remained to be determined whether cAMP was necessary to increase the firing of B48 in response to CBI-2 stimulation and/or peptide superfusion.

Figure 6. Intracellular injection of 8-br-cAMP increases B48 firing. **A**, Representative recordings of B48 firing before and after injection of 8-br-cAMP. Every 60 s, B48 was injected with a constant depolarizing current pulse (3 s duration). The change in the number of action potentials generated from the same size current pulse was measured after a 15 min injection with 8-br-cAMP. **B**, Aggregated data show that there is a significant increase in B48 firing 1 min after injection of 8-br-cAMP.

Figure 7. Effects of FCAP/CP2 and CBI-2 stimulation on B48 firing are blocked after injection of Rp-cAMP. **A1**, The number of B48 action potentials was not altered by the injection of Rp-cAMP. Representative recordings of B48 firing during 3 s current injections before and after Rp-cAMP injection are shown. **A2**, Injection of Rp-cAMP into B48 blocked FCAP- and CP2-induced increases in B48 firing. Representative recordings of Rp-cAMP-injected B48 firing during 3 s current pulses before and during the application of FCAP and CP2. **A3**, Aggregated data: average number of B48 action potentials elicited by the same current injection before and after injection of Rp-cAMP and the average number of action potentials in Rp-cAMP injected B48 before and during FCAP and CP2 application. **B1**, Representative recordings of 3 s current injections of B48 before and 1 min after CBI-2 stimulation. **B2**, Representative recording of Rp-cAMP-injected B48 current pulses before and 1 min after 10 s CBI-2 stimulation. Rp-cAMP injection into B48 blocked the CBI-2 stimulation-induced increase in firing. **B3**, Aggregated data: average number of B48 action potentials elicited by the same size current injection before and after CBI-2 stimulation, before and after injection of Rp-cAMP.

sion. To address this issue, we investigated whether the increase in the firing of B48, observed after CBI-2 stimulation and during FCAP/CP2 application, could be eliminated when a competitive antagonist of cAMP activation was injected into B48. Here, we measured the number of action potentials elicited during a constant current depolarization (3 s duration). We first performed

experiments to establish that Rp-cAMP, a competitive antagonist of the activation of cAMP-dependent protein kinase, did not alter the control level of the firing of B48. We found that injection of Rp-cAMP did not alter the number of B48 action potentials (Fig. 7A1) (control vs after injection of Rp-cAMP: $t = 0.751$, $p = 0.53$, $n = 5$). Using the same stimulation paradigm, we measured the effect of FCAP/CP2 perfusion on the firing of B48 after a 15 min injection of Rp-cAMP (Fig. 7A2). Analysis of aggregate data showed that, in the presence of the antagonist, FCAP/CP2 did not increase the number of B48 action potentials (Rp-cAMP injected vs Rp-cAMP injected during superfusion of FCAP/CP2: $t = 1.16$, $p = 0.33$, $n = 5$) (Fig. 7A3). This is in contrast with the increase in B48 firing that FCAP/CP2 produced in the absence of Rp-cAMP (Fig. 4).

Since the Rp-cAMP blocked the increase in the firing of B48 produced by exogenous application of FCAP/CP2, we reasoned that the effects of CBI-2 stimulation on the firing of B48 might also be eliminated by injection of Rp-cAMP. We tested this by measuring CBI-2 stimulation-induced changes in the number of B48 action potentials elicited by current injections. We found that in Rp-cAMP-injected B48 neurons stimulation of CBI-2 did not produce an increase in the number of action potentials (representative recording) (Fig. 7B2), an increase that otherwise was present in the absence of Rp-cAMP (Fig. 7B1). Statistical analysis of pre-CBI-2 Rp-cAMP-injected versus post-CBI-2 Rp-cAMP-injected B48 neurons showed that there was no difference between the two conditions ($t = 1.97$, $p = 0.14$, $n = 5$). Therefore, cAMP appears to be necessary for the increase in the firing of B48 after CBI-2 stimulation.

Motor programs: role of cAMP

The previous experiments demonstrated the role of cAMP in increasing B48 firing but did not address the role cAMP may have during feeding motor programs. To gain further insight into whether the cAMP/PKA pathway was involved in repetition priming of B48 firing, we examined B48 activity during CBI-2-elicited feeding motor programs. In the first set of experiments, we elicited a series of motor programs using the L-ISI CBI-2 stimulation paradigm. Under these conditions, no buildup of ingestiveness occurs and motor programs remained intermediate as defined by the low firing frequency of B8 during both protraction and retraction phase. Here, after a series of L-ISI CBI-2 intermediate programs were elicited, 8-br-cAMP was iontophoretically injected into B48 for 15 min. After the injection, a

second series of L-ISI CBI-2 programs was elicited. We found that B8 firing frequency during both the protraction and retraction phases was unchanged, whereas the firing frequency of B48 during the protraction and post-retraction phases was selectively increased during motor programs after injection (representative recording) (Fig. 8A). After a 20 min recovery period, B48 firing frequency returned to control intermediate levels.

After injections of B48 with 8-br-cAMP, the L-ISI CBI-2-elicited programs remained intermediate as defined by the lack of phase specificity of B8 firing (aggregate data) (Fig. 8B). A one-way ANOVA revealed that the protraction-phase and retraction-phase firing frequency of B8 did not significantly change from control, after B48 injection, or during programs elicited during the 20 min recovery (B8 protraction: $F_{(2,6)} = 3.03$, $p = 0.12$, $n = 4$; B8 retraction: $F_{(2,6)} = 0.32$, $p = 0.74$, $n = 4$).

Although the programs remained intermediate as defined by B8 activity, aggregate data showed a significant increase in B48 activity during the protraction and post-retraction phases (Fig. 8C). To determine the statistical significance of the differences among control, 8-br-cAMP-injected, and recovery groups, we performed a one-way ANOVA of B48 firing frequency in the protraction phase ($F_{(2,6)} = 19.94$, $p < 0.05$, $n = 4$) and B48 firing frequency in the post-retraction phase ($F_{(2,6)} = 12.19$, $p < 0.05$, $n = 4$). Individual comparisons revealed that B48 fired significantly more after injection of 8-br-cAMP than it did during the control or recovery period in both the protraction and post-retraction phases (protraction: control vs 8-br-cAMP injected: $t = 5.77$, $p < 0.05$; 8-br-cAMP injected vs recovery: $t = 5.10$, $p < 0.05$; post-retraction: control vs 8-br-cAMP injected: $t = 4.57$, $p < 0.05$, 8-br-cAMP injected vs recovery: $t = 3.90$, $p < 0.05$).

Further analysis of B48 activity during protraction phase revealed that the latency from the onset of CBI-2 stimulation to the first action potential in B48 decreased significantly after the injection of 8-br-cAMP ($t = 4.42$, $p < 0.05$). Consistent with this observation, we found the fraction of the protraction phase in which B48 was active also increased after the injection of 8-br-cAMP ($t = 11.6$, $p < 0.05$) (Fig. 8D).

Together, the data shown in Figure 8 indicated that injection of 8-br-cAMP upregulated B48 activity during feeding motor programs. Thus, 8-br-cAMP mimicked the increase in B48 firing frequency observed during intermediate CBI-2 programs elicited in the presence of FCAP/CP2 (Fig. 2) and during repetitive S-ISI CBI-2-elicited programs (Friedman et al., 2009).

The experiment described above indicated that it was possible to mimic the effects of repeated S-ISI CBI-2 stimulation and peptide application on B48 with injection of 8-br-cAMP. How-

Figure 8. 8-br-cAMP mimics the effect of S-ISI repetitive CBI-2-elicited programs on B48 firing frequency. A series of five L-ISI CBI-2-elicited intermediate programs were elicited (control). **A**, Representative recordings of L-ISI CBI-2-elicited intermediate program in which B48 fired weakly in the protraction phase and B8 fired weakly in both the protraction and retraction phases. After intracellular injection of 8-br-cAMP in B48, the L-ISI CBI-2-elicited program exhibited a high frequency of B48 activity and continued to exhibit intermediate firing of B8 during protraction and retraction. After 15 min of recovery, B48 protraction activity decreased to control levels. **B**, Aggregate data of the B48 protraction-phase and post-retraction-phase firing frequency during control, B48 8-br-cAMP-injected, and recovery L-ISI CBI-2 programs. **C**, Aggregate data of the B8 protraction- and retraction-phase firing frequency during control, B48 8-br-cAMP-injected, and recovery programs. **D**, Average latency of the first action potential of B48 from the start of CBI-2 stimulation was reduced after 8-br-cAMP injection into B48. The fraction of protraction with B48 activity was higher after 8-br-cAMP injection compared with control and recovery.

ever, it was not evident whether this was the signaling pathway that was used during repetition priming of B48 during repetitive CBI-2 stimulation. The possibility remained that a different signaling pathway may be responsible for the increase in activity of B48 during feeding motor programs. We thus sought to determine whether cAMP/PKA was not only sufficient but was also necessary for the increased B48 firing that followed repetitive S-ISI CBI-2 stimulation.

In each preparation, we first elicited a series of 10 CBI-2 programs using an S-ISI (30 s) (Fig. 9A). This produced repetition priming, i.e., progressively more ingestive programs as defined by the significant increase of B8 retraction-phase activity (1st vs 10th: $t = 5.68$, $p < 0.05$) and by the significant increase in B48 protraction-phase activity (1st vs 10th: $t = 11.67$, $p < 0.001$). After a 20 min recovery period, we initiated a 15 min iontophoretic injection of Rp-cAMP into B48. After the injection period, we again elicited a series of 10 S-ISI CBI-2 motor programs (Fig. 9B).

To determine the effects of the injections of Rp-cAMP into B48 on repetition priming, we compared the characteristics of

Figure 9. Effects of repetitive S-ISI CBI-2-elicited programs on B48 firing frequency are blocked by cAMP antagonist Rp-cAMP. **A**, Control S-ISI CBI-2 programs illustrate the increase in B48 protraction and B8 retraction firing from the 1st to the 10th program. **B**, After injection of Rp-cAMP into B48, B48 protraction activity no longer increased from the 1st to the 10th S-ISI CBI-2-elicited program, while B8 retraction activity increased similar to the control. **C**, Average firing frequency from the 1st to 10th program of B48 protraction activity and B8 retraction activity.

S-ISI CBI-2 programs elicited before (control) and after Rp-cAMP injection into B48. First, a comparison of B8 retraction-phase activity in the first programs in both series of stimulations (without and with Rp-cAMP) failed to reveal any differences between the two conditions ($t = 2.46$, $p = 0.10$, $n = 4$). The same was true for the 10th programs elicited in the two conditions ($t = 0.198$, $p = 0.86$, $n = 4$) (Fig. 9C). This indicated that, as defined by B8 activity, injections of Rp-cAMP into B48 had no effect on repetition priming of B8 firing. Next, we compared the effects of Rp-cAMP injection on the repetition priming of B48. In contrast to B8, we did not observe a progressive increase in B48 protraction-phase activity during the series of 10 programs. Comparison of B48 protraction-phase activity during the first program that was elicited in the absence Rp-cAMP and after its injection revealed no difference between the two conditions ($t = 1.92$, $p = 0.15$). In contrast, when the last programs were com-

pared we found that there was significantly less B48-protraction-phase activity during the 10th program after Rp-cAMP injection ($t = 14.44$, $p < 0.001$, $n = 4$) than before Rp-cAMP injection. Thus, the increase in B48 firing frequency during repetitive CBI-2 stimulation required cAMP/PKA signaling. Therefore, cAMP/PKA pathway appears to be critical for repetition priming of B48.

Discussion

Our data indicate that the repetition priming of B48 is mediated by two peptide co-transmitters of CBI-2, which act via cAMP/PKA. cAMP/PKA activation increases the firing of B48. Consequently, when B48 receives an excitatory drive during motor programs, its firing increases.

Motoneurons versus CPG

Repetition priming, whereby pre-exposure to a stimulus enhances subsequent sensory, cognitive, and motor behaviors, operates in both vertebrates and invertebrates (Epuru et al., 1995; Schacter and Buckner, 1998; Sinnamon et al., 2000; Proekt and Weiss, 2003; Proekt et al., 2008). Despite the pervasiveness of repetition priming, surprisingly little is known about the cellular mechanisms that alter network dynamics to produce this phenomenon (Grill-Spector et al., 2006; Wig et al., 2009). Studies of repetition priming in the feeding network of *Aplysia* began to identify network-level mechanisms responsible for repetition priming.

In free-feeding *Aplysia*, a manifestation of food-stimulation-induced repetition priming is a progressive increase of radula movements (Weiss et al., 1982). In the nervous system, repetition priming is observed after stimulation of CBI-2 and is expressed as an increase in firing frequency of motoneurons B8 and B48. The relative phasing of the activity of these two motoneurons, as well as other motoneurons, is controlled by the CPG. Repetition

priming of B8 activity is also controlled by the CPG and results from a change of balance of activity and synaptic efficacy of two CPG interneurons, B20 and B40, which promote egestive and ingestive motor programs, respectively (Jing and Weiss, 2001, 2002). Here, we obtain evidence that repetition priming of B48 might involve modification of intrinsic characteristics of B48. Our experiments link repetition priming of B48 firing to the direct upmodulation of B48. Together with previous findings, current data indicate that in a CPG-controlled network repetition priming can be implemented both through CPG-dependent and CPG-independent processes.

B48 versus B8

CPG-dependent priming operates in the case of B8, and CPG-independent priming operates in the case of B48. Could these

differences be related to the means that the CPG uses to drive these neurons and/or to their firing patterns? Studies in several systems show that there are different classes of motoneurons with different firing pattern characteristics (Morton and Chiel, 1993b; Jing and Weiss, 2001, 2005; d'Avella and Bizzi, 2005; Li et al., 2007). Here, B48 fires exclusively during protraction and only in CBI-2-elicited programs (Church and Lloyd, 1994; Evans et al., 1996). In contrast, B8 fires during retraction or protraction depending on the type of motor program (Morton and Chiel, 1993b; Nargeot et al., 1997). These differences are associated with two different modes with which the feeding CPG activates these motoneurons. B8 receives a balance of concurrent excitation-inhibition from the CPG throughout protraction and retraction, which determines the phase in which B8 fires (Sasaki et al., 2009). The CPG controls B48 firing through alternating excitation-inhibition that occurs during protraction and retraction, respectively. In Figure 10, to simplify the hypothetical discussion of increased firing we use the concept of excitability. In Fig. 10, we illustrate the consequences of increasing the excitability of a neuron that, like B8, receives concurrent excitation-inhibition. An increase of excitability of such a neuron would nonspecifically increase its firing. In the case of B8, this would result in radula closure during both protraction and retraction, thus producing a dysfunctional response. In contrast, an increase of excitability of a neuron that, like B48, receives alternating excitation and inhibition would result in more firing during protraction while remaining inhibited during retraction. In the case of B48, this would be appropriate for implementing stronger movements in a phase-specific manner.

The two modes of activating motoneurons, concurrent excitation-inhibition, and alternating excitation-inhibition (Fig. 10) have been identified as major modes of controlling network outputs in both vertebrates and invertebrates (Destexhe et al., 2003; Alaburda et al., 2005; Berg and Hounsgaard, 2009; Sasaki et al., 2009). Given that many networks are modulated, it should be possible to test the generality of the hypothesis that CPG-dependent vs CPG-independent modulation may be preferentially used based on the type of inputs that motoneurons, or for that matter other network outputs, receive.

Context versus experience

Experience-dependent states (Abraham and Willows, 1971; Allport et al., 1994; Sharma et al., 2003; Proekt et al., 2004, 2008) are not the only types of states that can modify CPG outputs. CPG states, and therefore outputs, can be modified by the context in which responses are being elicited (Nolen and Hoy, 1984; Esch et al., 2002; Gold and Shadlen, 2003; Friesen and Kristan, 2007). Context-dependent and experience-dependent CPG states differ (Nadim et al., 2008). In context-dependent states, the activity of

Figure 10. Effects of modulation of neuronal excitability differ depending on whether neurons are driven by concurrent excitation-inhibition or alternating excitation-inhibition. **A**, Diagrammatic illustration of the use of concurrent (left) and alternating (right) excitation-inhibition in two phases of behavior (dark and open bars). **B1, B2**, A neuron that receives an equal amount of excitation-inhibition in two phases of a program fires similarly in both phases of the program (**B1**), and an increase of its excitability results in its increased firing in both phases of behavior (**B2**). **C1**, A neuron that receives alternating excitation-inhibition has a weak preference to fire in the phase during which it receives excitation. **C2**, An increase of the excitability of such a neuron selectively increases its firing in the phase during which it receives excitation.

Figure 11. Hypothetical sequence underlying repetition priming of motoneuron B48 firing.

network elements can be controlled directly by the very hormones/modulators or modulatory networks that are part of the context. In contrast, to generate different responses experience-dependent states do not require the presence of modulators or activity of modulatory network inputs at the time when responses are generated. One cannot, however, assume that neuromodulators do not partake in establishing such experience-dependent states. The effects of neuromodulators are known to persist even when the neuromodulators are no longer present. Importantly, the presence of neuromodulators may be necessary for the acquisition but not for the subsequent expression of various forms of learning (Byrne and Kandel, 1996; Crow, 2004; Burrell and Sahley, 2005). Thus, at least in principle, neuromodulatory inputs may form a basis for transforming context-dependent network states into experience-dependent network states (Marder et al., 1996).

Our data indeed indicate that CBI-2, acting through its peptide cotransmitters, induces second messenger-mediated modulation of the intrinsic properties of B48 that persist when CBI-2 is no longer active (Fig. 11). As the effects of repeated CBI-2 stimulation can be blocked by Rp-cAMP and mimicked by 8-Br-cAMP, cAMP is sufficient and necessary for a priming of B48 activity. Second messengers are implicated in the regulation of several aspects of CPG outputs (Hempel et al., 1996; Heinrich et al., 2001; Katz and Clemens, 2001; Grillner, 2006; Doi and

Ramirez, 2008). Importantly, our data suggest that second messengers are involved in the formation of persistent changes of the CPG output, and, intriguingly, they can do so by acting on specific motoneurons rather than via CPG. Our data thus support the idea that persistence of experience-dependent network states reflects the persistence of effects of neuromodulatory inputs.

Feed-forward modulation

When food contacts the mouth of *Aplysia*, CBI-2 receives an excitatory input that elicits CBI-2 firing (Rosen et al., 1991). CBI-2 stimulation produces feeding motor programs that correspond to feeding behaviors that CBI-2 elicits in semi-intact preparations (Morton and Chiel, 1993b; Morgan et al., 2002; Jing and Weiss, 2005). The mediating action of CBI-2 is implemented by fast synaptic actions of ACh, the primary transmitter of CBI-2. Our data indicate that CBI-2-induced priming of B48 firing is likely to involve FCAP and CP2 (Fig. 11). The combination of current and previous results indicates that CBI-2 acts both as a cholinergic initiator and a peptidergic neuromodulator of feeding responses.

A similar duality of function exists in buccal motoneurons. Many buccal motoneurons use their primary transmitter to initiate/mediate buccal muscle contractions (Cohen et al., 1978; Church and Lloyd, 1994) and use their neuropeptides to upmodulate muscle contractions (Lloyd et al., 1984; Cropper et al., 1987; Lotshaw and Lloyd, 1990; Evans et al., 1999; Hurwitz et al., 2000). CBI-2-dependent upmodulation of motoneuronal firing and CPG-independent upmodulation of muscle contractions represent a feed-forward modulation. This has important consequences. First, the CBI-2-dependent increase of motoneuronal firing will result in larger muscle contractions (Cohen et al., 1978; Evans et al., 1996; Brezina et al., 2000a). Second, since greater amounts of peptides are released when motoneurons fire at higher frequencies (Vilim et al., 1996a,b), the increased firing of motoneurons will produce a greater upmodulation of muscle contractions. The dual effects of upmodulation of motoneuronal firing may provide an elegant mechanism for amplifying the neuromodulatory actions of CBI-2.

The effects of feed-forward modulation need not be limited to muscles and may be present in the nervous system. For instance, a chain of three neurons in which neuron 1 upmodulates neuron 2, and neuron 2 both upmodulates and elicits fast EPSPs in neuron 3 could produce similar amplifying effects on the firing of neuron 3 as those described for muscle contractions. Although feed-forward excitation and inhibition involving fast synaptic actions have been described in a number of networks, there appear to be no explicit descriptions of feed-forward modulation. Considering that invertebrate and vertebrate CPGs share a number of organizational motifs, feed-forward modulation may emerge as a motif in the organization of vertebrate networks.

Summary

Briefly, our study indicates that, acting via cAMP, peptidergic inputs to motor networks play an important role in inducing persistent experience-dependent network states that manifest themselves as repetition priming. Importantly, this study also suggests that in CPG-driven motor networks modulation of intrinsic motoneuronal characteristics may be used to generate persistent changes of network outputs. We suggest that utilization of CPG-dependent versus CPG-independent plasticity may be linked to whether a given motoneuron is driven by concurrent versus alternating excitation-inhibition, respectively.

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