Regeneration of Cerebral-Buccal Interneurons and Recovery of Ingestion Buccal Motor Programs in Aplysia After CNS Lesions

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Sánchez, José Antonio D., Yongsheng Li, and Mark D. Kirk. Regeneration of cerebral-buccal interneurons and recovery of ingestion buccal motor programs in Aplysia after CNS lesions. J Neurophysiol 84: 2961–2974, 2000. In the sea slug Aplysia, rhythmic biting is eliminated after bilateral cerebral-buccal connective (CBC) crushes and recovers within 14 days postlesion (dpl). The ability of cerebral-buccal interneuron-2 (CBI-2) to elicit ingestion buccal motor programs (iBMPs; i.e., fictive rhythmic ingestion) and to regenerate synaptic connections with target buccal neurons was assessed with intracellular recordings and dye injections. Isolated central ganglia were obtained from control animals and from lesioned animals at selected times after bilateral CBC crushes. Within 3 wk postlesion, transected CBI-2 axons sprouted at least 10 fine neurites confined to the core of the CBC that projected across the crush site toward the buccal ganglia. When fired with depolarizing current steps, CBI-2 was not observed to elicit iBMPs in preparations until 14 dpl. Thereafter a progressive enhancement in CBI-2’s ability to elicit iBMPs was observed with time postlesion. By 40 dpl, CBI-2-elicited iBMPs were indistinguishable from those of controls. CBI-2 regenerated monosynaptic connections with appropriate buccal premotor- and motorneurons by 14 dpl, and the strength of these connections increased with time postlesion. Dramatic frequency facilitation was exhibited by the regenerating CBI-2 buccal synapses; for instance, at early postlesion times, no observable excitatory postsynaptic potentials (EPSPs) were obtained with 1- Hz stimulation of CBI-2, while at 7 Hz, a dramatic increase in EPSP amplitude was obtained with successive spikes. The present study shows that the time course of axonal and synaptic regeneration by command-like interneuron CBI-2 is correlated with the recovery of ingestion buccal motor programs elicited by CBI-2. These results parallel our previous findings of functional neural regeneration in the feeding system and suggest that functional neural regeneration is at least in part mediated by regeneration of specific synaptic pathways.

INTRODUCTION

Molluscs exhibit robust recovery after lesions to the CNS (Moffett 1995, 1996). They offer distinct advantages for studies of CNS regeneration, including the presence of large, identifiable neurons with well-characterized synaptoc connective, and defined roles in the control of behavior (Kandel 1976, 1979; Kupfermann et al. 1991). Therefore it is possible to correlate recovery of specific behaviors with regeneration of particular neuronal connections. Often when molluscan neurons are axotomized or transplanted, they re-form appropriate synaptic connections leading to functional behavioral recovery in vivo (Benjamin and Allison 1985; Fredman and Nutz 1988; Hamilton and Fredman 1998; Murphy and Kater 1980; Scott and Kirk 1992; Scott et al. 1995; Syed et al. 1992). In addition, behaviorally relevant components of neural circuits can be studied in vitro using identified neurons isolated and grown together in tissue culture (Hamakawa et al. 1999; Magoski and Bulloch 1999; Schacher 1988; Syed et al. 1990).

Our studies have focused on functional CNS regeneration that leads to recovery of consummatory feeding behavior in the gastropod mollusc, Aplysia californica (Johnson et al. 1999; Scott and Kirk 1992; Scott et al. 1995, 1997). Consummatory feeding (biting, swallowing, and rejection) in Aplysia and related mussels, is largely controlled by the cerebral ganglion and paired buccal ganglia (Benjamin 1983; Kater and Rowell 1973; Kupfermann 1974a,b). Bilateral transections of the cerebral-buccal connectives (CBCs) eliminate rhythmic biting without disrupting the appetitive phases of feeding behavior (Kupfermann 1974a; Scott et al. 1995). Crush lesions applied bilaterally to the CBCs also eliminate rhythmic biting, but within 14 days postlesion, biting behavior recovers (Scott and Kirk 1992; Scott et al. 1995). Recovery of other behavioral parameters related to feeding progress with time postlesion, and by 50 days postlesion, consummatory feeding in lesioned animals appears normal (Scott et al. 1995). This recovery of feeding behavior is correlated with restoration of axonal projections in the connectives as shown by electron micrographs obtained from cross-sections of the crush site and anterograde labeling of regenerating axons across the lesion (Johnson et al. 1999; Scott et al. 1997).

Within 24 h after a CBC crush lesion, the axonal core of this nerve tract is no longer compressed and has assumed a circular shape, although it is considerably smaller than the adjacent intact regions of the CBC. At this time postlesion, the nerve core contains large areas of tissue debris and many microglia or amoebocytes with prominent nuclei, and approximately 50 regenerating axons with small diameters have grown across the crush site. The presence of cellular debris in regions of CBC adjacent to the crush site demonstrates that axons severed by the crush retract on either side of the lesion through a process of limited degeneration (Johnson et al. 1999; Scott et al. 1997).

Consummatory feeding in Aplysia consists of cyclic movements of the radula, a toothed apparatus used to grasp seaweed.
During rhythmic biting, radula protrusion (outward movement largely driven by the I2 muscle) (Hurwitz et al. 1996) is followed by closing and retraction (inward movement) of the radula. When the radula grasps a piece of seaweed, the latter is pulled into the buccal cavity, and cyclic swallowing occurs with repeated protrusion and retraction of the radula (Kupfermann 1974a; Weiss et al. 1982). Rejection occurs when inedible food is partially ingested, and during rejection radula closing is associated with protraction of the radula (Morton and Chiel 1993a,b). Ingestion and rejection motor programs can be distinguished one from another, and expression of these programs by isolated buccal ganglia can be modulated in vitro by a variety of biogenic amines and peptide transmitters (Kabotyanski et al. 2000; Sossin et al. 1987).

Four categories of neurons with cell bodies in the cerebral or buccal ganglia project axons through the CBCs (Chiel et al. 1988; Rosen et al. 1979, 1982, 1989, 1991a; Weiss et al. 1982) and are transected by CBC crush lesions (Johnson et al. 1999; Scott et al. 1997). One of these categories, the cerebral-buccal interneurons (CBIs), have cell bodies in the cerebral ganglion and are “command-like” or modulatory projection neurons for consummatory feeding behaviors (Kupfermann et al. 1991; Marder and Pearson 1998; Rosen et al. 1991a).

The most thoroughly studied CBI to date is CBI-2 (Church and Lloyd 1994; Hurwitz et al. 1999; Rosen et al. 1991b; Sánchez and Kirk 2000). When fired with prolonged steps of depolarizing current in semi-intact preparations, CBI-2 elicits ingestion buccal motor programs (iBMPs) (Church and Lloyd 1994; Rosen et al. 1991a, 1998). Sensory discharges in CBI-2 are elicited by tactile and chemical stimulation of the inner lips and perioral zone (Rosen et al. 1991a). Feedback pathways from the buccal ganglia to cerebral ganglion influence activity in CBI-2 and likely contribute to pattern generation for consummatory feeding (Rosen et al. 1991b; Sánchez and Kirk 1998). CBI-2 makes weak monosynaptic connections with multifunction neurons B31/32 (Rosen et al. 1991a) and strong monosynaptic connections with premotor neuron B34 and protractor motor neurons B61/B62 (Sánchez and Kirk 1998, 2000). However, the specific synaptic mechanisms whereby CBI-2 elicits iBMPs are not fully understood (Hurwitz et al. 1999).

Here we tested the ability of CBI-2 to elicit iBMPs and to regenerate synaptic connections with target buccal neurons after bilateral CBC crushes. CBI-2 was first able to drive iBMPs at 14 days postlesion (dpl). Fourteen days postlesion is the first time postlesion that all animals have recovered rhythmic ingesting after CBC lesions. Some of this work has appeared in abstract form (Sánchez et al. 1996).

**METHODS**

**Experimental animals and behavioral tests**

Adult Aplysia californica (100–250 g) were purchased from the Howard Hughes Medical Institute Marine Resources Facility (Miami, FL) and Marinus (Long Beach, CA) and maintained in recirculating Instant Ocean (Aquarium Systems, Mentor, OH) at 13–16°C. The animals were fed dried seaweed (laver) every 1–3 days. Subjects were deprived of food for a period of 2 or 3 days prior to behavioral testing. To select animals with normal feeding behavior, only subjects that met a prelesion rhythmic biting criterion of 10 bites within 3 min (Scott et al. 1995) were included in this study. This bite test was also performed on animals at all times postlesion prior to dissection. This enabled us to compare the properties of rhythmic biting in intact animals with CBI-2-elicited iBMPs tested in preparations taken from the same subjects. We quantified the time to the tenth bite and the average number of bites per minute during bite tests.

**Lesions**

The animals were immobilized by injecting chilled (4°C) isotonic MgCl₂ (40% of body wt) into the hemocoel. The relaxed animals were pinned dorsal side up and a small incision (~1 cm) was made in the dorsal skin and underlying muscles just anterior to the rhinophores at the midline. The CBCs were crushed bilaterally with No. 5 forceps midway between the cerebral and buccal ganglia. The crushes produced a clear separation (~0.5 mm in width) in the axonal core of the CBC, leaving intact a wafer of translucent nerve sheath (Scott et al. 1997). The body-wall incision was sutured closed in layers (6.0 suture silk), and animals were injected with streptomycin sulfate (400 µl at 10 mg/ml, diluted in 1 ml normal artificial sea water, NASW), tagged, and returned to the home tank.

**Physiology**

Semi-intact preparations (Fig. 1) were taken from control animals and animals taken at the following dpl: 7–9, 14, 20–29 (20 ± dpl), 30–39 (30 ± dpl), 40–49 (40 ± dpl), and 50–59 (50 ± dpl). Bite tests were conducted prior to dissection on the day of the experiment. Only lesioned animals that exhibited normal appetitive responses (Kupfermann 1974b; Scott et al. 1995) and, for animals at 14 dpl or later, that met the rhythmic biting criterion (Scott et al. 1995) were used for subsequent experiments.

Dissection procedures for in vitro experiments using semi-intact preparations have been described in detail elsewhere (Hurwitz et al. 1994; Plummer and Kirk 1990; Sánchez and Kirk 2000). Briefly, the animals were anesthetized as described in the preceding text by injection of isotonic MgCl₂, and the cerebral ganglia, pedal-pleural ganglia, and the buccal ganglia with a portion of I2 muscle attached (Fig. 1) were removed from anesthetized animals. The caudal surface of the buccal ganglia and ventral surface of the cerebral ganglia were surgically dehseated for intracellular recordings. The ped-pleural ganglia were left attached to the cerebral ganglion to uniquely identify CBI-2 in all preparations by its indirect inhibitory input elicited by neuron C-PR. This polysynaptic inhibitory input recruited by C-PR appears to be mediated by interneurons located in the pedal-pleural ganglia (Hurwitz et al. 1999; Teyke et al. 1997).

All experiments were performed at room temperature (22–24°C) with continuous perfusion of NASW [containing (in mM): 494 NaCl, 11 KCl, 19 MgCl₂, 30 MgSO₄, 11 CaCl₂, 10 Tris, pH = 7.4]. Saline containing 3 × Ca²⁺/3 × Mg²⁺ (Jordan et al. 1993) was used to block polysynaptic activity when testing for direct synaptic connections (Cohen et al. 1978).
Morphology

Conventional electrophysiological techniques were used to obtain simultaneous intracellular recordings from cerebral and buccal neurons. Cerebral-buccal neuron-1, CBI-1, and buccal neurons B4/5, B31/32, B61/62, and B8a, b were identified by previously established criteria, including their characteristic cell body locations and sizes, axonal projections, and spontaneous activity (Gardner 1971; Hurwitz et al. 1994; Rosen et al. 1991a; Susswein and Byrne 1988). In many cases, identification was confirmed with intracellular dye fills at the end of the experiment. Intracellular recordings were made with glass microelectrodes containing either 3 M KCl (5–15 M V) or Lucifer yellow (3% in distilled water, Sigma, St. Louis) in their tips with the shanks of the latter electrodes back-filled with 0.1 M LiCl (10–40 MFI). Two paradigms for CBI-2 intracellular stimulation were used. For precise control of CBI-2 firing frequency, we used 25-ms current pulses, each giving rise to a single action potential in CBI-2, delivered at 7 or 10 Hz. Firing in CBI-2 during these trains was not altered by synaptic input to CBI-2 coincident with iBMPs; therefore suprathreshold CBI-2 activity consisted of repetitive action potentials elicited at 7 or 10 Hz. When driven with current pulses, subthreshold depolarizing current was continuously applied to CBI-2 to facilitate one for one firing with current pulses. Alternatively, CBI-2 was stimulated with long-duration steps of depolarizing current that produced an average firing frequency 10 Hz.

Extracellular recordings were made with suction electrodes constructed from PE200 tubing pulled in a flame to an approximate tip diameter of 0.5 mm. The physiological data were digitized on a Neurorecorder (Neuro Data Instruments, New York) and recorded on VHS-VCR tape for subsequent analysis or were digitized with a computer-based data-acquisition and -analysis system (MacLab, CA).

Morphology

Neurons were filled with Lucifer yellow (LY) by passing negative current at 2–4 nA for 10–30 min. The preparations were fixed overnight in 4% paraformaldehyde, 0.3 M sucrose, in 0.1 M phosphate buffered saline (PBS), pH = 7.4. The fixed tissue was rinsed in 0.1 M PBS twice, dehydrated in an ascending ethanol series, and cleared in methyl salicylate. In several preparations, CBI neurons were filled using electrodes containing 4% carboxyfluorescene (in 1 M KCl) and cleared in NASW:glycerol (50:50) without fixing the preparations. The cleared ganglia were viewed with an Olympus BH2 epifluorescence microscope, and photographed with Ektochrome 400-ASA color slide film.

Statistics

Data are shown as means ± SE. Statistical analyses were performed as follows. Changes in EPSP and iBMP parameters at various days postlesion were performed by using one-way ANOVAs followed by a least-significant difference (LSD) post hoc test (significance established at P < 0.05). Non parametric tests for the ability of CBI-2 to elicit multiple iBMPs were done using Pearson χ² tests (P < 0.05).

RESULTS

Neurites sprout from the proximal stump of transected CBI axons

Using intracellular LY and carboxyfluorescene injections, we examined the cellular morphology of CBI-2 in control preparations and in preparations obtained 7–50+ (i.e., 50–59 dpl) days after bilateral CBC crushes. Within the cerebral ganglion, there was no apparent change in morphology of CBI neurons in lesioned preparations (Fig. 2, A and B); no overt sprouting occurred from the cell body or neuropilar processes. However, in all CBI-2 s that were dye-injected at 14, 20+, and later days postlesion (n = 5), processes had sprouted from the proximal axon stump in the CBC immediately adjacent to the crush site. Generally, three to five neurites emerged from the proximal axon and subsequently branched up to three times to form a projection of >10 fibers in the CBC (Fig. 2C). The majority of these regenerating neurites were confined to the axonal core of the CBC (see also Johnson et al. 1999; Scott et al. 1997).

CBI-2 re-establishes its command-like function and ingestion behavior recovers by 14 dpl

CBI-2 elicited ingestion buccal motor programs in all control preparations (n = 37; Figs. 3A and 4A) (Rosen et al. 1991a) (see Table 2 for a summary of physiological and behavioral parameters). Depolarizing current steps of long duration produced CBI-2 firing frequencies exceeding 10 Hz on average and elicited iBMPs that consisted of repeated cycles of fictive ingestion (ascertained by established criteria) (Morton and Chiel 1993a,b). Briefly, ingestion occurs when the radula is open during protraction and closed during retraction. Thus during fictive ingestion motor programs, onset of firing in protractor motor neurons, such as B61/62, begins prior to- and primarily precedes- that of closer motor neurons B8a, b (firing in B8a, b produces large axon spikes in the radular nerve, Fig. 3). Bursts of action potentials in radula retractor motor neurons (e.g., B4/5) coincide with firing in radula closer motor neurons. Bilateral CBC crushes abolish rhythmic biting when tested from 7–9 dpl (Scott and Kirk 1992; Scott et al. 1995), and CBI-2 s were unable to elicit iBMPs in preparations taken 7–9 dpl (Fig. 4A).
The ability of CBI-2 to elicit iBMs at 14 dpl and later times postlesion was further analyzed by determining the cycles per minute (cpm) of iBM elicited by CBI-2. The iBM cpm were dependent on the firing frequency of CBI-2 (Rosen et al. 1991a; Sánchez and Kirk 2000), and for precise control of firing frequency, in this set of experiments we fired CBI-2 at 10 Hz using depolarizing current pulses (25-ms durations, Fig. 4B). For analysis of cpm, only cases exhibiting at least three cycles of iBM in response to CBI-2 stimulation were included. In control experiments, CBI-2 elicited an average of 5.6 ± 0.4 cpm (n = 9) while at 14 dpl, mean cpm was significantly reduced (2.1 ± 0.1; n = 4, P < 0.01). Mean cycles per minute appeared to remain depressed until 30+ dpl, although by 20+ dpl there was no significant difference when compared with controls.

We also examined the latency between onset of CBI-2 firing and the first B61/62 burst at the beginning of an iBM. We found that the latency to the first B61/62 burst was proportional to the cpm of iBM elicited by CBI-2. For instance, at higher CBI-2 firing frequencies, CBI-2 produced an increase in iBM cpm, and the latency to the first B61/62 burst was decreased (Sánchez and Kirk 1998; data not shown). Therefore we drove CBI-2 at 10 Hz with current pulses and determined the latency to the first B61/62 burst at selected times postlesion (Fig. 4C). In control preparations (n = 9), the mean latency to the first B61/62 burst was 1.9 ± 0.3 s. At 14 dpl, this latency was significantly longer than that of controls (mean = 4.3 ± 0.6 s, n = 4, P < 0.01; Fig. 4C) and remained significantly elevated until 40+ dpl (see following text). Because CBI-2 driven iBMs always begin with bursts in B61/62 (Sánchez and Kirk 2000), the latency to B61/62 bursts under these conditions is likely a good measure of CBI-2’s effect on the central pattern generator (CPG) for iBMs. However, in cases where the strength of the direct CBI-2 to B61/62 synaptic connection was substantial (in preparations from control and lesioned animals; see following text), the direct connection likely contributed significantly to the B61/62 burst latency.

At 14 dpl, maintained firing of CBI-2 with steps of depolarizing current elicited at least one cycle of iBM in 81% of preparations (Fig. 4A). In 47.7% of the latter preparations, CBI-2 elicited multiple cycles of iBM (in this set of experiments, multiple cycles of iBM was defined as ≥2 cycles, Fig. 4A). However, the percentage of preparations in which CBI-2 elicited multiple cycles of iBMs was significantly less than that of controls (Pearson χ² = 9.16, P = 0.002). These results were consistent with a significant decrease in mean bites per minute and increased time to the 10th bite exhibited in these animals prior to dissection (Fig. 5). There was a progressive enhancement of CBI-2-elicted iBMs in preparations with increasing time postlesion (see following text).

Progressive enhancement of CBI-2 elicited iBMs after 14 dpl

In preparations isolated from animals 14 to 50+ dpl, there was a progressive enhancement of CBI-2’s ability to elicit iBMs (Fig. 4; including 4 preparations taken 60–86 dpl, data not shown). At 20+ days postlesion, CBI-2 was able to elicit at least one cycle of iBM in all of 16 preparations. However, the percentage of preparations exhibiting multiple cycles (i.e., ≥2 cycles) of iBM at 20+ dpl was significantly less than controls (Pearson χ² = 5.3, P < 0.02). Also the mean latency to the first B61/62 burst (4.4 ± 0.62 s) was still significantly longer than in controls (P < 0.01, Fig. 4C). By 20+ dpl, the mean cpm of iBM elicited by CBI-2 (4.0 ± 0.4, n = 6; restricted to cases where ≥3 cycles of iBM were elicited) was still decreased compared with controls but the difference was not significant (P < 0.05, Fig. 4B).

The ability of CBI-2 to reliably elicit multiple cycles of iBMs recovered to within control levels at 30+ dpl (Pearson χ² = 2.65, P = 0.103, n = 12, Fig. 4A), and iBM cpm were not significantly different from controls at 30+ dpl (5.0 ± 0.4, n = 6, P < 0.05, Fig. 4B). However, the latency to the first B61/62 burst remained significantly longer than controls (P < 0.01).
By 40+ dpl, the mean percentage of preparations exhibiting multiple cycles of iBMP, the mean number of cycles per minute of iBMP elicited by CBI-2, and the mean latency to the first B61/62 bursts were not significantly different from those of controls \((P > 0.05, \text{Fig. 4})\). In freely behaving animals at 30+ dpl and later times postlesion, there were no significant differences in the mean time to complete 10 bites or in the mean number of bites per minute when compared with unlesioned controls (Fig. 5).

**B61/62 burst duration and intraburst firing frequency recover with time postlesion**

In freely behaving animals, bite magnitude is determined largely by the extent of radula protraction (Rosen et al. 1989; Susswein et al. 1976), and in CBC lesioned animals bite magnitude recovers between 40 and 50 dpl (Scott et al. 1995). We quantified B61/62 burst duration and intraburst firing frequency at selected times postlesion. Since B61/62s drive contraction of the I2 muscle (Hurwitz et al. 1996), these parameters may influence bite magnitude. As described in the preceding text for tests of iBMP cpm, in this set of experiments we drove CBI-2 with current pulses (25-ms duration) at 10 Hz.

In control preparations, the mean duration of individual B61/62 bursts and the average intraburst firing frequency were 5.8 \pm 0.5 s \((n = 4)\) and 15.1 \pm 1.0 Hz \((n = 4)\), respectively (Fig. 6). At 14 dpl, mean burst duration increased significantly \((P < 0.05)\) and mean intraburst frequency decreased significantly \((P < 0.05)\) when compared with controls (Fig. 6). The decrease in mean intraburst firing frequency was consistent with the reduced ability of CBI-2 to elicit iBMP (Figs. 3 and 4) and with the decreased EPSP amplitude recorded in B61/62 at this time postlesion (see following text, Fig. 7). Mean burst duration returned to within control levels by 20+ dpl \((n = 5)\). However, mean intraburst firing frequency remained significantly decreased until 40+ dpl \((n = 3; \text{Fig. 6})\).

**CBI-2 re-establishes monosynaptic connections with B61/62**

We previously documented that monosynaptic connections are made by CBI-2 with protractor motor neurons B61/62 and that these connections exhibit strong facilitation (Sánchez and Kirk 1998, 2000). Here we have shown that CBI-2’s ability to drive iBMPs strengthens with days postlesion. One potential mechanism whereby CBI-2 recovers its ability to elicit iBMPs is by the regeneration of appropriate CBI-2 synaptic connections with target buccal neurons. To test this hypothesis, we used EPSP amplitude recorded in B61/62 to determine the
Changes in synaptic strength of CBI-2 to B61/62 connections were examined by driving CBI-2 with current pulses at 7 Hz in saline that contained $3 \times 10^{-3} \text{Ca}^{2+} / 3 \times 10^{-3} \text{Mg}^{2+}$ to suppress polysynaptic activity. Data were not analyzed at CBI-2 firing frequencies above 7 Hz because polysynaptic input was often recruited. In control preparations ($n = 9$), the average steady state B61/62 EPSP amplitude was 7.6 ± 0.3 mV (Figs. 7, A and B, and 8, A and B). Overt EPSPs were produced in B61/62 after single spikes in CBI-2; that is, repeated CBI-2 stimulation was not required to produce observable EPSPs in controls. At 7–9 dpl, no overt EPSPs were recorded in B61/62 at any frequency of stimulation ($n = 4$). By 14 dpl, the EPSPs in B61/62 were observable, but mean steady-state EPSPs were significantly smaller ($P < 0.05, n = 4$) than controls (Figs. 7B and 8B). At 14 dpl, no EPSPs were observed in B61/62 when CBI-2 was driven at frequencies of $\leq 3 \text{ Hz}$, with 5 Hz being the minimum CBI-2 firing frequency capable of eliciting an observable EPSP (Table 1). These results were likely due to a reduced strength of these connections and the dramatic frequency-dependent facilitation at stimulus frequencies of $\geq 5 \text{ Hz}$ at early times postlesion (Fig. 8D).

We have demonstrated that axon diameters of regenerating processes were initially reduced, and became larger with days postlesion (Fig. 2) (see also Johnson et al. 1999; Scott et al. 1997). Therefore one would predict that conduction velocity of...
regenerated CBI-2 axons would decrease at early times postlesion. Correlated with this would be a corresponding increase in the delay between CBI-2 action potentials in the cerebral ganglion and EPSPs recorded in B61/62. Therefore we examined the latency from CBI-2 action potentials to B61/62 EPSPs at selected times postlesion.

In control preparations, the mean latency from the peak of CBI-2 action potentials to the onset of B61/62 EPSPs was 27.3 ± 0.6 ms (n = 9, Fig. 7, A and C). Conduction delay within the cerebral-buccal connective accounts for most of this mean latency (Sánchez and Kirk 2000; and data not shown). At 14 dpl, the mean latency increased dramatically to 62.6 ± 0.6 ms (P < 0.05, n = 4, Fig. 7, A and C). This mean latency decreased with time postlesion, consistent with a progressive increase in axon diameter of regenerated axons; however, at all times postlesion, the mean latency was significantly longer than controls (P < 0.05, Fig. 7C).

Progressive enhancement of synaptic strength with time postlesion

At 20+ dpl, the mean amplitude of CBI-2 to B61/62 EPSPs tested at 7 Hz was still significantly smaller than controls (P < 0.05; Fig. 7B). In addition, the minimum CBI-2 firing frequency at which an observable EPSP could be recorded in B61/62 remained elevated relative to controls (Table 1). By 30+ dpl, mean steady-state EPSP amplitude (Fig. 7B), mean minimum firing frequency of CBI-2 required to elicit observable EPSPs and the number of spikes to the first observable EPSP had all returned to control levels (Tables 1 and 2).

Facilitation of CBI-2 to B61/62 synapses changes with days postlesion

We tested for changes in short-term synaptic enhancement (i.e., frequency facilitation) exhibited by CBI-2 to B61/62 synapses by using a facilitation index (FI). The FI in this study was defined as the steady state EPSP amplitude (averaged amplitudes of 3 responses at steady state) divided by the averaged amplitude of the first three observable EPSPs at 7-Hz stimulation of CBI-2 (Fig. 8). We used this definition of FI because at early times postlesion, EPSPs were not observed at low frequencies (e.g., 1 Hz, Table 1). Therefore FI as a ratio of steady-state EPSP amplitude at a high stimulus frequency (e.g., 7 Hz) divided by the steady-state EPSP amplitude at 1 Hz was undefined at 14 and 20+ dpl. In fact as described in the preceding text, at early times postlesion there was a minimum frequency required to elicit an overt EPSP (Table 1). Because some facilitation must have occurred before these synapses produced observable EPSPs at 14 dpl and 20+ dpl, FI in this study is likely to be an underestimate.
of the actual magnitude of facilitation, especially at early times postlesion (see DISCUSSION).

The average FI in control preparations was 6.81 ± 0.38 (Fig. 8, B and C). At 14 and 20+ dpl, mean FI was significantly decreased with respect to controls ($P < 0.05$). By 30+ dpl, the mean FI returned to within control levels ($P > 0.05$; Fig. 8C).

**Regeneration of CBI-2 connections to buccal premotor neurons**

Although our study focused primarily on synaptic input to motor neurons B61/62, we performed experiments testing regeneration of monosynaptic connections made by CBI-2 onto

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**TABLE 1.** Quantification of parameters related to CBI-2 regeneration at the indicated days postlesion

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<tr>
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<th>Control</th>
<th>7-9</th>
<th>14</th>
<th>20+</th>
<th>30+</th>
<th>40+</th>
<th>50+</th>
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<tr>
<td>Minimum frequency, Hz $^a$</td>
<td>S $^b$</td>
<td>—</td>
<td>5$^c$</td>
<td>3$^c$</td>
<td>S</td>
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<td>S</td>
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<tr>
<td>No. of spikes to observable EPSP $^d$</td>
<td>1</td>
<td>—</td>
<td>7.5 ± 1.4$^e$</td>
<td>4.2 ± 0.5$^e$</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>First observable EPSP, mV $^f$</td>
<td>1.19 ± 0.2</td>
<td></td>
<td>0.41 ± 0.1$^e$</td>
<td>0.24 ± 0.1$^e$</td>
<td>0.58 ± 0.1$^e$</td>
<td>0.53 ± 0.2$^e$</td>
<td>0.96 ± 0.1</td>
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Data are given as means ± SE. EPSP, excitatory postsynaptic potential. $^a$ Minimum CBI-2 firing frequency to elicit an overt B61/62 EPSP. $^b$ S, a single CBI-2 action potential elicited an observable EPSP in B61/62. $^c$ Significantly different from controls at $P < 0.05$; one-way ANOVA followed by least-significant difference (LSD) post hoc test. $^d$ Number of CBI-2 spikes to first observable B61/62 EPSP at the corresponding minimum frequency. $^e$ First observable B61/62 EPSP amplitude at the indicated minimum frequency.
premotor neurons B31/32 (data not shown). In control preparations, CBI-2 produced small amplitude EPSPs in B31/32 (mean EPSP amplitude $5.1.31 \pm 0.001 \text{ mV}$; $n = 4$) (see also Rosen et al. 1991a). We sampled these synaptic connections at selected times postlesion. These connections were not detectable at early times postlesion (i.e., 7–9 dpl; $n = 3$) and were significantly smaller than controls at 14 dpl ($0.45 \pm 0.01 \text{ mV}$; $n = 4$; $P < 0.05$). At 20+ dpl, the mean CBI-2 to B31/32 EPSP amplitude increased ($0.9 \pm 0.1 \text{ mV}$; $n = 3$) but remained significantly smaller when compared with controls ($P < 0.05$). The mean amplitude of EPSPs recorded in B31/32 ($1.25 \pm 0.002 \text{ mV}$; $n = 3$) returned to within control levels by 30+ dpl ($P > 0.05$).

**Regeneration of CBI-1 synaptic pathways to the buccal ganglia**

We also performed experiments on regeneration of synaptic connections made by CBI-1. This cerebral-buccal interneuron has been shown to produce single (or in rare cases 2) cycles of rejection-like BMPs associated with defensive withdrawal of the head (Rosen et al. 1998). In control preparations, CBI-1 makes monosynaptic EPSPs with multifunction neurons B4/5 that exhibit synaptic depression with repeated stimulation (Rosen et al. 1991a). The average B4/5 EPSP amplitude in control preparations was $1.67 \pm 0.3 \text{ mV}$ ($n = 11$). At 14 dpl after bilateral CBC crushes, firing CBI-1 at any frequency failed to produce observable EPSPs in B4/5. By 20+ dpl, CBI-1 produced overt EPSPs in B4/5, but they appeared to be smaller than controls ($0.62 \pm 0.04 \text{ mV}$; $n = 2$). The CBI-1 to B4/5 synapses increased in strength at 30+ dpl, however, the EPSPs still appeared to be smaller than controls ($1.19 \pm 0.5 \text{ mV}$; $n = 2$). Statistical tests were not performed in the latter two sets of experiments because of the low $n$ values, and this synaptic connection was not tested systematically at later times postlesion.
Consistent with previous studies by Rosen et al. (1991a, 1998), we found that CBI-1 could reliably drive at least one cycle of buccal motor program in control preparations (n = 15). CBI-1's ability to drive BMPs was immediately abolished when the CBCs were crushed. Unlike CBI-2, CBI-1 was not able to drive a motor program in the buccal ganglia at 14 dpl. CBI-1 regained its ability to drive a single cycle of BMP at 20+ dpl (n = 4) but required larger depolarizing currents (≥5 nA or greater) and thus higher (compared with controls) CBI-1 firing frequencies to elicit a single cycle of BMP. By 30+ dpl, CBI-1 reliably elicited a single cycle of BMP, resembling that observed in control preparations, and depolarizing currents of +2.5 nA or less were required to elicit a BMP (comparable to that required for control preparations; n = 5, data not shown).

In one preparation obtained at 86 dpl, the synaptic response elicited in B4/5 by CBI-1 was unusual. Driving CBI-1 produced what appeared to be the normal, rapidly depressing fast EPSPs observed in control preparations (Rosen et al. 1991a) (see inset, i). The inset is taken from the B4/5 trace at the time indicated by the bar (i). However, in addition to the fast EPSPs, with repetitive firing CBI-1 elicited a coincident slow inhibitory postsynaptic potential (IPSP). The peak IPSP amplitude occurred at the offset of CBI-1 firing (†). BMN, unidentified buccal motor neuron.

DISCUSSION

We previously demonstrated that functional neural regeneration occurs after bilateral crushes of the cerebral-buccal connectives (CBCs) in Aplysia and that recovery of consummatory feeding behavior (i.e., rhythmic biting) is likely due to regeneration of severed axons and reformation of synaptic connections (Johnson et al. 1999; Scott et al. 1995, 1997). In the present study, we quantify the nature and time course of axonal and synaptic regeneration by command-like CBI-2 of Aplysia following bilateral crush lesions of the CBCs. The regeneration of CBI-2 buccal connections is correlated with recovery of ingestion buccal motor programs elicited by CBI-2. This suggests that functional neural regeneration exhibited by this system is in part mediated by regeneration of specific synaptic pathways.

Potential mechanisms for functional neural regeneration and recovery of rhythmic biting

CBC lesions initially disrupt CBI-2 synaptic connections, and by 14 dpl CBI-2 synapses make weak but observable EPSPs in target buccal neurons. At this same time postlesion, the cycle frequency of iBMPs driven by CBI-2 is significantly lower than controls. This suggests that the weak regenerated buccal synapses may be responsible for the low bite frequency and decreased bite magnitude exhibited at 14 dpl in freely behaving animals (Fig. 5) (see also Scott et al. 1995).

The strength of CBI-2 synapses (i.e., mean EPSP amplitude) increases with time postlesion and attains control levels by 30+ dpl. This synapse regeneration is correlated with restoration of CBI-2's ability to elicit iBMPs. CBI-2's ability to elicit multiple cycles of iBMPs is initially eliminated after the CBC lesions but recovers rapidly and also achieves control levels by
30+ dpl. The number of iBMP cycles per minute (cpm) recruited by CBI-2 more rapidly returned to control levels and by 20+ dpl the average cpm is not significantly different from that of controls. Finally in our behavioral analyses of rhythmic biting, the time to achieve the 10th bite and the number of bites per minute during bite tests returned to control levels by 30+ dpl (Fig. 5, Table 2). These results are consistent with the hypothesis that recovery of rhythmic biting after bilateral CBC lesions is in part mediated by regeneration of CBI-2 buccal synapses.

Subsequent increases in strength of CBI-2 buccal synapses coincide with progressive enhancement of CBI-2-elicited iBMP cycle frequency, increases in bite frequency, and increases in bite magnitude (Scott et al. 1995). Except for the mean latency to the B61/62 EPSP, by 50+ dpl all measured parameters of CBI-2 buccal synapses, including EPSP amplitude, FI, as well as B61/62 burst duration and intraburst frequency are fully recovered (Table 2). This is consistent with the full recovery of consummatory feeding parameters between 50 and 60 dpl that we reported previously (Scott et al. 1995).

Of the preparations tested at 14 dpl, CBI-2 could drive at least one cycle of iBMP in 81% of these preparations, while 48% exhibited multiple cycles of iBMPs, leaving 19% of cases that failed to produce a single iBMP (Fig. 4). However, all subjects recover rhythmic biting at 14 dpl. Therefore if CBI-2 contributes to recovery of rhythmic ingestion at this time postlesion, why is CBI-2 unable to produce multiple cycles of iBMPs in all preparations obtained at 14 dpl? The answer may be that firing in both CBI-2s is required in the intact animal for rhythmic biting to be expressed at 14 dpl. We have shown in control preparations that two CBI-2s driven synchronously act cooperatively, producing an increased IBMP cycle frequency (when compared with driving a single CBI-2) (Sánchez and Kirk 1998; unpublished data). Therefore synchronous activation of both CBI-2s (that likely occurs with seaweed stimulation in intact animals) (Rosen et al. 1991a) may be required to elicit rhythmic biting, especially at early times postlesion. Consequently, driving a single CBI-2 in vitro would not be able to elicit and/or maintain rhythmic iBMPs in some preparations at 14 dpl due to weak synaptic CBI-2 connections in the buccal ganglia. In preparations obtained from lesioned animals, we did not test for recruitment of iBMPs in vitro while driving both CBI-2s synchronously. It will be important to test whether driving both CBI-2s synchronously increases the probability of eliciting and maintaining rhythmic iBMPs at 14 dpl.

The results presented here are consistent with our previous morphological results using anterograde transport of biocytin (Johnson et al. 1999). Using whole connective biocytin backfills, across the CBC crush site, we found that axons regenerating toward the buccal ganglia reach the edge of the buccal neuropil by 7 dpl. By 14 dpl, they have penetrated the ipsilateral and contralateral neuropil. It is at 14 dpl that all subjects have recovered rhythmic biting (Scott et al. 1995) and that CBI-2 has regained its ability to recruit ingestion buccal motor programs (Fig. 4, Table 2). Based on our current morphological and physiological results, we conclude that CBI-2 sprouts profusely from its proximal (with respect to its cell body in the cerebral ganglion) axonal stump and re-establishes synaptic connections with target buccal neurons by 14 dpl. These connections are sufficient for CBI-2 to elicit ingestion buccal motor programs in vitro and may be crucial for the recovery of rhythmic biting in lesioned subjects.

The distance required for axonal regeneration is equivalent for CBI-2 and CBI-1. However, the rate of recovery by CBI-1 buccal synapses appears to be more prolonged than that for CBI-2. Results that support the latter conclusion are as follows. The first observable CBI-1 to B4/5 EPSPs are obtained at 20+ dpl. EPSPs in B4/5 do not appear to return to control values by 30+ dpl, and CBI-1 is unable to elicit a BMP until 20+ dpl. One possibility is that CBI-1 and CBI-2 possess intrinsic differences in their rate of axonal regeneration that leads to the observed differences in time course for synaptic regeneration. Future morphological studies of their rate of axonal outgrowth could be used to address this issue.

**Mechanisms underlying changes in bite magnitude and latency to B61/62 EPSPs**

We previously hypothesized that the decreased bite magnitude observed early during functional neural regeneration (Scott et al. 1995) results from a decrease in the duration of motor neuron bursts during iBMPs and therefore a decreased neuromuscular drive resulting in smaller bite magnitudes. However, Scott et al. (1995) found that the duration of motor neuron bursts (recorded extracellulary with in vivo electrodes) is actually increased until the return of bite magnitude to control levels by 50 dpl. In the present study, we found that the mean duration of B61/62 bursts measured during CBI-2 driven iBMPs are also significantly increased at 14 dpl. Therefore the decrease in bite magnitude at early times postlesion cannot be explained by a decrease in motor neuron burst duration.

Intraburst firing frequency in B61/62 during iBMPs elicited by CBI-2 is likely determined both by input from the buccal CPG and the direct input from CBI-2. Intraburst firing frequency decreased at early times postlesion and returned to control levels at 40+ dpl (Table 2). This is consistent with our previous finding that bite magnitude in freely behaving animals recovers by 50 dpl (Scott et al. 1995). We suggest that the increase in intraburst firing frequency of B61/62 and the corresponding increase in neuromuscular drive to muscle I2 are responsible for the recovery of bite magnitude in freely behaving animals after CBC lesions.

One parameter that did not recover by 50+ dpl was the latency between the action potential in the CBI-2 soma and onset of an EPSP in B61/62. This latency is primarily due to axonal conduction time between the cerebral and buccal ganglia (Sánchez and Kirk 2000). We have shown that axon diameters within the CBC nerve core after bilateral CBC crushes are significantly smaller than controls even at 50+ dpl (Johnson et al. 1999; Scott et al. 1997). It appears likely that at 50+ dpl, the smaller CBI-2 axon diameter and its reduced conduction velocity accounts entirely for the increased latency observed.

**Frequency facilitation of CBI synaptic connections at early times postlesion**

CBI-2 re-establishes monosynaptic connections with B61/62 and B31/32 by 14 dpl. At this time postlesion as well as at 20+ dpl, CBI-2 to B61/62 connections exhibit a dramatic frequency-dependent facilitation. No overt EPSPs are detectable.
when CBI-2 is driven at low firing frequencies, while EPSP amplitude increases rapidly with repeated firing at high frequencies (Fig. 8D). Whereas in control preparations and at 30+ dpl and later times postlesion, EPSPs are detected with isolated CBI-2 action potentials (Tables 1 and 2).

It is thought that residual calcium in the presynaptic terminal underlies short-term increases in transmitter release with repeated presynaptic stimulation (Zucker 1999). Therefore the fact that no EPSPs are observed when CBI-2 is fired at <5 Hz at 14 dpl may be due to insufficient calcium entry to the presynaptic terminal (although facilitation occurs at the beginning of trains at 5 Hz, see following text). Another possibility is that the small-diameter CBI-2 presynaptic terminals (Johnson et al. 1999) require repeated activation before action potentials can penetrate the sites of transmitter release. Such a mechanism would be an unusual case of conduction block at low firing frequencies (Baccus et al. 2000).

An alternative explanation is that the immature CBI-2 synapses lack a sufficient pool of readily releasable presynaptic vesicles (Zucker 1989) to produce an overt EPSP. These synapses may remain silent until sufficient mobilization of vesicles occurs with repeated CBI-2 action potentials. The switching of individual synapses on and off during short-term synaptic plasticity has been documented recently at sensory to motor neuron connections in Aplysia (Royer et al. 2000).

**Short-term synaptic enhancement is likely underestimated at early times postlesion**

The CBI-2 connections with premotor neurons B31/32 and B34 and with motor neurons B61/62 exhibit short-term synaptic enhancement (STE), i.e., facilitation and augmentation/posttetanic potentiation (AUG/PTP) (Sánchez and Kirk 2000). We have demonstrated that in control preparations, STE can contribute to initiation of iBMPs driven by CBI-2 as well as to an increase in the cycles per minute of iBMPs elicited by CBI-2 (Sánchez and Kirk 2000). We focused here on facilitation during regeneration of CBI-2 buccal synapses. Although we did not test at all times postlesion for AUG/PTP at regenerating CBI-2 synapses, we observed it in seven preparations taken between 14 and 40 dpl (Sánchez and Kirk, unpublished observations).

In this study, we define facilitation index (FI) as the steady-state EPSP amplitude divided by the averaged amplitude of the first three observable EPSPs at 7-Hz stimulation of CBI-2 (see RESULTS). The FI is undefined at 7–9 dpl because no overt EPSPs were recorded. The FI remains decreased with respect to controls until 30+ dpl. It is at this latter time postlesion that the minimum firing frequency for CBI-2 to elicit an observable EPSP recovers to control levels (Tables 1 and 2). In controls as well as at 30+ dpl and later times postlesion, a single CBI-2 spike gives rise to an overt EPSP. In contrast, at 14 and 20+ dpl, substantial facilitation must occur before an overt EPSP is recorded. These results suggest that at early times postlesion, FI largely underestimates the magnitude of facilitation. Therefore it seems likely that STE, including facilitation and AUG/PTP, at synaptic connections with premotor neurons B31/32 (and potentially with B34) (Sánchez and Kirk 1998, 2000; unpublished observations) contribute to initiation—and/or modulation—of iBMPs in regenerating preparations. In fact, facilitation and AUG/PTP may be especially important at early times postlesion by compensating for the initially weak connections established by CBI-2 during regeneration.

**Significance with respect to other functional studies of neural regeneration**

Because of their large, identifiable neurons with well-characterized synaptic connections and defined roles in the control of behavior, molluscs provide distinct advantages for studies of CNS regeneration (Kandel 1976, 1979; Kupfermann et al. 1991; Moffett 1996). Normally when molluscan neurons are axotomized or transplanted, they re-form appropriate synaptic connections leading to functional behavioral recovery in vivo (Benjamin and Allison 1987; Fredman 1988; Hamilton and Fredman 1998; Murphy and Kater 1980; Scott and Kirk 1992; Scott et al. 1995; Syed et al. 1992). We confirm these previous results with the following exception. In one preparation at 86 dpl (the longest period postlesion that we tested), a CBI-1 connection to B4/5 exhibited a combined fast EPSP and slow IPSP, whereas in control preparations, this connection exhibits only a fast EPSP (Rosen et al. 1991a; Sánchez and Kirk, unpublished observations). It is possible that lesioned subjects, maintained for long periods, regenerate synaptic connections not found in control animals. However, the subject from which this preparation was obtained demonstrated normal feeding parameters. This suggests that the formation of apparently inappropriate synaptic connections at late times postlesion has no overt effects on feeding behavior. This is the only case of 114 CBI to buccal neuron combinations tested that an inappropriate synaptic response was observed. We conclude that formation of inappropriate connections between CBIs and buccal neurons is rare, and as with the one case we observed, does not adversely effect functional recovery.

Various factors can contribute to regeneration of appropriate synapses after injury, such as (Ambron and Walters 1996; Titmus and Faber 1990): cell body response to injury, intrinsic signaling mechanisms activated at the site of injury, response to extrinsic growth factors and cytokines, and target-derived factors. In addition, conditioning lesions can enhance neurite outgrowth and synaptogenesis during regeneration in both vertebrates and invertebrates (Fredman 1988; Fredman and Nutz 1988; Jacob and Croes 1998). Neurotrophic factors and cytokines are known to play a crucial role during regeneration (Lindholm et al. 1987; Rotshenker et al. 1992). In addition, glial cells may contribute directly to enhance synaptic strength during regeneration in vertebrates (Priefer and Barres 1997).

Although vertebrate CNS neurons have the capacity to regenerate and restore connections with their postsynaptic targets when provided, a permissive environment (Aguayo et al. 1991; Schwab 2000), the CNS of vertebrate animals in general has a very limited capacity for functional regeneration after injury. In contrast, invertebrates have a much greater capacity to repair CNS damage (Moffett 1996; von Bernhardi and Muller 1996) and in some cases can replace entire ganglia (Moffett and Austin 1982; Snyder and Moffett 1990). Functional neural regeneration requires the repair of severed connections between neurons to restore behavioral function (Guth et al. 1980). The large identifiable neurons and simple behaviors of invertebrate animals, such as Aplysia, allow us to correlate behavior with activity in specific neural circuitry in the CNS. Therefore it is possible to correlate recovery of...
behavior in lesioned animals with regeneration by identified neurons and their connections (Benjamin and Allison 1985; Bulloch and Kater 1982; Fredman and Nutz 1988; Murphy et al. 1985; Snyder and Moffett 1990; Vardi and Camhi 1982a,b; and the present report). Thus studies of regeneration in vivo and in vitro using invertebrate preparations will continue to be an important approach to determine the mechanisms of functional neural regeneration.

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