The Journal of Experimental Biology 199, 2631–2643 (1996)
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JEB0392

CONTRALATERAL SPROUTING AND COMPENSATORY INNERVATION FOLLOWING THE PERMANENT LESION OF A GANGLIONIC CONNECTIVE IN THE SNAIL

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Accepted 20 August 1996

Summary

The fate of sprouted fibres was examined following long-term recovery from lesions to the central nervous system of the snail *Achatina fulica*. Axonal dye-labelling of one of the cerebrobuccal connectives (CBC), following either a cut or a crush to the opposite CBC, revealed supernumerary labelling of neuronal elements in both the cerebral and buccal ganglia in the weeks following treatment. A part of this sprouting response involved the rerouting of axonal projections from injured neurones that project contralaterally into the uninjured CBC. In addition, intracellular dye-fills, immunocytochemistry for detection of serotonin and electrophysiological measurements all revealed that a contralateral, uninjured neurone, the metacerebral giant (MCG) cell, sprouted new processes to invade the buccal ganglion denervated by the lesion. The contralateral MCG also increased synaptic drive over a neurone in the denervated buccal ganglion, a cell that normally receives strong input only from the lesioned ipsilateral MCG. After 5 weeks of recovery, morphological and electrophysiological measurements returned to normal levels in animals receiving a crush to the CBC, suggesting a retraction of sprouted projections following successful regeneration across the lesioned pathway. In contrast, the measurements indicative of sprouted fibres continued for up to 5 months when the regenerative response was prevented by cutting the CBC. Together, these results suggest that both the cessation of sprouting and the eventual retraction of sprouted fibres in *Achatina fulica* is contingent upon successful regeneration of the damaged axonal pathway.

Key words: neuronal regeneration, axon elimination, competition, serotonin, gastropod, *Achatina fulica*, snail.

Introduction

The ability of invertebrate nervous systems to undergo regeneration and plastic changes following injury has been well documented over a wide range of species. Analyses performed at both the single cell and population levels have demonstrated that neurones respond to damage by following a characteristic sequence of events that can include: (1) regeneration from the site of injury, leading to reinnervation of original targets (Muller and Carbonetto, 1979; Benjamin and Allison, 1985; Macagno *et al.* 1985; Murphy *et al.* 1985; Fredman and Nutz, 1988); (2) sprouting of injured and uninjured neurones, leading to aberrant projections and possible compensatory innervation (Bulloch and Kater, 1982; Blackshaw *et al.* 1982; Murphey and Lemere, 1984; Arshavsky *et al.* 1985; Denburg, 1985; Allison and Benjamin, 1985, 1986; Gu and Muller, 1990; Syed *et al.* 1992); and (3) eventual retraction of aberrant fibres (Chase and Kamil, 1983; Denburg, 1985). Such studies support the view that multiple processes play a role in shaping the final outcome of neuronal injury.

Many of these events occur following a crush to the cerebrobuccal connective (CBC) in the snail *Achatina fulica*. Axonal dye-filling of the lesioned CBC has revealed the restoration of neuronal morphology and labelling patterns after 3–4 weeks of recovery (Croll and Baker, 1990). Electrophysiological measurements of regeneration support this morphological evidence, indicating the precise re-establishment of synaptic function. Following a crush to the CBC, the lesioned metacerebral giant neurone (MCG) has been shown to recover synaptic control of buccal follower cells during the first few weeks after injury (Chiasson *et al.* 1994). These regenerative responses in *A. fulica* are additionally accompanied by an extensive sprouting response by both injured and uninjured neurones. For example, dye-fills of the contralateral, uninjured CBC have revealed the presence of supernumerary neuritic projections and novel labelling of neuronal cell bodies in the weeks immediately following the crush (Croll and Baker, 1990). Importantly, however, the

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labelling of these supernumerary neuronal elements eventually disappears, suggesting that the retraction of aberrant fibres may depend upon successful regeneration by lesioned neurones (Croll and Baker, 1990).

In vertebrate neuronal systems, the initiation of neuronal sprouting and the retraction of those same sprouted fibres following regeneration have been viewed as indicative of neuronal processes involving intercellular competition (Yoon, 1972; Jackson and Diamond, 1981; Easter *et al.* 1985; van Mier and Litchman, 1994). However, surprisingly little evidence for, or against, such processes exists in invertebrate and particularly in gastropod systems (Blackshaw *et al.* 1982; Cohan *et al.* 1987; Gao and Macagno, 1987; Denburg *et al.* 1988). We therefore undertook in the present study to test whether regenerating neurites in *A. fulica* play a role in controlling a retraction of sprouted fibres. Specifically, we compared the long-term effects of a crush to the CBC, which permits regeneration of the lesioned pathway, with those of a cut to the connective, which prevents or delays such regeneration. Following the administration of lesions, axonal dye-labelling of the opposite, uninjured CBC was performed to compare sprouting responses among the populations of cerebral and buccal neurones. Analysis was additionally performed at the level of the single cell by monitoring changes in morphology of the MCG and its connectivity with a known buccal follower cell (Chiasson *et al.* 1994). Preliminary reports of this work have appeared elsewhere (Croll and Baker, 1994; Baker and Croll, 1995).

Materials and methods

Animals

Adult specimens of the terrestrial gastropod *Achatina fulica* Férussac, with shell lengths ranging between 40 and 60 mm, were obtained from Instrumix Suppliers, Inc. of Manila, Philippines. Animals were maintained at room temperature on a 12 h:12 h, light:dark cycle and were fed lettuce, carrot, squash and a slurry of crushed Purina rat chow in water.

Surgery

Surgical procedures were performed as reported earlier (Croll and Baker, 1990; Chiasson *et al.* 1994), except that a cut or a crush was administered to the exposed, right-hand CBC approximately mid-way between the cerebral and buccal ganglia. The crush, made with no. 5 jeweller's forceps, was judged to be complete when the site became transparent, indicating that only the connective tissue sheath remained. It has previously been demonstrated that such a crush to the CBC results in the complete transection of fibres in the connective and the initiation of a rapid regenerative response (Croll and Baker, 1990; Chiasson *et al.* 1994). Sham-operated snails were prepared following identical procedures but without damage to the CBC. Both lesion procedures resulted in an approximately 90 % survival rate.

Neuronal labelling

Normal, sham-operated and experimental snails were killed at varying intervals after surgery and the interconnected cerebral and buccal ganglia were removed and placed in saline (Chase and Goodman, 1977). The undamaged CBC (or simply the left-hand one in normal or sham-operated animals) was cut midlength and dye-filled towards both the cerebral and buccal ganglia with a solution of Ni^{2+}–lysine (Fredman, 1987; Croll and Baker, 1990). Ganglia were silver-intensified according to Croll (1986) and mounted whole in Permount (Fisher Scientific).

Injury-induced changes in projection patterns were evaluated by counting both the number of somata in the cerebral and buccal hemiganglia and the number of labelled fibres in trunks exiting the ganglia. While the use of whole-mounted preparations permitted evaluation of a large number of ganglia, it also imposed certain constraints. Some densely labelled fibre tracts and cell body populations could not be described quantitatively; thus, quantitative analyses of ipsilateral buccal cell bodies and ipsilateral buccal and cerebral trunk fibres were excluded. Fibre labelling in a few contralateral nerves and connectives also occasionally became too intense to quantify following injury. In such cases, a maximum count of 30 was conservatively scored for any single pathway. Quantitative descriptions were made only of those preparations that showed complete dye-filling of the CBC, as revealed by the presence of dye throughout the connective. A comparison of neuronal labelling in the cerebral and buccal ganglia following axonal dye-filling of the CBC revealed no differences between normal and sham-operated animals after 21 days of recovery (data not shown; see also Croll and Baker, 1990). As a result, the counts from normal and sham-operated animals were pooled for comparisons with the lesion treatments. Two-way analyses of variance (ANOVAs) were performed on the data across recovery time. Where significant differences were found ($P<0.05$), individual *t*-tests were performed and the Bonferonni correction was applied in the calculation of the significance levels of the contrasts.

Immunohistochemical procedures

The metacerebral giant neurone (MCG) is a large, bilaterally symmetrical serotonergic cell located in the cerebral ganglia. Each MCG has extensive, but primarily ipsilateral, buccal projections (Croll, 1988; Yoshida and Kobayashi, 1991; Chiasson *et al.* 1994) providing the only source of serotonin (5-HT) to these ganglia. This permitted the use of serotonin-like immunoreactivity as a measure of the buccal innervation of the MCG after the lesion treatments.

Indirect immunocytochemistry on whole-mounted ganglia was performed according to Croll (1988), using a polyclonal rabbit anti-serotonin antibody (Incstar Corp.) and a secondary, goat anti-rabbit antibody conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories). The tissue was mounted in a 3:1 solution of glycerol in phosphate-buffered saline (PBS) and viewed and photographed using a Leitz Aristoplan microscope equipped for epifluorescence with an L3 filter block.

Electrophysiological tests

Regeneration and sprouting by the MCGs was further examined by placing suction electrodes on the bilateral buccal salivary nerves in order to monitor 1:1 spikes triggered by action potentials elicited in the soma of either the MCG ipsilateral to the lesion or its contralateral homologue. Extracellular recordings from the salivary nerves and intracellular recordings of the MCG (using glass capillary microelectrodes containing 3 mol l^{-1} KCl; 10–30 MΩ resistance) were amplified and recorded using standard techniques and analyzed using a Computerscope enhanced graphics acquisition and analysis system, with signal-averaging software (RC Electronics Inc. Santa Barbara, CA, USA). Action potentials from the MCG (1–1.5 Hz; evoked by depolarizing d.c. current injection through the recording electrode) were used to trigger a 250 ms extracellular recording from the salivary nerve. Recordings were made in a saline bath containing high-[Mg^{2+}]/low-[Ca^{2+}] saline (Yoshida and Kobayashi, 1991) and were signal-averaged over 150–500 trials to verify any evoked signal and to eliminate background activity. The reliability of each recording was verified by the presence of spike activity in the salivary nerve from the salivary burster neurone, which appears to be homologous to that found in other gastropods (Prior and Gelperin, 1977; Croll *et al.* 1985; M. W. Baker, B. J. Chiasson and R. P. Croll, unpublished results). Recordings without repeated spike activity from the burster were excluded from the analysis.

We also examined the effects of the different lesion treatments upon a known connection between the MCG and a pair of bilateral buccal follower neurones, the left and right B1 cells. Normally, bursts of activity in the MCG elicit relatively large, compound excitatory postsynaptic potentials (EPSPs) in the ipsilateral B1 neurone but only weak depolarizations in the contralateral B1 neurone (Chiasson *et al.* 1994). Briefly, the interconnected cerebral and buccal ganglia were removed from the animal and placed in a Sylgard- (Dow Corning) coated Petri dish filled with saline. The epineural sheath was mechanically removed and the ganglia pinned to the dish, exposing the ventral surface of the cerebral ganglia and the dorsal surface of the buccal ganglia. Depolarizing square pulses of current (5–10 nA) were passed into the MCG, generating 2–3.5 Hz trains of action potentials lasting for 10 s. These 10 s trains (termed trials) were separated by 12 s intervals and were repeated 20 times. The amplitude of depolarization in B1 elicited by bursts of activity in the MCG was recorded. The electrical coupling coefficients between B1 neurones (V_{post}/V_{pre}; Bennett, 1966) were determined 21 days following administration of the crush by the injection of hyperpolarizing d.c. current *via* a balanced-bridge circuit for simultaneous current injection and voltage recording from a single microelectrode. The morphology of individual B1 and MCG cells was also observed in approximately 80 % of the animals by repenetrating the cells following the recording sessions and filling them with Ni^{2+}–lysine (Chiasson *et al.* 1994).

Results

Axonal dye-fills of the normal CBC

Axonal dye-filling of the CBC towards the cerebral ganglia, in sham-operated or unlesioned snails (N=10), labelled 42–57 fibres in all the major contralateral nerve trunks combined (the numbers of fibres in individual contralateral trunks ranged from 1 to 15). The number of stained cell bodies ranged from 63 to 71 in ipsilateral cerebral ganglia and from 8 to 12 in contralateral cerebral ganglia. The locations of the labelled cells on the dorsal surface of the cerebral ganglia are shown in Fig. 1A. (The locations of labelled cells, including the MCG, on the ventral surface have been described previously; Croll and Baker, 1990.)

Axonal filling of the CBC towards the buccal ganglia (N=11) labelled 43–66 fibres in all the contralateral nerve trunks combined (numbers of fibres in individual contralateral trunks ranged from 0 to 17). Too many ipsilateral buccal cell bodies were stained to quantify accurately in the whole-mounted ganglia (previously estimated as between 138 and 167; Croll and Baker, 1990), but approximately 55–87 cell bodies were consistently labelled within the contralateral ganglia. The locations of labelled cells on the ventral surface of the buccal ganglia are shown in Figs 2A and 3A. (The locations of labelled cells on the dorsal surface have been described previously; Croll and Baker, 1990.)

Labelling 21 days after the CBC cut or crush

Twenty-one days after the CBC had been cut, the severed ends of the connective had retracted towards the cerebral and buccal ganglia to form neuroma-like structures. On no occasion was there any evidence of CBC reconnection. In contrast, 21 days after crushing, the CBC on the damaged side appeared indistinguishable from that on the intact side when viewed under the dissecting microscope.

Twenty-one days after either cutting or crushing the CBC, dye-fills of the uninjured, contralateral connective resulted in augmented counts of most of the neuronal elements (Figs 1, 2, 3). After the cut, counts of contralateral neurites in both the cerebral and buccal trunks were significantly greater than control levels (P<0.001 for each measurement; Fig. 4A,B). Following the crush, counts of contralateral trunk fibres were significantly greater than control levels in the buccal (P<0.01) but not the cerebral (Fig. 4A,B) ganglia. The increase in trunk fibre staining was particularly evident within the injured CBC of the buccal ganglia following both lesions (Figs 2B,C, 3B,C).

Supernumerary cell bodies were also labelled 21 days after both lesion procedures. In the cerebral ganglia, novel somata were labelled in the E cluster on the dorsal surface (Croll and Baker, 1990; P<0.05 for both treatments; Figs 1B,C, 4C). In the buccal ganglia, a significant increase in the staining of contralateral somata was seen after cutting (Figs 2B, 3B), but not after crushing (Figs 2C, 3C), the CBC. In addition to the buccocerebral neurones which are normally present, a significantly greater number of small to medium-sized cell bodies (5–25 μm cell diameter) were stained after the CBC had

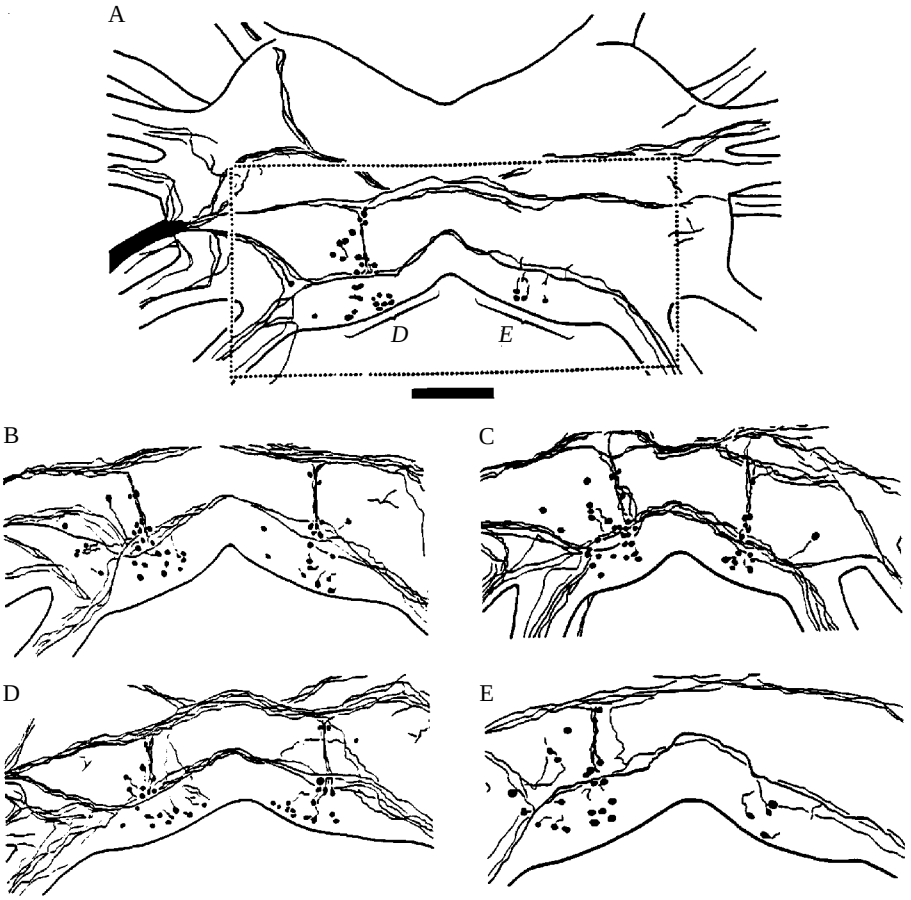

Fig. 1. *Camera lucida* tracings of the dorsal surface of representative cerebral ganglia following a dye-fill to the cerebrobuccal connectives (CBC) (darkened nerve on left side of ganglia). (A) Normal labelling on the dorsal surface of the cerebral ganglia showing the relative density of fibre labelling in commissural tracts and cerebral nerves. Two cell body clusters are also represented, indicating the ipsilateral D cell cluster (*D*) and contralateral E cell cluster (*E*). The dotted outline in A corresponds to the areas shown 21 days after either the cutting treatment (B) or crushing (C). Supernumerary labelling of cerebral neuronal elements was evident 21 days after either lesion treatment and could still be seen 49 days after the cut (D) but not the crush treatment (E). Scale bar, 300 μm.

been cut ($P<0.001$; Fig. 4D). These novel cell bodies were seen in six of the eight successful buccal fills obtained at day 21 and were restricted to the posterior-lateral margin of the ganglion, an area corresponding to the region normally occupied by labelled cells in the ipsilateral ganglion following a CBC dye-fill (cf. Fig. 2A,B and Fig. 3A,B). Both lesion types produce increased fibre labelling in the contralateral nerve trunks (Fig. 4B), especially the injured CBC and increased labelling of neuritic elements in the ganglionic neuropile (Figs 2B,C and 3B,C).

Labelling 49 days after the CBC cut or crush

By 49 days after cutting the CBC, two of the eight animals that were successfully dye-filled showed signs of reconnection of their connective. In both cases, a very thin connective was found originating from the neuromas on the stumps of the CBC and joining the cerebral and buccal ganglia. However, labelling of neuronal elements in these two preparations was similar to that seen in the other preparations 49 days after cutting, which showed no evidence of CBC reconnection (data not shown). As a consequence, all 49-day preparations were pooled for analysis.

Forty-nine days after the CBC had been cut, counts of fibres in the cerebral and buccal ganglia remained significantly greater than control values ($P<0.01$ for each region; Fig. 4A,B). There was, however, a significant drop in fibre labelling in both ganglia on day 49 compared with day 21 ($P<0.05$ for both ganglia). In contrast to the continued high counts observed after the CBC had been cut, the labelling of fibres returned to control levels in both the cerebral and buccal ganglia 49 days after the crush to the CBC (Fig. 4A,B).

Supernumerary somata also continued to be labelled in the cerebral and buccal ganglia 49 days after the CBC had been cut, and no significant differences were observed from the locations and cell body counts described 21 days after the connective had been cut (Figs 1D, 3D, 4C,D). In contrast, 49 days after the CBC had been crushed, a return to control labelling of cerebral and buccal somata was seen (Figs 1E, 3E, 4C,D).

When animals were allowed a 100-day ($N=6$) or 150-day ($N=4$) recovery period after the CBC had been cut, labelling of neuronal elements was unchanged from that described at day 49 (data not shown). None of these preparations showed any indication of reconnection of the CBC.

Taken together, these results suggest that both the cut and the crush treatments led to extensive sprouting responses by day 21. Most ganglionic trunks, especially the lesioned CBC, showed supernumerary labelling of neurites. Similarly, both lesion types led to novel labelling of somata among the same population of cells within the cerebral ganglia. It was, however, only after the CBC had been cut that newly labelled buccal cell bodies appeared. Supernumerary labelling

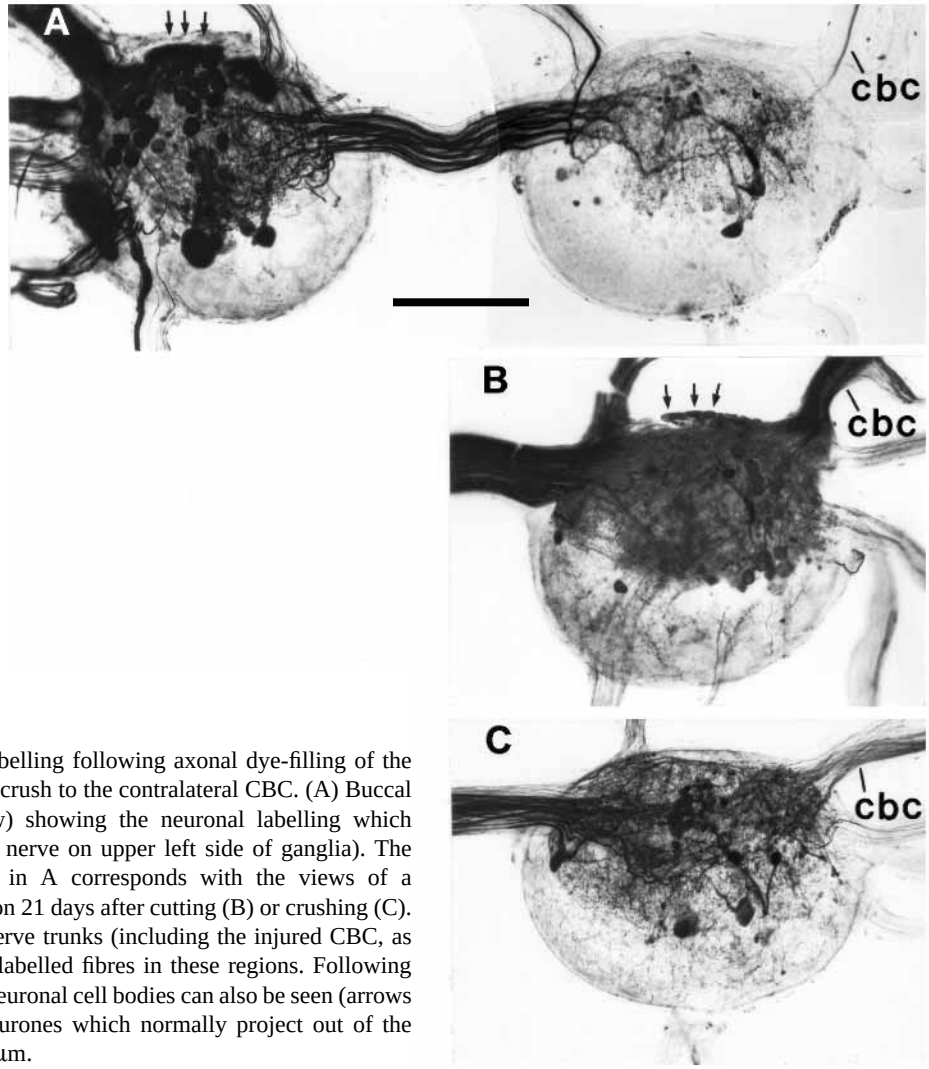

Fig. 2. Photomicrographs of normal buccal labelling following axonal dye-filling of the CBC (cbc) and labelling 21 days after a cut or crush to the contralateral CBC. (A) Buccal ganglia from a control animal (ventral view) showing the neuronal labelling which accompanies a dye-fill to the CBC (darkened nerve on upper left side of ganglia). The contralateral (right-hand side) hemiganglion in A corresponds with the views of a representative contralateral buccal hemiganglion 21 days after cutting (B) or crushing (C). The darkening of the neuropile and various nerve trunks (including the injured CBC, as indicated) results from increased numbers of labelled fibres in these regions. Following the cutting treatment, an increased number of neuronal cell bodies can also be seen (arrows in B), which appear to be homologous to neurones which normally project out of the ipsilateral CBC (arrows in A). Scale bar, 400 μm.

continued for up to 150 days after the CBC had been cut, whereas labelling returned to normal levels by day 49 after the CBC had been crushed.

Sprouting by the MCG: evidence from intracellular dye-fills and immunocytochemistry

The evidence presented above suggests that a retraction of sprouted fibres may be dependent upon the regeneration of normal connections. This hypothesis was tested further by examining the effects of the different lesion treatments upon the metacerebral giant neurone (MCG), a large serotonergic cell located on the ventral surface of the cerebral ganglia. The MCG projects multiple neurites through the CBC and innervates the ipsilateral buccal ganglia (Fig. 5A,B). The MCG is known to regenerate neurites rapidly to reinnervate the buccal ganglia after the CBC has been crushed (Chiasson *et al.* 1994).

Intracellular dye-fills were performed to determine whether lesions to the CBC led to sprouting by the contralateral MCG. Filling the contralateral MCG 16–21 days after cutting (N=12) or crushing (N=5) the CBC revealed no noticeable changes in its cerebral arborization, but pronounced differences were evident in the buccal projections of the cell. Increased numbers of small fibres in the buccal commissure, medial lateral, superficial pharyngeal and anterior lateral nerves were seen 16–21 days after both types of lesion (Fig. 5C). In addition, a general increase in contralateral neuropilar innervation was usually apparent after cutting the CBC.

Dye-fills of the lesioned (ipsilateral) MCG confirmed that the neurone failed to regenerate fibres across the cut site (N=8). Instead numerous retrograde neurites were seen extending back along the CBC to re-enter the cerebral ganglia (not shown). However, in none of these preparations was there any evidence for sprouting of fibres across the cerebral commissure and out through the contralateral CBC. Furthermore, no evidence was ever seen suggesting dye-coupling between the two MCGs.

Changes in the buccal innervation of the MGC were also revealed using serotonin-like immunoreactivity. When the CBC was cut, immunoreactivity in the connective across the cut site and in the buccal ganglion on the lesioned side was reduced over the first few days and was virtually undetectable

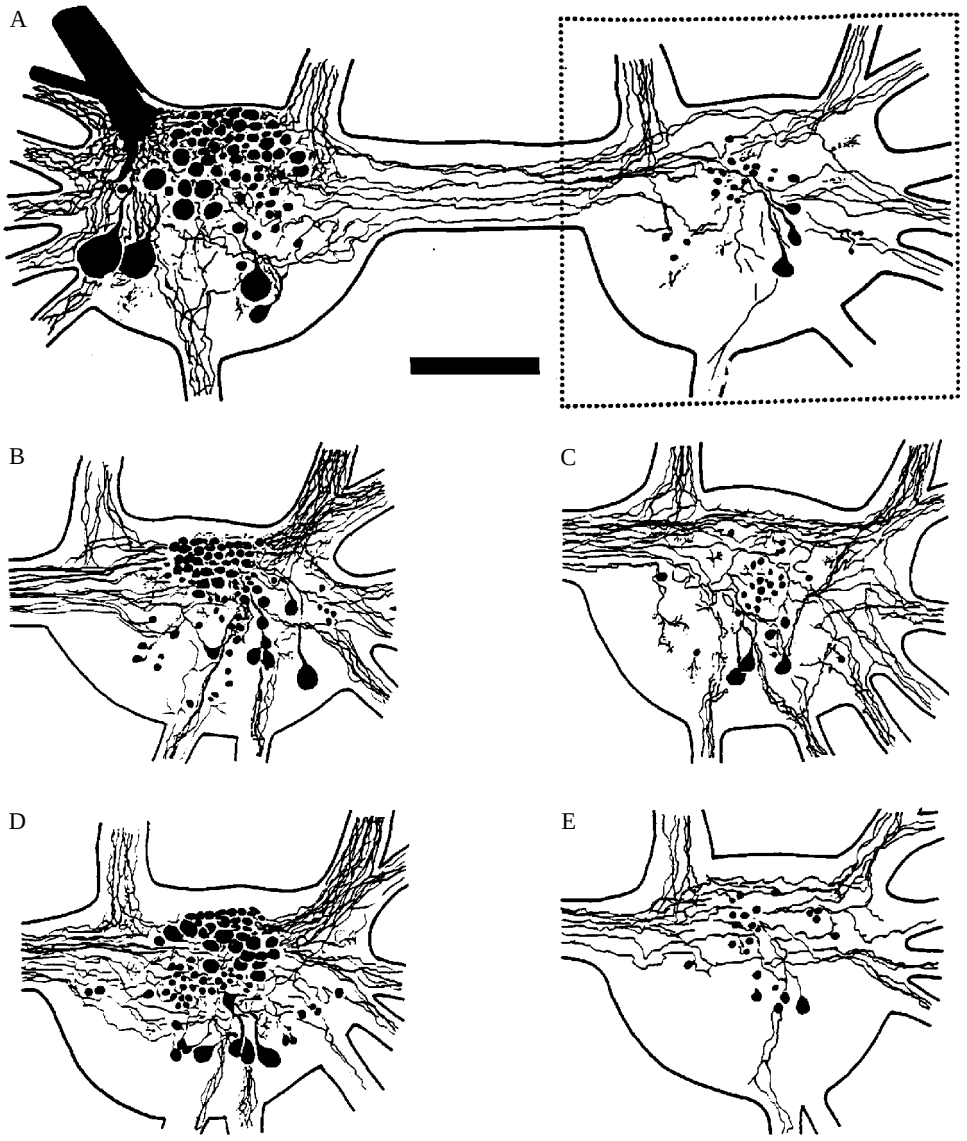

Fig. 3. (A) *Camera lucida* tracing of the ventral surface of representative buccal ganglia from a control animal showing the relative density of neuronal elements labelled following a dye-fill to the CBC (darkened nerve on left side of ganglia). The dotted outline in A corresponds with the ventral views of representative contralateral buccal hemiganglia 21 days after the cutting (B) or the crushing (C) treatment. Supernumerary labelling of buccal neuronal elements was evident 21 days after either lesion treatment and could still be seen 49 days after the cut (D) but not the crush (E) treatment. Scale bar, 400 μm.

by day 5–6 (*N*=5; cf. Fig. 6A,B). This disappearance was preceded by the break-up and 'blebbing' of the immunoreactive fibres, suggesting the distal degeneration of the lesioned MCG fibres, as previously described after a crush to the CBC (Chiasson *et al.* 1994). Beginning around day 5 after the cut, an increase in small-diameter immunoreactive fibres was noted in the buccal commissure and, at approximately 7 days (*N*=6), very small immunoreactive fibres (less than 0.5 μm in diameter) began to reappear in the denervated ganglion. After 28 days of recovery (*N*=8), the overall intensity of immunoreactivity had returned to normal levels within the ganglion on the injured side (Fig. 6C). Since regeneration by the lesioned MCG was effectively prevented by the cut to the CBC, this reappearance of serotonin-like immunoreactivity probably represents sprouting by the intact contralateral MCG into the denervated ganglion.

Electrophysiological evidence of regeneration and sprouting

Extracellular recordings were used to test for regeneration by the MCG and to confirm anatomical observations of sprouting. Normally, current injection into the soma of the MCG causing an action potential is correlated 1:1 with a relatively large, biphasic extracellular spike in recordings from the ipsilateral salivary nerve of unlesioned animals (*N*=8; Fig. 7B). In the first few days following the cut or crush to the CBC, no evoked response was seen in the salivary nerve (*N*=5, day 7; data not shown). Axonal regeneration was confirmed by the presence of triggered extracellular spikes which could be elicited both 21 days (*N*=8; Fig. 7C) and 49 days after the CBC had been crushed (*N*=3). The evoked spike was routinely smaller in amplitude (10–18 % of normal) and had a greater delay in onset compared with the spike seen in normal preparations (40 ms *versus* 20 ms). The evoked spike was also usually multiphasic. We interpret these changes as indicating that the regenerated fibres were smaller and more heterogeneous in size than the original projections into the salivary nerve.

Normally, action potentials in the contralateral MCG do not

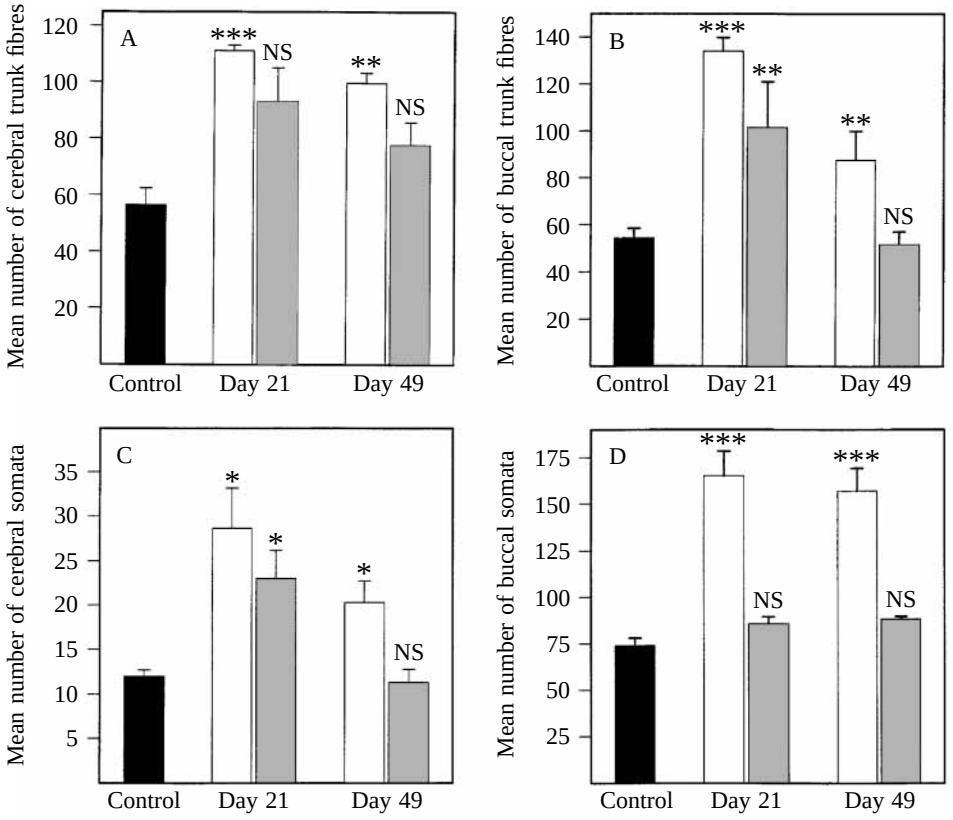

Fig. 4. Labelling of neuronal elements following a cut or a crush to the CBC (mean + S.E.M.). Filled black bars represent normal and sham controls, open bars represent cut treatment and hatched bars represent crush treatment. Labelling of contralateral cerebral trunk fibres is represented in A, contralateral buccal trunk fibres in B, contralateral cerebral somata in C and contralateral buccal somata in D. Values have been pooled for all trunks and neuronal populations in each ganglion and represent at least six successful dye-fills for each treatment condition and recovery time. ***P<0.001; **P<0.01; *P<0.05; NS, $P \geqslant 0.05$, Student's t-tests utilizing the Bonferonni correction in the calculation of significance levels.

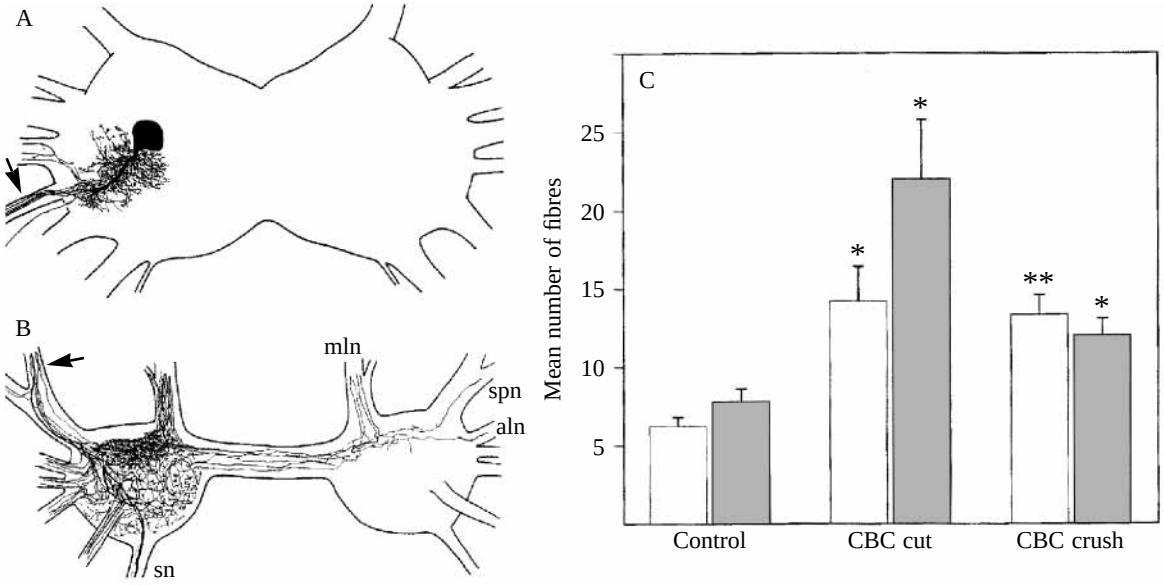

Fig. 5. Normal morphology of the metacerebral giant cell (MCG) and sprouting following a cut or crush to the contralateral CBC. (A) *Camera lucida* tracing of the ventral surface of the cerebral ganglia, showing the MCG morphology and projections in ipsilateral CBC (arrow) based upon an intracellular dye-fill with Ni^{2+}–lysine. (B) *Camera lucida* tracing of the buccal ganglia showing normal MCG innervation *via* the ipsilateral CBC (arrow). Fibres from the MCG ramify throughout the ipsilateral ganglia to project out through all ipsilateral nerves, including the salivary nerve (sn). Under normal conditions, only a minor contralateral arborization is present, with several fibres extending out through the medial lateral nerve (mln) and a single fibre in the superficial pharyngeal (spn) and anterior lateral (aln) nerve. (C) Histogram comparing the contralateral buccal fibre projections of the uninjured MCG 18–21 days following a cut or crush to the contralateral CBC. Values represent mean fibre number (mean + S.E.M.) of pooled projections for all contralateral buccal nerve trunks (open bars) and buccal commissural fibres (shaded bars). **P<0.001, *P<0.05, Student's t-tests utilizing the Bonferonni correction in the calculation of significance levels.

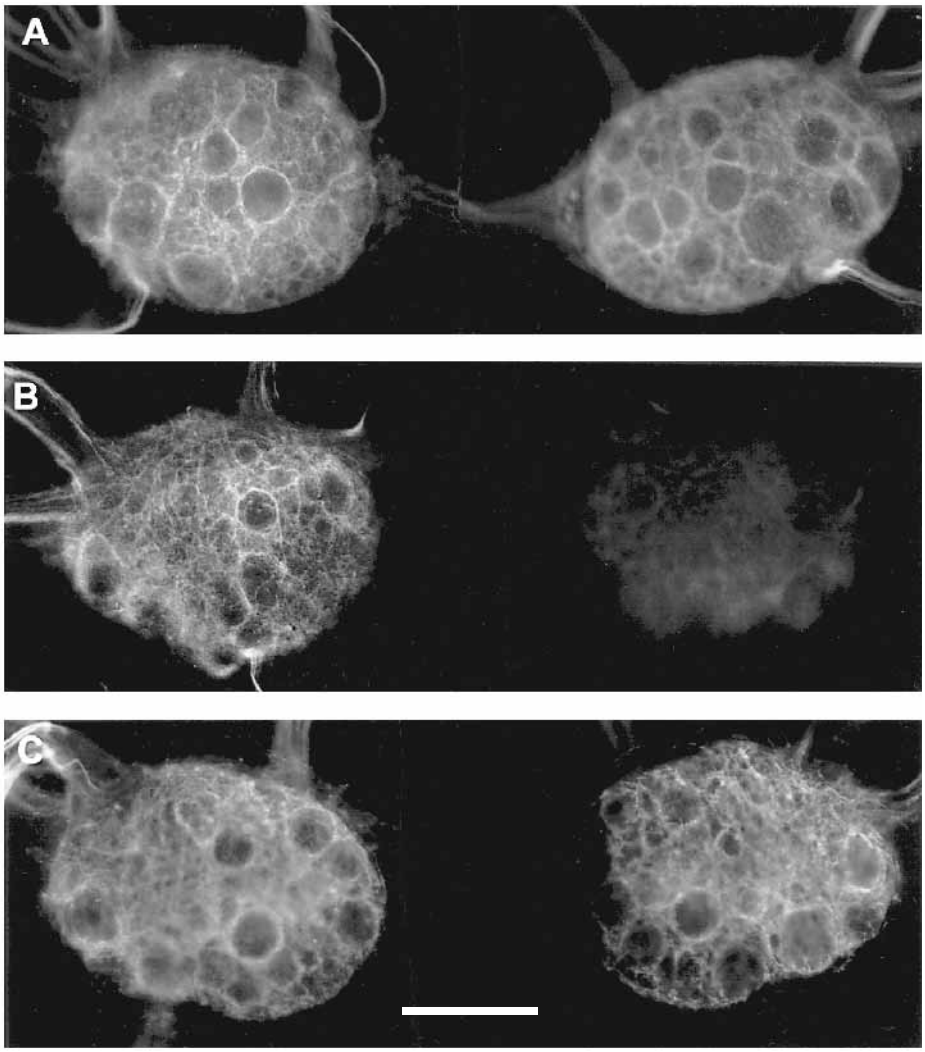

Fig. 6. Serotonin-like immunoreactivity of the buccal ganglia following a cut to the CBC. (A) Ventral view of normal buccal ganglia showing extensive bilateral innervation surrounding cortical somata. (B) Immunoreactivity 5 days after a midlength cut to the right-hand CBC. Immunoreactivity is almost completely absent in the lesioned side. (C) Serotonin-like immunoreactivity 28 days following a midlength cut to the right-hand CBC. Immunoreactivity has returned to essentially normal levels in the lesioned side of the ganglia, although it consists of much smaller reactive fibres. All composite photographs were made by metering the exposure time based upon the staining intensity of the intact ganglion. Scale bar is approximately 400 μm.

elicit extracellular spikes in recordings from the salivary nerve (Fig. 7D). However, 21–28 days after cutting the CBC, all preparations (N=6) exhibited long-latency, multiphasic evoked spikes in the salivary nerve on the side of the lesion when triggered by action potentials from the soma of the unlesioned, contralateral MCG (Fig. 7E). These novel extracellular spikes survived incubation in a saline with Mg^{2+} substituted for Ca^{2+}, suggesting that they did not involve an interposed synaptic relay. Forty-nine days after the CBC had been cut, four of five preparations persisted in showing evoked spike activity from the contralateral MCG in the salivary nerve on the side of the lesion. In contrast, 21–28 days after the CBC had been crushed, evoked spikes from the contralateral MCG were observed in only one of five preparations, and no such evoked responses were observed (N=4) following 49 days of recovery.

In a different set of animals, we monitored the ability of the uninjured, contralateral MCG to drive B1, a buccal follower neurone. Single action potentials in the MCG never correlated with detectable, discrete postsynaptic potentials in B1. However, evoked bursts of activity in the ipsilateral MCG (2.0–3.5 Hz trains of spikes lasting for 10 s) normally lead to a smooth depolarization of B1 with superimposed, discrete polysynaptic potentials occurring within the first 1–3 trials (intertrial interval 12 s). This compound response facilitates upon repeated bursts of activity in the MCG and culminates in a maximal depolarizing response of 4–5 mV, generally after 10–12 trials. The size of this facilitated, compound EPSP was maintained in later trials (up to at least 20 trials) and generally surpassed the threshold for activation of B1 (Fig. 8A; see Chiasson *et al.* 1994, for a more complete description of the influence of the MCG on B1). In contrast, stimulation of the contralateral MCG normally leads to little or no observed alteration in the membrane potential of B1 in the first 1–3 trials and, even after the response has facilitated, only a small (0.9±0.2 mV; N=7), compound EPSP is evoked (Fig. 8A). All subsequent values are compared with this control value.

Twenty-one days after the CBC had been cut, bursts of activity in the contralateral MCG depolarized B1 to a significantly heightened level, three- to fourfold above the control value within 3–5 trials (3.8±0.5 mV; P<0.0001; N=6), with B1 usually discharging action potentials during the compound EPSP (Fig. 8B). A similar increase in the ability of the contralateral MCG to drive B1 was seen 21 days after a crush to the CBC (3.9±0.5 mV; P<0.0001; N=4).

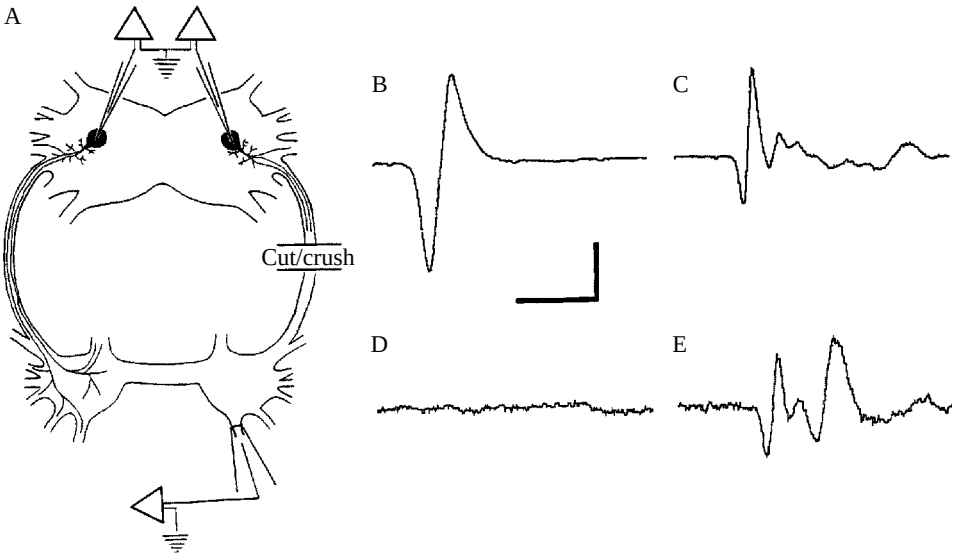

Fig. 7. Extracellular recordings from the buccal salivary nerve triggered by action potentials from the ipsilateral and contralateral MCG. (A) Schematic diagram illustrating the experimental design. Action potentials in one of the MCGs were used to trigger a 250 ms extracellular recording from the salivary nerve to test for regeneration by the lesioned MCG or sprouting by the contralateral MCG after a cut or crush to the CBC on the right side. (B) Averaged evoked response from the salivary nerve of a control preparation triggered by action potentials from the ipsilateral MCG. (C) Averaged evoked response from the salivary nerve triggered by action potentials from the ipsilateral MCG, 21 days following the CBC crush, indicating regeneration by the lesioned MCG axons into the buccal ganglia and out of the ipsilateral salivary nerve. (D) Averaged recording from the salivary nerve of a control preparation triggered by action potentials from the contralateral MCG. Normally, no evoked response is observed. (E) Averaged evoked response triggered by action potentials from the contralateral MCG 21 days following a cut to the CBC, indicating that the contralateral MCG has sprouted a projection(s) into the lesioned side of the buccal ganglia and out of the salivary nerve on that side. All recordings shown have been signal averaged for over 200 trials. Voltage scale in B, C, 500 mV, in D, E, 50 μV; horizontal time scale, 40 ms.

Forty-nine days after cutting the CBC, bursts of activity in the contralateral MCG continued to depolarize B1 to a significantly heightened level (3.8±0.3 mV; $P<0.0001$; $N=5$) and usually elicited a train of action potentials (Fig. 8B). However, 49 days after crushing the CBC, the effect of the contralateral MCG on B1 had returned to normal (1.5±0.5 mV; $P>0.05$; $N=5$).

Dye-fills of B1 ($N=3$–5 in each group) failed to reveal any changes in the morphology of the cell or any evidence of dye-coupling between the left and right B1 neurones, either 21 or 49 days following either the cut or the crush. Furthermore, we detected no change in the normally weak electrical coupling coefficient (Yoshida and Kobayashi, 1992; Chiasson *et al.* 1994) between the ipsilateral and contralateral B1 neurone (not shown), suggesting that the increased synaptic drive of the MCG upon the contralateral B1 neurone after the lesion treatments was not due to an increase in electrical coupling between the ipsilateral and contralateral B1 neurones.

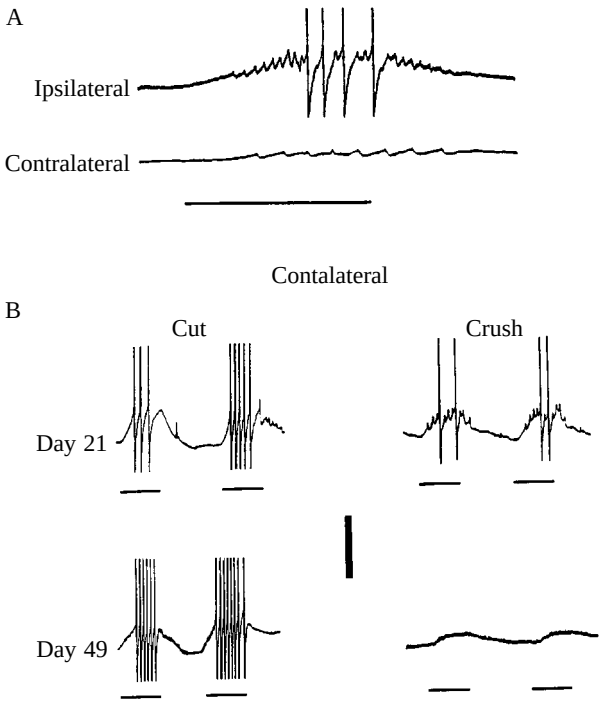

Fig. 8. Electrophysiological recordings from buccal neuron B1 during evoked bursts of activity in the MCG in normal (A) and lesioned (B) animals. In all recordings, the horizontal black bar indicates the time at which the MCG was stimulated to elicit a 10 s burst of action potentials at 2–3.5 Hz. Each such trial was repeated 20 times (12 s intertrial interval) for each preparation. (A) Simultaneous recordings from bilateral B1 neurones. Normally, following 10 trials, a burst of activity in the MCG evokes a 4–5 mV compound EPSP in the ipsilateral B1, consisting of an initial smooth depolarization followed by numerous discrete EPSPs with occasional superimposed action potentials. In contrast, the response in the contralateral B1 is normally much weaker, usually less than 1 mV. (B) Twenty-one days following a cut or a crush to the CBC, the response of B1 to a burst of activity in the contralateral MCG was greatly increased (trials 5 and 6 are shown for both treatments). Forty-nine days following the cut, the enhancement is maintained (trials 13 and 14 shown), whereas 49 days after the crush the ability of the contralateral MCG to drive B1 has returned to normal effectiveness (trials 19 and 20 shown). Vertical scale bar is 9 mV. The upper portions of the action potentials have been cropped.

In summary, the extracellular and intracellular recordings from the lesioned buccal ganglia support the morphological evidence showing that the contralateral MCG sprouts new projections into the lesioned buccal ganglion and suggest that these sprouted fibres may functionally compensate for lost inputs from the ipsilateral MCG. In addition, these responses were maintained for up to 49 days after the CBC had been cut, whereas after crushing they had returned to normal levels, suggesting a possible retraction of sprouted fibres in the latter case.

Discussion

This study used a variety of anatomical and physiological measurements to examine the long-term fate of sprouted neuritic projections following either a crush of the CBC, which permitted eventual regeneration, or a cut to the CBC, which effectively prohibited regeneration along this central pathway. Axonal dye-filling of the contralateral, uninjured CBC revealed that the two lesion treatments produced many of the same types of sprouting responses after 21 days. Both types of lesion led to supernumerary labelling of fibres in nerve trunks (especially within the injured CBC of the buccal ganglia) and novel labelling of somata within the cerebral ganglia. Two major sources appear to have contributed to the observed sprouting responses. First, some of the cells that were axotomized rerouted their projections directly across the midline to either ascend or descend the contralateral CBC. It has been suggested before that the appearance of novel cells in the cerebral E cluster after a CBC lesion might be due to this type of rerouting (Croll and Baker, 1990; Baker *et al.* 1993). Such contralateral rerouting by lesioned neurones has also been described in large individual gastropod neurones (Bulloch and Kater, 1981; Arshavsky *et al.* 1985; Cohan *et al.* 1987), but our results (also see below) reveal that such rerouting is a more generalized phenomenon that can also involve smaller neurones. Second, our results indicate that, in addition to rerouting major axons that have sustained direct injury, neurones can also sprout new collaterals from their axonal terminals into contralateral, denervated territories. For instance, within 21 days of either type of lesion to a CBC, the contralateral MCG increased the number of fibres projecting across the buccal commissure into the injured side of the buccal ganglia. Furthermore, the appearance of these sprouted fibres correlates with functional changes in synaptic connectivity. The contralateral MCG increased the strength of its synaptic drive upon the B1 neurone on the side of the lesion. We interpret this increase in synaptic drive as having arisen through the formation of new synaptic connections by the sprouted contralateral MCG upon B1 or, possibly more importantly, upon cells presynaptic to B1. Alternatively, however, an increase in synaptic efficacy may also have arisen through the strengthening of pre-existing (silent) synapses, the sprouting by other follower cells and/or the formation of new electrical synapses. For example, in the snail *Helisoma trivolvis*, axotomy has been reported to lead to the rapid formation of electrical synapses between lesioned buccal homologues (Hadley and Kater, 1983; Cohan *et al.* 1987). Thus, despite our lack of evidence for changes in the morphology of B1 or for changes in the electrical coupling coefficient between B1 neurones, we cannot exclude such changes in other buccal neurones. Further study is therefore required to confirm the role of MCG sprouting in these changes. The MCG in *A. fulica*, like its homologues in other gastropods, is known to excite many of the large buccal motoneurones and to facilitate the activity of the feeding central pattern generator (Weiss and Kupfermann, 1976; Rosen *et al.* 1989; Yoshida and Kobayashi, 1991). It is tempting, therefore, to speculate that the increased synaptic drive mediated by mono- and polysynaptic pathways between the contralateral MCG and B1 might reflect part of an overall compensatory change which helps to restore the activity of the feeding motor program. Similar types of compensatory changes among uninjured homologous neurones have previously been suggested to occur in both the cricket (Murphey and Lemere, 1984) and the leech (Blackshaw *et al.* 1982; Gao and Macagno, 1987; Gu and Muller, 1990).

While the sprouting responses were similar at 21 days following both types of lesion, the extent of sprouting was generally greater after the CBC had been cut. For example, the numbers of labelled fibres were generally greater after the CBC had been cut than after it had been crushed. In addition, we observed novel cells labelled in the buccal ganglia only after the CBC had been cut. On the basis of the sizes and locations of these cells, they appeared to belong to the same population that normally projects to the cerebral ganglia *via* the ipsilateral CBC. Thus, it appears that, when these neurones are prevented from projecting along their normal pathway, they eventually sprout new fibres that cross the buccal commissure and project to the cerebral ganglia *via* the contralateral CBC. Such differences between cut and crush treatments after 21 days of recovery suggest that regeneration of normal connections may play a role in the cessation of sprouting.

While some differences were seen in the sprouting responses at 21 days following the lesions, the most dramatic differences were seen at later times. Both the morphology and physiology of axotomized neurones appeared to have returned to normal 49 days after crushing. In contrast, supernumerary fibres and somata were still labelled 49 days, or even 150 days, after the CBC had been cut. Similarly, following extended recovery periods after the cutting treatment, the MCG was seen to maintain its increased synaptic drive over the contralateral B1 cell. Since previous studies have demonstrated that morphological and physiological regeneration after the CBC crush is complete within 35 days (Croll and Baker, 1990; Chiasson *et al.* 1994), the maintained supernumerary labelling response after the CBC cut may be directly correlated to the absence of normal ipsilateral regeneration. It should be noted, however, that some diminution in labelling with the axonal dye-fills was seen at day 49 compared with day 21 after the CBC was cut. Unfortunately, it was not possible to determine whether this drop in labelling represented a partial retraction of the total sprouted fibre population or the retraction of only

a select population. A partial retraction of sprouted fibres may indicate the selective pruning of non-functional connections (Lichtman and Balice-Gordon, 1990) or a compensatory retraction within some regions of the normal arborization of a neurone in order to accommodate sprouting by the neurone in other terminal regions (Murphey and Lemere, 1984).

Our results are thus consistent with a variety of other evidence, particularly from vertebrate preparations, which suggests that sprouted fibres can be selectively eliminated upon regeneration of the original innervation (Yoon, 1972; Purves, 1976; Jackson and Diamond, 1981; Easter *et al.* 1985; van Mier and Lichtman, 1994). While it is well documented that the retraction of transient neuritic processes can play an important role in invertebrate neurogenesis (Murphey, 1986; Gao and Macagno, 1987), the fate of sprouted fibres in the adult nervous system of invertebrates appears to vary depending upon the type of injury and the preparation examined. For example, in the adult leech, sprouted fibres are maintained for over a year after injury (Bannatyne *et al.* 1989; Blackshaw, 1994). In the cockroach, motoneurones selectively eliminate inappropriate connections after injury-induced sprouting into adjacent territory (Denburg *et al.* 1988; Denburg and Caldwell, 1992). In the snail, neurones can express a variety of responses ranging from retraction following osmotic stress (Bulloch, 1984) and injury (Chase and Kamil, 1983; Croll and Baker, 1990) to the long-term maintenance of sprouted fibres after injury (Altrup and Kold, 1982; Allison and Benjamin, 1985; Cohan *et al.* 1987). These latter results appear in contrast to our findings, which suggest that most sprouted fibres are retracted following successful regeneration in *A. fulica*. One reason for this discrepancy may lie in the type of sprouted fibre being assessed. Previous studies have generally provided detailed analyses of sprouted projections from single neurones following axotomy, whereas our evidence for retraction comes principally from axonal dye-filling experiments, which only labelled cells projecting into the contralateral, dye-filled CBC. As a result, neurones that sprouted projections in other directions and that might have been more resistant to retraction than those projecting in the CBC would have gone undetected in our preparation. Another reason for the discrepancy may be that previous studies have generally focused upon large, individually identifiable neurones, while our studies also included many smaller neurones. Large neurones may be able more easily to meet the extra metabolic demands of supernumerary neurites and, therefore, to be under less pressure to eliminate aberrant projections. In fact, the MCG in *A. fulica* is known to replace its severed fibres following a crush to the CBC by a multitude of smaller, parallel neurites which persist for at least 90 days following the crush (the longest recovery period examined; Chiasson *et al.* 1994). Thus, even in *A. fulica*, successful regeneration of large neurones does not always lead to a restoration of their normal morphology.

The initiation of sprouting and the retraction of those sprouted fibres following regeneration have often been cited as evidence for intercellular competition; sprouting results from the elimination of competitive influences, whereas retraction results from a regenerated neurone outcompeting neighbouring cells to regain its original targets. Target-supplied factors may mediate competition between neuronal projections (Murphey and Lemere, 1984; Denburg, 1985; Lichtman and Balice-Gordon, 1990; Muller and Gu, 1991). Regenerating neurites may also be capable of displacing the sprouted fibres through an activity-dependent mechanism, as envisioned in the classical 'Hebbian' synapse (Stent, 1973; Nelson *et al.* 1989). Alternatively, there may exist a hierarchical arrangement of target suitability for neurones, favouring connections which have previously existed over those that are newly established (Denburg *et al.* 1988; Cohan *et al.* 1987; Gu and Muller, 1990).

While competition models of sprouting and retraction remain attractive, certain aspects of injury-induced sprouting may also be explained in terms of other possible mechanisms. Inhibitory interactions between neurones have been suggested as a means by which constraints upon growth and connectivity can be achieved (Patterson, 1988; Lipton and Kater, 1989). Alteration in the level of inhibitory molecule(s), as a consequence of degenerating or regenerating neuronal elements, may help determine whether a neurone sprouts new projections or retracts existing ones. In the present study, sprouting was associated with the break-up and disappearance of serotonin-like immunoreactivity in the buccal ganglia on the side of the lesion. We have previously demonstrated (Baker *et al.* 1993) that the serotonin-depleting agents 5,7-dihydroxtryptamine and *p*-chlorophenylalanine can produce supernumerary labelling of neuronal elements similar to that documented here and also shown previously (Croll and Baker, 1990). This raises the possibility that the transient unilateral depletion of 5-HT in the buccal ganglia, which follows the degeneration of severed fibres from the MCG, may play an inductive role in some of the sprouting responses (Haydon *et al.* 1984; Murrain *et al.* 1990).

The experiments described in this report support the premise that both the cessation of sprouting and the eventual retraction of sprouted fibres by cerebral and buccal neurones in *A. fulica* is contingent upon successful regeneration across the lesioned CBC. In the absence of such a reconnection, neurones undergo contralateral and perhaps compensatory sprouting responses. These findings extend previously elucidated principles of neuronal plasticity (Yoon, 1972; Purves, 1976; Easter *et al.* 1985; Murphey, 1986; Denburg and Caldwell, 1992) to the adult central nervous system of gastropods and provide a framework for future studies examining the mechanisms by which regenerating and sprouted neurones interact to determine neuronal arborization patterns after an injury.

This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to R.P.C.

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