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MORPHOLOGICAL CHANGES AND FUNCTIONAL RECOVERY FOLLOWING AXOTOMY OF A SEROTONERGIC CEREBROBUCCAL NEURONE IN THE LAND SNAIL *ACHATINA FULICA*

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Summary

We have examined an identified serotonergic neurone in *Achatina fulica* and described the normal morphological and physiological characteristics of this cell. Injury-induced changes in this neurone following *in vivo* recovery are described and compared with *in vitro* gastropod models of regeneration. Nickel–lysine and biocytin dye-fills of the metacerebral giant (MCG) neurone, together with serotonin-like immunoreactivity, revealed an extensive innervation of the ipsilateral buccal ganglion, much greater than that previously reported. Labelled MCG fibres were seen to ramify throughout the ganglion, providing extensive neuropilar innervation. Serotonin-immunoreactive fibres were seen not only within the neuropile but also within the cell body layer of the buccal ganglia, surrounding many of the cell bodies with varicose fibres. Dye-fills also revealed a minor contralateral buccal innervation not previously described. This view of a predominantly ipsilateral innervation of the buccal ganglia by the MCG was supported by electrophysiological measurements. The ipsilateral buccal follower cell B1 displayed an increase in depolarization in response to repeated trains of action potentials to the MCG, whereas the contralateral B1 showed only a weak depolarization in response to the identical stimuli.

Following a crush to the cerebral–buccal connective (CBC), the MCG rapidly regenerated its injured projections, displaying both morphological and physiological recovery within 5–10 days. The original, severed fibres of the MCG were, however, replaced by a multitude of smaller neurites, which persisted for up to 3 months (the longest recovery period examined). Despite this morphological difference between normal and regenerated fibres, the MCG re-established functionally equivalent connections upon B1. In contrast with previous *in vitro* studies using gastropods, serotonin-like immunoreactivity revealed that severed distal fibres from the MCG rapidly degenerated (2–6 days), resulting in a transient unilateral depletion of serotonin in the

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buccal ganglia. We suggest that this loss of serotonin in the lesioned ganglion may play a functional role in regeneration, as has been suggested *in vitro*.

Introduction

The ability of gastropods to repair damage to their nervous systems is well documented. Neurones have been shown to undergo extensive regeneration, sprouting and synaptogenesis in response to injury. The majority of these studies have focused on single identifiable neurones *in vitro* (Murphy and Kater, 1980; Wong *et al.* 1981; Bulloch and Kater, 1982; Murphy *et al.* 1985; Murrain *et al.* 1990; Syed *et al.* 1990). Those studies that have investigated *in vivo* responses have mostly examined populations of neurones undergoing morphological recovery (Chase and Kamil, 1983; Moffett and Ridgway 1988*a*; Croll and Baker, 1990). Accordingly, relatively few studies on gastropod regeneration have focused upon *in vivo* regeneration of single identified cells (Benjamin and Allison, 1985; Cohan *et al.* 1987; Fredman and Nutz, 1988). Questions therefore arise as to the relationships between phenomena observed *in vitro* at the level of the single cell and those operating *in vivo*. For example, do all individual neurones *in vivo* behave in a similar way to the populations previously described? Does the extended period of recovery afforded by the *in vivo* preparation reveal differences not observable with *in vitro* preparations, because of their restricted longevity? Furthermore, *in vitro* studies are well suited for the demonstration of *potential* mechanism(s) involved in processes, such as the relationship between neurite outgrowth and synaptogenesis (Haydon, 1988; Haydon and Kater, 1988), and the role of neurotransmitters and trophic agents in the regulation of neuritogenesis (Kostenko *et al.* 1983; Haydon *et al.* 1984; Rehder *et al.* 1990; Ridgway *et al.* 1991). There remains, however, an important need for *in vivo* investigations to test the *sufficiency* and *necessity* of these mechanisms in the whole organism.

In a previous *in vivo* study, Croll and Baker (1990) described changes in neuronal labelling in cerebrobuccal and buccocerebral neurones of the land snail *Achatina fulica* by axonal dye-filling of the cerebral–buccal connective (CBC) at varying times after a crush. They reported robust regenerative and sprouting responses, which resulted both in reinnervation of target tissues and in the supernumerary labelling of somata and neurites in the weeks immediately following the crush. This supernumerary labelling eventually declined to normal levels, suggesting a retraction or displacement of aberrant neurites. These findings indicate that this preparation might be exploited to study both short- and long-term responses to injury and thus to provide a useful complement to the numerous *in vitro* studies of neuritic sprouting and regeneration of gastropod neurones. Furthermore, such a preparation should enable the study of numerous neurotrophic and neuritogenic agents which may operate *in vivo*. In fact, more recently, this population of cerebral and buccal neurones was used to test the effects of pharmacological depletion of serotonin on the neuronal projections of intact animals and the results suggest that this transmitter may play a significant role in the control of neuritogenesis *in vivo* (Baker and Croll, 1991; Baker *et al.* 1993), as it has been reported to do *in vitro* (Kostenko *et al.* 1983; Haydon *et al.* 1984; Murrain *et al.* 1990).

Using the *in vivo* preparation described above, the present study examines injury-induced responses of the bilaterally symmetrical metacerebral giant (MCG) cells (Croll, 1985, 1987, 1988), alternatively named v-DCNs (ventral distinct cerebral neurone; Ku *et al.* 1985; Yongsiri *et al.* 1986) and GCCs (giant cerebral cell; Chase and Tidd, 1991). This pair of cells has been reported to be the sole source of serotonin to the buccal ganglia (Croll, 1988), and injury to the neurones would, therefore, be expected to cause localized depletion of this transmitter. This depletion might in turn be expected to influence regeneration and sprouting by the MCG, along with perhaps other neurones. In order to address this point, we undertook the present study to establish a more complete background in which to assess the possible role of serotonin on MCG regeneration and sprouting. Previous descriptions of the general features of the MCG are consistent between various studies, but some differences in detail have appeared. We therefore attempt first to clarify some of the conflicts between previous studies of the MCG in *Achatina fulica*. Second, morphological responses of the MCG to a crush of the CBC, which include regenerative and degenerative events, are presented. These events are then correlated with the physiological recovery of synaptic effects upon an identified buccal follower cell.

Preliminary reports of aspects of this work have appeared elsewhere (Croll *et al.* 1987).

Materials and methods

Animals

Adult specimens of *Achatina fulica* Férussac (40–85 mm in shell length) were obtained from our laboratory colony which was maintained on a 12 h:12 h light:dark cycle at 20–25 °C. Original members of the colony were donated by Dr Ron Chase at McGill University or were obtained from John Takara in Honolulu, Hawaii, or Juanito Gementiza of Instrumix Suppliers, Manila, Philippines. Animals were fed a diet of lettuce and carrots supplemented with a slurry of crushed rat chow (Purina), crushed oyster shell and water.

Electrophysiological recordings and stimulation parameters

The interconnected cerebral and buccal ganglia were removed from the animal and placed into a Sylgard (Dow Corning) coated Petri dish filled with saline (NaCl, 67 mmol l^{-1}; $CaCl_2$, 11 mmol l^{-1}; $MgCl_2$, 10 mmol l^{-1}; KCl, 4 mmol l^{-1}; Tris, 5 mmol l^{-1}; Chase and Goodman, 1977). The epineurial sheath was mechanically removed from both sets of ganglia, which were then pinned to the dish, exposing the ventral surface of the cerebral ganglia and the dorsal surface of the buccal ganglia. Generally, one MCG and one ipsilateral buccal cell, B1, were simultaneously penetrated with microelectrodes (10–30 MΩ) filled with 3 mol l^{-1} KCl. Electrical signals were fed through A-M Systems Neuroprobe or WPI Micro-probe System amplifiers and displayed simultaneously on a Tektronix D12 dual-beam oscilloscope and a Brush multichannel chart recorder. Depolarizing square pulses of current (generally 5–10 nA, but occasionally as high as 18–20 nA) were passed into the MCG, generating 2.0–3.5 Hz trains of action potentials lasting for 10 s. The current was adjusted as necessary to

maintain this frequency of firing. Each 10 s train of activity in the MCG was termed a 'trial' and these trials were repeated 20 times separated by 12 s intervals. Amplitudes of postsynaptic responses were measured at the peak of the depolarization before action potentials were elicited in B1. Connectivity was also tested in the following saline containing increased concentrations of Ca^{2+}: NaCl, 34.1 mmol l^{-1}; KCl, 3.3 mmol l^{-1}; $CaCl_2$, 32.1 mmol l^{-1}; $MgCl_2$, 5.25 mmol l^{-1}; glucose, 5 mmol l^{-1}; Hepes, 10 mmol l^{-1} (Yoshida and Kobayashi, 1991). Occasionally two MCGs were simultaneously penetrated to test for interconnections. Statistical analyses were performed on electrophysiological data using Student's *t*-test. Values are reported as means and standard deviations. Significance was considered to be achieved when $P<0.05$.

Dye injections

After the electrophysiological data had been recorded, some of the cells were repenetrated with microelectrodes (60–130 MΩ) containing a solution of nickel–lysine (1.7 g $NiCl_2 \cdot 6H_2O$ and 3.5 g L-lysine free base in 40 ml of H_2O; modified from Fredman, 1987). Nickel was ionophoresed into B1 using 500 ms, 20–50 nA depolarizing current pulses repeated at 1.5 Hz. Following 1 h of dye injection, the electrode was removed and the dye was allowed to diffuse generally for 1–2 h while the ganglia were rinsed in fresh saline. Dye injections into the larger MCGs were extended for another 1 h while increasing the current pulses to 50–100 nA. In these cases, the dye was allowed to diffuse for 12–18 h. Subsequently, 0.5 ml of a saturated alcoholic solution of rubeanic acid (Sigma Chemical Co., St Louis, MO) was added to the saline (approximately 10 ml) bathing the ganglia. The nickel was allowed to precipitate for 20–40 min, and then the ganglia were rinsed briefly in saline before fixation in 10 % buffered formalin. Following silver intensification (Croll, 1986), the ganglia were dehydrated through an ascending ethanol series, cleared in methyl salicylate and mounted whole in Permount (Fisher Scientific Co., Fairlane, NJ). Specimens were viewed and photographed using a Leitz Aristoplan microscope and Orthomat E camera system. All *camera lucida* drawings were made using a Nikon SMZ dissecting microscope and drawing tube.

To double-label the MCG or B1, microelectrodes (150–200 MΩ) were filled with a 4 % solution of biocytin (biotin–lysine conjugate; Sigma Chemical Co., St Louis, MO; Horikawa and Armstrong, 1988) in 0.05 mol l^{-1} Tris buffer (pH 7.4). The dye was ionophoretically injected into the cell with 500 ms, 20 nA hyperpolarizing current pulses at 1.5 Hz. Following 45 min of dye-injection, the electrode was removed and another 2–3 h was allowed for dye diffusion. The ganglia were then processed for serotonin immunofluorescence using fluorescein isothiocyanate (described below) and lastly incubated in avidin–rhodamine isothiocyanate (Sigma; 1:100 dilution in 0.5 % Triton X100 in 0.1 mol l^{-1} phosphate buffer).

Immunohistochemical procedures

Following dissection of the cerebral and buccal ganglia and mechanical removal of the outer layers of connective tissue, the remaining epineural sheath was partially digested in protease (0.5 % Sigma type XIV in saline for 10–15 min). Procedures for indirect immunohistochemistry were according to the method of Croll and Chiasson (1989), using

polyclonal rabbit anti-serotonin antibodies (Incstar Corp., Stillwater, WI, USA) and goat anti-rabbit secondary antibodies conjugated to fluorescein isothiocyanate (Antibodies, Inc., Davis, CA, USA). Whole-mounted ganglia were viewed and photographed using a Leitz Aristoplan microscope equipped for epifluorescence with an L3 or N2.1 filter block for fluorescein or rhodamine fluorescence, respectively.

In order to test for specificity of staining, the primary antibody was preincubated for 24 h at 4 °C with 1 mg ml^{-1} of serotonin conjugated to bovine serum albumin with formaldehyde (Incstar Corp). All other procedures were carried out as described above. This procedure completely eliminated fluorescent staining in the cerebral and buccal ganglia and elsewhere in the central nervous system (CNS) as reported by Croll (1988).

Surgery

Snails were immobilized by an injection of 0.5 ml of 1 % succinylcholine in saline (Croll and Baker, 1990). Subsequently, a 5–8 mm incision was made between the superior optic tentacles. The edges of the incision were retracted, and another incision made through the capitocerebral membrane. Following displacement of the oesophagus, either the right or the left cerebral–buccal connective (CBC) was crushed approximately 2–3 mm from the buccal ganglia using Number 5 jeweller's forceps. (Earlier studies suggested that responses to either a left or a right crush did not differ in form or time course of subsequent regenerative events; Croll and Baker, 1990.) The oesophagus was then returned to its original position, and the incision was sutured. The experimental animals were wrapped in damp paper towels and placed back in their home cage. Animals were active, with no obvious behavioural deficits the following day. These surgical procedures resulted in a 90 % survival rate.

Results

Characterization of the normal MCG

Normal morphology

The MCGs are bilaterally symmetrical neurones (with a soma diameter ranging from 100 to 180 μm) located on the midventral surface of each cerebral hemiganglia (Fig. 1A). Dye injections (N=30) into the MCG revealed a major projection into the posterolateral cerebral neuropile where the MCG neurite ramifies into an extensive arborization of secondary and tertiary fibres. Near the lateral margin of the cerebral ganglion the main neurite branches, sending 2–3 medium-sized (4–6 μm) and one or two small (2–3 μm) fibres into the interior labial nerve. It also projects a large (10–15 μm) fibre into a subcerebral commissure. This commissural projection was usually severed in our preparation of the ganglia for dye-filling or recording, since all nerves except the cerebral–buccal connective (CBC) were generally cut within a few hundred micrometres of the ganglia. However, when the subcerebral commissure was intentionally left intact, dye-fills of the MCG revealed a single commissural fibre which extended posteriorly along a blood vessel to the aorta and then looped back to the contralateral cerebral ganglion. The fibre entered the lateral margin of the contralateral cerebral ganglion, where it branched to send a small projection into the contralateral interior labial nerve and

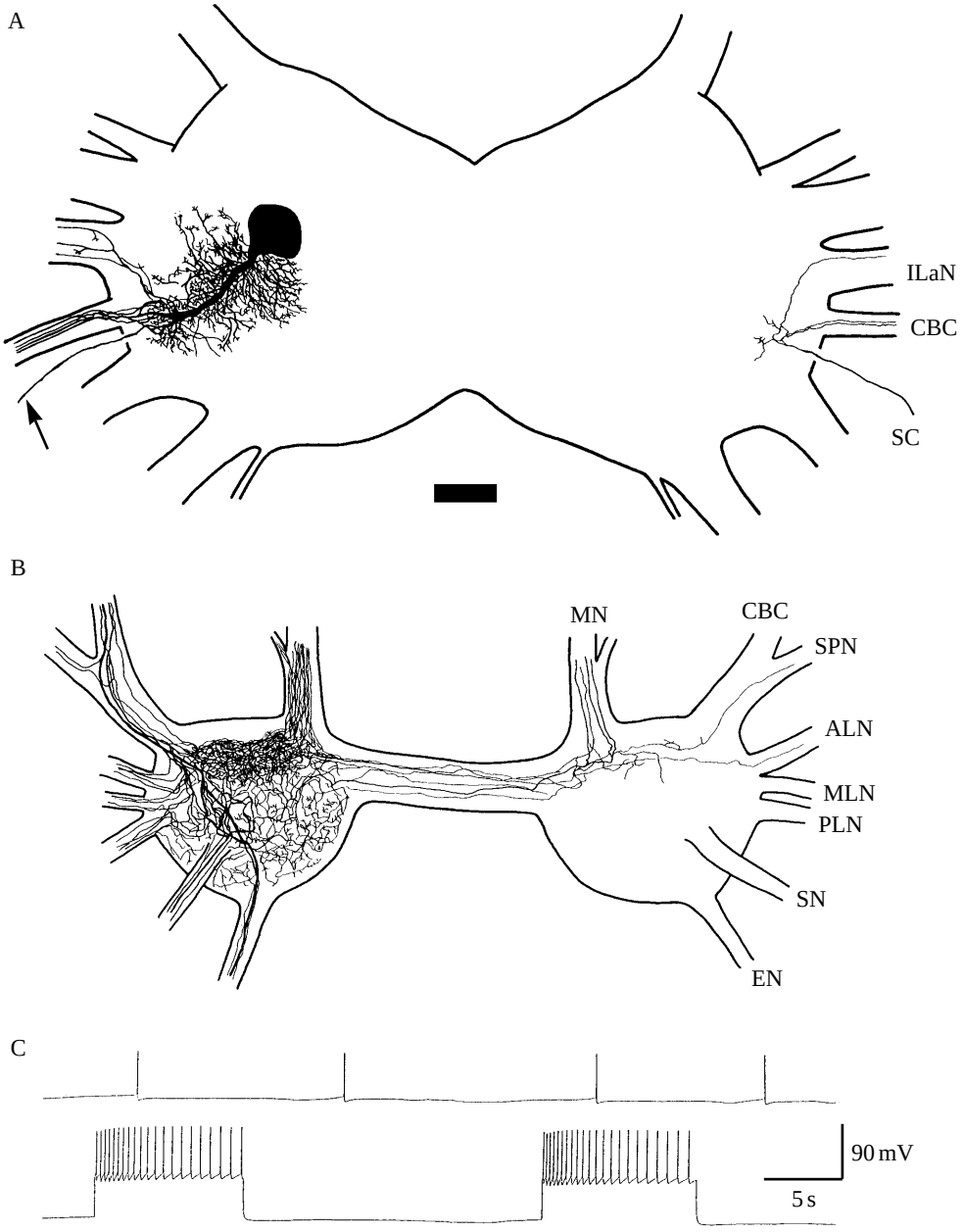


Fig. 1

1–2 small (<1 μm) fibres down the contralateral CBC (Fig. 1A). These neurites in the contralateral CBC entered the buccal ganglion, where they appeared to terminate near the posterior margin. Innervation of the buccal ganglia by the MCG was, however, predominantly *via* the ipsilateral MCG, from which 3–4 large (10–15 μm) fibres and 4–6 smaller (2–5 μm) fibres coursed into the CBC. Upon entering the buccal ganglia, the CBC

Fig. 1. Morphological and electrophysiological characteristics of the normal MCG in *Achatina fulica*. (A) *Camera lucida* tracing of the ventral surface of a cerebral ganglion, showing the MCG morphology based upon an intracellular dye-fill with nickel–lysine. The MCG projects neurites out of the ipsilateral interior labial nerve (ILaN), cerebral–buccal connective (CBC) and subcerebral commissure (SC; arrow on left). The MCG's subcerebral commissural projection can be seen where it enters the contralateral ganglion. (B) *Camera lucida* drawing of the buccal ganglia showing typical MCG innervation *via* the ipsilateral CBC. Fibres from the MCG ramify throughout the ipsilateral ganglion to project into all ipsilateral nerves, which include the superficial pharangeal nerve (SPN), anterior lateral nerve (ALN), medial lateral nerve (MLN), posterior lateral nerve (PLN), oesophageal nerve (EN), salivary nerve (SN) and medial nerve (MN). Fibres from the MCG also project into three contralateral nerves as shown. For clarity, the minor projection to the contralateral buccal ganglia *via* the contralateral CBC is not shown. (C) Electrophysiological tracings from simultaneous recordings of the two MCGs in a preparation in which the SC has been severed. This figure shows that there are no gross alterations in the activity pattern in one MCG when the contralateral cell is stimulated. Observations made at high sensitivity on oscilloscope sweeps also indicate that there is no alteration in the membrane potential of one MCG following either a depolarizing or a hyperpolarizing pulse to the contralateral MCG. Scale bar in A and B is approximately 120 μm.

neurites ramified, providing a dense network of fibres to the neuropile of the ipsilateral buccal ganglion, especially along the posterior margins (Fig. 1B). Several fibres greater than 2.5 μm in diameter and numerous fibres smaller than 1 μm in diameter projected into each ipsilateral buccal nerve. Only a few detectable fibres (2–4 μm) projected to the contralateral ganglion *via* the buccal commissure to form sparse branches within the contralateral posterior neuropile. Generally, these fibres sent a few projections into the contralateral medial nerve (MN) and a single, small projection into both the superficial pharyngeal nerve (SPN) and the anterior lateral nerve (ALN) (Fig. 1B).

Serotonin-like immunoreactivity and nickel–lysine dye-fills of the MCG revealed a similar number of fibres in the CBC. Additionally, following double-labelling procedures, no serotonin-like immunoreactive (SLIR) fibres in CBC were without the biocytin-positive stain following an intracellular injection of this dye into the ipsilateral MCG (compare Fig. 2A and 2B). The presence of only labelled MCG fibres in the CBC supports previous findings that no other SLIR neurones from the CNS contain projections to the buccal ganglia (Croll, 1987, 1988). Since the buccal ganglia is also devoid of any intrinsic serotonergic cells (Croll, 1988), serotonin-like immunoreactivity was exploited further in this study as an alternative technique for examining the MCG's buccal innervation. A rich network of varicose SLIR fibres was seen, both within the neuropile and among the nerves exiting the ganglia. Additionally, immunopositive fibres were seen within the cell body layer of the ganglia. Indeed, this cortical innervation was of sufficient density to provide outlines of buccal cell bodies (Fig. 2C,D), revealing a further level of organization not seen with intracellular dye injections.

Normal physiology

Following injury discharge produced by microelectrode penetration, activity within the MCG was of a variable frequency, but was generally less than 0.5 Hz. Simultaneous recordings from the two MCGs showed that when the subcerebral commissure was

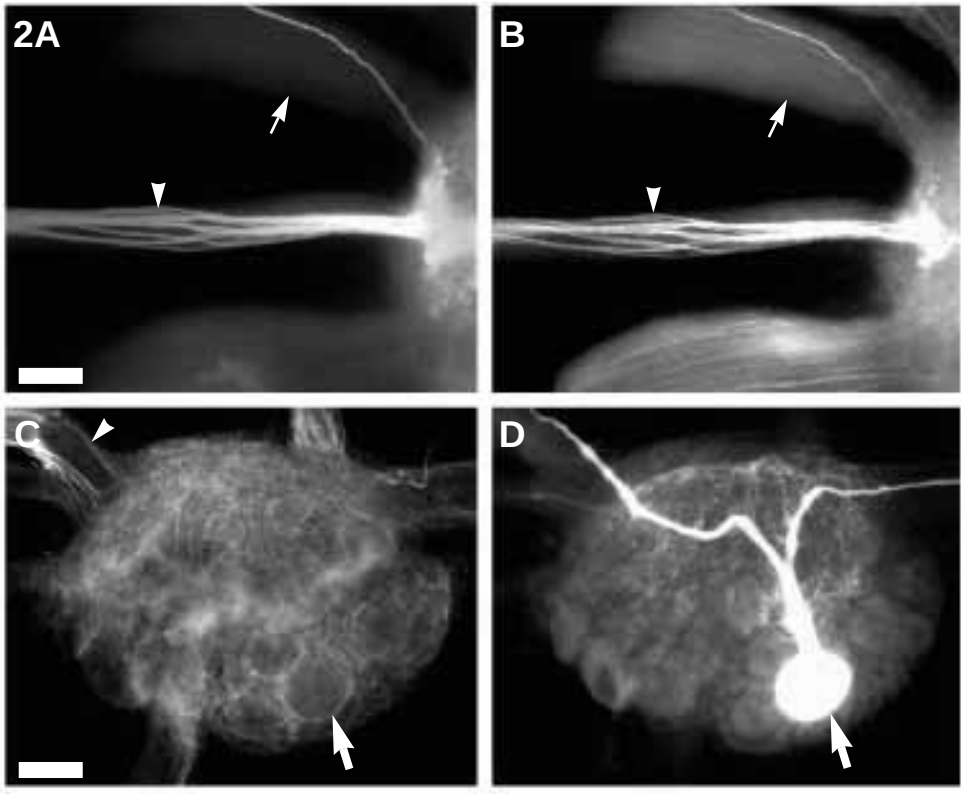


Fig. 2. Photomicrographs of double labelling for serotonin-like immunoreactivity and biocytin following intracellular dye injections of the MCG and buccal cell B1. (A) Biocytin fill of the MCG revealing fibres in the CBC (arrowhead) and ILaN (arrow). (B) Serotonin-like immunoreactivity in the same preparation as shown in A, revealing that all SLIR fibres in the CBC originate from the MCG. Note that labelled fibres seen in the lower nerve (cerebral–pedal connective) arise from other serotonergic cells. (C) A dorsal superficial view of a buccal hemiganglion revealing SLIR fibres from the MCG as they enter the ipsilateral ganglion from the CBC (arrowhead at the top left). Arrow indicates the SLIR fibres surrounding B1. (D) Double labelling of the same preparation as shown in C, showing a biocytin fill of neurone B1 (arrow). Scale bar in A is approximately 45 μm and also applies to B. Scale bar in C is approximately 70 μm and also applies to D.

severed no obvious correlation in their activity patterns was observed. This commissure was severed in order to isolate the lesioned MCG from its homologue, thereby allowing the study of the recovery of functional connections following lesion to the fibres in the ipsilateral CBC. Accordingly, in our preparation, the cells were not found to be electrically or chemically coupled. Brief or sustained depolarizing (Fig. 1C) or hyperpolarizing (not shown) square pulses in either MCG did not appear to alter the activity of the other cell or to change its membrane potential.

Effects on buccal cell B1

B1 is a large, bilaterally symmetrical cell (soma diameter ranging from 80 to 130 μm)

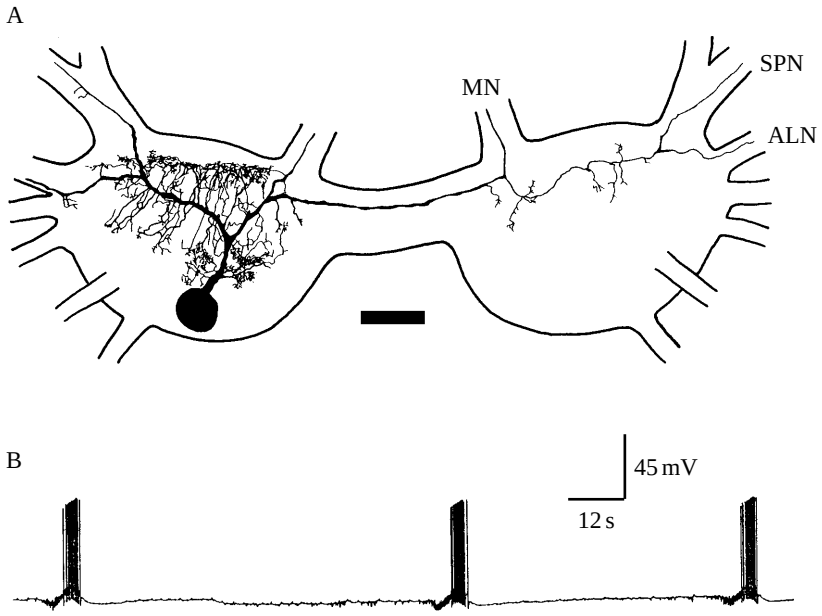


Fig. 3. Morphological and electrophysiological characteristics of the buccal cell B1. (A) *Camera lucida* tracing of B1 located on the dorsal surface of the buccal ganglion. Abbreviations are given in Fig. 1. (B) Electrophysiological tracing of the normal activity seen in B1 in the isolated cerebral and buccal ganglia preparation. Scale bar, approximately 120 μm.

located on the dorsal surface of the buccal ganglia. Its morphology is shown in Fig. 2D and Fig. 3A. In our preparations of interconnected cerebral and buccal ganglia, B1 exhibited a patterned activity characterized by a 10–100 s period of relative quiescence alternating with a 3–5 s period of high-frequency discharge of action potentials at 5–10 Hz (Fig. 3B). This pattern of activity is similar to that recorded previously in buccal motoneurones of *Achatina fulica* and other gastropods and appears to represent the spontaneous output of the central feeding motor programme generator (Yoshida and Kobayashi, 1991). Each B1 was weakly coupled to its contralateral homologue with a coupling coefficient of about 0.05 (data not shown), as reported by Yoshida and Kobayashi (1992).

Evoked activity (2.0–3.5 Hz trains of spikes lasting for 10 s) in the ipsilateral MCG depolarized B1 by 1–2 mV in the first 1–3 trials (Fig. 4A). The first depolarization elicited in B1 generally consisted of a smooth transition in the resting membrane potential, whereas subsequent trials exhibited facilitated responses which included rapid fluctuations in potential, presumably caused by discrete polysynaptic inputs. The discrete potentials, which generally commenced 2–3 s after initiation of MCG activity and persisted for 2–4 s beyond cessation of activity in the MCG (Fig. 4B), each averaged 1.5±0.4 mV in amplitude and occurred with a frequency of 2.2±0.8 Hz (N=10). The frequency of putatively polysynaptic potentials decreased to 0.5–0.6 Hz after the ganglia had been bathed in high-Ca^{2+} saline for 30 min. The amplitudes of both the individual,

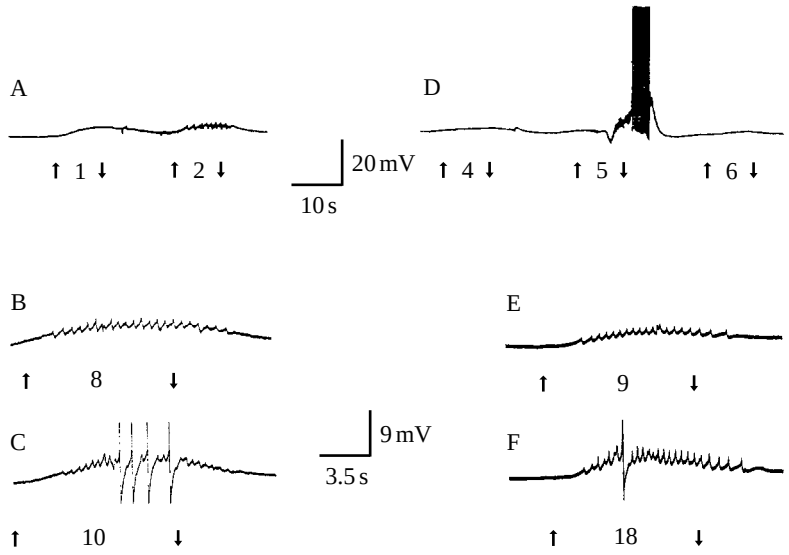


Fig. 4. Electrophysiological tracings of activity in the ipsilateral buccal cell B1 evoked by the MCG in normal (A–C) and injured (D–F) preparations. In all of the tracings, upward-pointing arrows indicate the onset of MCG firing and downward-pointing arrows indicate the cessation of MCG activity. The numbers located between the arrows indicate the trial shown. All action potentials have had their upper portion cropped. (A) The normal response of B1 to the first two trials of MCG stimulation. The first trial evokes a smooth depolarization with an increase in synaptic activity seen by the second trial. (B,C) Enhanced response of B1 to repeated trains of MCG stimulation. (D) Three days after the lesion, the injured MCG does not evoke a response in B1. Note that in normal or functionally regenerated preparations the MCG is able to prevent the spontaneously occurring high-frequency firing seen in this tracing (see text). (E,F) Seven days after a lesion to the CBC, the MCG is able to elicit nearly normal activity in B1. The upper scale bars apply to A and D. The lower scale bars apply to all other tracings.

discrete postsynaptic potentials and of the initial smooth depolarizations were either unaffected or were slightly increased in this saline.

The combined amplitude of the initial smooth depolarizations and later discrete polysynaptic potentials increased throughout later trials to reach a maximum of 4.2±0.8 mV. Action potentials in B1 first appeared after about 10–15 trials (Fig. 4C) and by trial 20, 75 % responded in this way to ipsilateral MCG stimulation (N=16). All influence of the MCG on the ipsilateral B1 was abolished when the ipsilateral CBC was cut (N=4). A second result of evoked activity in the MCG was a suppression of the spontaneous activity bursts recorded in B1. Since the later evoked compound synaptic potentials have similar time courses and amplitudes to the input from the feeding motor programme generator onto B1 (Yoshida and Kobayashi, 1991), our results suggest that activity in the MCG phase shifts the programme generator to synchronize its activity with that of the MCG (Weiss and Kupferman, 1978; Rosen *et al.* 1989).

The response of the contralateral B1 during and following the stimulation of the MCG differed substantially. Stimulation of the MCG did not result in any observed alteration (<0.2 mV) in the membrane potential of the contralateral B1 in the first 1–3 trials. In later

trials, the amplitude of the depolarization increased slightly, but was still confined to small depolarizations which did not initiate action potentials. The mean amplitude of the maximum depolarization observed in the contralateral B1 was 0.9±0.2 mV (N=7), which was significantly different (P<0.001) from the maximum depolarization observed in the ipsilateral B1. This small depolarization in the contralateral B1 following repeated stimulation of the MCG was abolished following transection of the CBC on the side of the stimulated MCG.

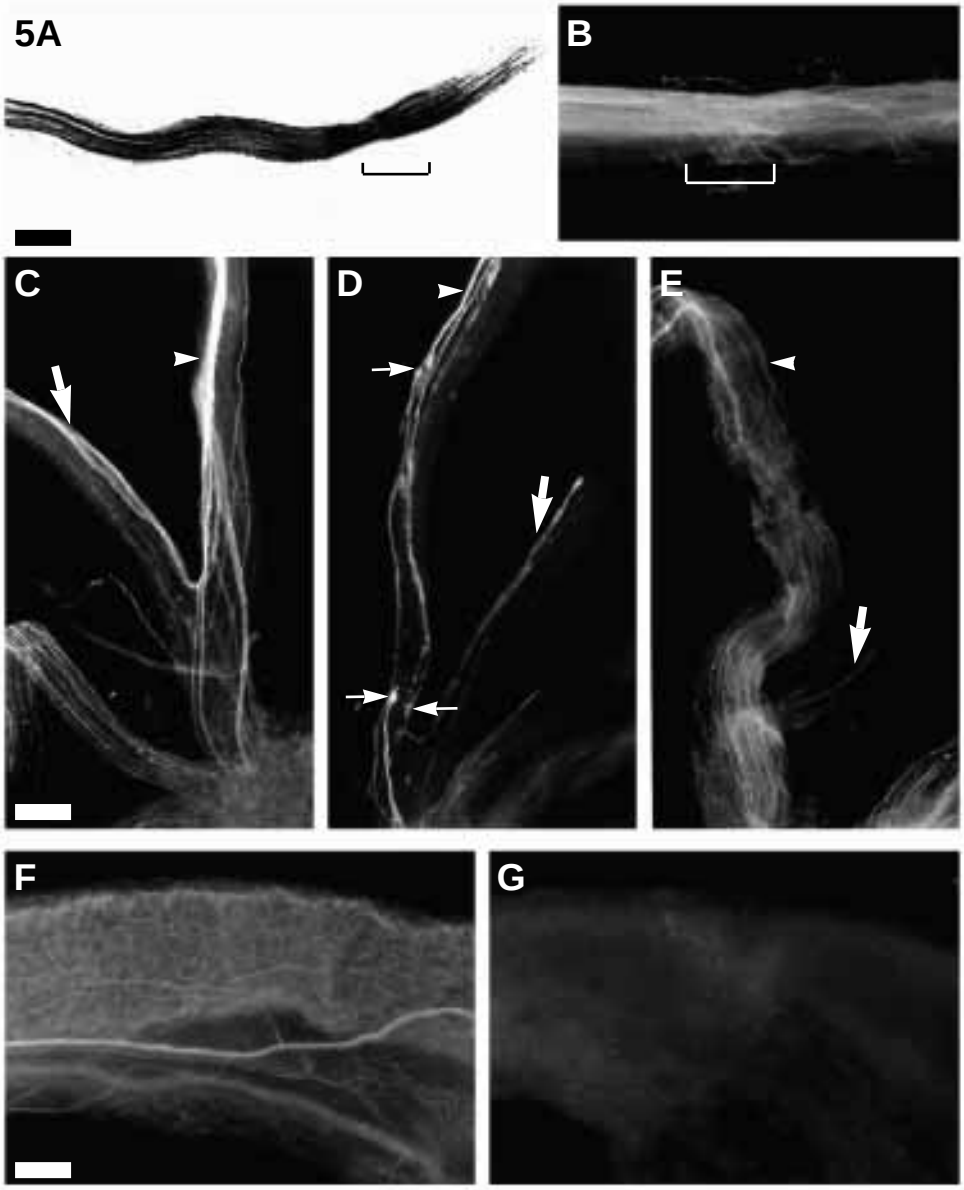
Characterization of the MCG following a crush to the ipsilateral CBC

Morphological changes

The MCG exhibited considerable morphological rearrangement following a crush to the ipsilateral CBC. Intracellular dye-fills of the MCG within 24–48 h of the crush (N=12), together with immunohistochemistry to detect serotonin, revealed that the crush completely severed the MCG neurites within the CBC (Fig. 5A). These techniques also revealed a multitude of small (0.5–1.0 μm) neurites, which had already begun to grow both anterogradely and retrogradely from the proximal stump of the severed axon. By 2–3 days following the crush, the regenerated fibres had extended further from the lesion site in both directions, but had not yet reached either the buccal or the cerebral ganglion. MCG dye-fills of preparations between 5 and 7 days post-lesion revealed that regenerated fibres had begun to grow into the ipsilateral buccal ganglion and that many of the ipsilateral buccal nerves possessed regenerated fibres. The buccal commissure had many small (0.5–1.0 μm) processes, some having reached the contralateral ganglion. Thus, by 5–7 days following a crush to the CBC, the injured MCG had regenerated a large number of fibres to reinvade its former target, the buccal ganglia. The regenerated fibres at this time, however, were principally confined to the neuropile and nerves with little evidence of cortical innervation (Fig. 6B). By 10 days following the crush, however, immunohistochemistry revealed complete reinnervation of the injured buccal ganglia with fine fibres surrounding buccal cell bodies to yield the somatic silhouettes characteristic of normal innervation (Fig. 6C).

Regenerated fibres in the CBC generally remained small and numerous, even after an extended period of regeneration (95 days). In six preparations, lesioned 73–95 days earlier, both anterograde- and retrograde-projecting fibres in the CBC could be seen (Fig. 5B), suggesting long-term maintenance of these morphological changes in response to injury. It was also possible to identify the crush site by the splayed fibres, which characteristically remained even after long periods post-injury (Fig. 5B). In contrast to the marked changes induced by injury to the MCG's neurites in the CBC, no dramatic changes were observed in the cell's arborization within the cerebral ganglion. We did not assess changes in projections of the MCG fibre within the subcerebral commissure.

While immunohistochemistry proved useful for studying regenerating fibres, it also permitted the visualization of degenerating MCG fibres. Within 2–3 days of the lesion, noticeably fewer, small SLIR fibres were present within the segment of the CBC distal to the crush. At about the same time, the larger fibres began to exhibit discontinuities, with obvious swellings (blebs) occurring at the ends of many of the isolated segments



(compare Fig. 5C and 5D). These isolated segments became shorter and then faded in staining intensity after another 1–3 days. By 3–6 days following the lesion, this pattern of degeneration was reiterated in all the various roots exiting the ipsilateral buccal ganglion.

Since degeneration commenced before regenerated fibres reached most areas, transient partial depletion of serotonin was often observed in localized areas. Immunohistochemistry suggests that the ipsilateral buccal ganglion was partially depleted of serotonin after about 3–9 days (Fig. 6A–C). In addition to degenerative

Fig. 5. Photomicrographs showing regeneration of the MCG along with degeneration of the MCG's distal severed neurites. (A) Nickel–lysine-labelled MCG fibres in the CBC 2 days after a crush. The crush effectively severed all MCG neurites (the crush site is indicated by the bracket) and regenerated fibres have by this time traversed the site of injury. The newly regenerated fibres extend anterogradely past the crush site (to the right) as well as retrogradely towards the cerebral ganglion (to the left). The regenerated fibres (both anterograde and retrograde) are clearly of smaller diameter than most non-lesioned MCG fibres within the CBC. (B) Serotonin-like immunoreactivity of MCG fibres 71 days after a crush to the CBC (note that the original crush site is still visible; indicated by bracket). (C–E) Serotonin-like immunoreactive fibres in the CBC (arrowheads) and SPN (large arrows) on the non-lesioned side of a buccal ganglion (C), on the lesioned side 3 days after the crush (D) and on the lesioned side 10 days after a crush (E). Compared with the normal immunoreactivity, the MCG fibres 3 days after the crush show characteristic signs of break-up and degeneration, including blebbings along the length of their processes (small arrows in D). (E) Rapid regeneration of distal projections occurs by replacement of the original large fibres with small neurites approximately 1 μm in diameter. (F) Serotonin-like immunoreactivity in the salivary duct. The MCG normally projects several large fibres into the ipsilateral salivary nerve to innervate the salivary duct, one of its peripheral targets. (G) Six days after the crush, SLIR fibres are no longer visible, suggesting complete degeneration of lesioned fibres. Scale bar in A, approximately 30 μm for A, 50 μm for B; scale bar for C–E, 30 μm; for F,G, 70 μm.

events located within the central nervous system, we examined a peripheral target, namely the salivary ducts. The ipsilateral salivary duct (innervated unilaterally by the salivary nerves of the buccal ganglia) was also partially depleted of serotonin after about 7–14 days (Fig. 5F,G).

Recovery of effects on the ipsilateral B1

Using the same stimulation parameters employed in non-lesioned preparations, the injured MCG was examined for its ability to induce activity within ipsilateral buccal neurone B1 at varying times after the administration of a crush to the CBC. In animals examined 3 days after the crush to the CBC (N=3), the MCG failed to evoke any response in B1 (Fig. 4D). In contrast, animals examined at 6–7 days (N=4) after the lesion demonstrated considerable recovery. The maximum depolarizations (3.9±0.8 mV) seen in B1 were not significantly different from those seen in normal animals (P>0.05; compare Fig. 4B and 4E), and in half the preparations an occasional action potential was elicited following stimulation of the previously injured MCG (Fig. 4F). Following these electrophysiological recordings, the MCGs in these preparations were injected intracellularly with dye; all cells exhibited regenerated fibres which had re-entered the ipsilateral buccal ganglion. Thus, by 6–7 days after the lesion, the injured MCG possessed a physiological action on B1 which was very similar to the normal response.

Eight animals were examined for functional recovery between 10 and 16 days after the lesion. In six of the eight animals examined, the injured MCGs were able to elicit action potentials in B1. In the two specimens where B1 did not discharge action potentials, one showed a maximal depolarization of 5.5 mV and the other of 1.5 mV.

Sixteen animals were also examined to ascertain whether functional recovery of the injured MCG was maintained for periods longer than 2 weeks. These animals covered a

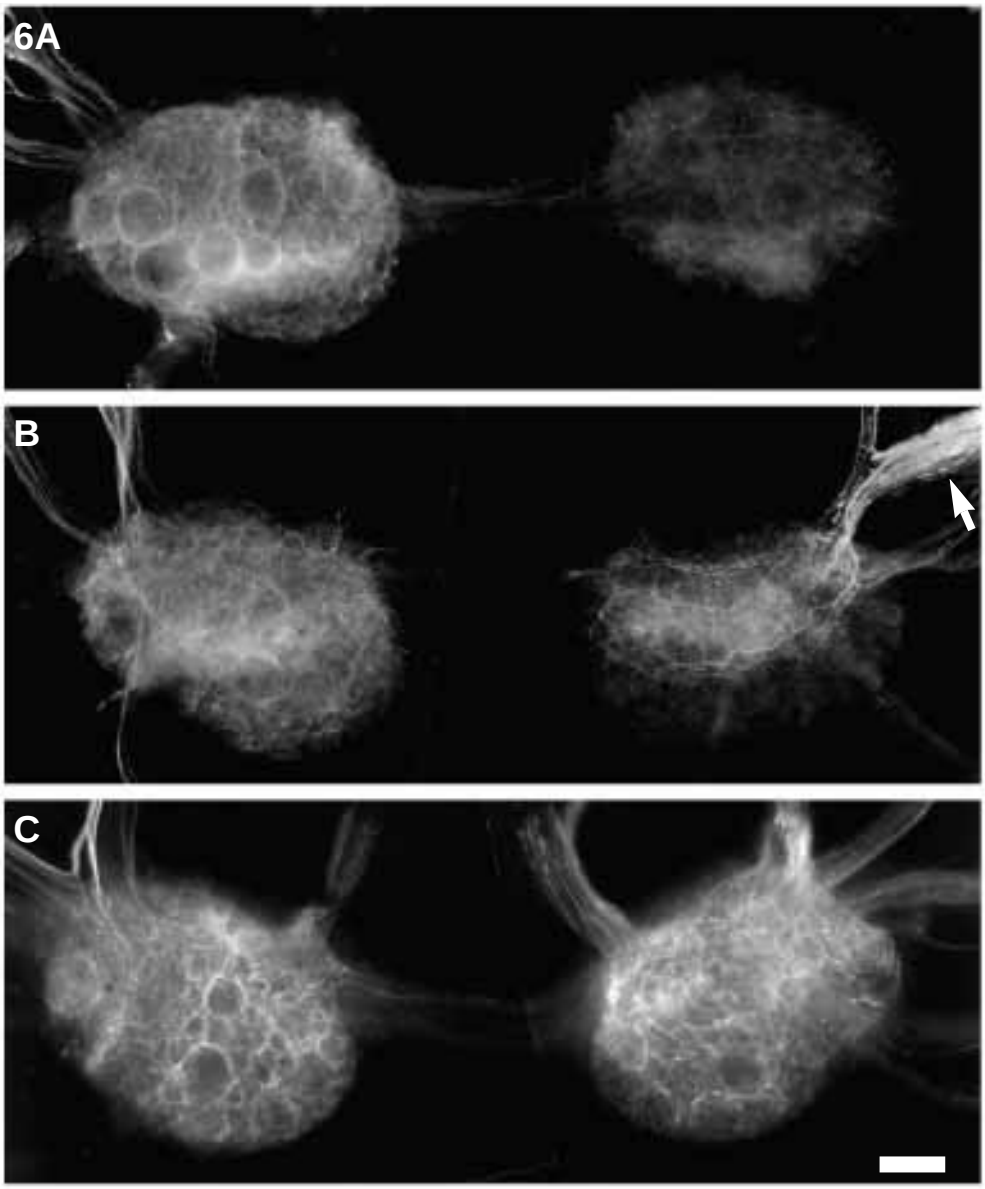


Fig. 6. Serotonin-like immunoreactivity suggests that after a crush to the CBC the lesioned ganglion undergoes a transient depletion in serotonin. (A–C) Serotonin-like immunoreactivity in the buccal ganglia: 3 days (A), 5 days (B) and 10 days (C) after a crush to the CBC on the right side. At 3 days, serotonin-like immunoreactivity is almost completely absent in the lesioned ganglion. By 5 days, serotonin-like immunoreactivity reveals that regenerating MCG fibres have begun to return to the lesioned ganglion, especially within the neuropile (arrow indicates CBC). 10 days after the lesion, serotonin-like immunoreactivity has returned to essentially normal levels. All composite photographs were made by metering upon the intact ganglion. Scale bar, approximately 100 μm.

range of approximately 3–12 weeks post-lesion. In all cases, the injured MCG was able to cause maximal depolarizations in B1 that were not significantly different from normal ($P<0.05$). In 12 of 16 specimens, the regenerated MCG was able to elicit action potentials in B1. The other 25 % of preparations demonstrated a mean maximal depolarization of 3.3±0.7 mV and, thus, were not significantly different from normal B1 responses.

Discussion

Normal morphology and physiology of the MCG

Descriptions of the normal morphology of the MCG and its innervation of the buccal ganglia provide a necessary backdrop for studies on axonal regeneration and sprouting of cerebral and buccal neurones in *Achatina fulica*. Our description of the MCG's buccal innervation differs in several important respects from previous characterizations. We have shown that the MCG possesses a much more extensive innervation of the buccal ganglia than had been previously reported (Croll, 1987, 1988; Yoshida and Kobayashi, 1991, 1992). Nickel–lysine fills of the MCG revealed a dense ipsilateral innervation of the ganglia; labelled fibres were seen in all ipsilateral nerves, as well as throughout the neuropile of the ganglion. This characterization of the MCG's buccal innervation was also supported by serotonin-like immunoreactivity. While we attribute the origin of all serotonin-like immunoreactivity in the buccal ganglia to fibres of the MCG, the possibility of minor peripheral monoaminergic input cannot be excluded. However, the unilateral loss of serotonin-like immunoreactivity in the lesioned buccal ganglion following the CBC crush (discussed below) suggests that the predominant source is from the MCG. Previous descriptions of serotonin-like immunoreactivity in *Achatina fulica* (Croll, 1988) and in *Helisoma trivolvis* (Murphy *et al.* 1985) demonstrated an extensive distribution of immunoreactive fibres around connective tissue layers of the buccal sheath, but not in the cell body layer of the ganglia. Serotonin-like immunoreactivity in the present study, however, suggests that the MCG provides an extensive innervation to the cortex of the ganglion, surrounding many of the buccal cell bodies with varicose fibres. Our results are, therefore, more in agreement with descriptions of serotonin-like immunoreactivity in the buccal ganglia of *Aplysia californica*. Schwartz and Shkolnik (1981) concluded after ultrastructural analysis that C1, the putative homologue to the MCG in *Aplysia californica*, made what appeared to be axosomatic contacts with buccal neurones.

While the MCG's buccal innervation is predominantly ipsilateral, the nickel–lysine fills also revealed minor projections extending across the buccal commissure to the contralateral buccal ganglia. Electrophysiological mapping of the MCG's buccal projections have previously described a possible MCG projection into the contralateral medial nerve (Yoshida and Kobayashi, 1992). We confirm morphologically the presence of these fibres in the contralateral medial nerve and, in addition, describe two new projections, one into the contralateral superficial pharyngeal nerve and another into the anterior lateral nerve. These projection patterns resemble qualitatively those described for the putative homologues of the MCG in *Helix aspersa* and *Aplysia californica* (Weiss and Kupfermann, 1976).

The contralateral buccal ganglia also receive MCG innervation from a second source: the subcerebral commissure provides 1–2 minor fibres to the contralateral CBC. Interestingly, this contralateral CBC innervation was not detected by the double-labelling technique employed, since all serotonin-containing fibres in the CBC appeared also to be filled with biocytin following intrasomatic injection of the ipsilateral MCG. This discrepancy could be due to the difficulty in visualizing such small fibres provided by the contralateral MCG or, alternatively, to interneuronal passage of the biocytin from the ipsilateral MCG into the fibres of the contralateral homologue. Our nickel–lysine fills of the MCG suggest that these small neurites in the contralateral CBC terminate in the posterior margin of the contralateral ganglion. Yoshida and Kobayashi (1992) recently reported electrophysiological evidence indicating that this MCG projection may extend into many of the buccal nerves. This inconsistency is probably the result either of the latter authors' recording of postsynaptic responses, which are difficult to control completely during electrophysiological mapping, or of our own incomplete dye-labelling of these small fibres, which constitute a projection extending over 30 mm. In any event, the morphological evidence presented here would indicate that these contralateral MCG projections to the buccal ganglia are minor and that most of the MCG's innervation is confined to the ipsilateral buccal ganglion.

Our physiological findings support this morphological description of a predominantly ipsilateral innervation to the buccal ganglia by the MCG *via* the ipsilateral CBC. With the subcerebral commissure severed, activity in the MCG neurone evoked a moderately strong depolarization in the ipsilateral B1 neurone and only a weak depolarization in the contralateral B1 neurone. The nature of this connection between the MCG and B1 was not explored in detail in this study, but it appears to have two components. We interpret the smooth early depolarization to be the result of a direct, monosynaptic connection between the MCG and B1 neurones. Both cells have extensive neuritic arborization throughout the ipsilateral buccal neuropile. MCG fibres also surround, and possibly terminate upon, buccal cell bodies, including that of B1. Neurone B1, alternatively named the dorsal buccal medial neurone (DBMn), has also been shown to be depolarized by bath application of serotonin (Matsuoka *et al.* 1987). The early depolarizing component of the connection described here is also consistent with features of what has been interpreted as a monosynaptic connection between MCG homologues and buccal follower cells in *Aplysia californica* and *Helisoma trivolvis* (Gerschenfeld and Paupardin-Tritsch, 1974; Gadotti *et al.* 1986). The slow time course of this putative direct connection is presumably due either to slow second-messenger-mediated neuromodulation or to diffuse synapses, which increase the serotonin level in the vicinity of postsynaptic receptors rather than through release at synaptic specializations (Schwartz and Schkolnik, 1981). The later depolarization following MCG activity appears to be polysynaptic. This interpretation is consistent with our finding that the initial, smooth depolarizing component of the postsynaptic response was preserved (or even enhanced), whereas the later, more rapidly fluctuating component was largely eliminated following a bath in saline with increased $[Ca^{2+}]$. As mentioned previously, we interpret the polysynaptic potentials as the output of the feeding central pattern generator, which was stimulated by the MCG. Thus, the MCG in *Achatina fulica*, like its homologues in other gastropods, not

only excites the buccal motoneurones but also facilitates the activity of the feeding central pattern generator during the first cycles of rhythmic outputs (Rosen *et al.* 1989)

Regeneration of the MCG following the crush

In addition to a detailed examination of the normal MCG, a second goal of this study was to examine the post-injury response of MCG neurites after a lesion to the ipsilateral CBC. Accordingly, we find that the MCG can rapidly and reliably regenerate its projections to the buccal ganglia. The time course for this regeneration was comparable to that observed in other *in vivo* and *in vitro* studies using gastropods (Murphy *et al.* 1985; Cohan *et al.* 1987; Allison and Benjamin, 1985; Benjamin and Allison, 1985; Croll and Baker, 1990). Also, as noted in other studies, the original severed axons were replaced with a multitude of smaller fibres (Murphy and Kater, 1980; Allison and Benjamin, 1985; Murphy *et al.* 1985). Importantly, these fibres were seen to persist even after survival times of more than 3 months in the present study (the longest time examined). This suggests that the eventual post-injury decline from a supernumerary to a normal staining pattern observed by Croll and Baker (1990) in *Achatina fulica* primarily involved the retraction of aberrant projections from cells other than the MCG. Long-term labelling of supernumerary and aberrant neurites has also been observed with *in vivo* studies using *Lymnaea stagnalis* and *Helisoma trivolvis* (Allison and Benjamin, 1985; Cohan *et al.* 1987).

Despite the differences in morphology between the normal MCG and those that have regenerated multiple parallel axons in the CBC, the regenerated cells do project back to normal central and peripheral targets. The MCG was thus able to make functionally equivalent connections in terms of both the presumably monosynaptic and polysynaptic depolarizing potentials evoked within B1. Indeed, both the initial response of B1 to MCG activity and the facilitated responses after repeated trains of action potentials were nearly indistinguishable in normal and regenerated preparations. The rapid re-establishment of functional connections is not entirely expected since injury can cause long-term changes, such as regrowth without physiological recovery (Janse *et al.* 1979). Successful regeneration can also be dependent upon the site of axotomy (Murphy and Kater, 1980; Allison and Benjamin, 1985) and the extent and timing of the lesion (Fredman and Nutz, 1988).

The present study also permitted examination of a less well described consequence of injury, namely, degeneration. We have observed the commencement of apparent degeneration within 2 days and its completion within 5–6 days following a nerve crush *in vivo*. While the break-up and loss of serotonin-like immunoreactivity in the buccal ganglion and its surrounding nerves is not absolute confirmation of the degeneration of severed neurites, the blebbing and disruption of MCG fibres as described here is consistent with other histochemical evidence for degeneration (Wilklund and Björklund, 1980). Ultrastructural evidence suggesting a rapid degeneration of severed neurites within 4–5 days of cutting a ganglionic connective has also been reported in the molluscan bivalve *Anodonta cygnea* (Borovyagin *et al.* 1972). A very similar type of degeneration, but with a time course of 1–2 weeks, has been described in the snail *Melampus bidentatus* by Moffet and Ridgway (1988*b*) following extirpation of an entire

cerebral hemiganglia. However, Murphy *et al.* (1985), using an organ culture preparation, reported that axonal degeneration of the MCG homologue in *Helisoma trivolvis* did not occur for at least the first 28 days after a nerve crush. While factors regulating axon survival vary between species (Bittner, 1991), it is also likely that the differences in survival time seen in this study and that of Murphy *et al.* (1985) are the result of *in vitro* techniques which isolate the CNS from processes normally contributing to axonal degeneration. The question of axonal degeneration is important, since such isolated segments appear to play important roles in axonal regeneration in other animals (Hoy *et al.* 1967; Muller and Carbonetto, 1979).

One consequence of a lesion to the CBC and the subsequent degenerative events is a unilateral depletion of serotonin in the buccal ganglia (see Fig. 6A,B). In other recent studies we have demonstrated that pharmacological depletion of serotonin in the uninjured CNS of *Achatina fulica* leads to widespread sprouting by cerebrobuccal and buccocerebral neurones (Baker and Croll, 1991; Baker *et al.* 1993), as does a lesion to the CBC (Croll and Baker, 1990). In addition, when regeneration of ipsilateral projections is prevented by extirpating a section of the CBC, the contralateral MCG appears to sprout new projections into the buccal hemiganglia on the side of the lesion (Baker *et al.* 1991). This sprouting response is inhibited by the systemic administration of serotonin (Baker and Croll, 1992) and disinhibited by serotonin receptor antagonists (Baker and Croll, 1993). Taken together, these studies suggest that unilateral depletion of serotonin, as documented here, may contribute to regenerative and sprouting responses observed after injury. This hypothesis is consistent with previous *in vitro* work, which suggests that serotonin may play a role in regulating neuritogenesis in some gastropod neurones (Kostenko *et al.* 1983; Haydon *et al.* 1984). Indeed, Murrain *et al.* (1990) showed that C1, the homologue of the MCG in *Helisoma trivolvis*, can be selectively inhibited from regenerating in organ culture by bath-application of serotonin. The mechanism(s) by which serotonin may modulate neurite outgrowth is currently unknown, but several possibilities exist, including activities resulting from an alteration in intracellular Ca^{2+} levels (Mattson and Kater, 1988; Rehder *et al.* 1990) and/or changes in the expression of cell adhesion molecules (Mayford *et al.* 1992).

In conclusion, elucidation of the underlying mechanisms controlling neuroplastic responses to injury requires multiple approaches, employing both *in vitro* and *in vivo* models. Here, we have described an *in vivo* gastropod preparation in which the role of a lesion-induced depletion of serotonin can be tested to determine its involvement in a regenerative response. The transient unilateral depletion of serotonin which results from a crush to the CBC occurs as a consequence of the unique morphology of the MCG in *Achatina fulica*. Unlike the giant serotonin cells of other well-studied gastropods, such as *Helisoma trivolvis* and *Lymnaea stagnalis* (see Croll, 1987), the MCG of *Achatina fulica* possesses major projections to the ipsilateral buccal ganglia with only minor contralateral components. In addition to examining the role of soluble factors, such as serotonin, this preparation presents the opportunity to investigate the role of competition between neurones. Following an injury to the CBC, the contralateral MCG sprouts new neurites into the partially denervated territory (Baker *et al.* 1993) in a manner resembling descriptions of competitive interactions between neurones in other systems (Blackshaw

et al. 1982; Murphey, 1986). Lastly, the use of a whole-animal preparation will provide the opportunity to examine how the recovery of function at the single-cell and cell-population levels coincides with the behavioural repertoire of the animal. A major challenge of future work will be to integrate the findings from the many levels of analysis possible in this and other preparations.

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