Conduction and coordination in deganglionated ascidians

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Abstract: The behaviour of Chelyosoma productum and Corella inflata (Ascidiae) was studied in normal and deganglionated animals. Chelyosoma productum lived for over a year after deganglionation and the ganglion did not regenerate. Electrophysiological recordings were made from semi-intact preparations. Responses to stimulation and spontaneous activity continued to be transmitted through the body wall and branchial sac after deganglionation. Spread was slow, decremental, and facilitative. Treatment with >10 μg·mL⁻¹ d-tubocurarine abolished all responses, indicating that nerves mediate conduction of excitation after deganglionation. Histological study using cholinesterase histochemistry and immunolabelling with antisera against tubulin and gonadotropin-releasing hormone showed no evidence of a peripheral nerve net in regions showing conduction, contrary to previous claims. The cell bodies of the motor neurones were found to lie entirely within the ganglion or its major roots. Their terminal branches intermingled to form netlike arrays. Sensory neurones were identified with cell bodies in the periphery, in both the body wall and the branchial sac. Their processes also intermingled in netlike arrays before entering nerves going to the ganglion. It is concluded that the "residual" innervation that survives deganglionation is composed of either interconnected motor nerve terminals, interconnected sensory neurites, or some combination of the two. In re-inventing the nerve net, ascidians show convergent evolution with sea anemones, possibly as an adaptation to a sessile existence.

Résumé: Le comportement de Chelyosoma productum et de Corella inflata (Ascidiae) a été étudié chez des animaux normaux et des animaux qui ont subi l’ablation de leur ganglion. Les C. productum ont vécu plus de 1 an après l’ablation du ganglion qui n’a pas été régénéré. Des enregistrements électrophysiologiques ont été faits à partir de préparations semi-intactes. Les réactions aux stimulations et l’activité spontanée continuent d’être transmises à travers la peau du corps et le sac branchial après ablation du ganglion. La transmission est lente, décroissante et subjet à la facilitation. L’administration de plus de 10 μg·mL⁻¹ de d-tubocurarine inhibe toutes les réactions, ce qui indique que les nerfs sont les médiateurs de conduction de l’excitation en l’absence du ganglion. Une étude histologique basée sur l’histochimie des cholinestérase et sur le marquage immunologique au moyen d’antisérums contre la tubuline et l’hormone libératrice de la gonadotrophine n’a pas mis en lumière de réseau de nerfs périphériques dans les régions où il y a conduction, contrairement à des affirmations antérieures. Les corps cellulaires des neurones moteurs sont entièrement contenus dans le ganglion ou dans ses racines principales. Leurs branches terminales s’entrecroisent selon des arrangements en réseau. Les neurones sensoriels ont été identifiés aux corps cellulaires périphériques, aussi bien dans la paroi du corps que dans le sac branchial. Leurs processus forment également des réseaux avant de pénétrer dans les nerfs qui aboutissent au ganglion. Nous concluons que l’innervation résiduelle qui persiste après l’ablation du ganglion se compose de terminaisons nerveuses motrices interreliées ou d’axones sensoriels interreliés ou d’une combinaison quelconque des deux. En réinventant le réseau de nerfs, les ascidies mettent en evidence leur évolution convergente avec des anémones de mer et peut-être devons-nous voir là une adaptation à un mode de vie sessile.

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Introduction

The behaviour of ascidians shows some remarkable parallels with that of cnidarians. Gentle stimulation of the outer surface of a siphon evokes the “protective” or “direct” response (Jordan 1907; Hecht 1918), which consists of small local contractions, while stronger or more sustained stimulation evokes responses that spread to the other siphon and eventually to the body-wall muscles. These contractions result in the closure or retraction of both siphons and contraction of the whole body, according to stimulus intensity. As in sea anemones (Pantin 1952), the spread of contractions is graded, decremental, and facilitative (Florey 1951; Hoyle 1952). Moreover, as in cnidarians (Batham and Pantin 1950), these responses occur against a background of spontaneous activity in the form of rhythmic contractions of the siphons and body wall accompanied by arrests of the branchial cilia. Isolated siphons continue to show rhythmic contractions (Day 1919; Yamaguchi 1931; Hoyle 1953) and respond to stimulation. The isolated branchial sac likewise responds to stimulation, giving propagated ciliary arrests, and shows spontaneous rhythmicity (Mackie et al. 1974; Kingston 1976). Thus, both muscular and ciliary effector systems show a high degree of interaction.
of regional autonomy in their responses, combined with dispersed pacemaker capability, much as in cnidarians (Passano 1963).

At the same time, ascidians (unlike cnidarians) have a single, seemingly well developed cerebral ganglion with substantial nerve bundles running out to the effectors, and some of their responses are quite unlike those of cnidarians. In the “ejection” or “crossed” reflex, for example, stimulation of the inside wall of one siphon evokes contraction of the opposite siphon, while the stimulated one remains open (Jordan 1907; Hecht 1918). The ganglion has been shown to contain cell bodies of the motor neurons (“ciliary-arrest neurons”) that innervate the branchial sac. Intracellular recordings from these cells show that they produce spikes which precede the electrical signals accompanying ciliary arrest in the branchial sac and appear, therefore, to act as the master pacemakers regulating ciliary activity in the sac (Arkett 1987). Sensory signals from the receptors in the siphons are believed to fire the ciliary-arrest motor neurons in a classic reflex response (Takahashi et al. 1973; Bone and Mackie 1982). Reflexes mediated by the ganglion typically show a short latency, indicating that the ganglion, whatever its other functions, plays a role as part of a fast pathway.

The picture, then, is one of a conventional nervous system with sensory and motor nerves centred in the cerebral ganglion, combined with a semi-independent peripheral conducting “net.” Spontaneity resides both within the central and peripheral conduction systems.

The independence of the peripheral net is vividly demonstrated in animals from which the ganglion has been removed. After a period of postoperative recuperation, the animal returns to a seemingly normal life. Tonus is restored in the body wall. Spontaneous contractions are exhibited, though at an altered frequency (Yamaguchi 1931; Bacz 1935). Deganglionated animals also respond to tactile and electrical stimulation with responses that spread decrementally and are graded according to stimulus strength and frequency. Stimulation of one siphon can still cause contraction of the other (Bacz 1934, 1935; Florey 1951), though stronger stimulation is needed than in intact animals, and the response is generally slower and less dramatic. Deganglionated animals behave, in fact, “like slightly fatigued intact animals” (Hoyle 1952).

What is the nature of the peripheral conducting system that coexists with and survives removal of the central nervous system? With the notable exception of M. Fedele, most workers in the field (and influential reviewers like von Buddenbrock 1928) have assumed that there is a peripheral nerve net of the cnidarian type, i.e., a system of neurons with their cell bodies in the periphery that provide a pathway for diffuse, unpolarized conduction. Florey (1951) concluded that ascidian muscle receives dual innervation consisting of central innervation (the ganglion and motor nerves) on the one hand and largely independent peripheral innervation having the character of a nerve net on the other. As histological support for this idea the work of Hunter (1898) is usually cited. However, Hunter himself never claimed to have found cell bodies in the peripheral nervous system and his Fig. 3, based on methylene blue preparations, is open to other interpretations. Other studies (Fedele 1923a, 1937a; Markman 1958; Mackie et al. 1974; Arkett et al. 1989) failed to reveal cell bodies in the nerves innervating the muscular and ciliary effectors. Fedele emphatically rejected the idea of a peripheral nerve net and argued that because the motor neurons have their cell bodies in the ganglion, the motor innervation would degenerate rapidly in deganglionated animals. Therefore, any “residual” responsiveness could not be nervous in origin but must be due to myoid conduction. The idea of epithelial excitability was suggested by ten Cate (1928), who drew a parallel with the non-nervous conduction seen in the skin of amphibian tadpoles at a stage prior to arrival of the innervation (Wintrebert 1904; Roberts 1971). Our objective in the present work was to clarify the nature of the peripheral conduction system that survives deganglionation.

Most of the older work was an exploration of the muscular responses of the siphons and body wall (reviewed by ten Cate 1931), but subsequent studies of the branchial sac are relevant to the same questions (Mackie et al. 1974; Kingston 1976, see footnote 3; Arkett 1987; Arkett et al. 1989). Motor neurons with cell bodies in the ganglion terminate on the ciliated cells and bring about coordinated ciliary arrests either in spontaneous rhythmic patterns or as a result of stimulation. When separated from the ganglion, the branchial sac continues to show coordinated, spontaneous arrests and to conduct responses in a diffuse and unpolarized manner. Here, too, a peripheral nerve net might be expected to be present, supplementing the innervation from the ganglion, but histological study has provided no support for a conventional nerve net. A rich motor innervation was revealed by cholinesterase staining (Arkett et al. 1989), but no nucleated nerve cells were observed. It was therefore proposed that the peripheral conduction system that was demonstrated physiologically consists of the synaptically interconnected motor-nerve terminals (Mackie et al. 1974; Bone and Mackie 1982). If this scenario is true it could serve as a model for the “peripheral nerve net” innervating the body-wall musculature.

*Chelysoma productum* (Stimpson) (Asciidiacea) was selected for most of the present investigation because it is easily deganglionated and survives for over a year without the ganglion regenerating (Hisaw et al. 1966). This is in marked contrast to *Ciona intestinalis* and *Ascidia mentula*, which regenerate their ganglia after 3–5 weeks (Schultze 1900; Day 1919; Lender and Bouchard-Madrelle 1964), a process that is explored in several recent articles, most recently by Bollner et al. (1997). We used electrophysiology to verify the earlier descriptions of peripheral conduction in the body wall both before and after deganglionation. The earlier accounts all date from the period before such techniques were available. We also used histological and immunocytochemical procedures to try to settle the vexed question of whether ascidians have a conventional peripheral nerve net. Finally, we consider alternatives to such a net. A preliminary report of this work has appeared elsewhere (Mackie and Wyeth 1999).

**Material and methods**

Specimens of *C. productum* and *Corella inflata* (Huntsman) were collected intertidally and from the undersides of floats and boats in and around Friday Harbor, Washington, and Victoria, British Columbia, and maintained in the seawater tanks at Friday Harbor Laboratories and the University of Victoria. Those at Victoria, where the seawater system is constantly filtered, were periodically given a particulate food supplement (Fritz Invertebrate Buffet, Fritz Chemical Co., Dallas, Texas). Most specimens of both species
lived for many months in captivity and remained in good condition, although there was some mortality in both control animals and those that had received ganglion surgery.

Ganglion surgery

The anatomy of C. productum is well described by Huntsman (1912) Specimens were anaesthetized in MS 222 (0.02% in seawater). The tunic was then incised to partially free the two large horny plates that lie between the siphons, and a small fish hook was used to retract the right-hand plate (p in Fig. 1A), allowing access to the ganglion, which was then transected or completely removed (Hisaw et al. 1966). The plate was then returned to its normal position in the tunic. The mantle-layer tissues healed rapidly, covering the lesioned area. Specimens of C. inflata were deganglionated after removal from the their tunics.

Histology

In the case of C. productum the animal was removed from its tunic and portions were dissected and pinned out in Sylgard-lined petri dishes. In some cases, after the branchial sac was removed, the mantle from the whole region around the siphons right out to the points of insertion of the disk-retractor muscles on the tunic was prepared as a single whole mount. In such preparations the motor innervation to the entire body-wall musculature could be seen. Essentially the same procedure was followed in the case of C. inflata, but more care was needed in removing the mantle from the tunic, especially around the siphons, where the tissues are easily torn. Pinning of the delicate tissues of C. inflata was best conducted using cactus spines, stretching the piece out progressively and evenly in all directions.

Preparations were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline at pH 7.3 for about 1 h prior to processing by the “direct colouring” thiocholine method for cholinesterases (Karnovsky and Roots 1964), following procedures published previously (Arkett et al. 1989). This method shows the motor innervation well but fails to show the peptidergic neurons of the dorsal strand plexus or the sensory neurons with peripheral cell bodies. Permanent mounts were studied using conventional bright-field microscopy.

For anti-tubulin fluorescence microscopy, tissues were fixed in either the same fixative or a picric acid – formaldehyde mixture (Zamboni and DeMartino 1967). The latter gave better reagent penetrability but poorer cytoplasmic fixation. The preparations were incubated in primary antibody followed by standard processing, with controls in which the primary antibody was omitted. Of several antibodies tried, the most useful was a mouse monoclonal raised against alpha tubulin (Amersham N356). Both sensory and motor neurons are well-labelled using this antibody. Treatment was completed with fluorescein isothiocyanate (FITC)-coupled goat antime secondary antibody. Brief immersion of the preparation in 1 mg mL\(^{-1}\) propidium iodide prior to mounting allowed the nuclei to be observed using the Texas Red filter set. In another set of experiments, paraformaldehyde-fixed preparations were treated using antisera raised against Tunicate I gonadotropin-releasing hormone (GnRH) (Powell et al. 1996), which labels sensory neurons as well as dorsal strand elements in C. inflata (Mackie and Marx 1999). The secondary antibody in this case was coupled to Alexa 488 (Molecular Probes, Inc., Eugene, Oregon). Labelled preparations were observed with a conventional fluorescence microscope or a laser-scanning confocal microscope (LSM 410, Zeiss). The filter combination used for Fig. 3D allowed both the long- and short-wave emissions to be visualized simultaneously, avoiding the need for an overlay.

In addition to these special procedures, tissues were studied alive with phase-contrast, polarization, and Nomarski optics, and some preparations were made by classical staining methods, e.g., fixation in Flemming’s solution (without acetic acid) followed by Heidenhain’s iron haematoxylin, a method used to good effect by Millar (1953). Methylene blue preparations were also made for supravital study of the nervous system in C. inflata, but penetration was often very slow, as noted by previous workers (Hunter 1898; Markman 1958).

Physiology

The reactions of intact specimens of both C. productum and C. inflata were observed, using tactile and electrical stimulation. Other specimens were anaesthetized and opened out to allow direct recording of neuromuscular activity. In the case of C. inflata, the animal was removed completely from its tunic and a large part of the mantle, including the siphons, was pinned out on a Sylgard platform after the viscera were removed. Chelyosoma productum “disk preparations” were made by cutting around the whole animal just below the disk and separating the disk from the lower part. The disk portion was then pinned out flat, with the tunic down, allowing access to the nerves and muscles of the mantle from above. The branchial sac was snipped away and discarded. The mantle was pinned out radially to extend the disk-retractor muscles, which otherwise tended to contract inwards. Stimulating and recording probes were placed at various points and surgical lesions were made using fine-pointed needles or scissors as shown in Fig. 1B. These preparations needed at least 8 h to recover from the stress of the operation, but after recovery remained responsive for 24–48 h when kept in running seawater at temperatures below 14°C. A perfusion system was employed for long-term experiments. This had the advantage that after anaesthesia or drug treatment, the preparation could be returned gently to seawater for further observation.

Tactile stimuli were delivered by means of a hand-held probe or a probe mounted on a loudspeaker activated by current pulses. Electrical stimuli were delivered through concentric bipolar stainless-steel electrodes (Model SNE-100, Clarke Electromedical Instruments, Reading, U.K.). Extracellular recordings were made using polyethylene suction electrodes pulled out to an internal diameter of 50–80 μm. Amplified signals were displayed on an oscilloscope and stored on an instrumentation tape recorder for later analysis. All electrical recordings were carried out in a Faraday cage, and special pains were taken to eliminate 60-cycle interference, as the signals recorded were typically in the microvolt range.

Results

Histology

Motor innervation

The large nerve trunks running out from the ganglion in tunicates are mixed nerves containing both sensory and motor fibres (Bone and Mackie 1982). In C. productum, fibres running in the body wall and branchial sac were found to fluoresce strongly with anti-tubulin antisera. The motor innervation could be visualized selectively by cholinesterase histochemistry using the procedure of Arkett et al. (1989). As can be seen in Fig. 2A (a control preparation from a freshly dissected animal), the nerves leaving the ganglion ramify repeatedly and eventually break down into small bundles that are especially abundant in muscular regions. At higher magnifications, small bundles or individual neurites were traceable to terminations on muscle fibres within the mantle. Nerve-cell bodies were not observed within the peripheral motor innervation.

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Animals from which the ganglion had been removed were kept for periods of up to 5 months before being fixed and processed with cholinesterase staining. In no case where we were sure that the entire ganglion had been removed was the ganglion found to have regenerated. While no attempt was made to quantify these observations, we found no evidence suggestive of degeneration in the peripheral ramifications of the motor neurons. Not only were the main nerve branches still strongly reactive (Fig. 2B), but small bundles and fine individual neurites were readily distinguishable running in all areas of the mantle, including tracts running around the zone from which the ganglion had been removed (Fig. 2C). In all regions where the motor innervation was followed out to its terminal ramifications, the fine endings of the motor neurons intermingled to form a plexus. This could be seen both in the vicinity of muscle fibres and in muscle-free zones (Fig. 2D). Similar observations were made in freshly prepared control tissues so this is evidently a normal feature of the anatomy, not the product of deganglionation. In sum, we found no evidence of degeneration, regeneration, regrowth, or reorganization of the motor innervation after complete ganglion removal.

In *C. inflata*, cholinesterase and anti-tubulin preparations gave a similar picture to that observed in *C. productum*, including vivid pictures of fine motor nerve endings on mantle-muscle fibres (Fig. 3A), but the effect of ganglion removal on the motor innervation could not be made studied in this species, as the animals did not survive the operation long enough for useful comparisons with control animals to be made.

Cholinesterase preparations of the branchial sac of both species showed the rich innervation of the stigmatal ciliated cells described by Arkett et al. (1989). Nuclei were absent from these nerves.

**Sensory innervation**

Using anti-tubulin and anti-GnRH labelling, we confirmed earlier descriptions (Fedele 1923a; Millar 1953) of scattered unipolar sensory cells whose cell bodies lie just beneath the mantle epithelia. The cells typically occur in pairs (Fig. 3B), but a few single ones were observed (Fig. 3C, 3D). In our preparations they were restricted to the siphons, and were most abundant in the outer mantle epithelium and siphon rims (Fig. 3E). A smaller population of these sensory cells was also observed in the inner mantle epithelium immediately adjacent to the siphon rim. Each mature sensory cell had a single cilium, 15–20 µm long when fully extended, projecting externally. In life, the cilia probably lie just below the tunic. As the cilia lie in a different focal plane, they were not seen in confocal images where the Z series stopped at the epithelial surface (Figs. 4C, 4D). Each sensory cell had a single neurite. The neurites of the sensory cells intermingled to form plexiform arrays in the periphery (Fig. 3F) before entering nerve bundles heading for the ganglion. Each sensory cell body contained a single nucleus. Seen as dark
(nonfluorescing) spaces in Figs. 3B and 3C, the nuclei appeared as bright objects after propidium iodide staining (Fig. 3D). Anti-tubulin immunofluorescence showed the microtubular organizing centres of the epithelial cells as bright spots (Figs. 3B, 3C), and their nuclei also stained with propidium iodide (Fig. 3D). The epithelial cells adjacent to
sensory cells did not appear to be specialized “supporting cells.”

A special effort was made to determine if sensory cells were present in the branchial sac, as such cells have never been reported in this location and were not seen in studies on C. inflata, using methylene blue staining, phase-contrast microscopy, and cholinesterase histochemistry (Mackie et al. 1974; Arkett et al. 1989). Anti-tubulin immunofluorescence microscopy on C. productum, however, showed a rich innervation stemming from multicellular sensory organs (“sensory papillae”) that are scattered through the epithelium covering the atrial surface of the branchial sac (Figs. 3G, 3H). They were most abundant on the dorsal side of the branchial sac and appeared quite distinct from the “cupular organs” reported in the epithelium lining the inner side of the atrial siphon of Ciona intestinalis (Fedele 1923a; Millar 1953; Bone and Ryan 1978), which are remarkable for their resemblance to sense organs in the vertebrate acoustico-lateralis system, having a “flag” or “cupula” of tunic-like material in which the sensory cilia are embedded. The sensory papillae in the branchial sac of C. productum consisted of a cluster of about 6–8 sensory cells, each with a single neurite. No cupula was present. The sensory neurites running out from the base of the papilla formed a compact bundle. Close to their origin, the neurites and sensory cell somata could be individually visualized (Fig. 3H). Propidium iodide showed the nuclei of the neurites and sensory cell somata could be individually visualized. With intact ganglia, cuts were made through the muscular and nonmuscular regions to determine whether or not there is a nerve net, in the sense of a plexiform array of nucleated bi-or multi-polar neurons, lying in these regions. Our preparations fully support the findings of Fedele (1923a, 1937a) in showing that the only neurons with cell bodies in the periphery are the sensory cells lying in the mantle epithelium (and, as we show here, in the branchial sac) and that there are no cell bodies in the motor terminal plexus in either the body wall or the branchial sac.

Lack of a “conventional” nerve net

The ability to detect the finest neurites of both sensory and motor neurons by means of anti-tubulin and anti-GnRH immunofluorescence and cholinesterase histochemistry, respectively, and to visualize their nuclei allowed us to scan large areas of the mantle and branchial sac of C. inflata. We examined both muscular and nonmuscular regions to determine whether or not there is a nerve net, in the sense of a plexiform array of nucleated bi-or multi-polar neurons, lying in these regions. Our preparations fully support the findings of Fedele (1923a, 1937a) in showing that the only neurites with cell bodies in the periphery are the sensory cells lying in the mantle epithelium (and, as we show here, in the branchial sac) and that there are no cell bodies in the motor terminal plexus in either the body wall or the branchial sac.

Physiology

Responses of intact animals and dissected preparations with intact ganglia

Both C. productum and C. inflata showed the classic protective (Jordan 1907) or direct (Hecht 1918) responses to external siphon stimulation. Light stimulation of the outer surfaces of either siphon caused graded contractions of that siphon, spreading, with stronger stimulation, to the other siphon and eventually to the body-wall muscles, causing a generalized retraction. A single sharp prod applied to a siphon typically caused rapid contraction of both siphons. In C. productum disk preparations set up with a recording electrode on the branchial siphon, a prod to the atrial siphon evoked contraction of both siphons accompanied by a burst of potentials recorded at the branchial siphon. The potentials summed to produce irregular compound events of up to 500 μV (Fig. 4A). Similar responses were observed when recording from the atrial siphon and stimulating the branchial siphon. When a nerve exiting the ganglion was cut and a recording electrode attached to its proximal stump, a stimulus to the siphon on the far side of the ganglion evoked a burst of action potentials (Fig. 4B) that again summed to produce a larger, irregular deflection. The size and duration of bursts evoked by tactile stimulation were found to vary roughly in proportion to the strength of stimulation, but the animals rapidly became less responsive with repeated stimulation and needed periods lasting several minutes to recover responsivity. The smallest events observed (10–20 μV) probably represent individual axon spikes. There was no evidence of consistently large, rapidly conducted units suggestive of giant axons and there are no reports of giant axons in the histological literature. As we were primarily concerned with the animal’s ability to show propagated responses after ganglion transection or removal, we did not explore the peculiar ejection or crossed reflex, as this response is well known to require an intact ganglion (reviewed by Goodbody 1974).

Responses to electrical stimulation resembled those to tactile stimulation. Low-voltage short-duration shocks evoked purely local responses that spread progressively with repetition, but a single strong shock resulted in immediate contraction of the other siphon, to the accommodation of a burst of potentials. Conduction velocities in the intersiphonal pathway were hard to estimate accurately because of uncertainty as to the arrival time of the first (typically small) potentials in the burst, but the fastest reliable values lay in the range 20–22 cm·s⁻¹.

Responses after ganglion transection or removal in C. productum

Disk preparations that had previously shown normal excitability were unresponsive for several hours after ganglion transection or removal but gradually recovered the ability to respond to mechanical and electrical stimulation. Direct responses of the siphons could be evoked by gentle stimulation and spread decrementally with repeated stimulation. In preparations in which the ganglion had been removed, single moderately strong shocks (which would have evoked contraction of both siphons in a preparation with an intact ganglion) failed to cause a response at the distant siphon (Fig. 4C). When repeated at 10 Hz, however, such stimulation caused responses that propagated right through (Fig. 4D). Single very strong shocks also produced responses that reached the other siphon. In experiments on animals with transected ganglia, response latencies varied widely but were always much longer than in control preparations with intact ganglia (Figs. 5A–5C). The fastest conduction velocities obtained in preparations with transected ganglia lay in the range 2.5–3.0 cm·s⁻¹.

In an attempt to determine the pathway taken by excitation in animals with transected ganglia, cuts were made through the entire mantle tissue at various points. It was found that a strip of intact tissue 3–5 mm wide on one side of the ganglion was sufficient to allow impulses to be conducted through to the second siphon (Fig. 5C).

Disk preparations from animals that had been deganglionated over a month previously showed a similar ability to conduct excitation between the siphons. This finding fits
Fig. 3. Nerves in *Corella inflata* (A–F) and *C. productum* (G, H) shown by immunofluorescence microscopy (A) and confocal microscopy (B–H). (A) Fine nerve processes running along muscle fibres (presumably motor terminals) in the mantle near the atrial siphon (anti-tubulin). (B) A pair of simple sensory cells in the outer mantle epithelium, showing their cilia, one curled and one extended (anti-tubulin). (C and D) Another sensory cell to the same scale as in B. In C the cell is shown by anti-tubulin immunofluorescence alone. In D propidium iodide staining shows nuclei. (E) Sensory cell pairs located around the rim of the atrial siphon (anti-GnRH). (F) Sensory neurites intermingling in plexiform array in the outer mantle epithelium (anti-GnRH). Arrowheads indicate sensory cells. (G) Two sensory papillae and sensory nerves in the branchial wall (anti-tubulin). (H) Sensory papilla enlarged (anti-tubulin).
with the lack of evidence of degeneration in the histological preparations.

Source of recorded potentials

In both *C. productum* and *C. inflata* it was found to be possible to record the characteristic bursts of electrical events not only with electrodes placed over muscle bundles but also from patches of mantle tissue where no muscle fibres were visible. The “best” (largest amplitude) recordings were made from muscular regions, but the patterns of impulses evoked by stimulation were similar in both places, and were also similar to the patterns recorded directly from large nerve roots (Fig. 4B). These observations indicate that the bursts of potentials are primarily nervous events that are augmented by muscle depolarizations in muscular regions. Ascidian body wall muscles are known from intracellular recordings to give twitch-type responses accompanied by all-or-none action potentials (Nevitt and Gilly 1986). Such events presumably sum with and augment the primary nervous signals.

Coordinated pacemaker activity in deganglionated *C. inflata*

After several hours’ recovery from ganglion removal, some pinned preparations of *C. inflata* started to show patterns of spontaneous activity in the form of regular bursts of small (50–70 \( \mu \)V) potentials roughly every 4 min. Recordings made with two electrodes, one on each siphon, showed similar patterns at the two sites despite the absence of the ganglion (Fig. 6). In the longer record from which Fig. 6 was taken, bursts lasted about 115 s, with 38 pulses per burst and interburst intervals of 127 s (mean values from a sequence of 13 bursts). Because these preparations were pinned out flat, it was not possible to see accompanying muscle contractions except at the edges of the siphons, where small twitches occurring in time with burst potentials were just visible. In one preparation a piece of the branchial sac was left attached to the mantle and it was seen that the cilia lining the branchial stigmata underwent arrest in synchrony with each of the early potentials in the bursts accompanying muscular contractions in the siphons, confirming observations by Mackie et al. (1974). Later in the bursts, the branchial cilia ceased to show discrete arrests but were maintained continuously in the fully arrested position until the end of the burst, when normal beating and metachronal rhythms re-emerged.

**Drug experiments**

In a series of controlled experiments using *C. productum* disk preparations, \( d \)-tubocurarine was found to block intersiphonal conduction in both normal and deganglionated preparations (Figs. 5D–5F). The lowest effective dose was 10 \( \mu \)g·mL\(^{-1}\) and the block was completely reversible at this level, though full recovery took at least 2 h. With stronger concentrations (20–40 \( \mu \)g·mL\(^{-1}\)) the preparation sometimes failed to recover or recovery was incomplete. In a deganglionated *C. inflata*, 10 \( \mu \)g·mL\(^{-1}\) curare blocked all pacemaker activity within 13 min, along with spontaneous muscle contractions and ciliary arrests, so the cilia beat continuously. Treatment with eserine (physostigmine) in concentrations up to 80 \( \mu \)g·mL\(^{-1}\) had no definite effect on the responses of either *C. inflata* or *C. productum*, as Bacq (1935) and Florey (1963, 1967) found with *C. intestinalis*. We were unable to demonstrate any effect of \( \alpha \)-bungarotoxin on *C. productum* at 10 \( \mu \)g·mL\(^{-1}\) even after 24 h of treatment. The times taken for curare to take effect and for responses to be restored on return to seawater varied considerably in our experiments, probably because of the slowness with which the drug penetrates intact tissues. Tunicate epithelia are well sealed, with extended tight junctions (Lane et al. 1995; Burighel and Cloney 1997). Preparations of *C. productum* in which the inner mantle epithelium was deliberately slit to assist penetration generally responded rapidly to the drug. The failure of Scudder et al. (1966) to obtain any blocking action with curare on whole, undamaged *C. intestinalis* may have been due to failure of the drug to penetrate, as these authors limited their drug tests to 1 h duration.

**Discussion**

**Motor innervation**

Motor nerves leaving the ganglion run out to the periphery, where they associate with muscles and ciliated cells. Cholinesterase histochemistry shows the finest processes, including synaptic boutons in the branchial sac of *C. inflata* (Arkett et al. 1989). The fine terminal neurites of the motor system form plexiform arrays in both the mantle (Fig. 2D) and the branchial sac, but there appear to be no cell bodies within this mass of neurites. Nickel backfills (Arkett et al.
1989) showed that in *C. productum*, all the cell bodies lay within the ganglion except for a small cluster close to the ganglion in one of the posterior nerve roots. As the nerve root in question (AM3) was the one that gives rise to the visceral nerve, it seems possible that the filled cells (which were located on the surface of the nerve root rather than within it) were part of the dorsal strand plexus (see below) and were not motor neurons. Lorleberg (1907) observed a few supposedly neuronal cell bodies (Ganglienzellen) in the nerve roots of *Styelopsis grossularia* close to the ganglion, but these became increasingly rare farther away from the ganglion. In *Polyandrocarpa misakiensis*, Koyama and Kusunoki (1993) saw “some neuron-like cells” in nerve roots close to the ganglion. Our preparations of *C. productum* and *C. inflata*, like those of Markman (1958) and Aros and Konok (1969) using *C. intestinalis*, failed to show cell bodies in the motor innervation outside the brain. It seems certain, therefore, that the great majority, and possibly all, of the motor neurons would be rendered anucleate by brain removal, and yet *C. productum* remains responsive to stimulation and shows spontaneous movements many weeks after deganglionation. We can only conclude that the distal fragments of the motor neurons stay alive and functional in the anucleate state. We have not addressed the question of how the distal fragments survive, but this is a well-documented phenomenon in other invertebrates, particularly crustaceans, insects, and annelids (reviewed by Bittner 1991). It may be noted that ascidian nerves run in blood spaces and lack...
cellular sheaths, so uptake of metabolites directly from the blood would seem likely.

Sensory innervation

Lorleberg (1907) failed to find any evidence of peripheral sensory cells and it was left to Fedele (1923a) and Millar (1953) to provide the first clear descriptions of them in *C. intestinalis*. We confirmed and extended these findings using anti-tubulin and anti-GnRH immunolabelling, both of which proved suitable for showing a wide variety of neurones, both sensory and motor, in contrast to the cholinesterase method, which was only effective in showing motor elements. Simple primary receptor cells lie in both the outer and the inner mantle epithelium of the siphons and have a sensory process or cilium that projects to the exterior. They typically occur in pairs or small clusters. Similar cells occur in ascidian larvae (Torrence and Cloney 1982) and pelagic tunicates (Bone 1959) and are generally regarded as mechanoreceptors (Goodbody 1974; Bone and Mackie 1982). Our investigation using immunolabelling shows the neurites of these cells running below the epithelium, intermingling and forming a netlike array (Fig. 3F) before entering larger nerve bundles leading to the ganglion. Sensory papillae occur in the outer layer of the branchial sac, where we describe them for the first time. Their afferent neurons form bundles that run to the ganglion via the visceral nerve roots but some bundles also leave the branchial sac and mingle with nerves in the mantle without passing through the ganglion. Such connections might provide a pathway for the coordination observed between mantle contractions and ciliary arrests in deganglionated preparations.

It is unclear if ascidians have sensory neurons with cell bodies in the ganglion and processes that end freely in the periphery. Free nerve endings are seen in many places where there are no primary sensory receptor cells and some of these might be sensory endings. In the oral tentacles, which are highly sensitive to tactile stimuli evoking the crossed response (Hecht 1918), Seeliger (1893–1907) described cells with processes projecting to the exterior that might be sensory structures, but his drawings are unconvincing, and subsequent workers, including ourselves, have been unable to confirm his finding. Burighel and Cloney (1997, Fig. 42) figure a presumptive secondary sensory cell in a tentacle of *Botryllus schlosseri*, raising the possibility that some of the “free nerve endings” described by other workers are actually postsynaptic or electrically coupled to secondary sensory cells lying in the epidermis. Secondary sensory cells occur...
in various larvacans (Bone and Mackie 1982) but have not been recognized in other tunicates, except for this one report on *B. schlosseri*.

**Pacemakers**

Intracellular recordings from the ganglion of *C. productum* demonstrate the presence of motor neurons that generate rhythmic patterns of impulses driving ciliary arrests in the branchial sac (Arkett 1987). However, the branchial sac continues to show spontaneous rhythmicity after deganglioneation (Mackie et al. 1974) and this persists for at least 3 weeks (Kingston 1976, see footnote 3). Pacemaker capability must therefore exist at the periphery. This is also clear from studies on the siphons and body wall of other ascidiains (Day 1919; Yamaguchi 1931; Bacq 1935; Hoyle 1953). Our present work on deganglioneated *C. inflata* has shown the emergence of a rhythmic pattern of muscle contractions coordinated between the two siphons and blocked by curare. The peripheral pacemakers are therefore presumably components of nerve cells but it is impossible to say whether they are located in sensory or motor nerves.

**Is there a peripheral nerve net?**

Most students of ascidian behaviour (e.g., Jordan 1907; Hecht 1918; ten Cate 1928; von Buddenbrock 1928; Schiller 1937; Florey 1937, 1939) and Markman (1931) have assumed that there is a peripheral nerve net in the mantle that survives deganglioneation and provides a basis for continuing responsiveness to stimulation. The physiological picture is fairly clear: peripheral conduction pathways showing the characteristics elsewhere associated with nerve nets certainly exist both in intact animals and after deganglioneation. We have confirmed previous reports that ascidiains respond to stimuli with contractions that spread decrementally for varying distances depending on the strength and frequency of stimulation, and produce graded responses in the muscles in a manner reminiscent of sea anemones. Conduction velocity in the “net” is slower than in the intact animal by about an order of magnitude. Florey (1951), the most recent physiologist to address this question, concluded that there is a twofold innervation of the musculature in *C. intestinalis*, the first consisting of motor nerves coming from the ganglion and the second consisting of a peripheral nerve net. After destruction of the ganglion, all remaining responses would be mediated by the nerve net.

The term nerve net is generally taken to refer to a plexiform array of bi- or multi-polar neurons lying outside the central nervous system and constituting a diffusely conducting (unpolarized) set of pathways (Mackie 1999). As evidence of such a net in ascidians, many workers have cited histological observations by Hunter (1898), whose Fig. 3 could be interpreted as showing cell bodies within the peripheral nervous system of *Molgula manhattensis*. Our findings, however, support Fedele (1923a; 1937a) and Markman (1958), who found no such nerve net in the mantle, and are in agreement with earlier reports (Mackie et al. 1974; Arkett et al. 1989) in showing that there is no nerve net in the branchial sac.

The assumption by various workers that there is a nerve net in the mantle, though emphatically denied by Fedele (1923a, 1937a), was (paradoxically) bolstered by Fedele’s own account of a nerve net in the dorsal fold of the branchial sac (Fedele 1923b, 1927, 1938). The system consists of a plexus of bi- and multi-polar neurons lying within the dorsal blood sinus and closely associated with the dorsal strand (dorsal cord) and visceral nerves. This plexus (“dorsal-strand plexus”) extends all the way from the cerebral ganglion back to the region of the gonads. It can be readily visualized by means of methylene blue staining and phase-contrast optics in living whole mounts (Mackie et al. 1974) and by its immunoreactivity with antiserum raised against GnRH (Mackie 1995; Powell et al. 1996; Tsutui et al. 1998). The presence of this nerve net in the dorsal fold made it quite plausible that another net existed in the mantle tissue, but, as noted above, there is no histological support for such a structure. Indeed, the fact that the nerve plexus in the dorsal fold can be visualized so readily means that if such a plexus were present in the mantle, it could hardly have gone undetected. The techniques used in the present study allowed us to visualize both the sensory and the motor innervation in considerable detail and to detect nuclei where present (i.e., in sensory cells), and would surely have revealed a nerve net if it were present.

**Alternatives to a conventional nerve net**

If there is no nerve net, we are left with four possibilities: (i) the transmission of excitation by mechanical forces between muscles in the body wall, acting as “independent effectors” (proposed by Loeb 1891; Fedele 1923b, 1937b), (ii) conduction in excitable epithelia (suggested by ten Cate 1928), (iii) conduction between the interconnected terminals of the motor nerves (suggested by Mackie et al. 1974), and (iv) conduction between interconnected branches of the sensory nerves.

Regarding *i*, myoid transmission is hard to reconcile with the observations of Magnus (1902) and Kinoshita (1910), who found that treatment with cocaine and other drugs that eliminate all nervous activity while leaving the muscles excitable by electrical stimuli blocked the spread of responses completely. Our own findings with curare, a potent blocker of cholinergic synapses, also show that conduction of excitation must depend not on muscles but on nerves, as muscles are not synaptically linked. This does not mean that the muscles are incapable of independent action when directly stimulated, or that they could not act mechanically upon other muscles in the locality, but the results of the experiments with drugs make it clear that the main peripheral conduction pathways surviving deganglioneation cannot be muscle-based.

Regarding *ii*, excitable epithelia that conduct behaviourally meaningful signals occur in some ascidian larvae (Mackie and Bone 1976), in the blood vessels linking the zooids in botryllid colonies (Mackie and Singla 1983), and in various pelagic tunicates (see Bone and Mackie 1982), raising the possibility that the mantle and branchial epithelia in adult solitary ascidiains are also excitable and conduct electrical signals. Recordings made with electrodes attached to the surfaces of the mantle epithelia, however, failed to show the large potentials typically associated with epithelial conduction, showing instead much smaller potentials in patterns similar to the bursts of action potentials recorded directly from cut nerves. Furthermore, curare would not be expected to block epithelial conduction, as transmission in all such
tissues is electrical not chemical (Anderson 1980). The small potentials recorded with suction electrodes attached to the mantle epithelia almost certainly derive from the plexiform mass of fine nerve terminals running under the epithelium.

Regarding iii, if the motor nerve terminals were interconnected synthetically, they could in theory produce the equivalent of a nerve net (Fig. 7A). Decremental spread would be exhibited if the synapses failed to transmit one-for-one but represented a partial barrier to the spread of excitation. This would occur, for example, if early impulses in a burst arriving at a synapse failed to evoke regenerative potentials on the postsynaptic side but evoked subthreshold depolarizations (excitatory postsynaptic potentials) that allowed later ones to produce spikes. The burst would diminish with each synapse crossed and gradually fade out or become ineffective in evoking effector responses. A consequence of an arrangement of this sort would be to reduce the selectivity of muscle excitation. Motor nerves in most animals function to excite specific effectors, leaving others unexcited (the concept of "labelled lines"), and if motor impulses invaded adjacent motor units, it would become hard to obtain precise local movements such as directional flexions. In fact, ascidian behaviour lacks directionality. Siphon closures and body-wall contractions are more or less symmetrical. In the case of the isolated branchial sac, impulses generally spread through the whole preparation, though not necessarily at the same velocity in all directions (Mackie et al. 1974). Creation of a motor net in which impulses are shared between adjacent motor units might have advantages in "smoothing out" responses.

In the scenario shown in Fig. 7A, sensory cells do not synaptically excite the motor nerves except in the ganglion, and it is assumed that the motor plexus itself is directly excitable by local stimulation but sensory units could synapse with the motor plexus in the periphery; some observations support such a picture. For instance, isolated siphons and siphons in deganglionated animals remain almost as responsive to vibrations as those in the intact animal (Magnus 1902), which implies that mechano-receptors are still functional parts of the response system with connections to local effectors.

Regarding iv, sensory cells lying in the mantle and branchial sac could send processes not only to the cerebral ganglion but also through branches in the periphery to one another (Fig. 7B). Synaptic links would regulate impulse traffic as in iii above. To excite the muscular or ciliary effectors, the sensory net would either have to contact the effectors directly or excite them indirectly through the motor innervation. We have shown them exciting the effectors indirectly by synapsing on motor terminals. However, direct connections between sensory cells and muscles are known in Hydra littoralis (Westfall 1973) and cannot be excluded. A net fashioned out of the interconnected branches of sensory neurons would satisfy the requirement for a set of peripheral conductive pathways that could survive deganglionation. A potential disadvantage of this arrangement would be some loss of precision in the ability of the animal to localize sources of stimulation, for sensory signals would tend to invade afferent pathways adjacent to those actually stimulated.

At present, the evidence is insufficient for deciding between the motor-net (Fig. 7A) and sensory-net (Fig. 7B) hypotheses. Both are highly unconventional and would reflect a unique situation among triploblastic animals but, given that there is no conventional nerve net, one or other of them, or some combination of the two, must be true.

**Evolutionary implications**

Regardless of the precise nature of the peripheral conduction system that endows ascidians with the functional equivalent of a nerve net, it is clear that ascidian evolution has been characterized by decentralization of certain functions that are more fully centralized in other tunicates. Doliolids and salps deprived of their ganglia show no spontaneous swimming or contractions of the body-wall musculature and do not respond to stimulation. However, doliolids show local autonomy in the ciliary control system in the branchial sac, like ascidians (Fedele 1923; Bone and Mackie 1982). The ganglion in ascidians is small and simple compared with, for example, that of salps. It still has a special role to play in maintaining muscle tone, as a fast conduction pathway and as modulator of certain complex responses, notably the crossed response (Day 1919; Bacq 1935; Florey 1951). These functions, however, are superimposed upon a fundamental neuromuscular organization characterized by the decremental, diffuse, facilitating spread of contractions within and between the siphons and in the body wall and branchial sac, often exhibited in rhythmic patterns (Hoyle 1952, 1953), and it is this residual innervation that survives deganglionation.

Evolutionary relationships within the Tunicata remain uncertain (Lacalli and Holland 1998; Lacalli 1999), and it is not clear whether the ancestral urochordate was planktonic or bottom-living, but a recent molecular phylogenetic analysis (Swalla et al. 2000) suggests that the ancestor “probably retained the tadpole tail, including the dorsal nerve cord, the notochord and flanking muscle cells through the adult life.” A good case can be made for the origin of ascidians from more active, mobile, free-living ancestors (Satoh and Jeffery 1995; Wada 1998) whose nervous systems would probably have been more fully centralized, as in present-day pelagic tunicates. The decentralization of functions seen in ascidians would therefore appear to be in some way adaptive in the context of their sessile habitat. It may be that processing all sensory information and motor output via a single nerve centre is not only unnecessary but metabolically wasteful in a situation where many responses can be managed initially by local action systems. Actinian nerve nets have the same potential for organizing actions locally that we see in ascidians and their nervous systems show a corresponding lack or low degree of centralization. Aside from the purely “visceral” activities carried out locally in the heart, gut, and gonoducts, ascidian behaviour consists essentially of opening, closing, and retracting the siphons, contracting the body-wall muscles, and arresting the cilia in the branchial sac. These components are functionally linked and are exhibited in various degrees depending on the strength of stimulation. There are no directional responses and little apparent need for precise localization of sources of stimulation. Whereas actinians have true nerve nets for organizing local action systems, it would appear that ascidians have created the equivalent of a peripheral nerve net by modifying the connectivity patterns of their motor and (or) sensory nerves. This has allowed them to
devolve certain functions to the periphery in a way not seen in pelagic tunicates. At the same time they have retained the cerebral ganglion and its major input–output pathways for organizing complex responses and as a fast pathway for rapid and coordinated defensive responses.

**Note added in proof:**
A forthcoming paper (Burighel et al. 2000) describes the distribution of nerves in the colonial ascidian *Botryllus schlosseri*, as visualized by cholinesterase histochemistry. Nerves extend to all organs except for the neural gland and gonads. A complex network of nerves is described, but no peripheral cell bodies were observed. The system does not interconnect different zooids within the colony. The picture of this component of the innervation in *B. schlosseri* thus corresponds in essential respects to what we describe here for the solitary ascidians *C. productum* and *C. inflata*.

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