GABAergic synaptic transmission modulates swimming in the ascidian larva

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Abstract
To examine the role of the amino acid GABA in the locomotion of basal chordates, we investigated the pharmacology of swimming and the morphology of GABA-immunopositive neurones in tadpole larvae of the ascidians Ciona intestinalis and Ciona savignyi. We verified that electrical recording from the tail reflects alternating muscle activity during swimming by correlating electrical signals with tail beats using high-speed video recording. GABA reversibly reduced swimming periods to single tail twitches, while picrotoxin increased the frequency and duration of electrical activity associated with spontaneous swimming periods. Immunocytochemistry for GABA revealed extensive labelling throughout the larval central nervous system. Two strongly labelled regions on either side of the sensory vesicle were connected by an arc of labelled fibres, from which fibre tracts extended caudally into the visceral ganglion. Fibre tracts extended ventrally from a third, more medial region in the posterior sensory vesicle. Two rows of immunoreactive cell bodies in the visceral ganglion extended neurites into the nerve cord, where varicosities were seen. Thus, presumed GABAergic neurones form a network that could release GABA during swimming that is involved in modulating the time course and frequency of periods of spontaneous swimming. GABAergic and motor neurones in the visceral ganglion could interact at the level of their cell bodies and/or through the presumed GABAergic fibres that enter the nerve cord. The larval swimming network appears to possess some of the properties of spinal networks in vertebrates, while at the same time possibly showing a type of peripheral innervation resembling that in some protostomes.

Introduction
Situated at the base of the chordate lineage, ascidians possess many features that prefigure those in vertebrates; the larval form of the life cycle, in particular, has long been considered to reflect a prototypical chordate body form (e.g. Berrill, 1950). With a recently sequenced genome and powerful new tools available for genetic manipulation, the simple larval nervous system of the ascidian Ciona intestinalis represents an exciting experimental model to understand ancestral features of the chordate nervous system, its development and physiology (see Meinertzhagen et al., 2004). We have examined the properties of the neural network controlling swimming behaviour in the ascidian larva with the aim of improving our understanding of the evolution and development of locomotory neural networks. In ascidians, pharmacological evidence already indicates that neuromuscular transmission is cholinergic (Ohmori & Sasaki, 1977). This resembles the situation in the more heavily populated nervous systems of vertebrates but in Ciona there are only five pairs of motor neurones in the motor region of the larval central nervous system (CNS), the visceral ganglion (Meinertzhagen et al., 2000), with axons that innervate the longitudinal muscle bands of the tail (Stanley MacIsaac, 1999). The bands themselves are innervated differentially (Bone, 1992) with both polyneuronal and multiterminal innervation (Stanley MacIsaac, 1999).

Ascidian larval swimming superficially resembles the undulating swimming produced by vertebrate spinal networks but in fact proceeds by rapid tail movements of two types, first analysed by Bone (1992). Alternating symmetrical contractions of the muscle bands impel forward progression of the larva through tail beats that are both rapid and undergo wide angular excursions. Asymmetrical tail flicks redirect the orientation of the head region or trunk prior to forward progression. Tail flicks also appear first when, in the embryo, they help to liberate the hatching larva. In both cases, the flexions are adapted to intermediate Reynolds’ numbers in sea-water (McHenry et al., 2003) and originate from the base of the tail’s point of insertion at the larval trunk. Thus, the ascidian larval tail which lacks segmentation (Lacalli, 1999), moves passively, like a whip. This is unlike swimming movements in mature tailed craniates, in which undulations are propagated down the length of the tail by active contractions of successive myotomes regulated by segmental circuits of neurones (Blight, 1977; Grillner et al., 1991).

The innervation of the tail muscles, by which these movements arise, has not been fully elucidated. Apparently, symmetrical and asymmetrical tail flicks are generated by different pathways from the trunk (Bone, 1992), presumably from the visceral ganglion. Cells that label with cholinergic phenotypic probes have been reported in the visceral ganglion (Takamura et al., 2002; Yoshida et al., 2004). In addition to these, cells that express phenotypic markers for the inhibitory neurotransmitter GABA also occur in the visceral ganglion (Takamura et al., 2002). According to the report of Yoshida et al. (2004), they appear to be located in a region of the visceral ganglion that also contains the...
candidate motor neurones. The exact number of these candidate cholinergic and GABAergic visceral ganglion neurones is unclear, however, as are their spatial relationships and the constancy of both of these features. Reconstructions from both confocal (Cole & Meinertzhagen, 2004) and serial electron micrograph (Stanley Mac-Isaac, 1999) images do, however, reveal that the cells are roughly aligned in two rows, one on either side of the dorsal midline. In order to compare the functional morphology and physiology of these two types of neurone, immunocytochemical labelling methods and muscle field potential recordings were carried out and interpreted with respect to the swimming behaviour of the intact larva.

Materials and methods

Larvae

_Ciona intestinalis_ adults were collected in the bay of Naples by the fishing service of the Stazione Zoologica and also from Nishiura port in Gamagori (Aichi, Japan). Adults were kept in the tanks of the Stazione Zoologica and Okazaki Institute for Integrative Bioscience. _Ciona savignyi_ larvae were obtained from local aquaculturists in Nova Scotia. For physiological experiments, gametes were collected from the gonoducts of several animals and used for _in vitro_ fertilization at 17°C. For physiological experiments, gametes were collected from the gonoducts of several animals and used for _in vitro_ fertilization at 17°C. Fertilized eggs were then raised in incubators in filtered sea-water at 17 °C. In Japan, developing embryos were reared in artificial sea-water (Jamarin U, Jamarin Laboratory, Japan). The next day, the embryos were transferred to the laboratory and maintained at 20 °C for the duration of the experiment. Under these conditions larvae hatch at around 11:00–12:00 h. Hatched larvae were then transferred to experimental chambers for recording.

Electrophysiological recordings

For electrophysiological recordings, larvae were placed in Petri dishes containing a base of 1% agar or, alternatively, dishes which had been coated with 1% bovine serum albumin that had been allowed to dry. Both approaches prevented the larvae contacting the plastic base of the Petri dish and commencing metamorphosis. All experiments were carried out in filtered (0.2 μm) natural sea-water. Glass micropipettes were drawn from borosilicate glass of 1.5 mm OD on a microelectrode puller (Model P87, Sutter Instrument Co., USA). The tips of the electrodes were broken under microscopic control so that their internal diameter was about four-fifths the diameter of the larval tail. Coarse manipulation of the microscope stage allowed the larval tail to be brought into close contact with the tip of the electrode. When this was achieved, negative pressure (suction) was applied and the larval tail was drawn into the pipette to about two-thirds of its length. Muscle action potentials were recorded differentially between the inside of the pipette and the sea-water of the bath and amplified (DAM 80, World Precision Instruments, New Haven, CT, USA) 10 000x with reference to a silver chloride pellet placed in the bath. Signals were AC-coupled and passed between 0.1 Hz and 10 kHz, digitized and stored using a Digidata 1200 data acquisition system and finally analysed using Clampfit software (version 9.0) (both from Axon Instruments, Inc., Union City, CA, USA). A custom-built shutter was controlled by 5-V control pulses delivered from the Digidata board allowing a step-down system (FAST-CAM Rabbit mini 2, PHOTORON, Tokyo, Japan) run at a capture rate of 400 frames/s and with an exposure time of 2 ms. Captured images were recorded on videotapes, digitized through a Digital Handycam (Sony, Japan) and loaded into a computer. Each frame was analysed using software (NIH Image, version 1.6 developed at the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/) to plot the position of the trunk tip as well as the centre of the pipette orifice. The XY coordinates derived were plotted and compared with the corresponding trace obtained from the electrophysiological recording.

GABA immunolabelling

_Ciona savignyi_ larvae were fixed at 22 °C for 60 min in 2% glutaraldehyde and 0.5% formaldehyde (as paraformaldehyde) in phosphate-buffered saline (PBS) at pH 7.0–7.4. Larvae were washed three times in 0.01 M PBS for 10–15 min each and then treated for 60 min at 22 °C with 1% ethanolamine in PBS, adjusted to pH 9.0 with 1 N HCl, and washed three times in PBS as before. To permeabilize the tunic they were then exposed for 60 min at 22 °C to proteinase K (10 μL in 2 mL of 100 mM Tris-HCl at pH 8.0 added to 50 mM EDTA) from an apoptosis fluorescein detection kit (G3250, ProMega). Larvae were mixed frequently to obtain uniform permeabilization. They were then washed five times for 10 min each in 0.2% Triton-X in PBS before blocking first in 1 mg/mL bovine serum albumin in 0.2% Triton-X PBS, all day or overnight at 4 °C, and then without rinsing in 1.8 mL of the preceding blocking solution to which 10% (200 μL) of goat serum had been added, for a further all-day period or overnight at 4 °C. They were then incubated in primary antibody, rabbit polyclonal antiserum GABA-9 (Hodgson et al., 1985), in 0.2% Triton-X PBS for 48 h at 4 °C at a dilution of 1 : 4000, followed by six washes in 0.2% Triton-X PBS. The larvae were then incubated in a Cy3-conjugated goat anti-rabbit secondary antibody at a dilution of 1 : 300 in 0.2% Triton-X PBS for 3 h at 4 °C and washed five times in PBS. Finally, larvae were mounted in Vectashield beneath 00 coverglasses, viewed either with 25/0.8 or 40/1.3 Plan Neofluar objectives and image stacks collected using a confocal microscope (LSM410, Zeiss). Images from the LSM410 software were finally prepared in Photoshop.

Drugs, compounds and solutions

Freshly filtered sea-water was continuously perfused over the preparation at a rate of 8 mL/min. Picrotoxin was purchased from Tocris but other drugs and compounds were from Sigma unless otherwise specified. The drugs were prediluted from concentrated stock solutions and added to the superfusate at the final concentrations indicated.

Results

Swimming activity

The recordings were made as previously described by Bone (1992). Activation of the muscles on either side of the tail produced large extracellular field potentials that were well resolved in recordings made by suction electrodes (Fig. 1A and B). In order to confirm the validity of these extracellular recordings, we made simultaneous intracellular microelectrode recordings from one side of the larval tail muscle and were able to correlate the alternating field potentials with the muscle intracellular action potentials (data not shown). Our results on this point fully confirmed those obtained by Bone (1992). No evidence was found for skin action potentials although occasional spikes were seen
which had a different sign. Figure 1A shows a typical recording to illustrate the uniformity of the swimming periods and the temporal relationship between swimming movements and electrical activity. Shortly after the larvae were drawn into the pipette it was noted that they showed some inhibition of swimming activity which lasted for at least 15 min. A similar effect was also observed on transferring the larvae to the bath. We therefore carefully checked that all of the experimental runs were made at least 20 min after the suction electrode was attached to the tail. During recording, the light illuminating the larva was occasionally switched off for 1–2-s intervals; this induced an increase in swimming activity (see Bone, 1992) (Fig. 2C). To verify that electrical signals taken with this method reflect muscle activity, simultaneous recordings of electrical activity and larval movements were performed. The results from these experiments show that one biphasic potential change is generated for each excursion of the larval body from the midline (Fig. 1). Thus, electrical activity reflects muscle activity and two electrical transients are generated for each alternating tail sweep. Each period of spontaneous or light-off-evoked swimming had a particular duration (swimming period duration) and a particular frequency (period frequency). Typical swimming periods around 2 h after hatching lasted 1 s (0.94 ± 0.09 s, mean ± SEM, n = 18, measurements from five animals), increasing in duration as larvae matured. After hatching, the frequency of the potentials associated with swimming periods was around 10–20 Hz while that of swimming evoked during ‘lights off’ was 30–40 Hz. During free swimming, these frequencies would be translated into tail beat frequencies of between 5 and 10 and 15–20 Hz, respectively.

Responses to GABA

Addition of 1 mM GABA to the superfusate had the effect of completely blocking any swimming periods (Fig. 2A and B), although single isolated muscle spikes continued. The effect was rapid and reversible. The surviving single spikes in GABA would have generated single unilateral twitches of the tail in an unrestrained larva. The detail of this effect is shown in Fig. 2B, i, ii and iii, which are time-expanded sections from the displays of the traces on the right-hand side in Fig. 2. The insets show that the regular pattern of swimming was absent in GABA. On washing, swimming activity reappeared and the intraperiod frequency resembled that before the application of GABA, often overshooting the preblock values (Fig. 2A and B). Similar responses were recorded from five of five larvae. GABA also had the effect of reversibly decreasing the amplitude of the muscle field potentials (Fig. 2B, i, ii and iii).

Responses to picrotoxin

Addition of picrotoxin, a non-competitive blocker of the chloride channel associated with the GABA_A receptor, had a dramatic effect on
swimming. Concentrations of between 1 and 20 µM increased both the duration and frequency of spontaneous periods of swimming (start of spontaneous swimming periods is marked by the arrows in Fig. 2C–E) in seven of seven larvae. In addition, it was noted that the ‘light-off’ effect, which normally produced potentials at frequencies of around 30–40 Hz, was apparently unaltered by the addition of picrotoxin.
**Immunoreactivity to GABA**

Immunoreactivity to GABA was observed in all regions of the larval CNS (Fig. 4a). The immunolabelling signal appeared weak in young larvae during the first hour after hatching but was stronger later on, although these differences were not quantified and could have arisen for technical reasons as well from changes in neurotransmitter content. In the sensory vesicle (sv, Fig. 4a, b and f), the labelling was widespread and the cell bodies difficult to visualize as individual cells. The pattern of labelling was consistently asymmetrical and, although it was not possible to visualize all cell somata clearly, label appeared to be concentrated in small cell populations in three regions. The first was a region of strong labelling on the right-hand side of the sensory vesicle (Fig. 4f), from what appeared to be a group of cell bodies possibly associated with the ocellus. The second was a region of weaker labelling on the left-hand side of the sensory vesicle, possibly associated with the region of the so-called coronet cells. These two regions were connected with each other across the midline by an immunoreactive tract of fibres that formed an asymmetrical pair of arcs on either side of the midline (Fig. 4e and f). The third was a region of labelling closer to the midline than these two, in the posterior wall of the sensory vesicle, and extending somewhat ventrally (arrowhead, Fig. 4f). At the confluence of the two lateral regions, a stout fibre tract extended caudally to the visceral ganglion. At the rostral end of the larva, there was also immunoreactivity to GABA in the palps (Fig. 4b).

In the visceral ganglion (vg, Fig. 4a), it was possible to discern immunolabelled cell bodies (Fig. 4d–f). At least four and up to six such cells formed two irregular rows (Fig. 4d), one left and one right, in many 4-h larvae. Often the cell bodies had a weak signal, however, with sometimes strongly and more weakly labelled cells occurring in the same larva (arrow and arrowhead, Fig. 4e and f). Two cells, the most anterior pair, situated some way back from the visceral ganglion’s anterior margin, were sometimes displaced laterally from these two rows (arrows, Fig. 4f). The rows themselves seemed to be fairly continuous, lacking gaps, suggesting that the cells were near-neighbours within their row.

From the visceral ganglion, varicose neurites descended down into the tail, presumably along the nerve cord (Fig. 4a). These comprised more than one neurite on each side, with frequent varicosities, for the first 50 μm or so of the anterior tail region (double arrowhead, Fig. 4c). A second line of immunoreactivity comprising just a single neurite or neurite bundle on each side, extended in a region more than 100 μm further along the tail and could represent either ascending or descending varicose axons (arrows, Fig. 4c). Along the length of this single neurite bundle were what appeared to be immunoreactive somata. We counted either two or three such somata, at least one on each side (asterisks, Fig. 4a). These may correspond to the two cells previously reported by Johnson et al. (1987) and now revealed in larvae transfected by neurone-specific driven green fluorescent protein to be bipolar cells with both an ascending and descending varicose axons (arrows, Fig. 4c). Along the length of this single neurite bundle were what appeared to be immunoreactive somata. We counted either two or three such somata, at least one on each side (asterisks, Fig. 4a). These may correspond to the two cells previously reported by Johnson et al. (1987) and now revealed in larvae transfected by neurone-specific driven green fluorescent protein to be bipolar cells with both an ascending and descending varicose axons (Fig. 4c). Along the length of this single neurite bundle were what appeared to be immunoreactive somata. We counted either two or three such somata, at least one on each side (asterisks, Fig. 4a). These may correspond to the two cells previously reported by Johnson et al. (1987) and now revealed in larvae transfected by neurone-specific driven green fluorescent protein to be bipolar cells with both an ascending and descending varicose axons (Fig. 4c). Along the length of this single neurite bundle were what appeared to be immunoreactive somata. We counted either two or three such somata, at least one on each side (asterisks, Fig. 4a). These may correspond to the two cells previously reported by Johnson et al. (1987) and now revealed in larvae transfected by neurone-specific driven green fluorescent protein to be bipolar cells with both an ascending and descending varicose axons (Imai and Meinertzhagen, unpublished). The results are summarized in Fig. 5, which shows in diagrammatic form the pattern of GABA immunolabelling common to all larvae examined.

**Discussion**

Our results support not only the presence but also the action of GABA in the motor circuits of larval Ciona and suggest that GABA is naturally...
released during swimming. The presence of immunoreactivity to GABA in the larval brain of Ciona is not in itself surprising. A prototypical gene for glutamic acid decarboxylase, the synthetic enzyme of GABA, has been isolated from Ciona (Bosma et al., 1999) in which the corresponding metabolic enzyme, GABA aminotransferase, is expressed in the larval stage (Mochizuki et al., 2003). Genomic evidence additionally indicates a number of GABA receptor genes (Okamura et al., 2005). Moreover, GABA immunoreactivity itself has previously been reported in Ciona, albeit only in an abstract and before confocal imaging methods were available to analyse the distribution patterns of immunoreactive cells (Johnson et al., 1987).

Thus, our finding of GABA-containing neurones not only substantiates existing data but does so in ways that were necessary to investigate GABA action during swimming.

Based on the observation of GABA-immunoreactive neurones in the visceral ganglion, the arrangement of cells within this region of the larval CNS begins to emerge. In one specimen there was a total of 29 somata in the visceral ganglion, arranged in two single files one on either side of the midline and containing five pairs of motor neurones. Extending between vg and tail are two types of varicose neurite, a coarse pair, presumably neuromuscular (arrow), and a more slender pair (double arrowheads). (b) Right lateral view, showing the same components as in A. Numbers of somata illustrated are approximate only. Dotted outlines circumscribe regions of weak immunoreactivity, presumably neuropilar, and continuous lines indicate fibre tracts and other regions of stronger immunolabelling.

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**Fig. 4.** GABA-immunolabelled whole-mounted Ciona larvae. Each image is from a different larva and illustrates a different feature as well as the uniform features of all labelling at two stages after hatching, either 6 h (d) or 4 h (all other images). (a) Entire larva, showing labelling in all three regions of the central nervous system (sensory vesicle (sv), visceral ganglion (vg) and dorsal nerve cord (nc)) as well as in three cell bodies (asterisk) in the tail, two on the right and the third on the left, along the length of GABA-immunoreactive fibres in the nerve cord. (b) Lateral view of the separate GABA-immunoreactive fibre tracts linking (arrow) the dorsal cell bodies of the vg and (arrowhead) the sv to the bilateral tracts of the caudal nerve cord. Note immunolabelling in the papillae (double arrowhead). (c) Somata of the vg form two groups in this larva, each comprising one strongly immunolabelled soma (arrowhead) and one other (asterisk). Varicose GABA-immunoreactive neurites in the caudal nerve cord comprise a single bilateral pair of long axons (arrows) that extends the length of the nerve cord and shorter neurites (double arrowheads) in the anterior 50 μm of the tail which are both more varicose and apparently multiple. (d) Somata of the vg, four strongly (one with arrow) and two more weakly (one with arrowhead) immunolabelled in this larva. (e) Profiles in the anterior tail consistent with the presence of about two immunolabelled somata (arrowhead) among varicose neurites. Note immunolabelled profiles. (f) In the sv, a cell group (arrowhead) extends ventrally. A bilateral pair of GABA-immunopositive cells (arrows) lies anterior to the vg. All larvae are shown with anterior to the left. (a–d) Left-lateral view; (e and f) dorsal view. Scale bars, 100 μm; b–f are at the same magnification. oc, ocellus.

**Fig. 5.** A summary diagram of GABA immunoreactivity in Ciona larvae. Diagrams are representative of the immunopositive components common to all preparations and illustrated in Fig. 4. (a) Dorsal view showing the three components (L, left; V, ventral and R, right) of the sensory vesicle (sv), connected by an immunoreactive tract to the visceral ganglion (vg). The latter contains dorsal paired rows of GABA-immunoreactive somata (d) and one pair of anterior neurones. Extending between vg and tail are two types of varicose neurite, a coarse pair, presumably neuromuscular (arrow), and a more slender pair (double arrowheads). (b) Right lateral view, showing the same components as in A. Numbers of somata illustrated are approximate only. Dotted outlines circumscribe regions of weak immunoreactivity, presumably neuropilar, and continuous lines indicate fibre tracts and other regions of stronger immunolabelling.
longer along the neuraxis than that occupied by cells expressing GABAergic markers (Yoshida et al., 2004). We found the latter to include from four to six neurones. Thus, in the predicted case that no neurone expresses both a cholinergic and a GABAergic phenotype, these two groups together account for 14–16 neurones, roughly half those of the visceral ganglion. Further analysis with double-labelled preparations will be necessary to confirm the spatial cohesion of both groups and their neighbourliness and the constancy of these features in different larvae.

The results of the electrophysiological experiments show that GABAergic effects are inhibitory with respect to swimming period and muscle potentials and that a GABA antagonist is excitatory, consistent with the view that at least some of the many GABA receptors identified in the genome (Okamura et al., 2005) show signature patterns of amino acids for anion selectivity. From the current evidence we cannot exclude that GABA transmission exerts effects other than those seen here over swimming activity. However, the evidence is consistent with the idea that GABAergic innervation is involved in modulating the duration and frequency of swimming periods, inhibiting both. No effect was noted on the interevent interval (regularity of timing) so that GABA does not appear to modulate the part of the network that coordinates alternating left/right activity. In this sense the results are similar to those obtained from studies on the vertebrate spinal cord, in which GABA receptors are involved in regulating swimming period and frequency by presynaptic and soma–dendritic effects (Schmitt et al., 2004). In vertebrates, these GABAergic effects are exerted on the glutamate–glycine pattern generator, in which GABA appears to act as a neuromodulator in the lamprey, amphibian and mammalian spinal cords (see Cazalets et al., 1998; Sillar et al., 2002; Schmitt et al., 2004). Thus, in the larval ascidian, as in vertebrates, locomotion persists in the presence of GABA receptor antagonists.

The mechanism and synaptic circuits by which the CNS modulates locomotor activity are less clear. The presence of GABA-immunoreactive neurites extending down the nerve cord provides a significant feature not at all typical of vertebrate neuromuscular innervation. The neurites are varicose, suggesting the presence of release sites along their length, but the postsynaptic targets have not been resolved. Pharmacological evidence indicates that neuromuscular transmission is cholinergic and transmission is both completely blocked by d-tubocurarine and its duration extended by eserine (Ohmori & Sasaki, 1977), indicating that the sole identified postsynaptic mechanism is nicotinic and suggesting that the muscle receptors are exclusively cholinergic. Thus, GABA apparently does not act postsynaptically at muscle cells. Where then does it act and could such action be responsible for any of the effects of GABA found here?

Peripheral GABAergic innervation is typical of arthropod muscles (Otsuka et al., 1966) which, unlike the muscles of chordates, use glutamate as an excitatory neuromuscular transmitter (Usherwood & Machili, 1966). The actions of GABA at this site are both presynaptic (Dudel & Kuffler, 1961) and postsynaptic (Fatt & Katz, 1953) and have been identified with special clarity in crustacean muscle (Atwood, 1982), although GABA-immunoreactive innervation is also seen in the muscles of many other protostomes. Evidence for such GABAergic innervation is also reported in deuterostomes other than Ciona, in the muscles of a distant relative, the larvacean Oikopleura (Bollner et al., 1991) and of a representative of the yet more basal group, echinoderms (Devlin, 2001).

In vertebrates the situation differs somewhat. While neuromuscular innervation by GABA has not been reported in vertebrates, it is clear from both physiological and anatomical studies that GABAergic innervation plays an important role in the vertebrate spinal cord. GABA-positive fibres and cells are present and GABA_A receptors are involved in providing strong presynaptic inhibitory control over the motor neurone pool that is excited by glutamate and that drives locomotion (see Schmitt et al., 2004). In this respect, the presence of GABAergic-positive fibres in the Ciona larval nerve cord presents the possibility that they may exert presynaptic inhibition peripherally at the level of the neuromuscular synapse.

The modulation by GABA of the amplitude of the muscle field potentials, which causes a reduction in amplitude, indicates that the GABAergic network may exert control over swimming not only at the level of the visceral ganglion but also by altering the amplitude of the muscle junction potential. Muscle cells in the ascidian larval tail lack Na channels (Ohmori & Sasaki, 1977; Nakajo & Okamura, 2004) and so produce junction potentials that may vary in amplitude. The decrease in amplitude could be the result of two types of mechanism, a presynaptic effect through a decrease in the amount of neurotransmitter released or inhibition at the level of the cell bodies of selected neurones, with the possibility that only one or two of the three bands of muscle on either side of the notochord (Katz, 1983) are activated. In the first situation the main effects of GABA innervation would be at the level of the neuromuscular junction itself, while in the second it would be within the CNS, at the level of the motor neurone cell body. As GABA-positive fibres appear to be present at both these levels our evidence does not allow us to rule out either of these possibilities.

In addition, the fact that period duration is also modified suggests that inhibitory effects also occur at the level of the circuit generating period length. This may be at the level of the motor neurone cell bodies in the visceral ganglion and/or in GABAergic premotor interneurones descending upon these. However, the means by which the Ciona larval CNS generates swimming patterns are not yet known. It is possible that a central pattern generator provides a rhythmic input to the ‘largely’ motor visceral ganglion, integrating sensory activity from photoreceptors and otolith (‘balance organ’) and sensory neurones in the tail and trunk. The GABA network could provide a modulatory inhibitory input to this network. We cannot rule out the possibility that such a central pattern generator also resides in both the sensory vesicle and part of the visceral ganglion. In order to evaluate better the mode of action of GABA on the network controlling swimming it will be necessary to perform electrophysiological measurements at least at the level of the muscle fibres and motor neurones.

In summary, our results are consistent with the possibility that GABAergic peripheral inhibition might have been an ancestral feature of neuromuscular mechanisms which, in chordates, became associated with cholinergic excitation. They also suggest that a system of peripheral inhibition was lost at the level of the neuromuscular junction somewhere in the chordate lineage after its divergence from urochordate groups such as ascidians, while the mechanism of GABAergic presynaptic inhibition remained associated with a glutamatergic–glycinergic neurone pool that in vertebrates came to reside exclusively within the CNS. It is notable that in all vertebrate spinal preparations so far studied, GABAergic inhibition strongly modulates locomotor activity (Cazalets et al., 1998; Sillar et al., 2002; Schmitt et al., 2004) either by presynaptic inhibition or by postsynaptic inhibition on the motor neurone soma. Thus, the ascidian system for which we report initial evidence appears to incorporate a combination of features from both non-vertebrate and vertebrate motor systems, possibly those more closely related to prototypical chordate or ancestral bilaterian brains.
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Abbreviations

CNS, central nervous system; PBS, phosphate-buffered saline.

References


