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GABA-like immunoreactivity in nonspiking interneurons of the locust metathoracic ganglion

M. Wildman, S. R. Ott† and M. Burrows*

Department of Zoology, University of Cambridge, Cambridge CB2 3EJ, UK

*Author for correspondence (e-mail: mb135@hermes.cam.ac.uk)

†Present address: School of Biological Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK

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Summary

Nonspiking interneurons are important components of the premotor circuitry in the thoracic ganglia of insects. Their action on postsynaptic neurons appears to be predominantly inhibitory, but it is not known which transmitter(s) they use. Here, we demonstrate that many but not all nonspiking local interneurons in the locust metathoracic ganglion are immunopositive for GABA (γ-aminobutyric acid). Interneurons were impaled with intracellular microelectrodes and were shown physiologically to be nonspiking. They were further characterized by defining their effects on known leg motor neurons when their membrane potential was manipulated by current injection. Lucifer Yellow was then injected into these interneurons to reveal their cell bodies and the morphology of their branches. Some could be recognised as individuals by comparison with previous detailed descriptions. Ganglia were then processed for GABA immunohistochemistry. Fifteen of the 17 nonspiking interneurons studied were immunopositive for GABA, but two were not. The results suggest that the majority of these interneurons might exert their well-characterized effects on other neurons through the release of GABA but that some appear to use a transmitter other than GABA. These nonspiking interneurons are therefore not an homogeneous population with regard to their putative transmitter.

Key words: GABA, immunohistochemistry, premotor interneurons, motor control, locust, *Schistocerca gregaria*.

Introduction

The nervous systems of arthropods contain interneurons that do not spike during normal behaviour. These nonspiking interneurons play a prominent role in many integrative processes, including the processing of sensory signals and the generation of movements. The advantage of graded transmission by neurons that do not generate action potentials may lie in the increased bit rate afforded by such transmission (Laughlin et al., 1998). In insects, early integration of visual signals from the compound eye relies entirely on graded transmission, with both the photoreceptor cells and their postsynaptic targets being nonspiking neurons (Shaw, 1968; Zettler and Jarvilehto, 1971). In the terminal ganglion of the ventral nerve cord of insects and crustaceans, nonspiking interneurons process signals from mechanosensory receptors on the cerci and the tail, respectively (Kondoh et al., 1991; Nagayama, 1997; Reichert et al., 1982). Arthropod motor control also relies strongly on graded transmission. For the control of limb movements in insects, nonspiking interneurons (Pearson and Fourtner, 1975) organise sets of motor neurons in appropriate combinations (Burrows, 1980; Büschges and Wolf, 1995; Wolf and Büschges, 1995). In crustaceans, they are essential components of the motor networks that generate ventilatory movements (Dicaprio, 1989; Mendelson, 1971) and movements of the tail (Nagayama and Hisada, 1987; Nagayama et al., 1984), while nonspiking interactions between motor neurons are involved in movements of the gut (Graubard, 1978).

In the thoracic motor networks of insects, nonspiking interneurons mediate powerful effects on postsynaptic neurons by the graded release of chemical transmitter (Burrows and Siegler, 1978). Both inhibitory and excitatory effects on motor neurons occur (Burrows and Siegler, 1976, 1978), but all connections between interneurons appear to be inhibitory (Burrows, 1979a; Burrows, 1987). Many nonspiking neurons release transmitter tonically, so that single synaptic potentials are sufficient to modulate this graded release (Burrows, 1979b). Despite the extensive studies on nonspiking interneurons in these networks, the identity of their transmitter(s) remains unknown. GABA (γ-aminobutyric acid) is extensively associated with inhibition and is widely distributed in insect thoracic ganglia (Watson, 1986; Watson and Pflüger, 1987). Three common inhibitory motor neurons that innervate leg muscles (Hale and Burrows, 1985; Hoyle, 1966; Pearson and Bergman, 1969) show GABA-like

immunoreactivity (Watson, 1986), and GABA is used by these neurons as an inhibitory transmitter in the muscles (Usherwood and Grundfest, 1965). GABA is also present in one population of spiking local interneurons that have inhibitory actions on nonspiking interneurons and motor neurons (Burrows, 1987; Burrows and Siegler, 1982; Watson and Burrows, 1987), in a population of intersegmental interneurons (Watson and Laurent, 1990) and in unidentified interneurons that mediate presynaptic inhibition of the central terminals of proprioceptors (Burrows and Laurent, 1993; Watson et al., 1993) and exteroceptors (Watson and Pflüger, 1994). In the terminal abdominal ganglion of the crayfish, an identified nonspiking interneuron that inhibits other spiking interneurons (Nagayama et al., 1994; Reichert et al., 1983) is immunoreactive for GABA (Nagayama et al., 1996), as are some other nonspiking interneurons (Nagayama et al., 1997).

In the present paper, we show, by intracellular dye injection into physiologically characterized nonspiking interneurons in a locust thoracic ganglion and subsequent immunohistochemistry with an antibody raised against GABA, that many of these interneurons are GABA-immunopositive. The diversity of these local nonspiking interneurons is, however, emphasized by the finding that not all of them show GABA immunoreactivity.

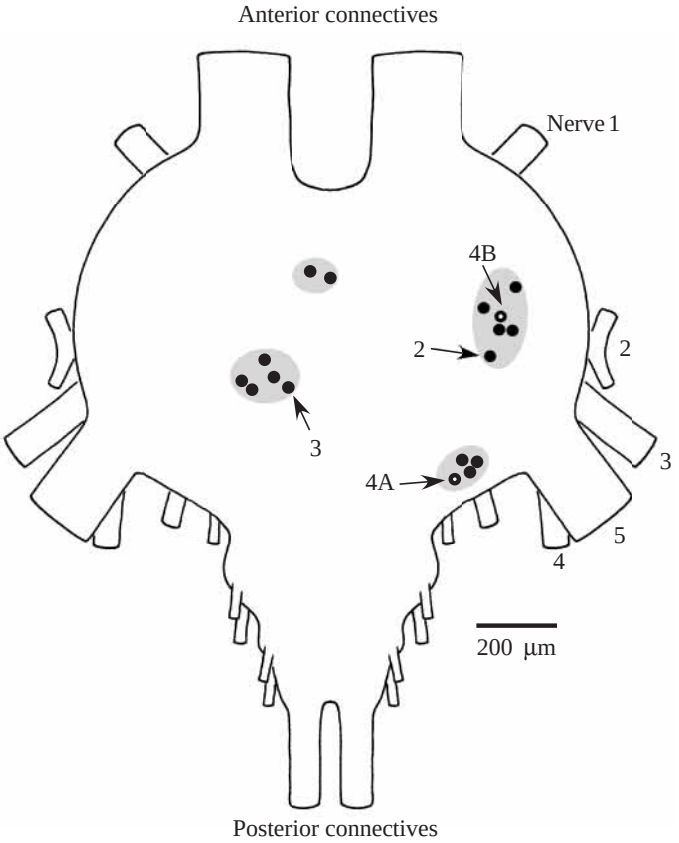

Fig. 1. Location of the cell bodies of nonspiking interneurons in the metathoracic ganglion analysed in this study. The interneurons were first characterized physiologically by intracellular recordings from their neurites and were then stained by intracellular injection of Lucifer Yellow. The ganglion is viewed ventrally and the lateral nerves innervating the left hind leg and left hind wing are numbered. All the stained interneurons affected motor neurons innervating muscles in the left hind leg. The filled circles represent the 15 interneurons that were GABA-immunopositive, and the open circles represent the two interneurons that were not GABA-immunopositive. The numbering of the cell bodies refers to the figures in which these interneurons are illustrated.

Materials and methods

Animals and preparation

Adult male and female locusts *Schistocerca gregaria* Forskål (gregarious phase) were taken from our crowded culture and restrained ventral side uppermost in Plasticine, with the tibia and tarsus of the left hind leg free to move. The metathoracic ganglion was exposed through an opening in the ventral cuticle, stabilized on a wax-coated silver platform, and the thorax perfused with physiological saline at a temperature of 20–22°C. The ganglionic sheath was treated for 1 min with crystals of protease (Sigma type XIV) to facilitate the penetration of microelectrodes.

Electrophysiology and intracellular dye injection

Intracellular recordings from the neuropilar processes of nonspiking local interneurons were made in the left half of the metathoracic ganglion with thin-walled glass microelectrodes, filled at their tips with a 5% aqueous solution of Lucifer Yellow and in their shanks with 0.5 mol l^{-1} lithium chloride so that they had resistances of approximately 25 MΩ. Interneurons were identified as nonspiking according to established criteria (Burrows and Siegler, 1978). In short, an interneuron was considered to be nonspiking if (1) it was never observed to spike in response to any injury inflicted by the initial penetration of the microelectrode, to mechanosensory stimulation of exteroceptors or proprioceptors of the legs, to injection of depolarizing current, or on rebound from a hyperpolarizing current pulse and (2) if depolarizing and/or hyperpolarizing injection of current produced motor effects without the occurrence of spikes in the interneuron. Motor effects were monitored by observing movements of the left hind leg and by recording from selected leg muscles with implanted pairs of fine steel pins or 50 μm-diameter silver wire, insulated except for the tips.

Following physiological characterization, an interneuron was filled with Lucifer Yellow by the application of hyperpolarizing current pulses (–7 nA, 500 ms duration at 1 Hz) for 20–30 min, superimposed on a constant current of –2 nA. The mesothoracic and metathoracic ganglia were then removed from the locust and fixed for 30 min at 5°C in 4% paraformaldehyde and 0.1% gluteraldehyde in 0.1 mol l^{-1} phosphate buffer (PB; pH 7.2) (Nagayama et al., 1996). They were postfixed overnight at 5°C in 2% paraformaldehyde and 15% picric acid in PB and then washed in two changes of PB, each for 10 min.

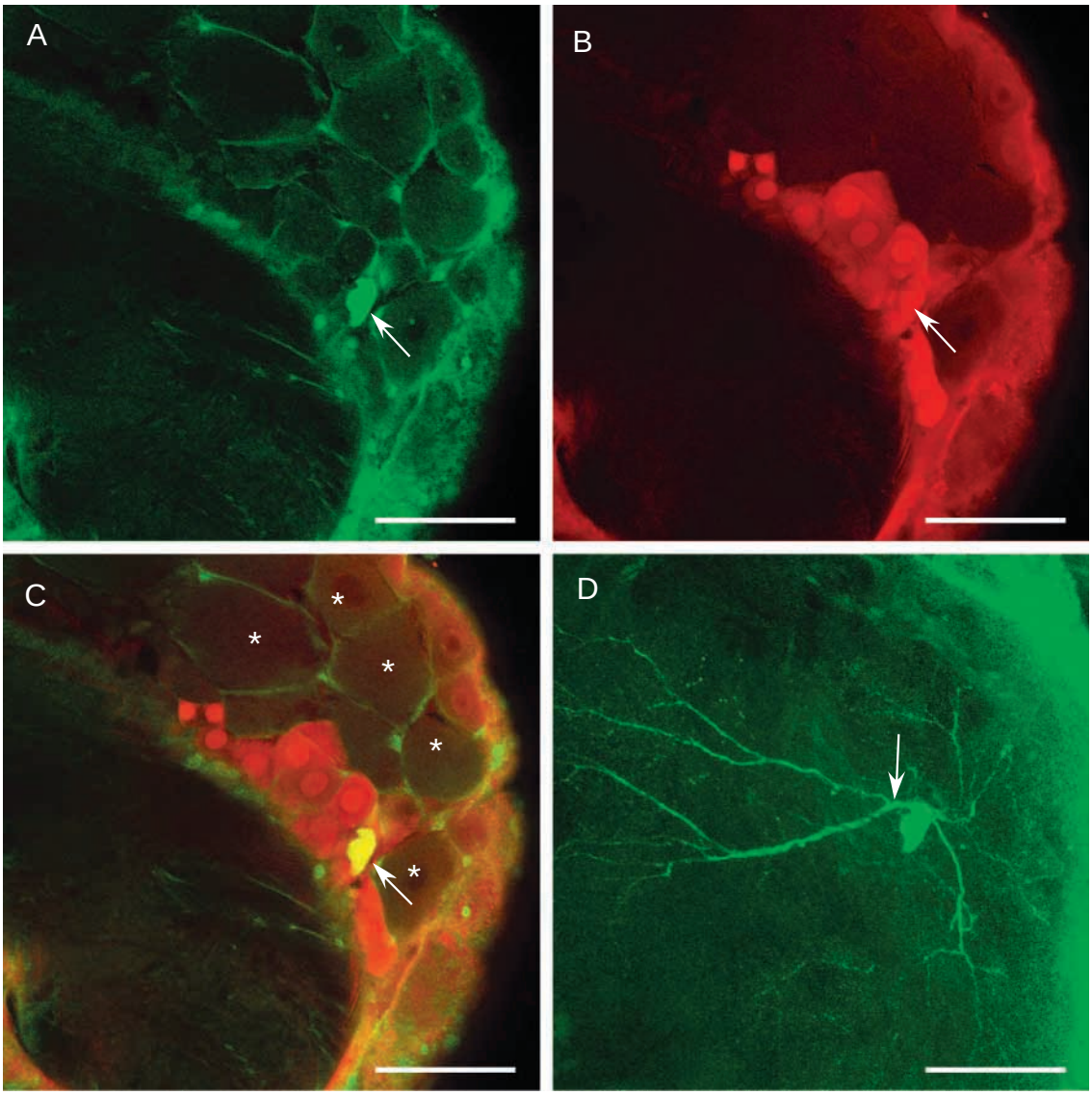

Fig. 2. Colocalisation of Lucifer-Yellow-fluorescence and anti-GABA-immunofluorescence in a nonspiking local interneuron in the metathoracic ganglion, demonstrated by confocal microscopy (all images are from whole mounts of ganglia, viewed ventrally). (A) A single cell body (arrow) in the anterior lateral region of the ganglion shows intense fluorescence for Lucifer Yellow (in green pseudocolour in this and following figures; slight background is due to tissue autofluorescence). (B) The same cell body (arrow) shows intense fluorescence for Cy3 in the same optical section, indicating binding of the GABA antibody (Cy3/GABA in red pseudocolour). A number of other small cell bodies are also GABA-immunopositive. A and B each show a composite of two confocal planes separated by 3.1 μm. (C) A merging of the two images in A and B results in a single yellow cell body, indicating colocalisation of the two fluorophores in the interneuron. The surrounding large cell bodies belong to glutamatergic motor neurons (five are indicated with asterisks) that showed no immunoreactivity with the GABA antibody. (D) The morphology of the neuron as revealed by the Lucifer Yellow dye (branches reconstructed from 29 confocal planes, each separated by 5.4 μm; cell body from a single confocal plane 56 μm more ventral than the ventralmost plane used in the reconstruction of the branches). A single primary neurite (arrow) emerges from the cell body to give rise to a profusion of fine branches in the neuropil. The ganglion is viewed ventrally with anterior to the top. Scale bars, 100 μm.

GABA immunohistochemistry in intact ganglia

To improve penetration of the antibody, the ganglia were dehydrated through a graded ethanol series (10 min each in 30%, 50% and 70% ethanol) and stored for 1–5 days in 70% ethanol. They were then placed in 90% ethanol for 1 h, rehydrated through the same series of ethanol and washed three times, each for 10 min, in PB at 37°C. The permeability of the ganglionic sheath was improved by treatment with 0.1% collagenase (Sigma type IV) and 0.1% hyaluronidase (Sigma type I-S) in PB for 1 h at 37°C, followed by three washes, each for 10 min, in PB and a further 10 min wash in PB containing 0.3% Triton X-100 (PB-Tx). Non-specific antibody binding was blocked by pre-incubation with 5% normal goat serum in PB-Tx (PB-Tx-NGS) for 6 h. The ganglia were then incubated

with a polyclonal anti-GABA primary antibody (Sigma, Poole, UK; product number A2052; diluted 1:750 in PB-Tx-NGS) for approximately 90 h at 5°C on a rotator. The antibody was raised in rabbit using a GABA–BSA (bovine serum albumin) conjugate as the immunogen. To remove immunoglobulins that do not specifically bind to GABA the antibody was affinity-purified. Dot blot assays show positive binding with GABA and GABA-keyhole limpet haemocyanin conjugate but not with BSA. This antibody has been used extensively in previous studies on locust (*Schistocerca gregaria*; Judge and Leitch, 1999; Seidel and Bicker, 1997), bee (*Apis mellifera*; Ganeshina and Menzel, 2001), crayfish (*Procambarus clarkii*; Pearlstein et al., 1998; Watson et al., 2000) and crab (*Cancer borealis*; Kilman and Marder, 1996; Swensen et al., 2000), in each of which the specificity of the antibody was established. Following three washes in PB-Tx, each for 2 h, the ganglia were incubated for approximately 42 h at 5°C on a rotator in Cy3-conjugated secondary antibody ['affinity-purified, cyanine fluorophore Cy3-conjugated goat anti-rabbit immunoglobulin G (IgG) (H+L)', where H+L refers to whole antibody molecules with full-length heavy and light chains; Jackson ImmunoResearch Laboratories, West Grove, PA, USA] diluted 1:200 in PB-Tx-NGS. Finally, ganglia were washed in three changes of PB-Tx, each for 2 h, dehydrated through an ethanol series (30%, 50%, 70%, 90% and two changes of 100%, each for 10 min), and cleared and mounted in methyl salicylate.

The ganglia were viewed under a Leica DMR confocal microscope running Leica TCS NT software (Leica, Nussloch, Germany). Observation conditions that avoid cross-talk between the Lucifer Yellow and the Cy3 fluorescence signal were established in control preparations (see below). The excitation wavelength was set to 488 nm for Lucifer Yellow and 568 nm for Cy3, and the detector range was set to 500–530 nm for Lucifer Yellow and 550–600 nm for Cy3. All images presented are from optical confocal sections of whole ganglia. Images from different focal planes were stacked as layers and combined in Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA, USA) or in the public domain software NIH-Image (U.S. National Institutes of Health; http://rsb.info.nih.gov/nih-image/index.html).

GABA immunohistochemistry in frozen sections

Eight ganglia containing a Lucifer-Yellow-injected interneuron were sectioned rather than treated as whole mounts. After fixation as above, ganglia were cryoprotected overnight at 5°C in 20% sucrose in PB, embedded in 20% gelatine and frozen. Cryosections were cut at 20 µm, collected on chrome alum-gelatine-coated slides and air-dried for approximately 30 min. The embedding gelatine that surrounded the sections was removed by dipping the slides in warm PB (approximately 40°C). After pre-incubation in PB-Tx-NGS for 1–2 h at room temperature, the sections were incubated in the primary anti-GABA antibody (1:750 in PB-Tx-NGS) overnight at 5°C, washed three times for 15 min each in PB-Tx, and incubated in the Cy3-conjugated anti-rabbit-IgG antibody (1:200 in PB-Tx-NGS) for 1 h at room temperature. Finally, the sections were washed three times in PB-Tx, each for 10 min, mounted in buffered glycerol and viewed with a Zeiss Axiophot compound microscope (Zeiss, Oberkochen, Germany).

Controls

The following control experiments were performed. First, to rule out the possibility that Cy3 labelling was due to binding of the secondary antibody to endogenous epitopes, the primary antibody was omitted in negative controls. No positive staining was observed under these conditions. Second, as a positive control for GABA immunodetection, we used the GABAergic common inhibitor motor neurons (Hale and Burrows, 1985; Watson, 1986; Wolf and Lang, 1994). A common inhibitor was identified by intracellular recording and subsequent injection of Lucifer Yellow. After processing for GABA immunohistochemistry, Lucifer-Yellow-fluorescence and GABA-immunofluorescence were consistently colocalized in the common inhibitors. Third, to exclude possible crossreactivity of the antibody with glutamate, identified flexor tibiae motor neurons that are glutamatergic (Bicker et al., 1988; Usherwood, 1994; Watson and Seymour-Laurent, 1993) but do not contain GABA (Watson, 1986; Watson et al., 1985) were injected with Lucifer Yellow and then processed for GABA immunohistochemistry. No fluorescence signal was detected in the flexor tibiae neurons with the excitation/emission filter settings used for Cy3 detection. These conditions were also used to exclude the possibility that Lucifer-Yellow-fluorescence gave cross-talk with the filter settings used for Cy3 or that the primary antibody was binding non-specifically to neurons that do not contain GABA.

The results are based on successful identification and staining of 17 nonspiking interneurons in 14 locusts.

Results

Morphological and physiological properties

Injection of Lucifer Yellow into a neuropilar process of a nonspiking interneuron usually gave unequivocal staining of a single cell body. Ganglia where the dye had diffused into other neurons, presumably because of damage at the site of impalement, were discarded. The cell bodies thus revealed were in four different regions of the ventral cortex of the metathoracic ganglion (Fig. 1) that correspond to those previously described for nonspiking interneurons (Siegler and Burrows, 1979). Two groups of cell bodies were ipsilateral to the leg that was affected by their action, one group was at the midline and the fourth group was just contralateral to the midline. All the neurons stained were local interneurons with processes restricted to the metathoracic ganglion, and none had axons in any of the lateral nerves innervating muscles in the body or limbs or in the connectives that link the ganglion to others in the ventral chain. The recordings from these interneurons showed that they were nonspiking. The continuous barrage of synaptic potentials, many

hyperpolarizing, that they received could be changed by imposed movements of particular joints of the left hind leg or by stimulation of mechanoreceptors on the body and legs. During spontaneous movements of the left hind leg, or when movements were evoked by mechanical stimulation of the animal, the interneurons also underwent changes in their patterns of synaptic potentials that were correlated with movements of the left hind leg. At no time did these changed patterns of synaptic inputs lead to spikes. Manipulation of the membrane potential of an impaled interneuron by injection of depolarizing current led to changes in the spike output of specific sets of motor neurons to muscles in the left hind leg, even though no spikes were generated in the interneuron. Injection of hyperpolarizing current into some interneurons also altered the motor output, indicating that at rest these interneurons normally released transmitter tonically. Upon release from hyperpolarization, no spikes were observed in any of the interneurons.

GABA immunostaining in nonspiking interneurons

The metathoracic ganglion treated with either our wholemount or frozen section immunostaining procedures revealed a large number of GABA-immunoreactive cell bodies, the distribution of which corresponded to that previously described with a different antibody (Watson, 1986). The three common inhibitory motor neurons to each hind leg, which have been identified as GABAergic in previous studies, were consistently labelled by our GABA immunohistochemistry. The excitatory motor neurons that contain glutamate were, however, not labelled, demonstrating that the GABA antiserum did not crossreact with glutamate (see Fig. 2A–C and below).

Fifteen out of a total of 17 nonspiking interneurons that were injected intracellularly with Lucifer Yellow showed strong GABA immunoreactivity. In several preparations, the Lucifer Yellow dye diffused throughout the recorded nonspiking interneuron to reveal the shape and distribution of its fine neurites. This staining was retained during the immunohistochemical processing, and, by confocal microscopy, the morphology of the neurones could be reconstructed from a series of optical sections (Figs 2D, 3B, 4A). Fig. 2 shows a GABA-immunopositive nonspiking interneuron in the anterior lateral group, injected with Lucifer Yellow following physiological characterization. A single cell body (Fig. 2A, arrow) showed intense fluorescence with the excitation/emission filter setting for Lucifer Yellow detection (shown in green pseudocolour in this and all following figures; the background is due to the autofluorescence of locust nervous tissue at these shorter wavelengths). The same cell body showed intense fluorescence when the same optical section was viewed with the excitation/emission filter setting that detects the Cy3 fluorophore tagged to the GABA antibody (Fig. 2B, red pseudocolour in this and all following figures). The cell body (Fig. 2B, arrow) was a member of a group of similar sized somata that were also GABA-immunopositive. When the two images were merged, the cell body of the nonspiking interneuron was yellow, indicating colocalization of the Lucifer Yellow and Cy3/GABA fluorophores (Fig. 2C, arrow). By contrast, the cell bodies of neighbouring immunopositive neurons remained red. The larger and more peripherally located neurites of these neurons were also GABA-immunopositive, but deeper neurites were not labelled presumably because, in the whole ganglia used, the antibody failed to penetrate. Importantly, the large cell bodies of glutamatergic motor neurons (five are indicated by asterisks in Fig. 2C) were not stained, demonstrating that the antiserum did not bind to glutamate. Confocal reconstruction of the Lucifer Yellow staining in this interneuron showed a single primary neurite emerging anteriorly from the cell body and then turning dorsally to give rise to a profusion of fine branches in the neuropil (Fig. 2D).

Fig. 3 shows an interneuron among a different group of neurons, just contralateral to the midline, which was also found to be GABA-immunopositive. Colocalization of Lucifer Yellow (green) and Cy3/GABA (red) are indicated by the cell body appearing yellow (Fig. 3A, arrow). When hyperpolarizing current was injected into this interneuron, the tibia of the left hind leg extended slowly and the spike frequency of the slow extensor tibiae motor neuron was increased, but no effect of depolarizing current could be discerned in tibial muscles. The cell body was contralateral to the leg muscles that were affected. Confocal reconstruction of the interneuron based on the Lucifer Yellow staining showed that the primary neurite crossed the midline in a dorsal commissure (Siegler and Burrows, 1979; Watkins et al., 1985; Wilson, 1981) and then gave rise to a profusion of fine branches in the neuropil in the left half of the ganglion (Fig. 3B; ventral view, arborizations are hence in the right half of the image). Interneurons with this morphology were encountered in five of the 14 locusts.

Examples of other GABA-positive interneurons with differing physiological effects were also found in both the anterior lateral and posterior lateral groups (Fig. 1). For instance, when depolarizing current was injected into an interneuron in the anterior lateral group, the tibia of the left hind leg was slowly extended and muscle recordings showed that the slow extensor tibiae motor neuron increased its spike rate. Similarly, when depolarizing current was injected into an interneuron with a cell body in the posterior lateral group of nonspiking interneurons, the tibia of the left hind leg was flexed and muscle recordings showed that flexor tibiae motor neurons increased their spike rate. The cell bodies of both of these interneurons showed colocalisation of Lucifer Yellow and GABA staining (not illustrated).

Some nonspiking interneurons do not stain for GABA

Two of the 17 interneurons did not show GABA immunoreactivity in our double-labelling experiments. The cell body of one of these interneurons in the posterior lateral group was clearly labelled with Lucifer Yellow but not with the GABA antibody and therefore appeared green when the two pseudocolour images were merged (Fig. 4A).

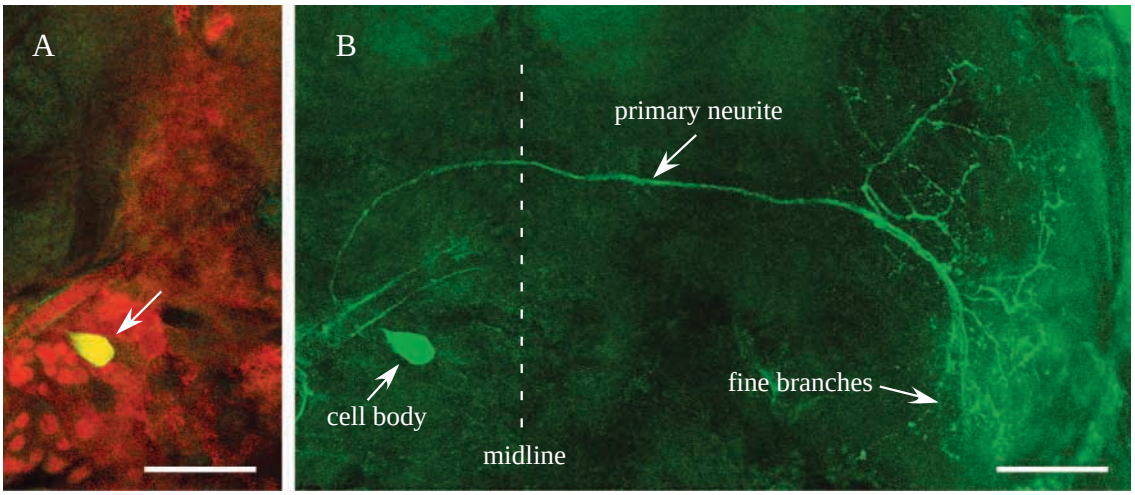

Fig. 3. A GABA-positive nonspiking interneuron in the metathoracic ganglion whose morphology can be directly related to previous descriptions; ventral view. (A) The cell body of the interneuron (arrow) appears yellow when the Lucifer-Yellow-fluorescence (green) and the Cy3/GABA-immunofluorescence (red) are merged, indicating colocalization of the dyes. Combined from three confocal planes each separated by 5.4 μm. (B) Morphology of the interneuron as revealed by the Lucifer Yellow dye (reconstructed from 41 confocal planes each separated by 5.4 μm). The primary neurite crosses the midline and then forms an extensive array of fine branches in neuropil close to the contralateral edge of the ganglion. A tracheole anterior to the cell body is strongly autofluorescent. Scale bars, 100 μm.

Morphological reconstruction of the Lucifer-Yellow-filled interneuron revealed the primary neurite running anteriorly before giving rise to a profuse array of fine branches in the neuropil. A few nearby cell bodies of similar diameter stained red and were clearly GABA-immunopositive.

The second interneuron was in the anterior lateral group (Fig. 4B). Its cell body again appeared green when the Lucifer-Yellow- and Cy3/GABA-fluorescence was combined, indicating that it was not GABA-immunoreactive. In the same optical section, a large group of small-diameter cell bodies stained red and were therefore GABA-immunopositive. When hyperpolarizing current was injected into the Lucifer-Yellow-labelled nonspiking interneuron, the frequency of spikes in flexor tibiae motor neurons was increased, whereas there was little effect on the spikes in the slow extensor tibiae motor neuron and in an unidentified motor neuron to a coxal muscle (Fig. 4C).

Discussion

We have combined intracellular electrophysiology and dye injection with subsequent GABA immunohistochemistry in the locust metathoracic ganglion to show that approximately 90% (15 out of 17) of the nonspiking interneurons we sampled showed GABA immunoreactivity. In these interneurons, the Lucifer Yellow and GABA labelling was clearly colocalised. The remaining nonspiking interneurons that we characterized were not immunopositive for GABA, even though, in the same preparations, GABA immunoreactivity was detected in other neurons known to show GABA immunoreactivity (Watson, 1986; Watson and Burrows, 1987) and in common inhibitory motor neurons that use GABA as their transmitter (Usherwood and Grundfest, 1965). The GABA staining was always seen in the cytoplasm of the cell bodies and in the larger neurites. It was sometimes also seen in the nuclei, resulting perhaps from a fixation-related change, or from the overlying cytoplasm of this or other neurons in these preparations of whole ganglia.

The nonspiking interneurons analysed in this study do not represent the sampling of a particular population of nonspiking interneurons. The recordings were made from the neuropil and therefore different types of nonspiking interneurons were encountered largely at random. This is reflected in the fact that their cell bodies were in four different locations within the ganglion where cell bodies of nonspiking neurons are known to reside (Siegler and Burrows, 1979). On the basis of their morphology and physiological action, some of the interneurons encountered in the present study can be directly related to previous and more detailed descriptions (Siegler and Burrows, 1979; Watkins et al., 1985). For example, the interneuron in Fig. 3 is one in a group of three nonspiking interneurons described first in locusts (Siegler and Burrows, 1979; Wilson, 1981) and then in stick insects (*Carausius morosus*; Büschges, 1990). Two of the nonspiking interneurons with cell bodies in this position have the pattern of branches shown here, whereas the third has an additional ipsilateral field of branches. In stick insects, the two former interneurons were originally called E4 neurons (E for excitatory) but the name was subsequently revised so that one was called E4 and the other I4 (I for inhibitory) in both locusts and stick insects (Büschges and Wolf, 1995) to reflect the finding that there were two neurons with similar shapes (Wilson, 1981) but different actions on the same motor neurons (Wolf and Büschges, 1995). Interneurons with cell bodies in this position that we stained in five locusts always showed GABA immunoreactivity, suggesting that at

least one of them has an inhibitory action mediated by GABA but leaving open the question of whether the other mediates its effects by disinhibition or is really excitatory.

The GABA immunoreactivity in some nonspiking interneurons suggests that they may use GABA as their transmitter, while its absence in others suggests that they must use a different transmitter(s). This further emphasizes the diversity of nonspiking interneurons already revealed by their morphology and physiological actions. All of the known interconnections between nonspiking interneurons involve inhibition (Burrows, 1979a), but both inhibitory and excitatory effects of nonspiking interneurons on motor neurons have been described (Burrows and Siegler, 1976, 1978). The inhibitory actions of the nonspiking interneurons at these synapses involve conductance increases in postsynaptic neurons with a time course that suggests the use of conventional transmitters such as GABA. Some of the excitatory effects on motor neurons may be explicable by disinhibition through the web of connections that a particular nonspiking interneuron makes with other nonspiking interneurons. Alternatively, GABA itself may exert a direct depolarizing effect on postsynaptic neurons as it does in some crustacean networks (Swensen et al., 2000). The finding that a few of the nonspiking interneurons do not show GABA-like immunoreactivity does, however, suggest that some interneurons might mediate direct excitation of postsynaptic neurons by the release of transmitters other than GABA.

If the properties of the nonspiking interneurons in our sample are representative of the overall thoracic population, then this suggests that GABAergic inhibition is a predominant feature of the processing by these interneurons in the local circuits that control leg movements. The exclusively inhibitory nature of the local interactions between nonspiking interneurons (Burrows, 1979a) supports this interpretation. While the orchestration of motor output by nonspiking interneurons seems to be predominantly mediated by inhibition, the nature and postsynaptic action of the transmitter used by the GABA-negative nonspiking interneurons now

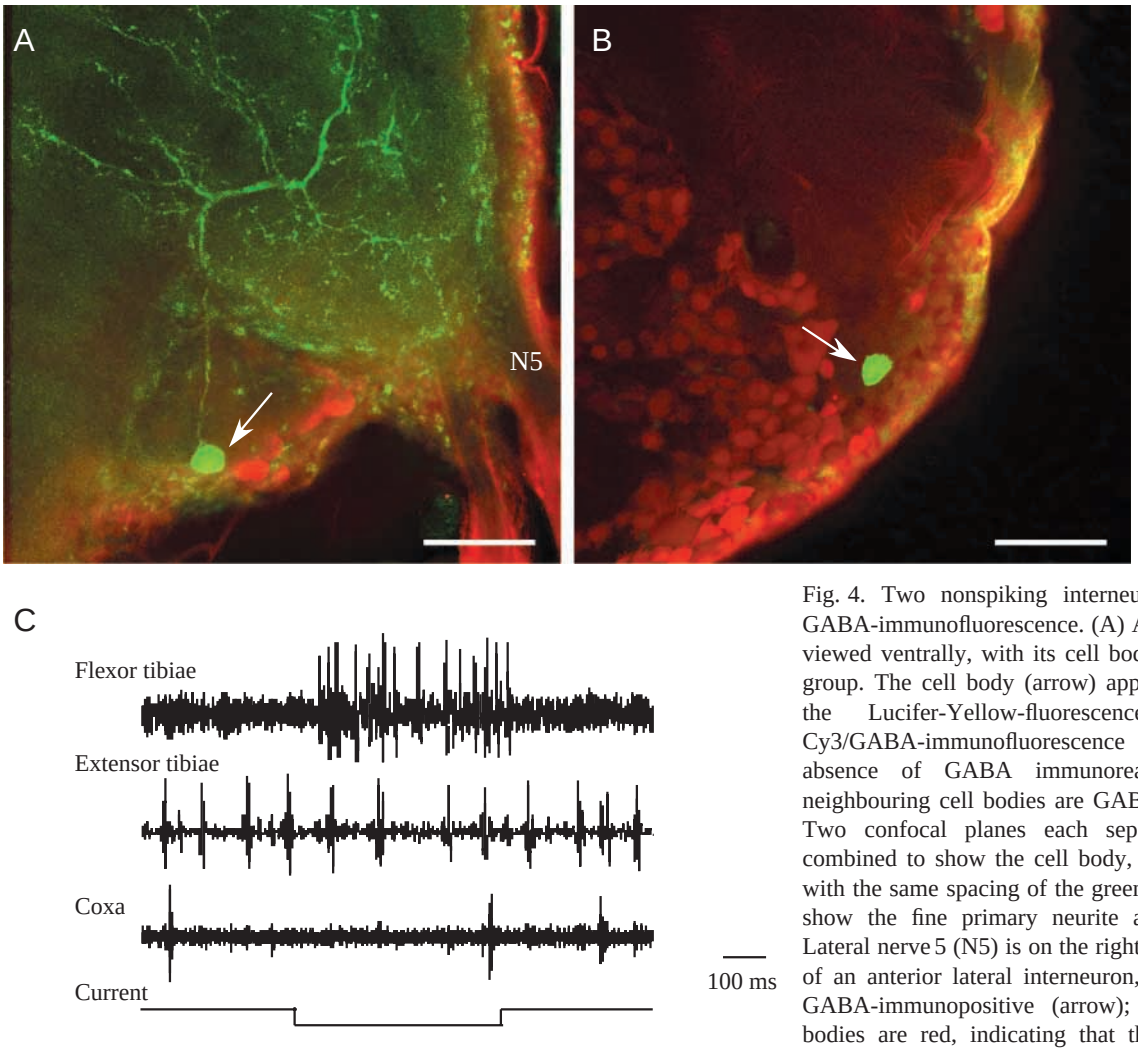

Fig. 4. Two nonspiking interneurons that do not show GABA-immunofluorescence. (A) A nonspiking interneuron, viewed ventrally, with its cell body in the posterior lateral group. The cell body (arrow) appears green after merging the Lucifer-Yellow-fluorescence (green) and the Cy3/GABA-immunofluorescence (red), indicating the absence of GABA immunoreactivity. A few small neighbouring cell bodies are GABA-immunopositive (red). Two confocal planes each separated by 8.8 µm were combined to show the cell body, and seven further planes with the same spacing of the green alone were combined to show the fine primary neurite and neuropilar branches. Lateral nerve 5 (N5) is on the right. (B) The green cell body of an anterior lateral interneuron, indicating that it is not GABA-immunopositive (arrow); some other small cell bodies are red, indicating that they are immunopositive. Combined from two confocal planes separated by 4.5 µm. (C) Injecting a pulse of hyperpolarizing current into the interneuron shown in B causes an increase in the frequency of motor spikes recorded in the flexor tibiae muscle but has little effect on spikes in the slow extensor tibiae motor neuron or in an unidentified motor neuron in the coxa. Scale bars in A and B, 100 µm.

needs to be determined to decide between two possibilities; either the transmitter used by the nonspiking interneurons that do not show GABA immunoreactivity may only exert inhibitory effects, so that any excitatory effects are due to disinhibition, or some nonspiking interneurons may use a transmitter that has direct excitatory effects on motor neurons.

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