

Common and specific inhibitory motor neurons innervate the intersegmental muscles in the locust thorax

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Accepted 16 March 2006

Summary

The inhibitory innervation of the intersegmental (body wall) muscles between the first and the second thoracic segment of the migratory locust, *Locusta migratoria*, was investigated using neuroanatomical, immunocytochemical and electrophysiological techniques. Three neurons located in the prothoracic ganglion show GABA-like immunoreactivity and project into the intersegmental nerve. Two are common inhibitors. One of those innervates the oblique intersegmental muscle M59 and two dorsal longitudinal muscles (M81 and M82). The second common inhibitor also innervates M59 and the

ventral longitudinal muscle M60. The third neuron innervates M60 exclusively and, for that reason, has to be regarded as the first specific inhibitor ever observed in insect neuromuscular assemblies. According to their innervation pattern, we term these neurons CI_{59/60}, CI_{59/81/82}, and SI₆₀. CI_{59/81/82} and CI_{59/60} appear to be segmentally homologous to CI_a and CI_b neurons, respectively, in the other body segments.

Key words: insect, inhibitory motor neuron, neuroanatomy, electrophysiology, locust, *Locusta migratoria*.

Introduction

Inhibitory motor neurons are integral elements of arthropod motor control strategy (reviewed in Rathmayer, 1998) (see also Wolf, 1990). In keeping with this essential role, both limb (reviewed in Wiens, 1989) and body wall (Schmäh and Wolf, 2003a) muscles receive inhibitory innervation, in addition to their standard excitatory drive (e.g. Hoyle and Burrows, 1973). Inhibitory innervation is usually by common inhibitory motor neurons (CIs), which supply several or even all muscles of an appendage (e.g. Rathmayer and Erxleben, 1983). These common inhibitors function in the adjustment of muscle performance to behavioural requirements, ranging from postural maintenance to fast and forceful predatory strikes or escape movements (Rathmayer, 1998). Such adjustment is necessary due to the small number, small size and overlapping composition of arthropod motor units, which are in turn dictated by the limited potential of muscle fibres for miniaturisation. In fact, the diameters of muscle fibres are not very different in elephants and fruit flies; adult skeletal muscle fibres range from 5 to 100 µm in vertebrates, and actually up to several 100 µm in invertebrates (e.g. Dudel et al., 2001), endowing the smaller invertebrates with correspondingly smaller numbers of muscle fibres.

Contrasting with the ubiquitous occurrence of common inhibitors, specific inhibitors that innervate just a single muscle

are rare. In fact, just two muscles in the distal leg segments of decapod crustaceans are supplied by specific inhibitory motor neurons (in addition to their common inhibitory supply). These are the propodite stretcher and the dactylopropodite opener muscles (Wiersma and Ripley, 1952; reviewed in Wiens, 1989), the muscles that move the two most distal leg segments dorsally. Peculiarly, these two muscles share their only excitatory motor neuron. Specific inhibitory innervation is needed, therefore, to uncouple these two muscles' contractions, as elicited by discharges of the shared excitor.

In the present study, we provide the first description of specific inhibitory innervation in an insect (the locust) as a representative of the second major arthropod group. It appears, in fact, that we have encountered a situation that may reflect an evolutionary transition from (vestigial) common inhibitory to specific inhibitory supply. The motor neuron that is a specific inhibitor in the first thoracic segment of the locust has segmental homologues in other body segments that are common inhibitors.

Materials and methods

Insects

Adult and larval locusts (*Locusta migratoria* L.) were obtained from crowded laboratory cultures maintained at the

University of Ulm, the Technical University of Munich and the RWTH Aachen. Adults were taken a few days after the final moult, hoppers shortly after the moult from the 4th to the 5th instar. Hoppers turned out to yield superior results with neuroanatomical and immunocytochemical techniques. This is most likely due to the fact that their muscles are smaller and less compact, which might facilitate the penetration of histological reagents. Prior to any manipulations insects were anaesthetised by cooling on ice.

Dissection

After removal of head and abdomen, the thorax was bisected by parasagittal longitudinal cuts such that one half still contained the ganglia of the ventral nerve cord (similar to the situation shown in Fig. 1). This half was pinned medial side up into an Elastosil[®]-lined (Wacker Silicones, Munich, Germany) Petri dish and covered with ice-cold locust saline. Ganglia, nerves and muscles were exposed by removing the salivary gland, parts of the tracheal system and fatty tissue. Some preparations were made by opening the locust with a dorsal midline incision and removing gut, salivary glands and fatty tissue. All further steps were specific for the method applied, and are described below.

Neuroanatomy and muscles

Nerves of interest were severed, their stumps isolated from the rest of the preparation in small Vaseline[®] (Fluka, Buchs, Switzerland) vessels, and immersed in 0.5–1.5% CoCl₂ or NiCl₂ (Fluka, Buchs, Switzerland). After incubation for 18–36 h at 6°C, ganglia and muscles were removed. Further histological treatment followed standard procedures for cobalt staining and silver intensification (e.g. Bräunig, 1997). In some preparations, nerves were stained using biocytin (Molecular Probes, Eugene, Oregon, USA) or neurobiotin (Vector Labs, Burlingame, California, USA) following the procedures described (Stevenson and Meuser, 1997). Biocytin and neurobiotin stainings were visualised using CY3-tagged avidin (Dianova, Hamburg, Germany) at dilutions ranging between 1:500 and 1:2000. Both staining techniques yielded similar results.

Muscles and nerves are termed according to Snodgrass (Snodgrass, 1929) and Campbell (Campbell, 1961) (Fig. 1). Campbell described two dorsal longitudinal muscles, the large M81 and the much smaller M82. We confirm the presence of a third small dorsal longitudinal muscle (see Fig. 1, top right) described by Wiesend and termed DLM3 (Wiesend, 1957). In Wiesend's terminology DLM1 corresponds to M81, DLM2 to M82. DLM3 degenerates in the adult locust.

Immunocytochemistry

Preparations consisted of the pro- and mesothoracic ganglion, the peripheral branches of the intersegmental nerve

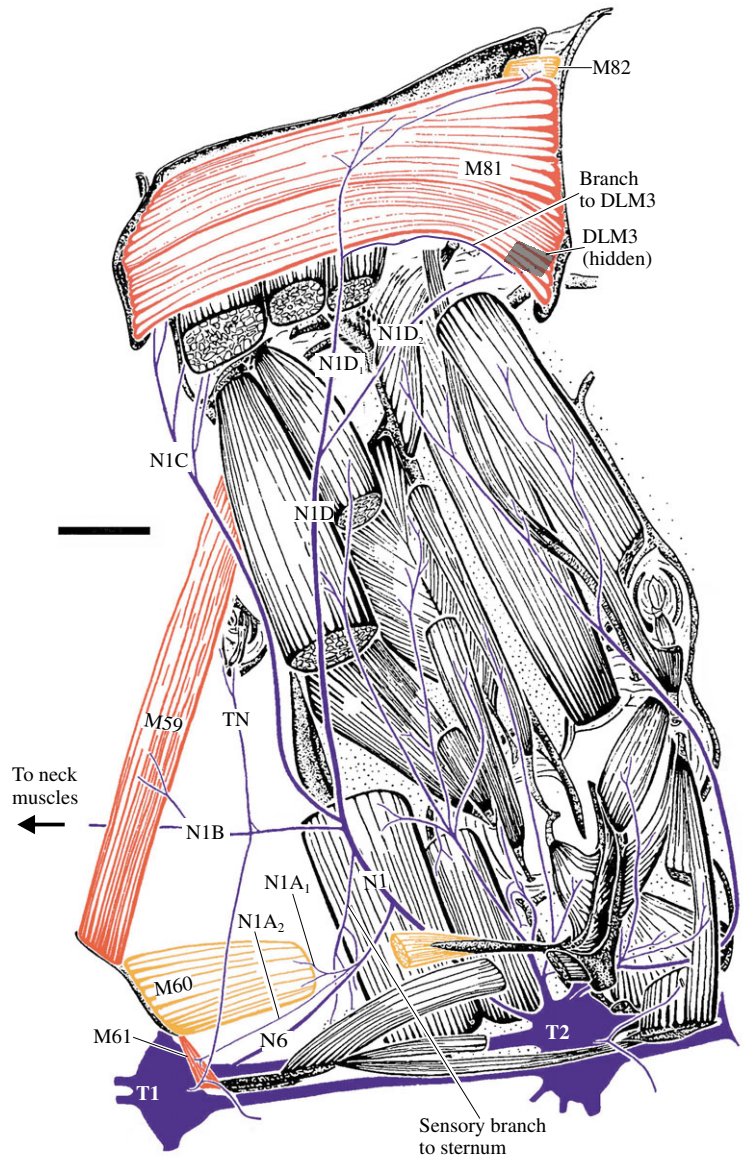


Fig. 1. Anatomy of the locust (pro- and meso-) thorax. Muscles relevant in the present report are highlighted in reddish colours (adapted from fig. 8 in Campbell, 1961). Muscle numbers follow the Snodgrass terminology (Snodgrass, 1929). The nervous system is marked in blue. Note nerve supplies of muscles: M59 (sterno-pleural intersegmental muscle) supplied by a branch of N1B, M60 (2nd ventral longitudinal muscle) supplied by N1A₁, M61 (sterno-spinal muscle) supplied by N1A₂, M81 (dorsal longitudinal muscle) and M82 (oblique dorsal muscle) supplied by N1D₁ (for details, also see Fig. 6). The larval dorsal longitudinal muscle DLM3 (hidden) is located just below the posterior ventral corner of M81, indicated by grey shading. Scale bar, 1 mm.

(nerve 1) between the two ganglia from one half of the body, and muscles innervated by these branches. In 5th instar hoppers, the phragma, which provides the anterior attachment surface for the main dorsal longitudinal muscle (M81), was cut from the scutum, the muscle deflected posteriorly, and fixed in this position. In this way it was possible to expose the underlying small larval muscles (Wiesend, 1957; Bernays, 1972).

After dissection, tissues were fixed *in situ* with GPA (1 part glutaraldehyde, 25% aqueous solution; 3 parts picric acid, saturated aqueous solution, and acetic acid to 0.5%) (Boer et al., 1979) for at least 2 h at room temperature, or overnight at 6°C. In order to improve antibody penetration, the preparations were dehydrated in an ascending series of isopropanol after fixation, immersed in propylene oxide for 30 min, and rehydrated again in a descending series of isopropanol. Subsequently, tissues were rinsed several times in phosphate-buffered saline (in mmol l⁻¹: NaCl 140, KCl 10, CaCl₂·2H₂O 2, NaH₂PO₄·H₂O 4, Na₂HPO₄·2H₂O 6) containing 0.5% Triton X-100 (Merck, Darmstadt, Germany) (PBS-TX). Ganglia, nerves and muscles of interest were removed by further careful dissection and transferred into small Petri dishes for further processing. Care was taken during all subsequent histological procedures not to disturb the natural orientation of nerves and muscles.

To reduce non-specific background staining, tissues were incubated in blocking medium (PBS-TX containing 2% normal goat serum) (Sigma, Taufkirchen, Germany) and 5% bovine serum albumin (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA) prior to incubation in primary antiserum for at least 1 h. Two primary antisera were used in the present study, both yielding the same results. Sera were donated by Sabine Kreissl and Werner Rathmayer, and also by Hans Agricola (see Blechschmidt et al., 1988). Primary antiserum was diluted in the same blocking medium at 1:3000 v/v. Tissues were incubated in primary antiserum for 1–2 days at 6°C under constant gentle irritation. Immunostainings were developed using Vectastain[®] ABC kit (Vector Laboratories, Burlingame, CA, USA). After thorough washing in PBS-TX (1 day at room temperature) tissues were incubated overnight at 6°C in biotinylated secondary antibodies (dilution according to Vectastain protocol). This was followed by a second washing period of 1 day, this time using phosphate-buffered saline (PBS) without detergent, and incubation in avidin–horseradish peroxidase complex (overnight at 6°C, dilution according to Vectastain protocol). After washing for 4 h in several changes of PBS at room temperature, tissues were transferred into Tris-HCl-buffered diaminobenzidine solution (DAB; Sigma; 2.5 mg in 10 ml buffer, pH 7.6) for 1 h. After this pre-incubation period, tissues were transferred into fresh DAB solution, now containing H₂O₂ (1 µl per 10 ml buffer). Development was stopped by washing in Tris-HCl buffer (pH 7.4). Subsequently, preparations were dehydrated in an isopropanol series, cleared in methyl salicylate, and mounted in Canada Balsam.

Combining immunocytochemistry and backfilling for double-labelling

The backfill and anti-GABA immunocytochemistry procedures were combined to double-label those motor neurons in the intersegmental nerve that possess GABA-immunoreactive cell bodies in the prothoracic ganglion. Neurobiotin was used for backfilling in these cases, and the backfill was developed with Cy3-coupled streptavidin (Sigma).

Anti-GABA immunocytochemistry was performed with Cy2-coupled secondary antibody (Dianova, Hamburg, Germany). All other details were as stated above.

Electrophysiology

Spike activity in nerves was recorded by *en passant* electrodes, usually bipolar hook electrodes insulated with Vaseline[®]. Synaptic potentials in muscle fibres, both EPSPs and IPSPs, were observed in intracellular recordings performed with glass microcapillaries. The microelectrodes were filled with 3 mol l⁻¹ KCl and had tip resistances of about 30 MΩ. Electrophysiological data were recorded with an npi SEC-10L amplifier (Tamm, Germany) and stored on computer disc using a biologic DRA-800 analogue-digital converter (CED Cambridge Electronic Devices, Cambridge, UK). For evaluation of electrophysiological recordings, SPIKE2 software (CED Cambridge Electronic Devices) was used. Further details are described elsewhere (Schmäh and Wolf, 2003a).

Results

Immunocytochemistry

Anti-GABA immunocytochemistry revealed three stained axons entering the posterior intersegmental nerve (N6) from the prothoracic ganglion (T1) (Fig. 2A). One of these three axons entered the first lateral branch of N6, N1A, which innervates muscles 60 and 61 (M60, M61; see Fig. 1).

This branch is here termed N1A although it does not actually branch off N1 (the mesothoracic intersegmental nerve) but rather buds off N6. However, it had been observed previously that between the second (T2) and third (T3) thoracic ganglion a side branch of the intersegmental nerve innervates muscle 116, a muscle that is serially homologous to M60. This side branch may originate from N6 in T2 or from N1 in T3. This side branch was called N1A in previous publications (e.g. Bräunig et al., 1994) and for this reason, and to avoid inconsistencies in the nomenclature, we decided to term the homologous branch innervating M60 and M61 N1A, also, although in no case did it actually originate from N1.

N1A divides further into two branches: N1A₁, innervating M60, and N1A₂, innervating M61. The GABA-immunoreactive axon proceeded into N1A₁ and innervated M60 (*N*=11). In well-stained preparations the axon could be observed to subdivide further on the muscle and to form blebby terminal ramifications on the muscle surface (similar to the situation shown in Fig. 2C for M59, below). We never observed GABA-immunoreactive terminals on the fibres of M61, nor any stained axon or axon collateral in N1A₂ (*N*=16).

The other two axons proceeded further in N6. After this nerve had fused with N1 of T2 (the two nerves forming the intersegmental nerve proper), both axons ran into N1B, which is also called the paramedian nerve (Fig. 2B). Nerve 1B may originate from N1 individually or it may form a common root with N1C (the nerve innervating the wing; the latter situation is depicted in Fig. 2B). N1B runs anteriorly, innervates M59

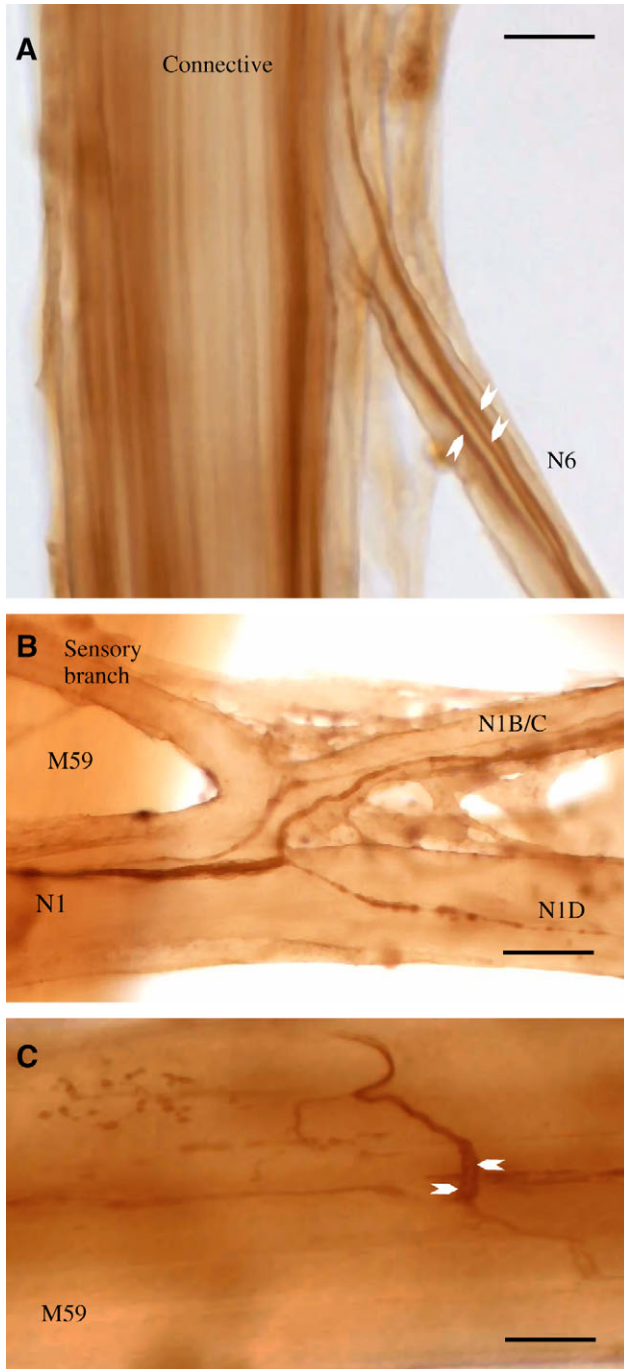


Fig. 2. Anti-GABA immunocytochemistry of peripheral nerves and muscles. (A) Three GABA-immunoreactive axons (arrowheads) enter nerve 6 (N6) as it branches off the posterior pro-mesothoracic connective. (B) Branching point of mesothoracic nerve 1 (N1) as it divides into N1B (here still including N1C: N1B/C) and N1D (compare Figs 1 and 6). A sensory branch that supplies the sternum (see ventralmost label in Fig. 1) also buds off at this point. Two immunoreactive axons run from the prothoracic ganglion through N1 and into N1B to supply M59. Only the larger-diameter axon gives off a branch into N1D to supply M81 and M82. (C) GABA-immunoreactive axonal branches and terminal boutons on M59. Two immunoreactive axons supply the muscle (arrowheads). Scale bars, 35 μm (A,B); 100 μm (C).

(usually with two distinct side branches), and proceeds further into the neck region. About half way between its origin from N1 and its side branches innervating M59, N1B forms an anastomosis with the transverse nerve. Within N1B and its side branches the two axons could be followed into M59 where they further divided and formed terminals on fibres of this muscle (Fig. 2C) ($N=6$).

In no case could we observe the immunoreactive axons in N1B to proceed beyond M59 ($N=16$), which confirms the result of the electrophysiological examination described below. These axons (and the anterior segment of N1B) may thus be eliminated as potential sources of inhibitory innervation to neck muscles (Bräunig, 1997). Likewise, the axons did not form axon collaterals to enter the transverse nerve that innervates the spiracular muscles. One of the two GABA-immunoreactive fibres, however, formed a collateral, which proceeded into nerve 1D (Fig. 2B) ($N=8$). Nerve 1D innervates the dorsal longitudinal muscles (DLMs) (N1D₁) and sense organs associated with the wing hinge (N1D₂). This axon collateral was rather thin when compared to the main axons proceeding towards M59 (Fig. 2B). In 5th instar larvae, this collateral could be followed to proceed into N1D₁ (and not into the sensory N1D₂) and into its side branches, which innervate M81, the major dorsal longitudinal muscle, and M82. In a few preparations, GABA-immunoreactive terminals could be observed on these muscles. In no case was there any indication that DLM3 (see Fig. 1) (Wiesend, 1957), a larval muscle that degenerates in the adult, was innervated by this axon.

Neuroanatomy

The results obtained by staining the intersegmental nervous system with anti-GABA antisera, as described above, may be summarised as follows. Three immunoreactive axons leave the prothoracic ganglion. One of these enters N1A₁ and innervates M60; the two remaining axons proceed into N1 and further distally into N1B to innervate M59. One of these two axons sends a collateral towards the dorsal longitudinal muscles and innervates M81 and M82, though not DLM3 (see summary in Fig. 6).

In order to scrutinise these results, individual motor nerves were stained using cobalt chloride, nickel chloride, biocytin or neurobiotin. In cases of common innervation, staining the motor nerve of one muscle should result in stained terminal ramifications on muscles innervated by the same (common) neuron. This backfilling technique can also reveal numbers and locations in the CNS of the motor neuron somata giving rise to these axons and collaterals.

Backfills of the motor nerve to M59

In backfills of the motor nerve that supplies M59 (branches of N1B), one axon collateral was seen clearly in nerve 1D ($N=49$), and in well-stained preparations ($N=9$) this collateral could be followed to its terminal ramifications on M81 and M82 (Fig. 3B). *Vice versa*, when staining N1D ($N=4$), an axon collateral was observed to innervate M59 *via* N1B.

The situation was less obvious in the case of M60.

Backfilling the M59 motor nerve sometimes revealed terminals on M60 ($N=7$). One has to be careful, though, to interpret this as common inhibitory innervation since a DUM neuron that supplies both muscles was described previously (Bräunig, 1997). In six preparations this DUM neuron, termed DUM1B, with its soma located in the mesothoracic ganglion, stained exceptionally well such that its ramifications could be followed individually. These backfills confirmed the peripheral branching pattern as revealed previously by intracellular staining (Bräunig, 1997): DUM1B innervates M60, M61 and M59 but it does not project into N1D.

In the present backfills of the motor nerve to M59 ($N=34$) at least four axons were discernible, with diameters much larger than DUM1B. Two of these entered N1 of the mesothoracic ganglion (together with the axon of DUM1B), another two entered N6 of the prothoracic ganglion. The fibres entering N1 could be followed to two mesothoracic somata and presumably belong to the excitatory motor neurons to M59; they are clearly discernible by their anterior and contralateral soma location (Steffens and Kutsch, 1995). The axons in N6 originated from two somata in the prothoracic ganglion. These cell bodies occupied a conspicuous posterior ventral position, just contralateral to the ganglion midline. They exhibited looping primary neurites in preparations where internal structures of the ganglia were discernible (Fig. 3A; arrowheads indicate primary neurites). One of the two axons in N6 formed a small-diameter collateral entering N1A₁ (Fig. 3C), which projected to M60 and formed terminal ramifications in the anterior medial region of this muscle. In contrast to the less intensely labelled axons of the DUM1B neuron this collateral did not project into N1A₂ and to M61.

Backfills of the M60 motor nerve

Backfills of the nerve supplying M60 ($N=13$) often ($N=9$) labelled a collateral axon in N1B and the nerve branches innervating M59. This confirms the common innervation of muscles M59 and M60 observed in the backfills of M59. The common axon appeared distinctly thicker than that of DUM1B described above. Occasionally, an additional much smaller axon was observed ($N=3$). In these preparations a thin axon proceeding through mesothoracic N1 and/or a posterior medial soma in the mesothoracic ganglion was also present, indicating that this thin axon belonged to DUM1B.

We did not observe axon collaterals in the nerve supply of M61, other than a very thin axon ($N=4$), to be interpreted as a DUM1B collateral according to the criteria mentioned above.

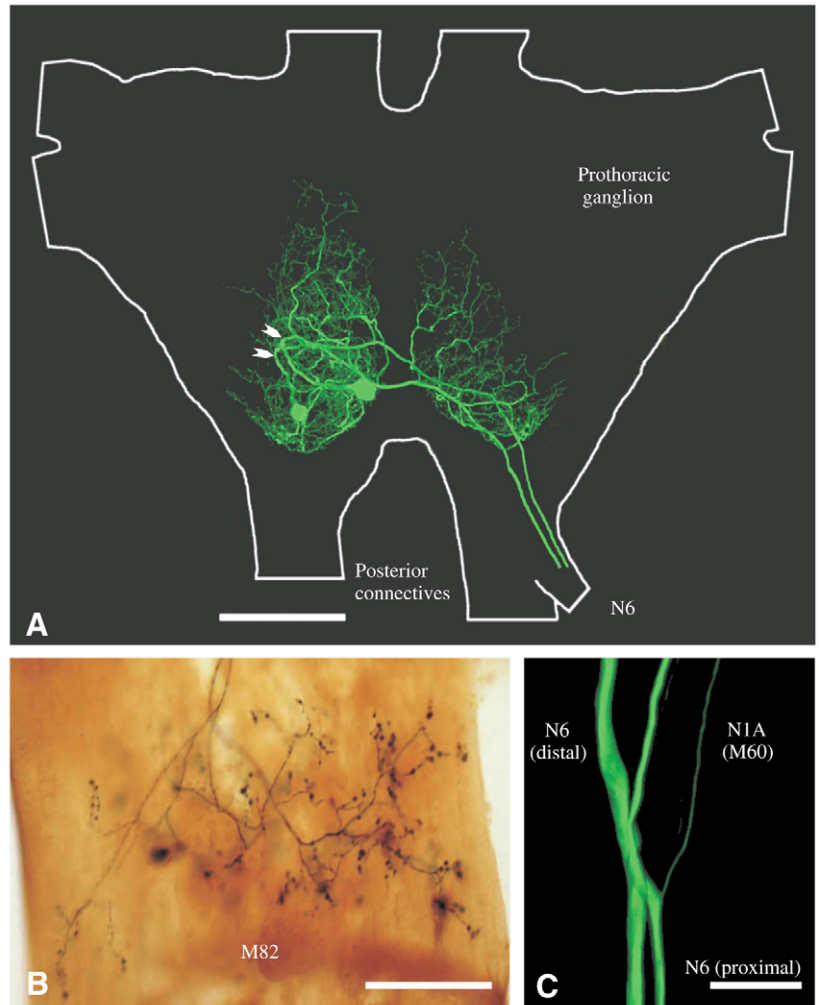


Fig. 3. Backfills reveal anatomical details of (inhibitory) innervation in the locust prothorax. (A) Backfilling the motor nerve to M59 stains two (presumptive inhibitory, see text) motor neurons in the prothoracic ganglion, the axons of which leave the ganglion through nerve 6 (N6) (confocal image of neurobiotin fill, developed with Cy2, ganglion outline indicated). Arrowheads indicate looping primary neurites. (B) Backfilling the motor nerve to M59 also stains axonal branches and terminal boutons on M82 (Ni^{2+} -backfill, silver-intensified). (C) Backfilling the motor nerve to M59 finally reveals the axons of the two (inhibitory) motor neurons shown in A as they project into the periphery through N6. Only the smaller diameter axons send a branch into N1A to innervate M60 (confocal image, as in A). Scale bars, 200 μm (A); 150 μm (B); 25 μm (C).

Six to nine axons were counted in N6 of the prothoracic ganglion in backfills of the M60 motor nerve. This variability was apparently produced by a number of small-diameter axons, presumably belonging to a corresponding number of similarly small somata in the prothoracic ganglion, that were sometimes labelled, and sometimes not. The larger cell bodies (30–45 μm diameter) were labelled reliably ($N=13$). These were four somata in the ipsilateral ventral soma cortex, which presumably belong to the excitatory motor neurons of M60, and two posterior somata near the ganglion midline, which gave rise to looping primary neurites where discernible

(compare Fig. 3A, arrowheads). Three smaller cell bodies (20 μm or even smaller) located ventrally on the ganglion midline were stained occasionally. In addition, a DUM soma was labelled in the mesothoracic ganglion of well-stained preparations. According to the above description this was DUM1B.

Backfills of the M61 motor nerve

The motor supply of M61 was backfilled to scrutinise the results reported above, in particular, the lack of common innervation with muscles 59 and 60, except for DUM1B. These backfills ($N=21$) corroborated the above data. Two axons were reliably observed in the nerve supply of M61, running through N1A₂, prothoracic N6, and mesothoracic N1 into the mesothoracic ganglion to connect to two cell bodies located anteriorly and contralaterally. These two somata were reminiscent of the excitatory motor neurons that supply M59, and they presumably represent the excitatory motor neurons to M61. In a few ($N=9$) well-stained preparations DUM1B was also labelled, with the characteristics outlined above, namely, a very thin axon supplying M59 and occasionally M60.

Double-labelling by combined backfills and immunocytochemistry

The cell bodies of putative inhibitory motor neurons were identified by combining anti-GABA immunocytochemistry with

backfills of (motor) nerves ($N=13$). Backfills of prothoracic nerve 6 labelled the cell bodies of all neurons noted above, except those located in the mesothoracic ganglion (i.e. the excitors of M59 and M61, and DUM1B). Processing such backfill preparations for anti-GABA immunocytochemistry produced a double label in those neurons that have an axon in nerve 6 and are GABA-immunoreactive, and thus are presumptive inhibitory motor neurons. This is illustrated in Fig. 4, which presents an area of the prothoracic ganglion just contralateral to the ganglion midline, with regard to the filled nerve. Four somata in the centre of the image show red fluorescence and thus have axons in N6 (Fig. 4Ai; in a sample cross section of the same ganglion area, though from a different animal, only two of these somata are visible, Fig. 4Aii). Three of the four cell bodies are also marked by anti-GABA immunocytochemistry, that is, green fluorescence (Fig. 4Bi,Ci; cross section in Fig. 4Bii,Cii). These are the three presumptive inhibitory motor neurons described above, namely, the cells with looping primary neurites and somata contralateral to the ganglion midline and posterior ventral soma locations. The fourth cell body located in close proximity to these three inhibitors was regularly marked in backfill preparations. It never stained for the inhibitory transmitter GABA, however.

All backfilled cells were visible, of course, in the whole-mount preparations (Fig. 4Ai,Bi,Ci). Comparison with sectioned ganglia revealed double-labelling more accurately (Fig. 4Aii,Bii,Cii) in cases where superpositions like in

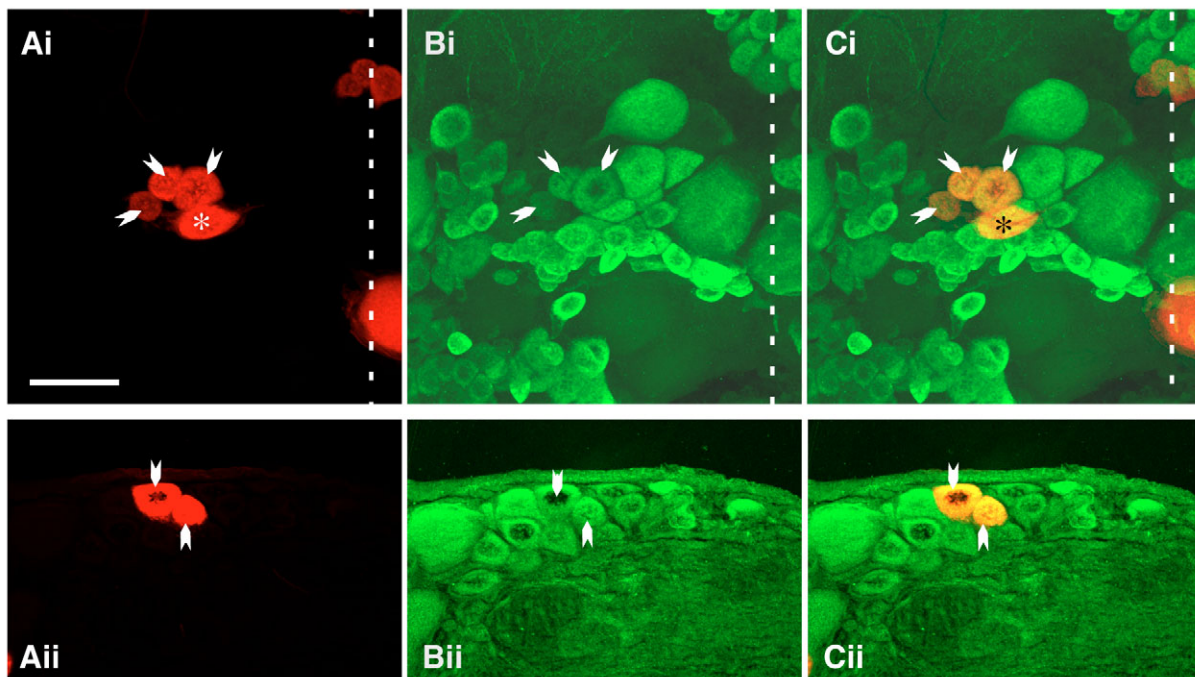


Fig. 4. Double labelling of inhibitory motor neurons through combination of backfilling nerve 6 (A; red fluorescence, Cy3) and anti-GABA immunocytochemistry (B; green fluorescence, Cy2). (Ai,Bi,Ci) A whole-mount preparation (anterior is to the top; ganglion midline indicated by dotted line, backfill labels are contralateral to the side of nerve 6 exit); (Aii,Bii,Cii) a cross section through the same area of the prothoracic ganglion in a different animal (ventral is to the top). The overlays in Ci and Cii show which neurons have acquired both labels (arrowheads indicate the same cells in Ai–Ci, and Aii–Cii, respectively). Note that the cell marked with an asterisk in Ci does not stain for GABA in Bi. It only appears orange because it is located on top of cells labelled in green. Scale bar, 50 μm .

Fig. 4Ci appeared ambiguous. For instance, the most posterior backfilled cell body in Fig. 4Ai is not labelled by the GABA antiserum (marked by asterisk). This is discernible by a detailed comparison of soma locations between Fig. 4Bi and 4Ci (see arrowheads). And it holds true despite an overlay of green fluorescence with the red backfill label, with the two labels occupying different depths (Fig. 4Ci). In cross sections no such ambiguity is present (the same is true of optical sections in the confocal microscope, of course; not shown).

Electrophysiology

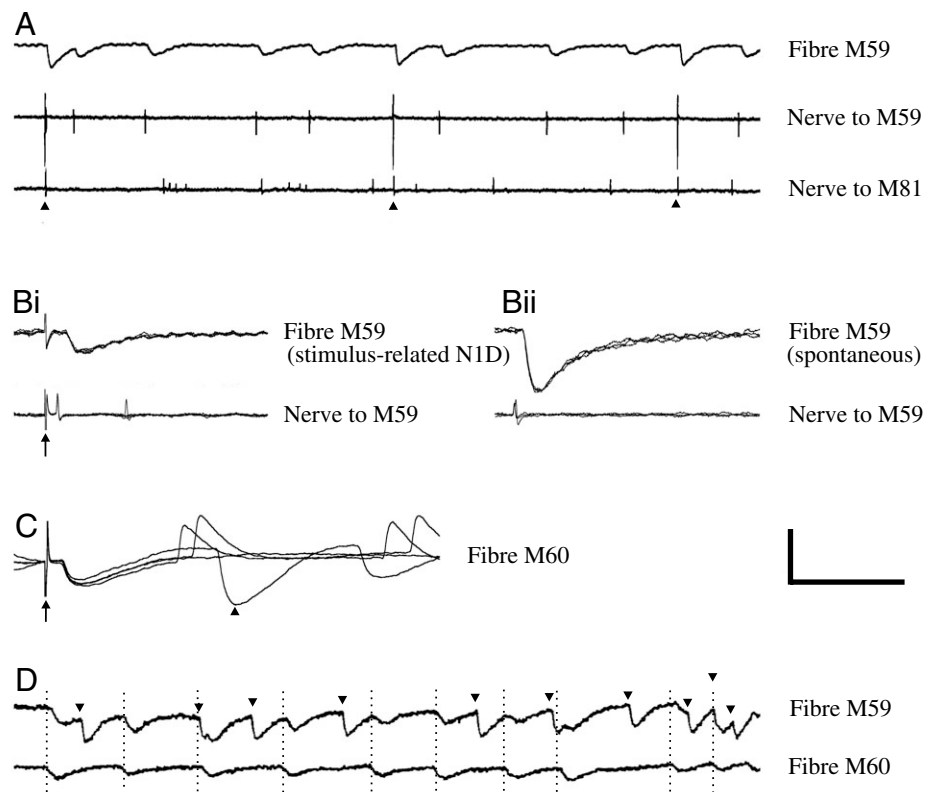
The results described above show that, in the intersegmental nervous system, there are neurons that innervate more than one muscle, in addition to the DUM1B neuron [and possible axon collaterals of an allatostatin-immunoreactive cell (Kreissl et al., 1999); we never encountered any indication for those in our preparations]. One neuron clearly innervates M59, M81 and M82. It was not unequivocally clear, however, whether this neuron also forms the collateral innervating M60, or whether this innervation originates from the second prothoracic cell, which stains after filling the M59 motor nerve. To resolve this matter conclusively, we further examined the innervation of muscles 59, 60, 61 and 81 using

electrophysiological techniques. These experiments also elaborated and scrutinised the other results obtained with the immunocytochemical and neuroanatomical techniques.

Electrical stimulation of nerve 1D (see Figs 1 and 6) resulted in phase-locked spikes in the motor nerve to M59 (Fig. 5Bi), but not in N1A, the efferent nerve supplying M60 and M61 ($N=4$). The phase-locked spikes were followed by phase-locked inhibitory postsynaptic potentials recorded from individual muscle fibres in M59. *Vice versa*, stimulation of the motor nerve to M59 ($N=1$) resulted in phase-locked discharges of one unit in N1D and phase-locked IPSPs in muscle fibres of M82. This confirmed the immunocytochemical and neuroanatomical results, indicating that there is one inhibitory motor unit common to M59 and the DLMs, and not M60.

In some preparations, the inhibitory units were spontaneously active (cf. Schmäh and Wolf, 2003a). In such cases ($N=2$), two units were recorded from the motor nerve supplying M59. Both were followed in a 1:1 fashion by IPSPs of distinct shapes and amplitudes in the muscle fibres (see also Fig. 5B,D; see figure legend for details of the experimental situation). Only one of these units could also be recorded in N1D₁, the motor nerve to the DLMs (Fig. 5A). This was the unit producing the larger-amplitude IPSPs in M59. These

Fig. 5. Electrophysiological examination of motor nerve supplies and intracellular muscle fibre recordings support anatomical and immunocytochemical data. (A) Extracellular recordings from the nerves supplying M81 (bottom trace) and M59 (middle trace), and intracellular recording from an M59 muscle fibre (top trace) during spontaneous activity illustrate two major features. First, there are two classes of IPSPs in M59, with different amplitudes and shapes. Second, the large-amplitude IPSPs exhibit a 1:1 relationship to spikes in the nerve supplies of both, M59 and M81 (marked by arrowheads). The small-amplitude IPSPs are related only to spike activity in the M59 motor nerve. (Bi) Stimulating the motor nerve to M81 and M82 in a different experiment (N1D; stimulus artefacts marked by arrow) elicits spikes in N1B (bottom trace) and small-amplitude IPSPs in a fibre of M59 (top trace). (Bii) Large-amplitude IPSPs in this same muscle fibre are not stimulus-related but occur in 1:1 relationship to spontaneous spikes, with slightly different shape and amplitude when compared to those seen in Bi, bottom trace. Four traces each from the same recording are superimposed in Bi and Bii. (C) Stimulating the motor nerve to M59 (stimulus artefacts indicated by arrow) elicits (small-amplitude) IPSPs in fibres of M60. Large-amplitude IPSPs (arrowhead) occur spontaneously. (D) Simultaneous intracellular recordings from fibres of M59 (top trace) and M60 (bottom trace) illustrate common inhibitory input. 1:1 relationship of IPSPs in the two muscle fibres is clearly discernible (dotted lines mark the beginnings of IPSPs in M60), as is the unrelated occurrence of larger-amplitude IPSPs in M59 (arrowheads; uncorrelated IPSPs were also observed in M60 in other experiments). Scale bars, 20 mV, 275 ms (A); 10 mV, 32.5 ms (B); 10 mV, 25 ms (C); 20 mV, 250 ms (D).



results indicate that two inhibitory units innervate M59. One of these also innervates the DLMs but not M60, since stimulation of N1D never elicited phase-locked spikes in the motor nerve to M60 (see above).

In a corresponding set of experiments, we observed common inhibitory innervation of muscles 59 and 60 ($N=5$) (Fig. 5C). By inference from the above data, this had to be the unit not common to muscles 59 and 81/82. Common innervation of M59 and M60 was most clearly illustrated by paired intracellular recordings from fibres of these two muscles. IPSPs occurred in 1:1 relationship in both recordings (Fig. 5D; dotted reference lines). In keeping with the above data, this (common) inhibitory innervation to M59 gave rise to the smaller-amplitude IPSPs (while the common innervation to M59 and M81 generated the larger-amplitude IPSPs in M59, above; and note arrowheads). Stimulating the motor nerve to M59 corroborated these findings. IPSPs occurred in M60 in a 1:1 fashion with regard to the stimulus (Fig. 5C). It is particularly noteworthy in the present context that IPSPs of different amplitude, and not related to the stimulus on the motor supply of M59, often occurred in the intracellular recordings from M60 fibres (arrowhead in Fig. 5C). This is strongly indicative of double inhibitory innervation of M60, with only one of these inhibitory motor neurons being common to M60 and M59.

IPSPs were never recorded in fibres of muscle 61, despite probing several muscle fibres in each of the 6 preparations examined.

Discussion

The main results of the present paper may be summarised as follows (Fig. 6). The locust prothoracic ganglion contains three GABA-immunoreactive neurons (per hemiganglion, or side of the body), which innervate body wall muscles and elicit IPSPs in the muscles' fibres. The muscles are supplied by branches of the intersegmental nerve. Two of the inhibitory neurons are common inhibitory motor neurons. One of these common inhibitors innervates muscles M59 and M60, the other one muscles M59, M81 and M82. The third neuron appears to be a specific inhibitor that innervates M60 exclusively. According to this innervation pattern, we term these neurons $CI_{59/60}$, $CI_{59/81/82}$ and SI_{60} .

All the three techniques that we applied – immunocytochemistry, neuroanatomy, electrophysiology – alone and in combination, support this interpretation (summarised in Fig. 6). Backfills of the prothoracic part of the intersegmental nerve (nerve 6), apart from numerous ipsilateral somata, which presumably belong to excitatory motor neurons (cf. Kutsch and Heckmann, 1995), marked four somata, which are located ventrally in the ganglion near the midline, but contralateral to the filled nerve. The primary neurites leave these somata in characteristic loops, which had already been noted in previous reports (Kutsch and Heckmann,

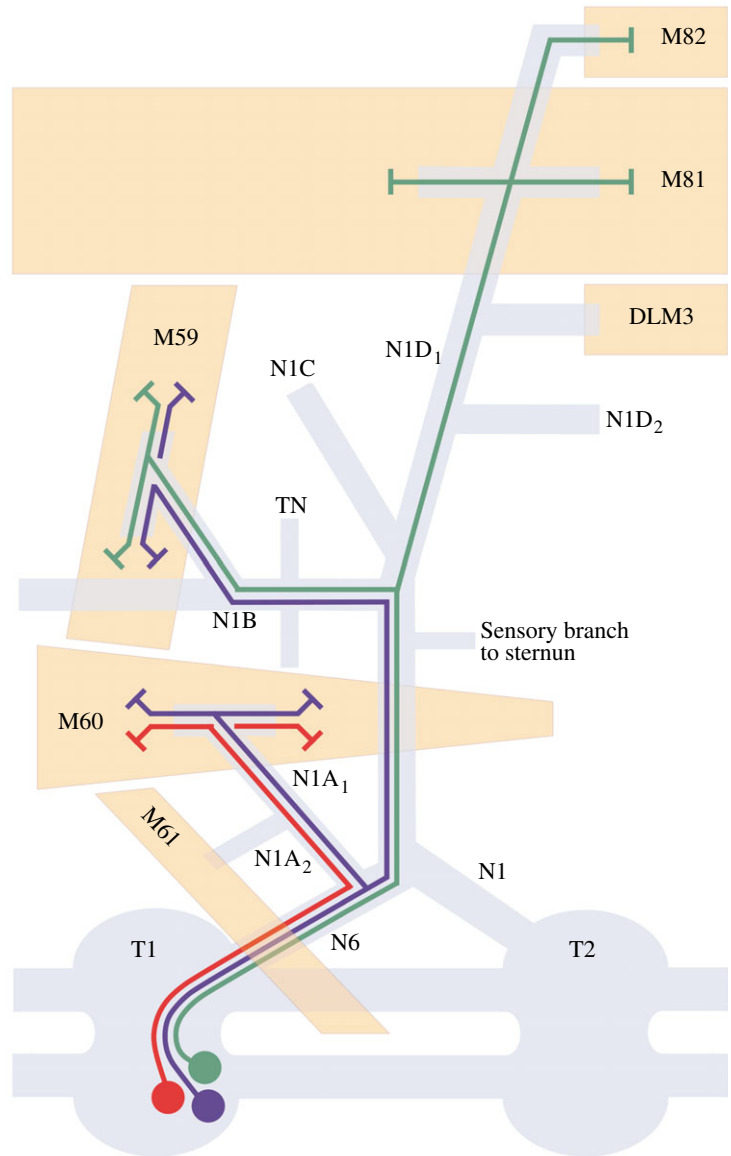


Fig. 6. Summary diagram of the inhibitory motor neuron supply to prothoracic muscles, as revealed by the combination of immunocytochemical, neuroanatomical and electrophysiological techniques. The three inhibitory motor neurons are each marked by a particular colour: $CI_{59/81/82}$, green; $CI_{59/60}$, blue; SI_{60} , red. Compare also to the more realistic representation of nerves and muscles in Fig. 1. T1, T2, pro- and mesothoracic ganglia; TN, transverse nerve (remaining nerve and muscle labels see text).

1995; Schmäh and Wolf, 2003a). Three of the four contralateral somata are relatively small with diameters ranging from 20 to 25 μm . In double-labelling experiments these three neurons exhibit GABA-like immunoreactivity. The fourth soma is somewhat larger (close to 30 μm diameter) and does not stain with GABA antisera. It appears to supply M60 (data not shown) (cf. Kutsch and Heckmann, 1995).

The existence of three GABA-immunoreactive cells is also demonstrated by immunocytochemistry in the periphery. Three axons are visible in N6; one of these innervates M60, the other

two proceed further into N1B to innervate M59. One of the latter two axons branches into N1D to supply the DLMs. The other axon of the two should form a collateral innervating M60, according to the neuroanatomical and electrophysiological data. However, this was never observed with immunocytochemical staining. This additional collateral showed up only in backfills and here it was distinctly smaller than the main axon in N6 (Fig. 3C). It has previously been reported that very small axons show variable staining in peripheral nerves with anti-GABA immunocytochemistry (Watson and Pflüger, 1987).

Not all muscles innervated by branches of the intersegmental nerve receive GABA-immunoreactive inhibitory innervation. One example is DLM3, and the other exception is M61. In the case of DLM3, not one of the more than 50 backfills from M59 revealed labelled axons in its specific motor branch (a small side branch of N1D₁; see Fig. 1), nor terminals on the muscle. It is very unlikely that this is due to a failure of the backfill technique since axon collaterals and terminals were clearly stained in the case of M82, which is located much more dorsally than DLM3, that is, farther away from the site of the nerve fill.

The case of M61 is less clear. A previous study of the intersegmental muscles plainly shows a prothoracic neuron, resembling one of the inhibitors described here, stained in a backfill from the motor nerve to M61 (Kutsch and Heckmann, 1995). By contrast, we could not find any indication for an inhibitory innervation of this muscle in the present study. There are several possible explanations for this discrepancy. First, the innervation pattern might differ between various locust strains (compare e.g. Goodman et al., 1974) for differences in neuronal arborisations between breeding strains). Second, there might be differences between individuals. That is, occasionally M61 does receive an axon collateral from one of the two inhibitors that innervate M60, and sometimes it does not. The fact that we never observed inhibitory innervation of M61 in 70 preparations would seem to argue against this interpretation. Third, there might be problems caused by the staining technique, for instance, dyes entering axons damaged in the course of dissection. In this respect, researchers often tend to interpret backfill data with a certain bias, regarding preparations that exhibit the maximum number of neurons as being the most representative. This is based on the assumption that in all the other preparations particular neurons have failed to stain (which may indeed easily happen, depending on the nerve studied). This interpretation, however, is problematic when axons are exposed for prolonged periods to cytotoxic staining solutions such as heavy metal salts.

Assuming that there is indeed an occasional innervation of M61 by one of the inhibitors, the question arises which neuron sends axons or axon collaterals into N1A in the respective cases. There are two possible candidates: the common inhibitor CI_{59/81/82}, and the specific inhibitor, SI₆₀. In the latter case, the specific inhibitor would turn into an occasional third common inhibitory neuron. This raises the interesting possibility that this first specific inhibitor ever found in insects might represent

a common inhibitor, too, the number of its targets being in the process of reduction to just one muscle, for unknown reasons.

In the remaining body segments of the locust, there are just two inhibitory motor neurons that supply the body wall muscles (Schmäh and Wolf, 2003a). What are the segmental correspondencies between these neurons and the three prothoracic inhibitors? That is, which are the segmental homologues, if any, of CI_{59/60}, CI_{59/81/82} and SI₆₀? The morphologies of all three inhibitory motor neurons in the prothoracic ganglion are rather similar (although SI₆₀ appears to possess the smallest cell body of the three inhibitors), thus offering few clues for specific homologisation. Their morphologies also agree closely with those of the previously described body wall inhibitors in other body segments: 'loop cell' (Kutsch and Heckmann, 1995), corresponding to CI_a, and 'common contralateral A-type cell' (Steffens and Kutsch, 1995), corresponding to CI_b. That is, they feature small somata, compared to those of other motor neurons, located in the posterior half of the ventral soma cortex close to the ganglion midline and just contralateral with regard to their axons; and the primary neurites exhibit characteristic loops before crossing the midline (see Schmäh and Wolf, 2003a).

Thus, it is the inhibitors' innervation patterns which provide the main characteristics that one may employ for segmental comparison. According to these criteria, CI_{59/81/82} corresponds to CI_a. This homology appears safe to infer, based in particular on the (common) innervation of the dorsal longitudinal muscles, which are the most clearly similar and homologous between different body segments. By the same token, when considering the (common) innervation of ventral body wall muscles, CI_{59/60} may correspond to CI_b. Due to the more derived structure of M59 and M60, however, this inference is more tentative. For one of these muscles, M60, segmental homology with ventral longitudinal muscles in the more posterior segments is safe to assume (Steffens and Kutsch, 1995), supporting homology of the inhibitory innervation. However, M60 is supplied not only by CI_{59/60} but also by SI₆₀. This makes the assignment of homology problematic, although the common innervation exhibited by CI_{59/60} argues for correspondence to CI_b. SI₆₀, thus, has no corresponding neuron in the other body segments, at least in the locust.

Under an evolutionary perspective, the plesiomorphic condition appears to be the presence of three body wall inhibitors per segment. The prothoracic ganglion is the only ganglion of the locust in support of this hypothesis. The abdominal ganglia of the dragonfly, by contrast, regularly possess three body wall inhibitors per segment (Schmäh and Wolf, 2003b; M.S. and H.W., unpublished data). Three body wall inhibitors also seem to be present in the abdomen of bushcrickets and crickets (Consoulas and Theophilidis, 1992; Consoulas et al., 1993). Perhaps this holds even for the cricket thorax (Böser, 1999).

As indicated above, specific inhibitors may originate from common inhibitory motor neurons by restriction of their innervation field to just a single muscle. The latter may occur due to the absence of target muscles, for example, when

muscles are lost in the course of segment fusion (tagmatisation). The sturdy thoracic tagma of locusts has quite obviously entailed such muscle loss; compare, for instance, the thoracic muscle endowment of locusts (Snodgrass, 1929) and crickets with their much more moveable thorax (Du Porte, 1920). The apparent presence of three body wall inhibitors per segment in the cricket thorax (Böser, 1999; M.S. and H.W., unpublished data) seems to point in this direction. Perhaps the most plausible explanation for a possible occasional common innervation of M61 – as discussed above – would be an axon collateral of SI₆₀. That is, SI₆₀ would not just innervate M60 but send an occasional, and perhaps vestigial, collateral through N1A₂. This situation would represent the ongoing loss of inhibitory innervation in a very small muscle (M61) that spans between fused sclerites that can no longer be moved relative to each other (spine of spinasternum and sternal apodeme). This scenario appears more parsimonious than an attempt to infer some functional necessity for a specific inhibitory neuron in just one of the numerous longitudinal intersegmental muscles.

Sandra Cestnik and Tove Heller provided essential help with the backfills, Ursula Seifert finished figures and text, and Steffen Harzsch, Uwe Rose, and above all, Tobias Müller were important partners for discussion. We thank Sabine Kreissl, Werner Rathmayer and Hans-Jürgen Agricola for the donation of anti-GABA antisera.

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