The Journal of Experimental Biology 199, 2447–2450 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JEB0546

GABA-LIKE IMMUNOREACTIVITY OF AN IDENTIFIED NONSPIKING LOCAL INTERNEURONE IN THE CRAYFISH TERMINAL ABDOMINAL GANGLION

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Accepted 12 July 1996

Summary

Using an antiserum directed against γ-aminobutyric acid (GABA) to label neurones with GABA-like immunoreactivity, approximately 70 central neurones (68±9; mean ± S.E.M., *N*=9) were labelled in the terminal abdominal ganglion of the crayfish *Procambarus clarkii*. This mean number of neurones with GABA-like immunoreactivity represents approximately 10 % of the total number of neurones in the terminal ganglion. A combination of intracellular staining using Lucifer Yellow and immunocytochemical staining revealed that an identified nonspiking local interneurone (the local directionally selective interneurone, LDS) showed GABA-like immunoreactivity.

Key words: nonspiking local interneurone, local directionally selective interneurone, γ-aminobutyric acid, immunoreactivity, lateral inhibition, identified neurone, crayfish, *Procambarus clarkii*.

Introduction

Nonspiking local interneurones are widely distributed in the central nervous system of arthropods, especially insects and crustaceans (Nagayama and Hisada, 1987; Siegler and Burrows, 1979), and are one of the essential neural elements producing and controlling movements (e.g. Burrows, 1992; Nagayama *et al.* 1994). Some nonspiking local interneurones organize motor outputs while others process sensory information. In the crayfish, the local directionally selective interneurone (LDS) has bilateral arborizations restricted within the terminal (sixth) abdominal ganglion and is identifiable as a unique neurone by means of its characteristic shape and physiological properties (Nagayama and Hisada, 1988; Reichert *et al.* 1982). This interneurone is depolarized by headward water currents and was named the local directionally selective interneurone by Reichert *et al.* (1983). Branches on the soma side are input sites receiving sensory input directly from mechanosensory afferents, while branches on the opposite side of the cell body are output sites making inhibitory connections onto intersegmental ascending interneurones (Nagayama *et al.* 1994; Reichert *et al.* 1983). LDS has no measurable output effect upon the motor neurones innervating the tailfan muscles, although other nonspiking interneurones affect the activity of these motor neurones significantly (Nagayama and Hisada, 1988; Nagayama *et al.* 1984). Thus, LDS acts as a sensory integrator to mediate transverse lateral inhibition of the mechanosensory interneurones (Reichert *et al.* 1983).

γ-Aminobutyric acid (GABA) is the most widely distributed inhibitory transmitter in both the vertebrates and invertebrates (Sattelle, 1990). Immunological analyses have revealed the distribution of GABAergic neurones in the central nervous system of both insects and crustaceans (Mulloney and Hall, 1990; Watson, 1986). The somata of the inhibitory motor neurones of the lobster have far higher cytoplasmic concentrations of GABA than do the somata of excitatory motor neurones (Otsuka *et al.* 1967). Furthermore, pharmacological studies have been carried out to establish GABAergic inhibitory transmission in crayfish (El Manira and Clarac, 1994; Pfeiffer-Linn and Glantz, 1991; Takeuchi and Takeuchi, 1965; Vu and Krasne, 1993). We have, however, no information about inhibitory transmitters released from nonspiking local interneurones, in particular from LDS. In this paper, we show, for the first time, that LDS has GABA-like immunoreactivity by double marking using intracellular staining and immunocytochemical labelling.

Materials and methods

Crayfish *Procambarus clarkii* (Girard) measuring 8–12 cm in body length from rostrum to telson were used for all experiments. Details of the dissection procedure have been described previously (Nagayama *et al.* 1984). The isolated nerve chain including the last two (fifth and sixth) abdominal ganglia was fixed for 20 min in a primary fixative containing

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4 % paraformaldehyde and 0.1 % glutaraldehyde in Dulbecco's phosphate-buffered saline (DPBS, Sigma). Before fixation, the ventral ganglionic sheath in the terminal abdominal ganglion was surgically removed. The tissue was then immersed in secondary fixative containing 0.2 % picric acid and 2 % formaldehyde in DPBS at 4 °C. The tissue was washed for 30 min in 0.1 mol l^{-1} glycine in DPBS, dehydrated through an ethanol series to 70 % ethanol and stored at 4 °C overnight. The tissue was then dehydrated through an alcohol series to 90 % ethanol for 1 h, rehydrated through an ethanol series to DPBS, and preincubated in several changes of wash buffer, DPBS containing 0.3 % Triton X-100 (Sigma) and 5 % goat serum (Chemicon), for 6 h. In some preparations, LDS or the flexor inhibitor (FI) motor neurone was stained intracellularly by the injection of Lucifer Yellow (5–10 nA hyperpolarizing current pulses of 500 ms duration at 1 Hz for 15–30 min) into the isolated abdominal preparation. LDS showed spontaneous depolarizing and hyperpolarizing postsynaptic potentials of large amplitude and elicited no significant change of activity in the uropod motor neurones following depolarizing current injection (Nagayama and Hisada, 1988). FI was readily identified by its large-diameter cell body located near the midline (Wine, 1984). The gross morphology of LDS and FI was confirmed by *in situ* observations using brief blue–violet illumination from a high-pressure mercury lamp (Aonuma *et al.* 1996). After identification of stained neurones, the terminal abdominal ganglion was desheathed and the abdominal nerve chain was isolated for immunocytochemical staining.

The primary antibody, rabbit anti-GABA (Sigma) at a dilution of 1:750, was preincubated with dried crayfish nerve and muscle powder (Mulloney and Hall, 1990) for 6 h at 4 °C in wash buffer, 0.3 % Triton X-100 in DPBS. The solution was centrifuged for 10 min at 2000 ***g*** and the supernatant was collected and used. The abdominal nerve chain was incubated in the primary antibody solution for approximately 90 h at 4 °C on a rotator and then washed in several changes of wash buffer for 6 h. Anti-rabbit-IgG Cy-3 (Chemicon) at a dilution of 1:50 was used as the secondary antibody. The tissue was incubated in the secondary antibody solution for approximately 40 h at 4 °C on a rotator, then washed in several changes of wash buffer for 5 h. The tissue was then dehydrated in an alcohol series and cleared in methyl salicylate.

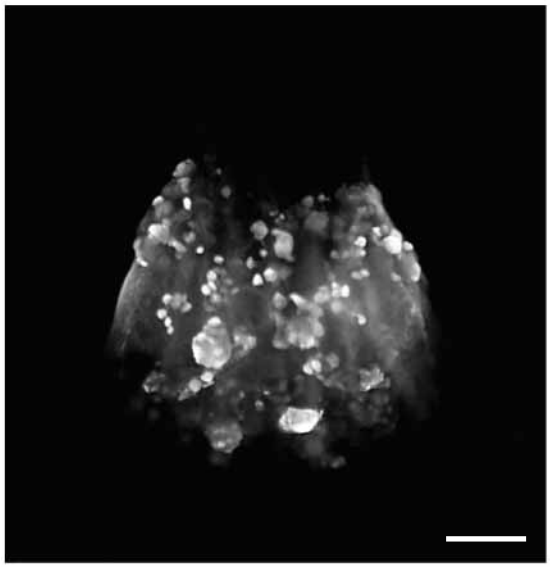

Fig. 1. Distribution of cell bodies showing GABA-like immunoreactivity in the terminal abdominal ganglion of the crayfish, viewed from the ventral side. Anterior is to the top. Note that this preparation is the same as that shown in Fig. 2 although photographed using different emission and barrier filters. Scale bar, 200 μm.

Fluorescence was detected using Zeiss or Olympus fluorescence microscopes. The light employed for excitation was passed through 450–490 nm (for Lucifer Yellow) and 510–560 nm (for Cy-3) bandpass excitation filters. The resulting fluorescence was passed through LP 520 (for Lucifer Yellow) and LP 590 (for Cy-3) barrier filters attached to the fluorescence microscope. The fluorescent images were photographed or recorded in the same focal plane using a cooled CCD video camera (Imagepoint, Photometrics) and stored on an IBM-compatible computer as files of TIF format *via* a parallel interface for later image analysis.

Results and discussion

Previous histological studies have shown that the total number of cell bodies of central neurones in the terminal abdominal ganglion of the crayfish *Procambarus clarkii* is about 650 (Kondoh and Hisada, 1986*a*; Reichert *et al.* 1982). Fig. 1 shows a typical distribution of cell bodies with GABA-like immunoreactivity in the terminal abdominal ganglion. Approximately 70 labelled cell bodies were counted on the ventral surface of the terminal abdominal ganglion (68±9; mean ± S.E.M., *N*=9). No labelled cell bodies were distributed on the dorsal surface. The mean number and distribution of labelled neurones in *Procambarus clarkii* were quite similar to those of the crayfish *Pacifastacus leniusculus* reported previously by Mulloney and Hall (1990). In their report, 67±17 neurones (mean ± S.E.M., *N*=7) showed GABA-like immunoreactivity.

To confirm the selectivity of staining against GABA, one flexor inhibitor motor neurone (FI) was stained intracellularly using Lucifer Yellow and then processed using the anti-GABA immunohistochemical procedure (Fig. 2). FI is known to possess a large cell body (approximately 100 μm in diameter) within the terminal abdominal ganglion (Fig. 2A) and has been physiologically and pharmacologically identified as a GABAergic motor neurone (Otsuka *et al.* 1967). The Lucifer-Yellow-filled cell body of FI (Fig. 2B) showed GABA-like immunoreactivity (Fig. 2C; see also Figs 1, 2A). The largest cell body in the terminal abdominal ganglion is a motor giant neurone (MoG), an excitatory motor neurone innervating the abdominal flexor muscle. The cell body of MoG is located just anterior to the cell body of FI and showed no measurable GABA-like immunoreactivity (arrowhead in Fig. 2C). The immunocytochemical staining used in this study thus selectively labelled GABAergic neurones.

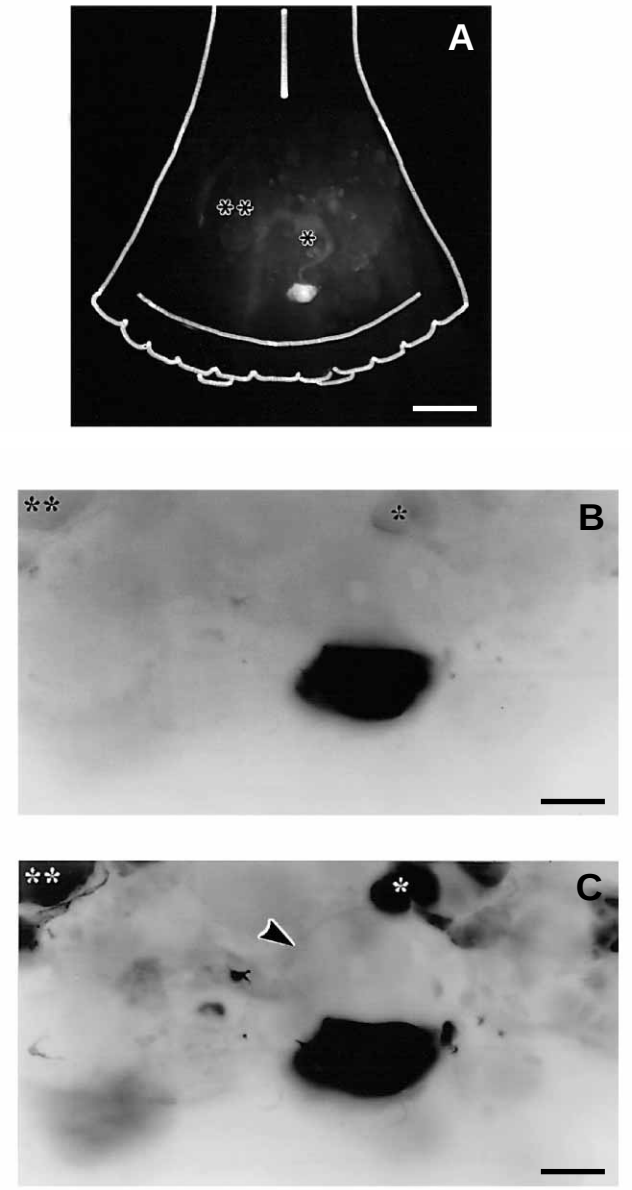

Fig. 2. GABA-like immunoreactivity of the flexor inhibitor motor neurone FI. (A) Photograph of FI viewed from the ventral side. The image is focused on the cell body of FI. The outline of the terminal ganglion is indicated in white. (B) Reversed computer image of A showing higher magnification of the cell body of FI labelled with Lucifer Yellow. (C) Distribution of cell bodies with GABA-like immunoreactivity (stained dark) in the same focal plane as in B. In each photograph, the cell bodies of two neurones used as landmarks are indicated by asterisks to show the relative position of the cell body of the Lucifer-Yellow-filled FI in the ganglion. Note that the cell body of a motor giant interneurone (MoG) (indicated by an arrowhead) showed no immunoreactivity. In B and C, the contrast was enhanced by computer image analysis. Scale bars: A, 200 µm; B, C, 50 µm.

The local directionally selective interneurone LDS is a bilateral nonspiking local interneurone in the terminal abdominal ganglion (Fig. 3A). One of the most characteristic features of LDS is its thick transverse process leading to extensive bilateral branches. In three preparations, LDS was

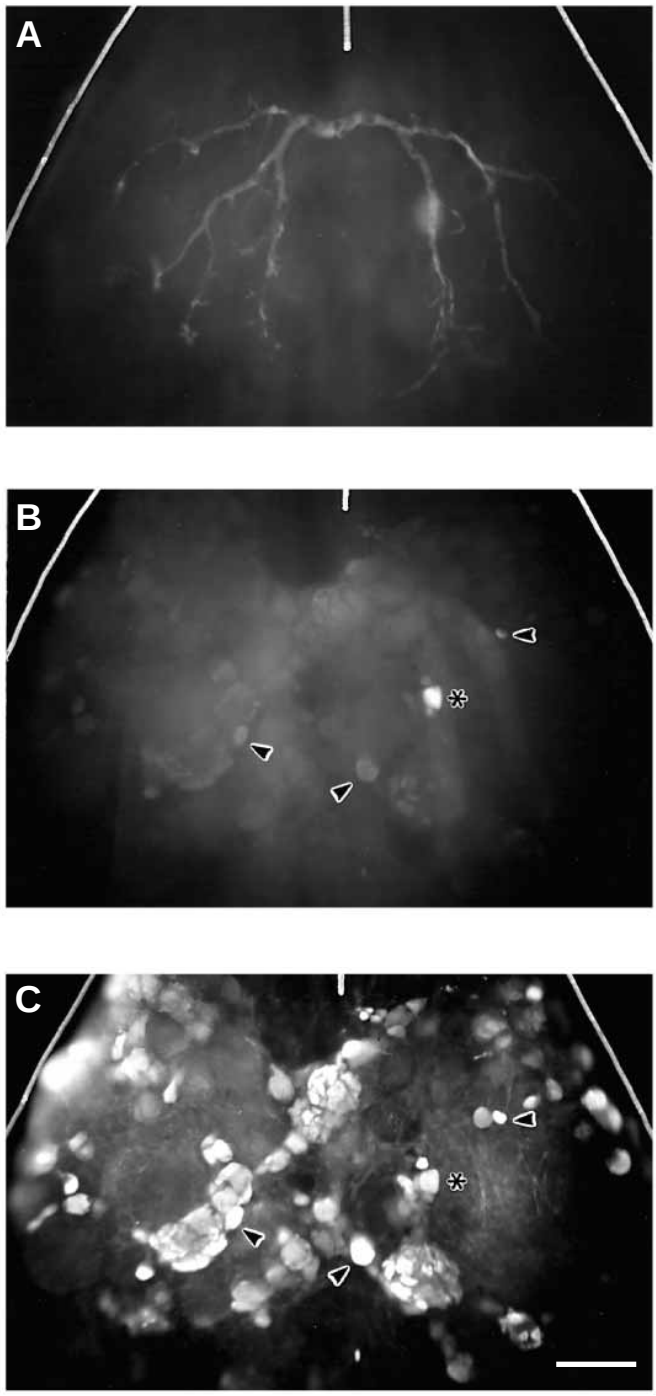

Fig. 3. GABA-like immunoreactivity of the local directionally selective interneurone LDS. (A) Morphology of LDS viewed from the ventral side. The image is focused on the transverse process of LDS leading to bilateral branches. Anterior is to the top. The vertical white line indicates the midline of the ganglion. (B) Cell body of LDS labelled by intracellular injection of Lucifer Yellow. (C) Distribution of cell bodies with GABA-like immunoreactivity in the same focal plane as in B. The terminal ganglion is outlined in white. The cell body of LDS in B and C is indicated with an asterisk, and the cell bodies of three neurones used as landmarks are indicated by arrowheads at different angles to show the relative position of the cell body of the Lucifer-Yellow-filled LDS in the ganglion. Scale bar, 100 µm.

identified by intracellular injection of Lucifer Yellow and subsequently treated with immunocytochemical staining against GABA. The Lucifer-Yellow-labelled cell body of LDS, which ranged in diameter between 20 and 30 μm, was located on the ventral surface of the medial portion of the ganglion near the midline (asterisk in Fig. 3B). This Lucifer-Yellow-filled cell body of LDS was also labelled by immunocytochemical staining against GABA (indicated by an asterisk in Fig. 3C). In all preparations, the cell body of LDS showed GABA-like immunoreactivity.

This study, therefore, strongly suggests that LDS is a GABAergic interneurone and that its synaptic interactions are mediated by GABA. In a previous study, depolarizations of LDS caused membrane hyperpolarization in several ascending interneurones in a graded manner dependent upon the membrane potential change in LDS (Nagayama *et al.* 1994). Moreover, numerous synaptic vesicles have been observed on branches on the output site of LDS (Kondoh and Hisada, 1986*b*). These observations support the proposed chemical nature of the action of LDS. This is also supported by a preliminary study in which local injection of GABA into the neuropile of the crayfish mimicked the membrane hyperpolarization of ascending interneurones elicited by contralateral sensory stimulation (H. Miyata and T. Nagayama, unpublished data).

This study is the first to demonstrate that GABA is a neurotransmitter of an identified nonspiking local interneurone, LDS. Further pharmacological studies are now needed to characterize the GABAergic nature of the lateral inhibition from LDS to ascending interneurones.

We are grateful to Professor Brian Mulloney for his kind advice on immunocytochemical staining techniques. We are also grateful to Dr Philip L. Newland for his critical reading of this manuscript. T.N. was supported by a grant (08640856) from the Ministry of Education, Science and Culture.

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