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SHORT COMMUNICATION

ULTRASTRUCTURAL EVIDENCE FOR GABAERGIC INPUT ONTO CERCAL AFFERENTS IN THE LOCUST (*LOCUSTA MIGRATORIA*)

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Ultrastructural studies have revealed input synapses on the central terminals of many sensory afferent neurones in both vertebrates (Maxwell *et al.* 1982; Ralston *et al.* 1984) and invertebrates (Altman *et al.* 1980; Watson and Pflüger, 1984). Indeed presynaptic modulation of sensory information flowing into the central nervous system appears to be a very widespread phenomenon. It may take the form of either inhibition (Eccles, 1964) or facilitation (Klein and Kandel, 1980), but it is the former that is most widely known from physiological experiments. Several mechanisms can bring about presynaptic inhibition. (1) Depolarization of an afferent terminal may reduce the amplitude of the action potential, leading to a reduction of the calcium influx and, consequently, of transmitter release (Miledi and Slater, 1966; Blagburn and Sattelle, 1987). (2) A conductance increase in the terminal may reduce the height of an action potential regardless of the direction of the potential change (Baxter and Bittner, 1981; Hue and Callec, 1983) and block spike conduction through small-diameter axonal branches (Atwood, 1976). The most widely suggested mechanism for this in both vertebrates (Nicholl and Alger, 1979) and invertebrates (Kennedy *et al.* 1980) is an increase in chloride conductance mediated by GABA. (3) The presynaptic calcium current may be reduced by the direct action of a neurotransmitter (Shapiro *et al.* 1980).

In the insect nervous system the sensory afferents of cercal hairs have proved important for the study of presynaptic inhibition. The cerci project posteriorly from the last segment of the abdomen and are covered with sensory hairs. Filiform hairs are very sensitive to air currents and even to low-frequency sound (Plummer and Camhi, 1981; Kämper, 1984). Their afferents run into the terminal ganglion where they synapse with giant interneurones that ascend the ventral nerve cord (Shankland and Goodman, 1982; Boyan *et al.* 1986). Wind stimuli to the cerci can initiate running, jumping or flying in various insects as part of escape behaviour (Huber, 1965; Camhi *et al.* 1978; Boyan *et al.* 1986). For the sensory input evoking

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such vital behaviour to be interpreted unambiguously, it is necessary that hair displacement brought about by air currents is distinguishable from that caused by movement of the cercus. In the locust, filiform afferents are inhibited by presynaptic depolarization during passive displacement of the cercus (Boyan, 1988). This is thought to be evoked by the activity of a stretch receptor at the base of the cercus acting *via* an unidentified interneurone. In the cricket, sensory hair afferents are also presynaptically inhibited by the activity of other afferents belonging to hairs with different directional sensitivity (Levine and Murphey, 1980). This sharpens the directional sensitivity of the giant interneurones and guides the escape response in an appropriate direction. A similar interaction between cercal afferents occurs in first-instar cockroach nymphs (Blagburn and Sattelle, 1987).

The insect terminal ganglion contains numerous neurones immunoreactive for GABA (Jacobs *et al.* 1985; Watson and Pflüger, 1988; Bernard and Thomas, 1988) and this transmitter appears to mediate at least some of the presynaptic inhibition of the cercal afferents (Hue and Callec, 1983). In vertebrates, immunocytochemistry has been used in conjunction with anterograde degeneration (Barber *et al.* 1978) or intracellular staining (Maxwell and Noble, 1987) to demonstrate that sensory afferent terminals receive inputs from processes that are labelled with antibodies against glutamate decarboxylase, the enzyme that catalyses the synthesis of GABA. No comparable investigations have been carried out on invertebrates.

The objectives of the present study are twofold. (1) To demonstrate that filiform hair afferents in the locust receive presynaptic input. (2) To discover whether some or all of the presynaptic neurones are immunoreactive for GABA.

Adult locusts (*Locusta migratoria*) from a crowded culture at the Australian National University were restrained and the cerci immobilized with cyanoacrylic glue. A Vaseline well was made around hairs on the lateral face of the cercus and filled with distilled water. Four to six long filiform hairs were cut at their bases and the water replaced with 5 % horseradish peroxidase (HRP) (Sigma type VI) in 0.2 mol l^{-1} Tris buffer (pH 8.0). The well was sealed with Vaseline and the insects kept in a moist atmosphere at 4°C for 4 days followed by 2 days at room temperature. Terminal ganglia were then fixed *in situ* for 5 min in 5 % glutaraldehyde in 0.05 mol l^{-1} phosphate buffer (pH 7.4) containing 6.8 g of sucrose per 100 ml. After excision they were further fixed for 2 h and reacted with diaminobenzidine (DAB) according to the method of Watson and Burrows (1981). Briefly, after washing in phosphate and Tris buffer, the ganglia were immersed in 0.5 mol l^{-1} $CoCl_2$ in Tris buffer and after further washing incubated for 1 h at 37°C in 10 ml of phosphate buffer containing DAB (5 mg), β-D-glucose (20 mg), ammonium chloride (4 mg) and glucose oxidase (Sigma type V, 6 units). The ganglia were fixed in OsO_4, block-stained with uranyl acetate, dehydrated in alcohol and embedded in L.R. White resin. Ultrathin sections through the afferent terminals were picked up on pioloform-coated nickel slot grids and stained using antibodies against GABA (Sera Laboratories) in an immunogold procedure (see

Watson, 1988, for details). Unetched sections were washed and floated on droplets of 5% goat serum in Tris buffer for 30 min, primary antiserum (1/800) for 2 h, washed again and transferred to 15 nm gold-labelled goat antirabbit antiserum (Janssen, 1:15) for 1 h. After further washing the grids were contrasted with uranyl acetate and lead citrate. The immunogold labelling could be abolished by preabsorption of the primary antiserum with a GABA–BSA conjugate (see Watson, 1988).

Ultrastructural examination of sections through the terminals of the filiform hair afferents reveals numerous synapses upon them from processes containing small agranular synaptic vesicles (Fig. 1). Some processes presynaptic to the afferent terminals show immunogold labelling (Fig. 1A,C) though this is quite light compared with processes in ganglia of control animals, possibly due to the effects of the prolonged immobilization at low temperatures required for the backfills or to the histochemical processing of the HRP. Because of the lightness of labelling, a statistical test was used to demonstrate that immunoreactive processes could be clearly distinguished from background labelling. Gold particles over 16 immunoreactive processes were counted and their density calculated per unit area. For comparison, particles were counted in 16 1-μm^2 areas taken at random from the same micrographs. The density of particles in immunoreactive processes was $42.4 \pm 14.0\,\mu m^{-2}$ (mean±standard deviation) which is significantly greater ($P<0.001$, unpaired t-test) than background levels ($7.1 \pm 2.73\,\mu m^{-2}$). Labelling was highly consistent from section to section, as can be seen by comparison of two non-adjacent sections (Fig. 1A,B).

The vesicles in the immunoreactive presynaptic processes have a diameter of approximately 32–40 nm. Some of the non-immunoreactive neuropilar processes (including the one presynaptic to the hair afferent in Fig. 1D) contain vesicles that are larger, being about 37–53 nm in diameter. This is consistent with ultrastructural observations of GABA-immunoreactive processes in the thoracic ganglia of *Schistocerca*, in which vesicles were smaller than those in unlabelled processes (Watson, 1988). Flattened agranular vesicles have often been taken as indicators of the presence of GABA (Uchizono, 1965, 1967). However, vesicle shape can vary according to the tonicity of the fixative (Tisdale and Nakajima, 1976) and it will be noted that in this preparation the larger agranular vesicles in the unlabelled processes of Fig. 1C also exhibit some flattening. Furthermore, it has recently been reported that, even within the same tissue, GABA-immunoreactive processes may differ in their vesicle content (Hamori and Takacs, 1989), demonstrating the importance of immunocytochemical support for the nature of the transmitter.

The presence of non-immunoreactive inputs (Fig. 1D) onto the terminals suggests that at least two presynaptic effects may be experienced by the afferents. There is some physiological support for this. Hue and Callec (1983) have presented evidence for a presynaptic inhibition of cercal afferents in the adult cockroach that can be blocked by picrotoxin or low-chloride saline. Using a mannitol gap technique on the cercal nerve they also demonstrated that GABA

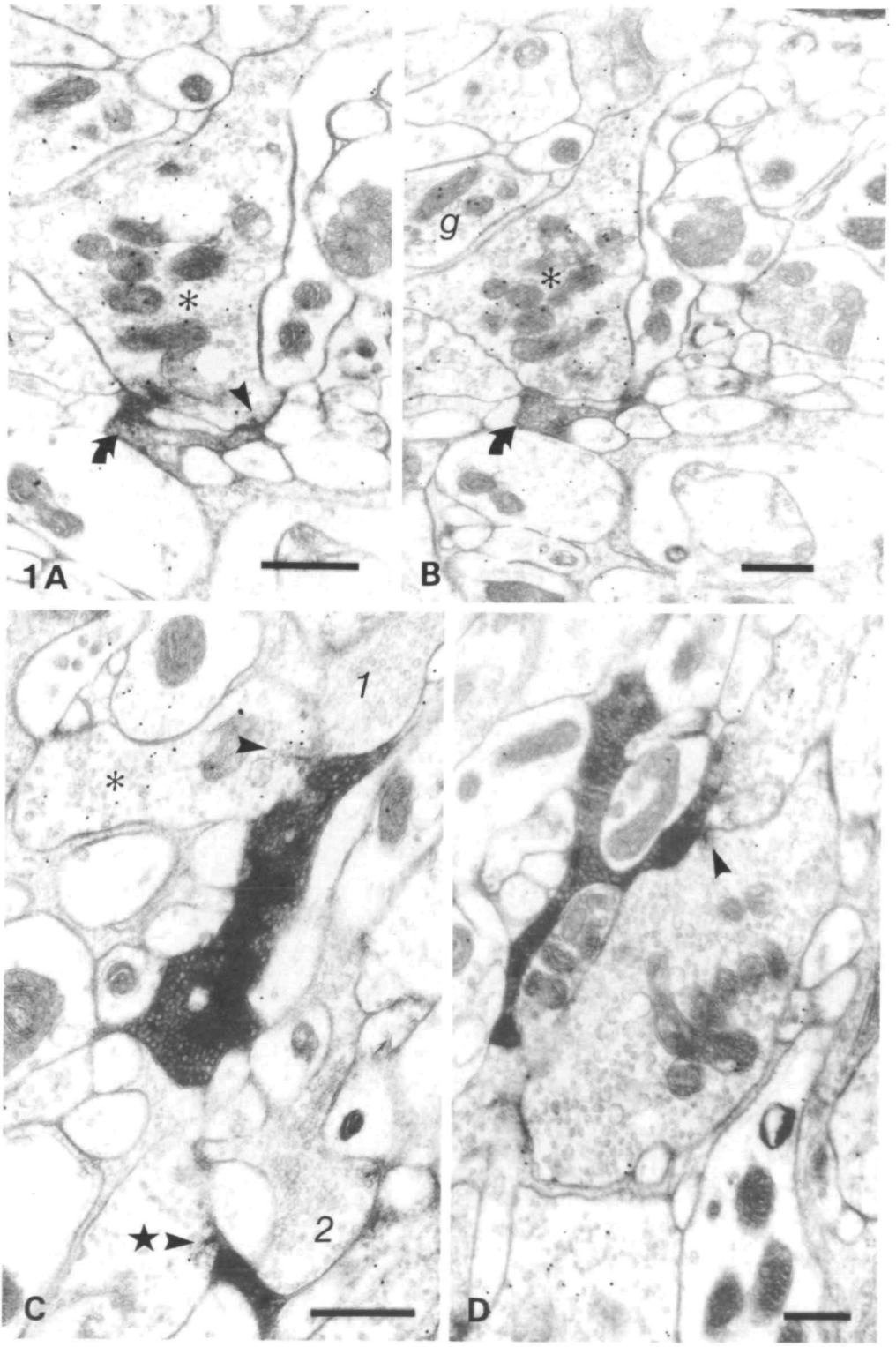
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Fig. 1. (A,B). Two non-adjacent sections through a process showing GABA-like immunoreactivity (asterisk) which makes a synapse (arrowhead in A) with an HRP-labelled filiform hair afferent (curved arrow). Note the consistency of gold particle distribution between the two sections. In B, which is at lower power to show the level of background labelling, a second immunoreactive process is visible (*g*). (C) An HRP-labelled afferent receives an input (arrowhead) from a process immunoreactive for GABA (asterisk) while a smaller afferent branch receives an input (arrowhead) from a non-immunoreactive process with similar synaptic vesicles (star). Note that larger agranular vesicles in two unlabelled processes (*1* and *2*) show some flattening. (D) An HRP-labelled afferent receiving an input synapse (arrowhead) from an unlabelled process containing larger and rounder agranular vesicles than are seen in labelled processes. Scale bars, 0.5 μm.

induced a chloride-dependent hyperpolarization. In an intracellular analysis of presynaptic inhibition between two identified afferents in the first-instar cockroach, Blagburn and Sattelle (1987) observed inhibitory postsynaptic potentials in the afferents. However, in this case the IPSPs had no effect on spike height or synaptic transmission. The effect of one afferent upon the other was due to a depolarization whose reversal potential was probably more positive than −35 mV and therefore unlikely to be due to chloride ions.

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