Input and Output Synapses on Identified Motor Neurones of a Locust Revealed by the Intracellular Injection of Horseradish Peroxidase

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Summary. Physiologically characterised motor neurones in the thoracic ganglia of the locust were injected with horseradish peroxidase in order that the spatial relationship between their input and output synapses could be observed with the electron microscope. A modification in the development procedure for the peroxidase ensured that the internal fine structure of the stained neurones was not obscured by the diaminobenzidine reaction product. Input and output synapses may occur within 1 µm of each other on the neuropilar processes of the motor neurones. This supports physiological evidence that motor neurones may be involved in local circuit interactions within the thoracic ganglia.

Key words: Horseradish peroxidase - Locust - Motor neurone - Synapse - Ultrastructure.

The discovery of the existence of serial and reciprocal synapses on invertebrate neurones has been seen as evidence for their involvement in local circuit interactions of the type previously reported in vertebrates (for review see Pearson 1979). Such interactions are mediated by graded potentials and may be confined to discrete regions of a neurone. Reciprocal and serial synapses in vertebrates are generally observed on interneurones (Shepherd 1979) but in invertebrates they have also been reported on the central processes and axons of some motor neurones (King 1976, 1977). This suggests that invertebrate motor neurones function not only to control the force produced by the muscles but also as integrative elements in local circuits within the central nervous system. Physiological studies on the thoracic ganglia of locusts have shown that spikes in leg and flight motor neurones can have effects on other neurones within the ganglia (Burrows 1973; Hoyle and Burrows 1973). We report here that these motor neurones have closely apposed input and output synapses which could support such interactions. The individual physiologically characterised motor neurones were injected with horseradish peroxidase so that they could be identified in the electron microscope. The
modification of this method used here has several advantages over other techniques for the ultrastructural labelling of neurones.

Materials and Methods

Glass microelectrodes were made from thin-walled capillary tubing (O.D. 1.0 mm, I.D. 0.9 mm) containing a glass fibre. They were filled with a solution of 4% horseradish peroxidase (HRP) (Sigma VI) in 0.2 M Tris buffer (pH 7.4) with KCl added to 0.5 M. Their D.C. resistance was 30–40 MΩ in saline.

The locust, *Schistocerca americana gregaria* (Dirsh), was prepared for recording from neurones in the mesothoracic or metathoracic ganglia according to the methods previously described (Hoyle and Burrows 1973; Burrows 1975). The thorax was perfused with saline (Usherwood and Grundfest 1965) to which was added 90 mM sucrose. Electrodes were driven across the sheath of the ganglion to record intracellularly from the cortically placed somata of the neurones. Motor neurones were identified according to well-established criteria (Hoyle and Burrows 1973; Burrows 1975). Those studied here innervate the dorso-ventral flight muscles in the meso- or metathoracic segments, and extensor and flexor muscles of the tibia of a hind leg. A motor neurone which had been unequivocally characterized according to variety of physiological criteria was intracellularly injected with HRP for 50 min by passing 5 nA of positive current in 500 ms pulses every second. At the end of the injection period, the synaptic and spike potentials were only slightly smaller than those initially recorded, permitting further physiological tests to confirm the identity of the neurone. After filling the neurone, 1 h was allowed for the HRP to diffuse before fixation for 2 h in 2.5% glutaraldehyde in 0.05 M phosphate buffer pH 7.4 containing 6.8 g sucrose/100 ml at 4°C. After 10 min in the fixative the ganglion was bisected. The half-ganglion was then washed in the same sucrose phosphate buffer and transferred to 0.2 M Tris buffer (pH 7.4) at room temperature before being placed in 0.5% cobalt chloride in Tris buffer for 10 min (Adams 1977). It was then washed in two changes of Tris buffer, returned to the phosphate buffer and trimmed to a size commensurate with the expected distribution of known processes of the neurone. This was necessary as the glucose oxidase reaction medium did not penetrate satisfactorily over distances greater than 0.5 mm. The ganglion was then placed for 1–2 h at 37°C in an incubation medium of 0.05 M phosphate buffer (without sucrose) 50 ml, diaminobenzidine (DAB) 25 mg, ammonium chloride 20 mg, β-D-glucose 100 mg, glucose oxidase (Sigma V) 20–30 units. After incubation the cell body of the injected neurone is black and the neurites reddish-brown. It is possible at this stage to observe the gross structure of the neurone with the light microscope and thereby determine the extent of the labelling and confirm that the desired neurone has been stained. This is followed by osmication (1% in phosphate buffer with sucrose, pH 7.4) for 1 h, uranyl acetate (2% aqueous) block staining, rapid ethanol dehydration and embedding in Spurr’s resin. Thin sections were stained with 2% uranyl acetate and Reynold’s lead citrate and examined with a Philips 200 electron microscope.

Results

The main neurite and some smaller branches of an injected neurone are visible in 0.5–1 μm thick unstained sections in the light microscope (Fig. 1). When the labelled neurone is examined with the electron microscope the cytological details of the neurite (Fig. 2) and its fine branches (Figs. 3 and 4) are clear, while the distinction between these and unlabelled processes in the neuropil is unequivocal. By contrast, in neurones similarly injected with 4% HRP but treated with hydrogen peroxide and DAB, the main neurite could only with difficulty be distinguished from adjacent unlabelled ones. No smaller processes were observed to be labelled.

By tracing labelled processes of neurones in glucose oxidase treated ganglia, through serial sections we conclude that the stain is confined to one neurone. We have no evidence to suggest transport of the stain across synapses to other neurones. No non-specific labelling was observed in ganglia incubated in DAB without HRP injection.