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ADIPOKINETIC HORMONE STIMULATES NEURONES IN THE INSECT CENTRAL NERVOUS SYSTEM

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Summary

A simple preparation designed to screen and compare the central action of putative neuroactive agents in the moth *Manduca sexta* is described. This approach combines microinjections into the central nervous system with myograms recorded from a pair of spontaneously active mesothoracic muscles. Pressure injection of either octopamine or *Manduca* adipokinetic hormone (M-AKH) into the mesothoracic neuropile increases the monitored motor activity. Under the conditions used, the excitatory effects of M-AKH exceed those of the potent neuromodulator octopamine. This suggests that M-AKH plays a role in the central nervous system in addition to its known metabolic functions and supports recent evidence that neuropeptides in insects can be multifunctional.

Key words: neuromodulation, peptide, octopamine, microinjection, thoracic ganglia, flight muscle, *Manduca sexta*.

Introduction

Improvements in purification techniques along with new approaches using molecular biology have led to a steady increase in the number of putative chemical messengers characterized in insects. This progress has been most pronounced for neuropeptides that may act as transmitters, modulators or hormones controlling various metabolic, developmental and behavioural events (Adams, 1990; Nagasawa, 1993). Despite our rapidly growing knowledge concerning insect neuropeptides, the exact functions of peptides in the central nervous system (CNS) of insects remain to be clarified. Here we introduce a preparation designed to test the central action of putative neuroactive substances within the CNS of an established experimental animal, the lepidopteran *Manduca sexta*. The significance of this approach is exemplified by comparing the effects of octopamine, a biogenic amine well known for its strong modulatory effects on the CNS of arthropods (Evans, 1985; Orchard *et al.* 1993), with *Manduca* adipokinetic hormone (M-AKH), a peptide primarily viewed as a neurohormone controlling energy metabolism (Ziegler *et al.* 1990).

Octopamine is regarded as the invertebrate counterpart of noradrenaline and plays a key role in modulating behaviour and nervous functions (Evans, 1985; Orchard *et al.* 1993). For example, it increases the level of excitation in flight motor circuits in the hawkmoth *Manduca sexta* (Claassen and Kammer, 1986), initiates motor activity such as walking and flying in locusts (Sombati and Hoyle, 1984) and affects the escape circuitry of cockroaches (Pollack *et al.* 1988).

The adipokinetic hormones (AKHs) are a family of structurally related peptides that occur widely in insects (Gäde, 1990). AKHs regulate the release of stored energy (Orchard, 1987). In locusts and lepidopteran insects, AKHs are produced by the intrinsic neurosecretory cells of the corpora cardiaca and released into the circulatory system to mobilize lipid from the fat body for flight (Ziegler *et al.* 1990; Goldsworthy, 1983; O'Shea and Rayne, 1992). In addition, other functions have been ascribed to peptides of the AKH family, including acceleration of the heartbeat in the cockroach *Periplaneta americana* (Baumann and Gersch, 1982), stimulation of cytochrome haem synthesis in *Blaberus discoidalis* (Keeley, 1978) and inhibition of protein synthesis in crickets (Cusinato *et al.* 1991).

Materials and methods

Male and female *Manduca sexta* (L.) were selected from a laboratory colony. After cold anaesthesia, the legs and the wings were amputated and the moth was waxed to a holder with its ventral side up. A window was cut in the ventral thoracic cuticle and the underlying tissues were carefully removed, exposing the pterothoracic ganglion.

Putative neuroactive substances and other agents were ejected from a multi-barrelled pipette into the mesothoracic

neuropile (Fig. 1). These pipettes were pulled from triple-barrelled capillary tubing (WPI) using a vertical puller (David Kopf Instruments) to a total tip diameter of 5–10 μm. After filling each barrel with a different solution, PE tubes were placed into the shanks, glued in place and connected to three-way valves. Each valve could be independently regulated and the switching of the valves was manually controlled. The pressure required for injection varied between 0.1 and 0.4 MPa (1–4 bar) and was produced using syringes filled with silicone oil.

All agents were dissolved in insect saline with a pH of 6.9 (Itagaki and Hildebrand, 1990), which also served as the control. Solutions used for the experiments were 5×10^{-2} mol l^{-1} octopamine (DL-octopamine, Sigma), 5.6×10^{-4} mol l^{-1} synthetic *Manduca*-AKH (pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-NH_2, Peninsula Laboratories, CA, USA) and 5×10^{-2} mol l^{-1} phentolamine methanesulphonate (Aldrich). The calculated amounts of the injected substances were derived from the concentration of the solution in the micropipette and the injection volume. The injection volume was calculated from the injection pressure, the duration of the injection and the diameter of the pipette.

To monitor the motor activity, steel wire electrodes (40 μm diameter) were implanted through holes in the cuticle into the right and left middle unit of the II PD 2 muscle. A silver wire in the abdomen served as the indifferent electrode. The middle unit of the mesothoracic third axillary muscle is involved in flight steering. Extracellular recordings from this muscle in resting moths show that it is spontaneously active, but also sensitive to various external and internal changes (Rheuben and Kammer, 1987). Stimulating the animals with optomotor patterns elicits responses in this muscle that are directionally selective (Wendler *et al.* 1993). Because this muscle unit is innervated by a single motoneurone, the frequency of the recorded muscle potentials is identical with the spike frequency of the motoneurone.

Recordings were amplified, displayed and stored using standard equipment and the data were evaluated with specially written software (Wendler *et al.* 1993).

Results

Injection of either octopamine or synthetic *Manduca*-AKH into the circulatory system of *M. sexta* fails to change the spontaneous activity or the directional selectivity of the motor response. This is true despite using 10^3–10^5 times the total octopamine found in the haemolymph (Davenport and Wright, 1986) and 10^3–10^5 times more M-AKH than required for an adipokinetic response in adults (Ziegler, 1990). These results probably demonstrate the effectiveness of a 'blood–brain barrier' because pressure injections into the mesothoracic ganglion resulted in strong responses in the muscle. Following injections of octopamine into the neuropile invaded by the dendrites of the monitor muscle's motoneurone (Fig. 1), clear responses could be obtained. The spontaneous activity (approximately 2 Hz) was initially reduced and later exhibited a continuous increase (Fig. 2A). Additionally, adjacent flight muscles that are normally silent in resting moths became active for a short period after the injection of octopamine. These results support an earlier study on the effect of octopamine on the thoracic ganglia and the flight motor in *Manduca sexta* (Kinnamon *et al.* 1984). The excitatory response to octopamine, as well as the spontaneous activity, could be completely abolished by injection of the octopamine antagonist

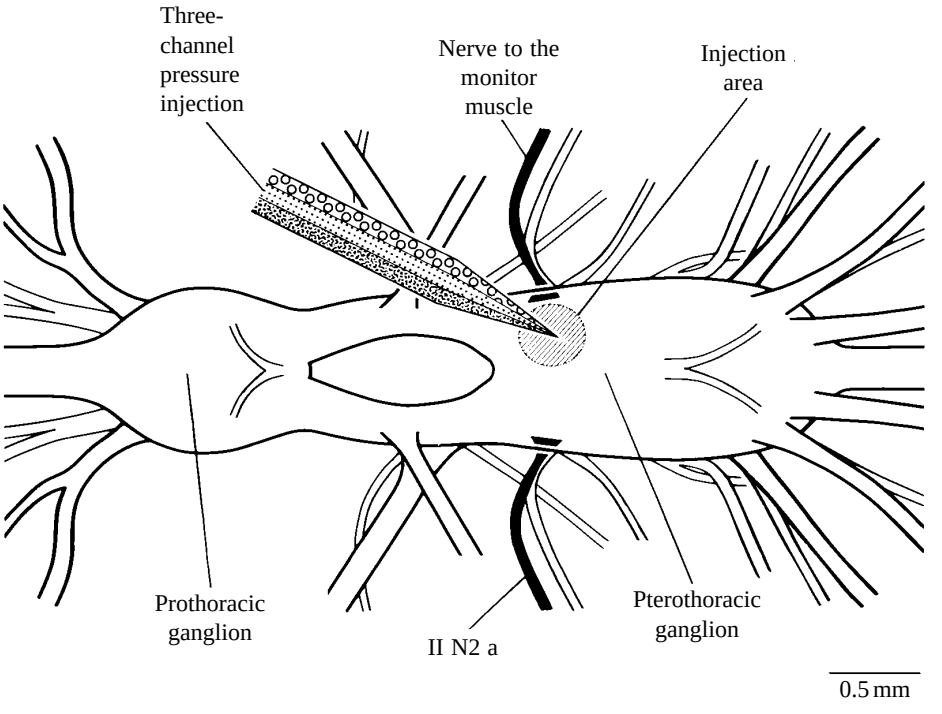

Fig. 1. Thoracic central nervous system of *Manduca sexta* with peripheral nerve roots, dorsal view. The location of the pressure injections is indicated for the right-hand side of the mesothoracic ganglion. This area of neuropile contains the dendrites of the motoneurone that innervates the recorded monitor muscle *via* nerve II N2 a. Anterior is to the left.

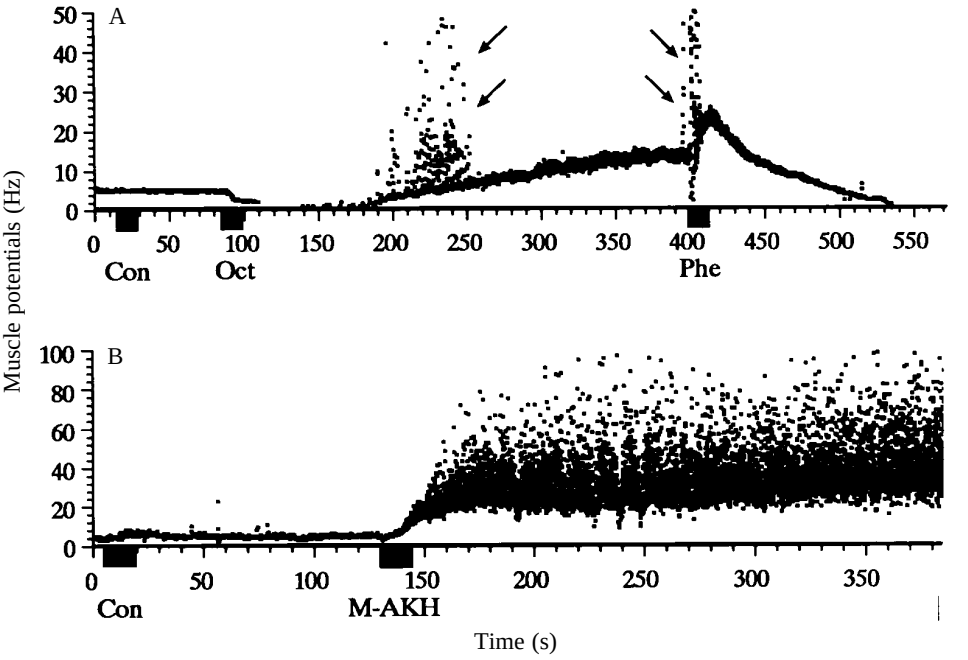

Fig. 2. Typical responses to the applied substances shown as frequency histograms of the electrical activity recorded from the monitor muscle ipsilateral to the injection site in the CNS. (A) Injection of 9×10^{-13} mol of octopamine (Oct) and 9×10^{-13} mol of phentolamine (Phe). (B) Injection of 1×10^{-15} mol of *Manduca*-AKH (M-AKH). The black boxes on the abscissa indicate the times and durations of the injections. All substances were dissolved in a saline that also served as the control (Con). Arrows indicate phases where motor units adjacent to the monitor muscle became active for a short period.

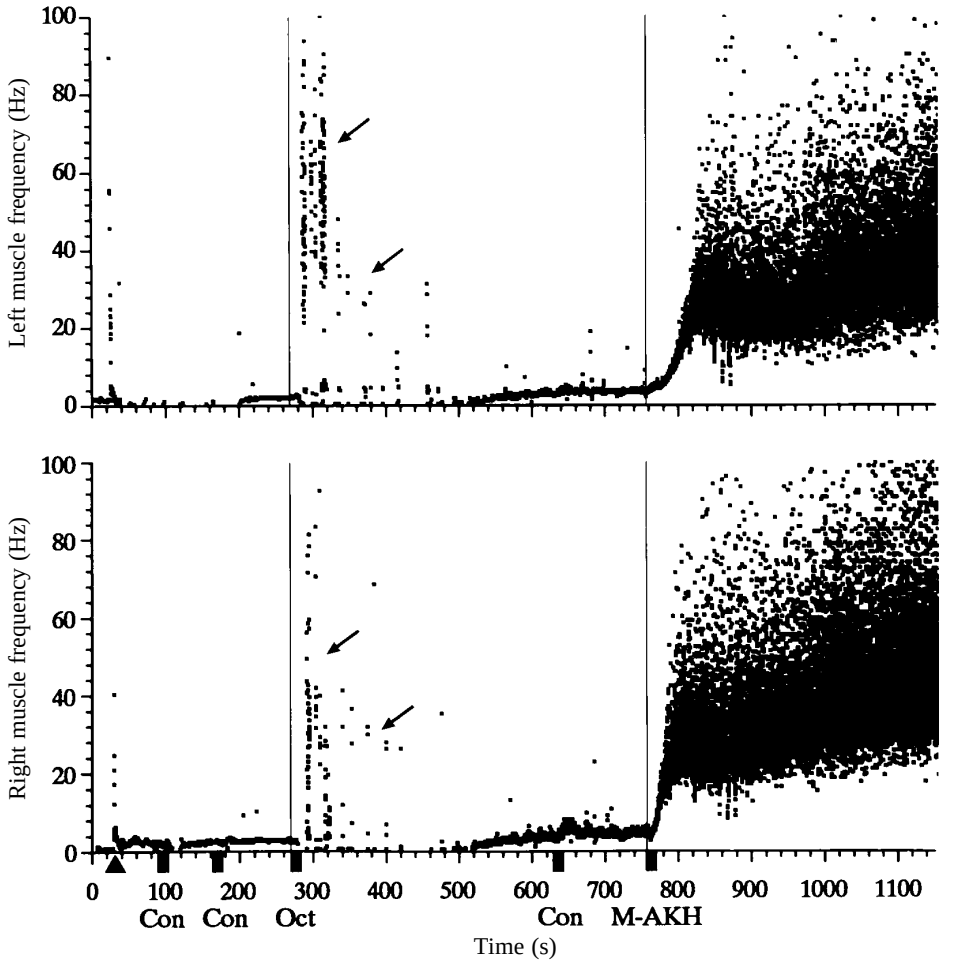

Fig. 3. Direct comparison of the responses to octopamine and M-AKH. The frequency histograms are for the bilateral pair of monitor muscles in the same individual. Injections were made exclusively on the right side of the CNS. The typical response patterns, however, appeared on both sides. The excitatory effect of 1×10^{-15} mol of M-AKH exceeded that of 9.2×10^{-14} mol of octopamine. The black triangle indicates the time of penetration of the ganglion and the vertical lines mark the onset of the injections. All other abbreviations and symbols are the same as in Fig. 2.

phentolamine methanesulphonate, which initially led to a further increase in activity and a brief activation of adjacent motor units (Fig. 2A). Control injections with insect saline failed to elicit any changes.

Although administered in lower doses than octopamine, M-AKH induced even greater activity in the monitor muscle, with the spontaneous activity becoming more irregular (Fig. 2B). To verify this observation and to minimize the influence of factors such as deviations from the optimal injection site or different conditions of individual test animals, triple-barrelled microcapillaries were used, which permitted successive tests of octopamine, M-AKH and saline at the same location in the same individual (Fig. 3). In all experiments, the excitatory response following M-AKH injection was stronger than the one elicited by octopamine. In contrast to octopamine, the effects of M-AKH were clearly restricted to the monitor muscle.

The strength, timing and duration of the responses were dependent on the concentrations of the substances, the duration of the injection and the injection site in the ganglion. In most cases, the activity changes also appeared within 20–50 s on the side contralateral to the injection site (Fig. 3). The shortest latency observed was approximately 10 s for injections of M-AKH and 8 s for octopamine.

There was no indication that the marked increase in motor activity induced by M-AKH in resting animals was due to damage to the motoneurones. Comparably high frequencies have been measured in the monitor muscles during tethered flight (Suder, 1994), and in all experiments the activity returned to the spontaneous level after some minutes. Octopamine and M-AKH both completely altered the directional selectivity of the motor response when the moth was stimulated with optomotor patterns.

Discussion

The muscles that were employed to monitor motor output have proved to be well suited to detect and quantify changes of neural activity following the injection of test substances into the haemolymph or the thoracic neuropile. With pressure injections at multiple sites in the nervous system, further targets for the test substances could be localized at different levels of the neuronal circuitry between the sensory systems and the thoracic motor system.

Our study clearly demonstrates strong effects of M-AKH in the CNS of *M. sexta*. This is supported by a recent study on neurohormone D, a member of the AKH family, which has been shown to elicit an increase in the spontaneous spike frequency of dorsal unpaired neurones isolated from the terminal ganglion of the cockroach (Wicher *et al.* 1994). There remain, however, several unanswered questions. M-AKH is thought to be exclusively synthesized in the corpora cardiaca (Ziegler *et al.* 1991). It is rather unlikely that M-AKH released from the corpora cardiaca into the haemolymph could reach targets in the mesothoracic neuropile. Injections into the circulatory system failed to elicit an effect in the monitor muscle. It seems more likely that peptidergic neurones within the ganglia are a source for M-AKH. Preliminary studies using an antibody against locust-AKH resulted in the labelling of three somata in the pterothoracic ganglia. Peptides can occur in multiple forms in a given species and, at present, we cannot exclude the possibility that the observed responses in the CNS could be due to M-AKH mimicking an AKH-like peptide not yet identified. Preliminary receptor-binding studies with crude membrane preparations from the pterothoracic ganglion in *Manduca sexta* indicate that M-AKH might be specifically bound. The concentration of binding sites, however, appears to be very low; only about 10 % of that found on fat body membranes. This result could indicate that there are few cells in this ganglion which respond to M-AKH. It does not exclude the possibility, however, that the active agent in the ganglion is not M-AKH, but an M-AKH-like substance. This problem will be further investigated by the use of synthetic M-AKH analogues, which should also provide some information on the characteristics of the receptor.

Although the neuronal targets for the injected substances within the ganglion remain unknown, a few conclusions can be drawn from the bilateral responses elicited by a single injection. Latencies of less than 10 s between the effect on the ipsilateral and contralateral monitor muscles appear to be too rapid to be accounted for by diffusion of the injected agents to the contralateral side of the ganglion and, together with the low doses required, seem to argue that M-AKH and octopamine may act upon bilateral premotor networks rather than upon the motoneurone itself. However, the strength and duration of the contralateral response is always diminished compared with those of the injection side, which would be expected for effects mediated by diffusion across the ganglion. An exception is the short-term activation of adjacent flight muscles by octopamine. In this case, a bilateral network seems to become activated because the effects have the same strength on both sides and appear simultaneously (Fig. 3).

Preliminary studies with another peptide (from the FLRFamide family) on the monitor muscles have revealed strong inhibitory effects, thus underlining the usefulness of this preparation in studying the action of other neuroactive substances in the CNS of an insect.

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