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CENTRAL NERVOUS PROCESSING OF SEX PHEROMONES IN TWO STRAINS OF THE EUROPEAN CORN BORER *OSTRINIA NUBILALIS* (LEPIDOPTERA: PYRALIDAE)

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Summary

Antennal lobe neurones were investigated in the pyralid moth *Ostrinia nubilalis* using intracellular recording and staining techniques. Response characteristics of antennal lobe neurones from males in the so-called E and Z strains, in F_1 hybrids and in parental backcrosses were studied. The antennal lobe of a male *O. nubilalis* comprises approximately 30 ordinary glomeruli and three enlarged glomeruli making up the macroglomerular complex (MGC). Receptor neurones enter the antennal lobe *via* the antennal nerve and arborize in single glomeruli. Intracellularly stained, pheromone-responding projection neurones in both parental strains arborized in different glomeruli within the MGC, irrespective of their response characteristics. Neurones were grouped according to their specificity to single pheromone components and to pheromone blends. Component-specific, blend-specific and generalist neurones were found. Specificity only occurred at low stimulus concentrations and disappeared as concentrations increased. Although all neuronal types were present in both pheromone strains and crossings, differences in abundance and sensitivity were found. In the parental strains, neurones responding to the major pheromone component and to the respective strain-specific blend were more abundant than neurones responding to the minor component and the blend produced by the other strain. Neurones investigated in Z×E hybrids responded similarly to those of E-strain males, whereas neurones in EZ×Z paternal backcrosses responded similarly to those of Z males. In the hybrids and paternal backcrosses, hybrid-blend-specific neurones were present that were not found in parental-strain males.

Key words: olfaction, antennal lobe, electrophysiology, neuroanatomy, *Ostrinia nubilalis*, moth, European corn borer.

Introduction

The European corn borer *Ostrinia nubilalis* (Hübner) appears in two strains that use different ratios of (*E*)-11- and (*Z*)-11-tetradecenyl acetate (11-14:OAc) as a sex pheromone (Anglade *et al.* 1984; Klun and Robinson, 1971). Another acetate, (*Z*)-9-tetradecenyl acetate (Z9-14:OAc), has been found to be a behavioural antagonist (Glover *et al.* 1989). Females of the E-strain produce 99 % E11-14:OAc and 1 % Z11-14:OAc (E blend), and females of the Z-strain produce 3 % E11-14:OAc and 97 % Z11-14:OAc (Z blend) (Klun *et al.* 1973). Hybrid females produce intermediate ratios between sex pheromone components (Klun and Maini, 1979; Zhu *et al.* 1996*a*,*b*).

Males of the Z and E strains respond with upwind flight preferentially to the pheromone blend of females of their own strain. The E-strain males, however, respond to a wider range of pheromone component ratios and with lower sensitivity than the Z-strain males (C. E. Linn Jr, personal communication). F_1 hybrid males (E×Z and Z×E) respond behaviourally to the Z blend and different hybrid blends, but rarely to the E blend, whereas paternal backcross males (EZ×Z and ZE×E) reveal the same behaviour as the respective paternal-strain males (Roelofs *et al.* 1987; C. E. Linn Jr, personal communication). Males of both parental strains have sensilla containing receptor neurones responding to the major and the minor pheromone component. The receptor neurones responding to the major pheromone component show a larger spike amplitude and a higher spike frequency than the receptor neurones responding to the minor component (Hansson *et al.* 1987; Hallberg *et al.* 1994; Cossé *et al.* 1995).

The production of the pheromone blend is genetically controlled primarily by one autosomal factor, the pheromone-responding receptor neurones in the male are controlled by another autosomal factor, and the behavioural response to pheromone is controlled by a sex-linked gene (Roelofs *et al.* 1987; Löfstedt *et al.* 1989). This implies that backcross or F_2 males could possibly have antennal receptors responding like one strain or like hybrids but, nevertheless, show a behavioural response to sex-pheromone blends like that of the males of the

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other strain (Roelofs *et al.* 1987). An elegant crossing experiment by Cossé *et al.* (1995) confirmed that male sex-pheromone preference is independent of the make-up of their peripheral sensilla. Central nervous processing, therefore, must be involved to explain this discrepancy. Understanding central nervous processing in general makes it possible to link processes that occur at the periphery with behavioural responses.

Sex pheromone receptor neurones, like other olfactory receptor neurones in insects, project into the first olfactory neuropile, the antennal lobe, through the antennal nerve (Bretschneider, 1924). The antennal lobe comprises a number of glomeruli in which synaptic contacts between receptor neurones and antennal lobe interneurones are made (for a review, see Hansson, 1995). Male moths have a few enlarged glomeruli, the macroglomerular complex (MGC), situated at the entrance of the antennal nerve. These glomeruli are dedicated to receiving information regarding female-produced sex pheromones (Bretschneider, 1924; Koontz and Schneider, 1987). The number of glomeruli within the MGC varies from species to species and seems, in some noctuid moths, to be correlated with the number of female-produced sex pheromone components (Todd *et al.* 1995, and references therein). Receptor neurones responding to specific pheromone components may arborize in distinct glomeruli within the MGC. There are, however, different projection patterns of receptor neurones into the MGC in different moth species (Hansson, 1995, 1996, and references therein).

The anatomical and physiological characteristics of sex-pheromone-processing antennal lobe interneurones have been studied extensively in a number of moth species (Hansson, 1995, and references therein). In *Manduca sexta* L., the response characteristics of projection neurones correlate with arborization patterns within the MGC (Hansson *et al.* 1991). Blend-specific neurones have been found in several noctuid species (Christensen *et al.* 1989, 1991; Hansson *et al.* 1994*a*; Anton and Hansson, 1994, 1995; Wu *et al.* 1996). Pheromone processing in closely related heliothine species, which use at least some of the same pheromone components, was studied by Christensen *et al.* (1989, 1991). In males of the noctuid *Agrotis segetum* (Schiff.), antennal lobe interneurones in two strains using different blends of the same pheromone components were characterized while stimulating with single pheromone components and strain-specific blends. In each of these two strains, neurones specifically responding to the strain-specific pheromone blend were found more frequently than neurones responding to the blend of the other strain (Wu *et al.* 1996).

In the current study, we examined the response characteristics and morphology of antennal lobe interneurones in *O. nubilalis* when stimulating with single sex pheromone components, pheromone blends and plant odorants. The characteristics and abundance of certain neurone types were compared between the E- and Z-strain males, F_1 hybrids and males produced in a paternal backcross.

Materials and methods

Insects

Laboratory cultures of E and Z strains of *O. nubilalis* were established in Lund from insects obtained from NYS Agricultural Experiment Station, Geneva, New York, USA. These cultures had originally been established from larvae, pupae and adults collected from corn stubble in areas of New York state where a particular race was known to be predominant (Eckenrode *et al.* 1983). The cultures were maintained on a bean diet (Zhu *et al.* 1996*a*) at 23 °C under a 16 h:8 h light:dark cycle. The genetic purity of the cultures was monitored by gas chromatographic analysis of pheromone production in females to avoid accidental mixing. Reciprocal F_1 hybrids were produced by crossing E females with Z males and *vice versa*. A paternal backcross was produced by mating E×Z females with Z males. Supplementary data were obtained on Z-strain males originating from a culture at Iowa State University.

For the electrophysiological experiments, a male moth was restrained in a plastic pipette tip, with the head (width approximately 2 mm) protruding from the tip. The head was immobilized with dental wax (Kerr) and the scales were removed from the cuticle. The cuticle between the eyes was cut off, together with the proboscis, and the muscles and tracheae as well as the sheath overlying the antennal lobes were carefully removed. The plastic holder with the moth was placed in an electrophysiological apparatus, and the opened head was superfused with a saline solution at pH 6.9 (Christensen and Hildebrand, 1987).

Stimulation

The antennae of the moths were exposed to a charcoal-filtered and moistened airstream flowing at 0.5 m s^{-1} through a glass tube. The odorants were applied on a piece of filter paper inside a Pasteur pipette. The Pasteur pipette was inserted into the glass tube approximately 80 mm from the antenna, and a 0.5 s air pulse (4 ml s^{-1}) was sent through the Pasteur pipette by means of a stimulation device (Syntech). A Pasteur pipette with a clean filter paper was used as a blank stimulus. Responses to solvent blanks did not differ from those to the filter paper blank. The odour stimuli were given randomly with 10 s inter-stimulus intervals, but smaller amounts were usually tested first. The stimuli tested were the two pheromone component isomers, E11-14:OAc and Z11-14:OAc, the behavioural antagonist, Z9-14:OAc, the E-strain blend 99:1 (E:Z), the Z-strain blend 3:97, and the two F_1 hybrid blends, 65:35 and 85:15, as well as one grean leaf volatile, (*E*)-2-hexenal, and a flower odorant, phenylacetaldehyde (PAA). All compounds were dissolved in hexane. The pheromone components and blends were applied at amounts between 1 ng and 10 μg; the plant odours were used at 100 μg. Larger amounts of pheromones were not used because they are unnatural and often caused central neurones to die after extremely high discharges. The pheromone components were purchased from the Institute for Pesticide Research, Wageningen, The Netherlands. Contamination by the opposite

geometric isomer was less than 0.3 % (below the limit of quantification) according to gas chromatographic–mass spectrometric analyses. The plant-related odours originated from Sigma Chemical Co. and were 98 % pure.

Intracellular recording and staining techniques

Standard intracellular recording and staining techniques were used (Kanzaki *et al.* 1989). Briefly, using a micromanipulator, a glass recording electrode with the tip filled with Lucifer Yellow CH (4 % aqueous solution, Sigma) and filled with lithium chloride, or an electrode filled with KCl, was inserted into the antennal lobe close to the entrance of the antennal nerve. When intracellular contact was established, the ipsilateral antenna was stimulated and the activity of the neurone before, during and after stimulation was observed on a Tektronix digital oscilloscope. The physiological data were stored on video tape (Vetter) and visualized on an electrostatic recorder (Gould) or fed into an analyzing computer program (Data Wave, Data Wave Technologies Corporation).

Physiologically characterized neurones were stained with Lucifer Yellow by passing 0.5–1 nA of constant hyperpolarizing current through the recording electrode for 10–15 min. The brains were fixed in 2.5 % buffered formaldehyde solution, dehydrated, embedded in Spurr's resin, and sectioned at 10 µm. Serial sections were photographed on Fuji Sensia 400 colour slide film, and the neurones were reconstructed from the slides.

Data analyses

Spikes were counted manually from the storage oscilloscope. The number of spikes counted during a 600 ms period after the stimulus had reached the antenna minus the number of spikes counted during the preceding 600 ms (representing the spontanous activity of the neurone) was noted as the net number of spikes. The net number of spikes produced in response to the blank stimulus was subtracted from the net number of spikes produced in response to an odour stimulus to quantify the response to a specific stimulus in one neurone. Histograms were created from the quantified responses to single components and to blends for the different amounts tested. However, interpretations of quantitative differences in responses cannot easily be made (see Figs 5, 6, 7, 9, 10), as responses to the same amount of a given stimulus are variable (Fig. 1).

The sensitivity of neurone types was compared using a log-likelihood ratio test for contingency tables. High sensitivity (response at 1 ng) opposed to lower sensitivity (response at 10 ng or higher) was tested for groups of 2–4 neurone types. *G*- and *P*-values are given for the groups of cell types compared. Differences in the abundance of neuronal types in the different male moth types could not be tested statistically because of the small sample sizes.

Neuroanatomical techniques

Brains were dissected, fixed in alcoholic Bouin's and embedded in paraffin wax. Sections (20 µm) were silver-stained according to the method of Rowell (1963). The number of MGC glomeruli and their position were determined by using photographs and *camera lucida* drawings of Rowell-stained serial sections and unstained or propidium-iodide-stained (2.5 µmol l^{-1} in phosphate buffer) sections in the fluorescence microscope.

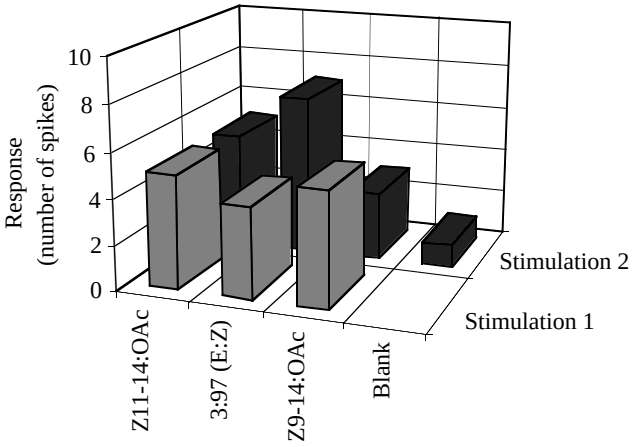

Fig. 1. Variability of responses to the same stimuli (10 µg) in an antennal lobe neurone in an E-strain *Ostrinia nubilalis* male. The responses of one neurone to three different olfactory stimuli presented on two occasions are shown. The response to Z11-14:OAc is the same for both stimulations, whereas the responses to Z9-14:OAc and to the Z blend (3:97) differ.

Mass fills of antennal receptor neurones were made by inserting a cut antenna into a glass capillary containing a 2 % solution of Neurobiotin in 0.25 mol l^{-1} KCl for 4–6 h. The moth heads were fixed in 4 % paraformaldehyde in phosphate buffer. After dissection, the brains were incubated in Avidin–Lucifer Yellow conjugate, embedded in Spurr's resin, and sectioned at 10 µm.

Results

Antennal lobe organization

The antennal lobe of *O. nubilalis* males in both the E and the Z strains consists of approximately 30 ordinary glomeruli and three enlarged glomeruli. These three large glomeruli, situated at the antennal nerve entrance into the antennal lobe, constitute the MGC (Fig. 2A,B,D). MGC glomerulus *a*, the largest glomerulus in the male antennal lobe, is situated anteriorly at the entrance of the antennal nerve (Fig. 2A). The anterior part of glomerulus *a* appears as a homogeneous structure; posteriorly, many invaginations give it an inhomogeneous appearance. MGC glomerulus *b* is situated laterally to *a* and farther from the entrance of the antennal nerve. Like glomerulus *a*, it is inhomogeneous in its posterior part. Glomerulus *c* is located medially and posteriorly to glomerulus *a* (Fig. 2B). Each receptor neurone arborizes in one single glomerulus within the antennal lobe, as was shown by mass fills of receptor neurones with Neurobiotin from the periphery and by single stained

receptor neurones, filled accidentally during intracellular recording experiments (Fig. 2C). Olfactory output neurones (projection neurones), stained intracellularly in this study, have dendritic branches in the MGC and their axons project through the inner antennocerebral tract (IACT) to the calyces of the mushroom bodies and the inferior lateral protocerebrum or the lateral horn of the protocerebrum (Fig. 2D). Their cell bodies are situated in the lateral or the medial cell group (Fig. 2B).

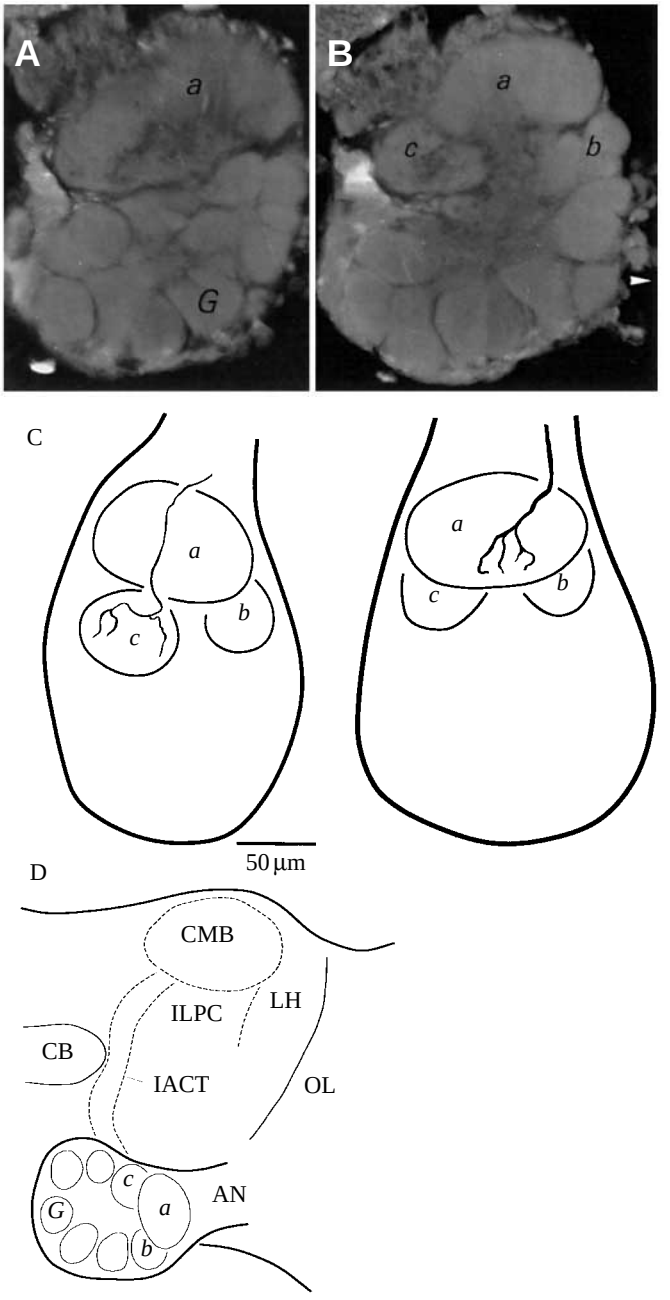

Fig. 2. Anatomy of the antennal lobe in male *Ostrinia nubilalis*. (A,B) Frontal sections through the antennal lobe, showing glomeruli *a*, *b* and *c* of the macroglomerular complex (MGC) and ordinary glomeruli (*G*). (C) Two reconstructions from serial sections of intracellularly stained receptor neurone axons show them projecting into glomeruli *c* and *a*. (D) Schematic drawing of a hemi-brain in frontal view. AN, antennal nerve; CB, central body; CMB, calyces of the mushroom body; IACT, inner antennocerebral tract; ILPC, inferior lateral protocerebrum; LH, lateral horn of the protocerebrum; OL, optic lobe. The arrowhead in B points laterally. Scale bar, 50 µm (for A–C).

General physiological characteristics of antennal lobe neurones

We recorded intracellularly from 113 pheromone-responding antennal lobe neurones in 74 male *O. nubilalis* and from seven neurones responding only to plant-related odours in seven animals. Recordings were made from the parental E and Z strains, the hybrids and the parental backcross moths (Table 1). Three pheromone-responding projection neurones and one local interneurone were stained at least partially in E-strain moths, and three pheromone-responding projection neurones were stained in Z-strain moths.

Antennal lobe neurones had a spontaneous activity of between 0.5 and 15 Hz. The action potential amplitude varied between 8 and 25 mV. All responses of antennal lobe neurones to pheromone and plant-related stimuli were excitatory. No neurones displaying a purely inhibitory response to the test odours were observed. Some neurones showed only an excitatory response, sometimes lasting for more than 1.5 s (Fig. 3A), while others reacted with excitation followed by a period of inhibition (Fig. 4). Neurones were categorised according to their response pattern to the different pheromone stimuli and plant-related odorants. Seven neurones responded exclusively to one or both of the plant-related odorants, as in the examples shown in Fig. 5. Forty-six of the pheromone-responding neurones also responded to one or both plant-related odorants as in the example shown in Fig. 6A. The response and specificity of most neurones investigated was strongly dose-dependent. In 94 % of the specific pheromone-responding neurones, differences in responses to single compounds and to mixtures only occurred at relatively low stimulus levels (see Fig. 9). With increasing levels of the stimulus, even specific neurones responded to the whole stimulus range. In 56 % of the specifically responding neurones, responses to all pheromone stimuli were found at amounts only one order of magnitude higher than the amount eliciting the specific response (Figs 4, 7).

Neurones that responded to E11-14:OAc at a lower level than to Z11-14:OAc were defined as ‘E neurones’ (Fig. 7A,B) and neurones that were more sensitive to Z11-14:OAc than to E11-14:OAc were defined as ‘Z neurones’ (Figs 4, 7C,D). Neurones that responded to one or several of the blends at a lower level than to the E and Z isomers were defined as ‘E-blend neurones’, ‘Z-blend neurones’, ‘E&Z-blend neurones’ or ‘hybrid-blend neurones’ (or ‘blend-neurones’). The remaining neurones, responding to the E and Z isomers and blends at the same level, were defined as unspecific neurones (‘generalist neurones’). Neurones only responding to Z9-14:OAc were defined as ‘Z9-14 neurones’.

The sensitivity of different neurones to the same pheromone component was highly variable, irrespective of the strain. Some responses were obtained at a stimulus amount of 1 ng

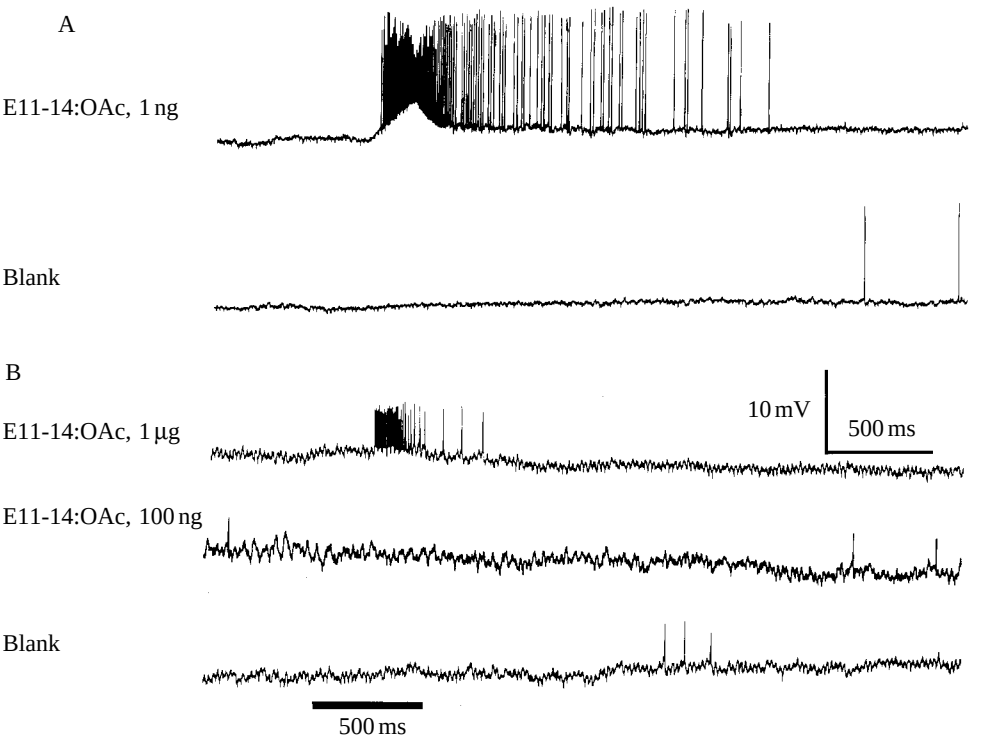

Fig. 3. Responses of projection neurones with different sensitivity to E11-14:OAc in *Ostrinia nubilalis*. (A) Strong response of an E neurone in a Z-strain male to 1 ng of E11-14:OAc. (B) Response of an E neurone in an E-strain male to 1 µg of E11-14:OAc. The neurone does not respond to 100 ng of E11-14:OAc. The solid bar indicates the period of stimulation. Blank, airpuff without odour stimulus.

(Fig. 3A) while other neurones only reacted to 1 µg (Fig. 3B). Sensitivity, however, was different for different neurone types when four types were statistically tested (G=4.63; P<0.04). High sensitivity was more abundant in generalist neurones and E neurones than in Z neurones and blend neurones (Fig. 8).

E neurones

E neurones were found in all strains and crossings investigated (Table 1). In all but three E neurones, a response to the Z isomer occurred at one or two orders of magnitude above the specific response to the E isomer in all strains and crossings (Fig. 7A,B). In three E neurones in the E-strain males, however, specificity was encountered over a larger range of stimulus levels. High sensitivity was more abundant in E neurones than in blend neurones (G=3.76; P<0.06) (Fig. 8).

Z neurones

Z neurones also were found in all strains and crossings (Table 1). All Z neurones responded to the E isomer at one or two orders of magnitude above the level for the specific response to the Z isomer (Figs 6, 7C,D). Z neurones had a significantly lower sensitivity than generalist neurones (G=8.93; P<0.003). Z neurones with high sensitivity occurred only in Z-strain males (Fig. 8).

Blend neurones

A few blend neurones were found in all strains and

Table 1. *Abundance of the different neurone types in* Ostrinia nubilalis *males*

Neurone type	Moth type					Total number of neurones
	Z strain	E strain	E×Z hybrid	Z×E hybrid	EZ×Z backcross	
Z neurones	8	3	1	1	6	19
E neurones	5	11	1	7	3	27
Z-blend neurones	3	0	0	0	1	4
E-blend neurones	1	1	0	2	0	4
E&Z-blend neurones	1	0	0	0	0	1
Hybrid-blend neurones	0	0	0	2	1	3
Generalist neurones	9	20	7	11	6	53
Z9-14 neurones	0	0	0	1	1	2
Total number of neurones	27	35	9	24	18	113
Number of moths	21	25	5	14	9	74

For definitions see text.

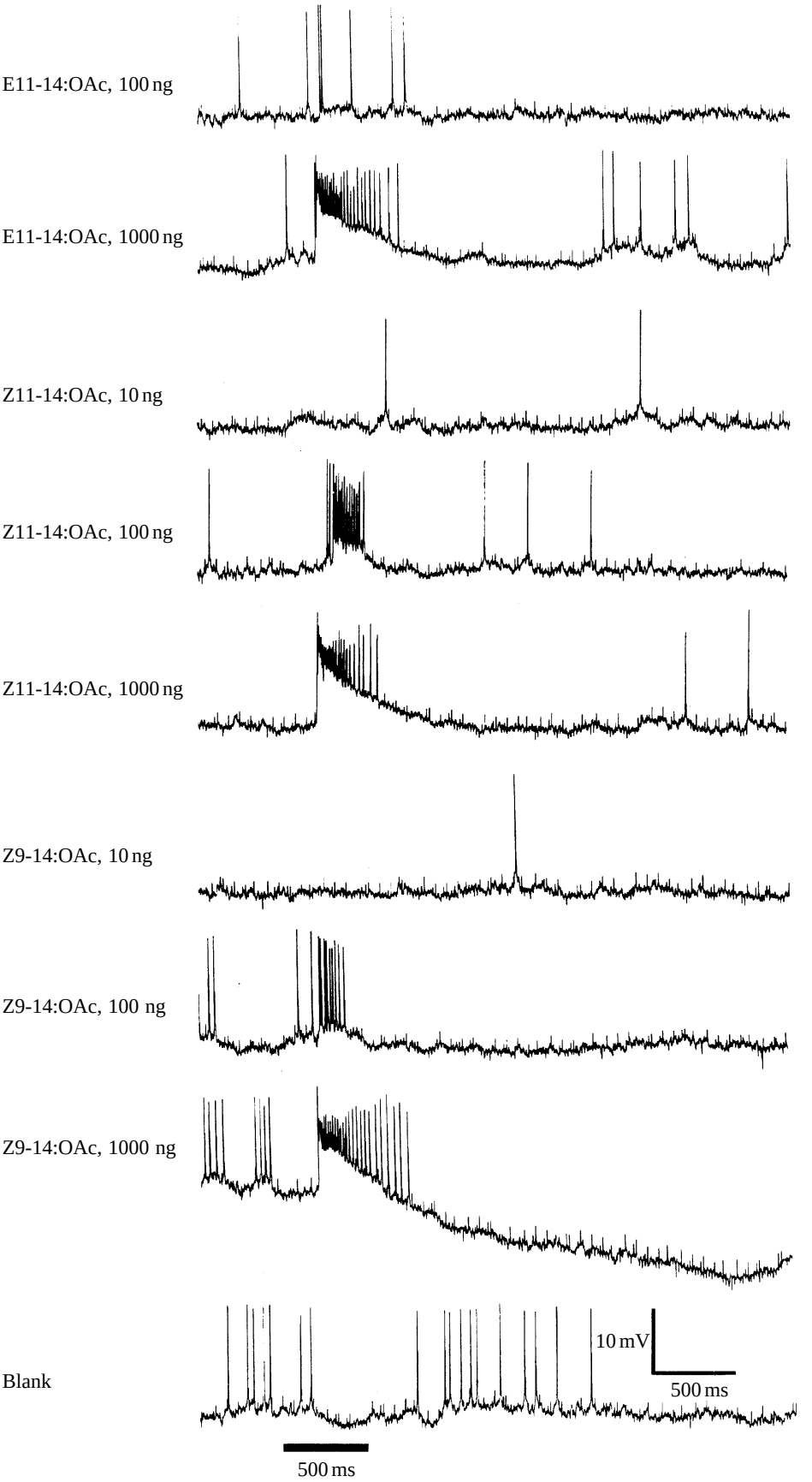

Fig. 4. Specificity of antennal lobe neurones in *Ostrinia nubilalis*, based on sensitivity differences. Responses of a typical Z neurone to pheromone stimuli in an E-strain male, showing excitatory responses to 100 ng of Z11-14:OAc and Z9-14:OAc and to 1 µg of E11-14:OAc. Blank, airpuff without odour stimulus. Small spikes are extracellularly registered activity of a neighbouring neurone. The inhibitory response to the blank is probably due to mechanosensory input to the neurone. The solid bar indicates the period of stimulation.

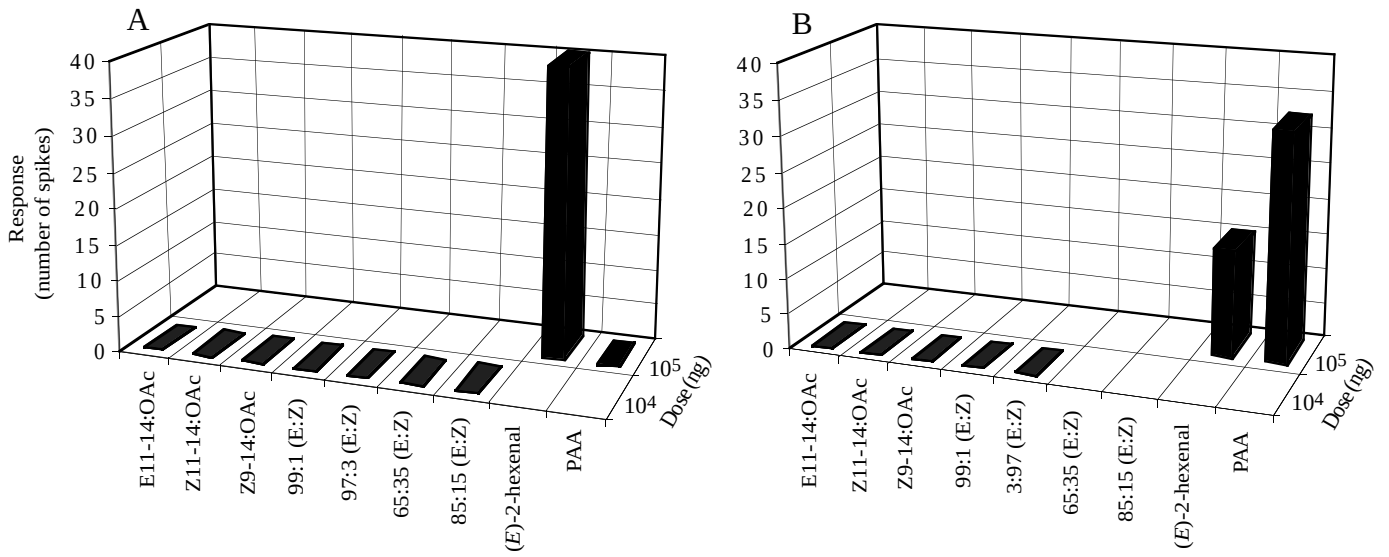

Fig. 5. Histograms of responses of two plant-odorant-responding antennal lobe neurones in an *Ostrinia nubilalis* Z-strain male (A) and an E-strain male (B). The number of response net spikes minus blank net spikes is given (see Materials and methods). Empty squares indicate amounts/stimuli that were not tested. PAA, phenylacetaldehyde.

crossings except E×Z hybrids (Table 1). Blend neurones responded most often to the strain-specific blend (Table 1). All blend neurones responded to single pheromone components at levels one order of magnitude above that for the blend response (Fig. 9). On average, blend neurones had intermediate sensitivity compared with the other neurone types. The sensitivity of blend neurones was significantly lower than that of generalist neurones (G=5.88; P<0.02) and E neurones (G=3.76; P<0.06), but did not differ from that of Z neurones (G=0.0042; P=0.95) (Fig. 8).

Generalist neurones

Generalist neurones were the most abundant neurone type in all strains and crossings (Table 1). Most generalist neurones responded to all pheromone stimuli at the same concentration (Fig. 6A,B). The number of action potentials increased with increasing stimulus concentration and often levelled out at high stimulus concentrations (Fig. 6A). Fifty-five per cent of the generalist neurones responded to plant stimuli in addition to pheromone components, as shown in the example in Fig. 6A. Thirty-eight per cent of the generalist neurones did not respond to Z9-14:OAc (example shown in Fig. 6D) and 28 % did not respond to some of the blends, as in the example shown in Fig. 6C. The sensitivity of generalist neurones was relatively high in all strains and crossings. The sensitivity of generalist neurones was significantly higher than that of blend neurones (G=5.88; P<0.02) and Z neurones (G=8.93; P<0.003) but did not differ from the sensitivity of E neurones (G=0.23; P=0.63) (Fig. 8).

Z9-14 neurones

Two Z9-14:OAc-specific neurones were found, one in a hybrid male showed medium sensitivity, the other in a paternal backcross male showed low sensitivity, and neither responded to the other pheromone components (Fig. 10).

Abundance of neurone types in the different insect types

In males of both pheromone strains, in hybrids and in parental backcrosses, all the neurone types described above were encountered. The abundance of the different neurone types was, however, different in the different males tested. As too few neurones of each type were found to allow statistical analyses, we describe some tendencies, which can be seen in Table 1. In the parental strains, more neurones responded preferentially to the major pheromone component than to the minor component (Table 1). This difference was more pronounced for the E-strain than for the Z-strain males. In the Z×E hybrids, more E neurones than Z neurones were found. In the E×Z hybrids, too few neurones could be characterised to indicate any differences. In the parental backcross of EZ females with Z males, Z neurones were more abundant than E neurones. In all tested groups, most neurones were generalist neurones, responding to the same levels of both pheromone components (Table 1).

Anatomical characteristics of antennal lobe neurones

The only local interneurone filled was stained in an E-strain male and responded to all pheromones tested. This local interneurone had a lateral cell body and arborizations spread over the entire antennal lobe, including all glomeruli of the MGC (Fig. 11).

In the E-strain males, three pheromone-responding projection neurones were stained. These neurones had arborizations within the MGC. Two of the MGC neurones were completely stained and revealed arborizations in the protocerebrum. In the Z-strain males, projection neurones were stained in three preparations. In two moths, projection neurones were stained completely to the protocerebrum, and one preparation showed an incomplete stain.

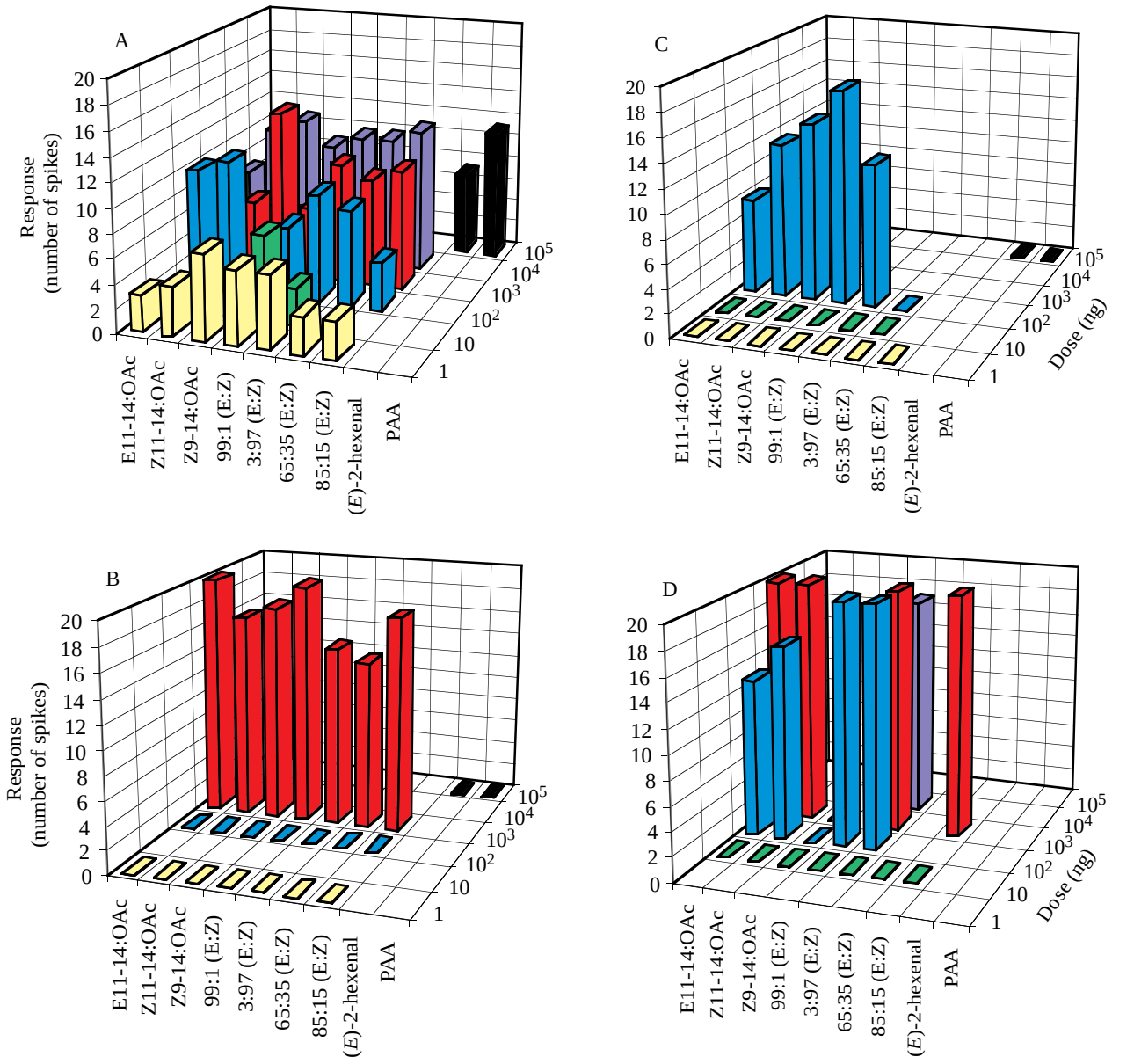

Fig. 6. Histograms of responses of generalist neurones in *Ostrinia nubilalis*. Generalist neurones responded to all stimuli tested (A); to all (B) or most (C) pheromone stimuli, but not to plant odours, or to all tested pheromone stimuli except the behavioural antagonist (D) at the same stimulus levels. (A,C) Z-strain males, (B) Z×E hybrid male, (D) EZ×Z backcross male. The number of response net spikes minus blank net spikes is given (see Materials and methods). Empty squares indicate amounts/stimuli that were not tested. PAA, phenylacetaldehyde.

Projection neurones in E-strain males

One incompletely stained projection neurone had a medial cell body, some arborizations within glomerulus *a*, and dense arborizations within glomerulus *c*. The axon was incompletely stained, but was clearly leaving the antennal lobe (Fig. 12A). This projection neurone was categorised as a Z neurone, responding to Z11-14:OAc and to all blends with more sensitivity than its response to E11-14:OAc (Fig. 7C). The two completely stained projection neurones both had a medial cell body. One projection neurone had dense arborizations within MGC glomerulus *a* and some branches in glomerulus *b* (Fig. 12B), the other neurone had dense arborizations within glomerulus *a* and some branches within glomeruli *b* and *c* (Fig. 12C). The axons of both neurones projected through the inner antennocerebral tract to the calyces of the mushroom bodies, where they arborized mainly in the central part, and to the inferior lateral protocerebrum, where they arborized extensively (Fig. 12B,C). Both completely stained projection neurones were found to be E cells, responding to E11-14:OAc and to some blends with more sensitivity than to Z11-14:OAc.

Projection neurones in Z-strain males

One incompletely stained projection neurone showing responses characteristic of a Z neurone had a medial cell body

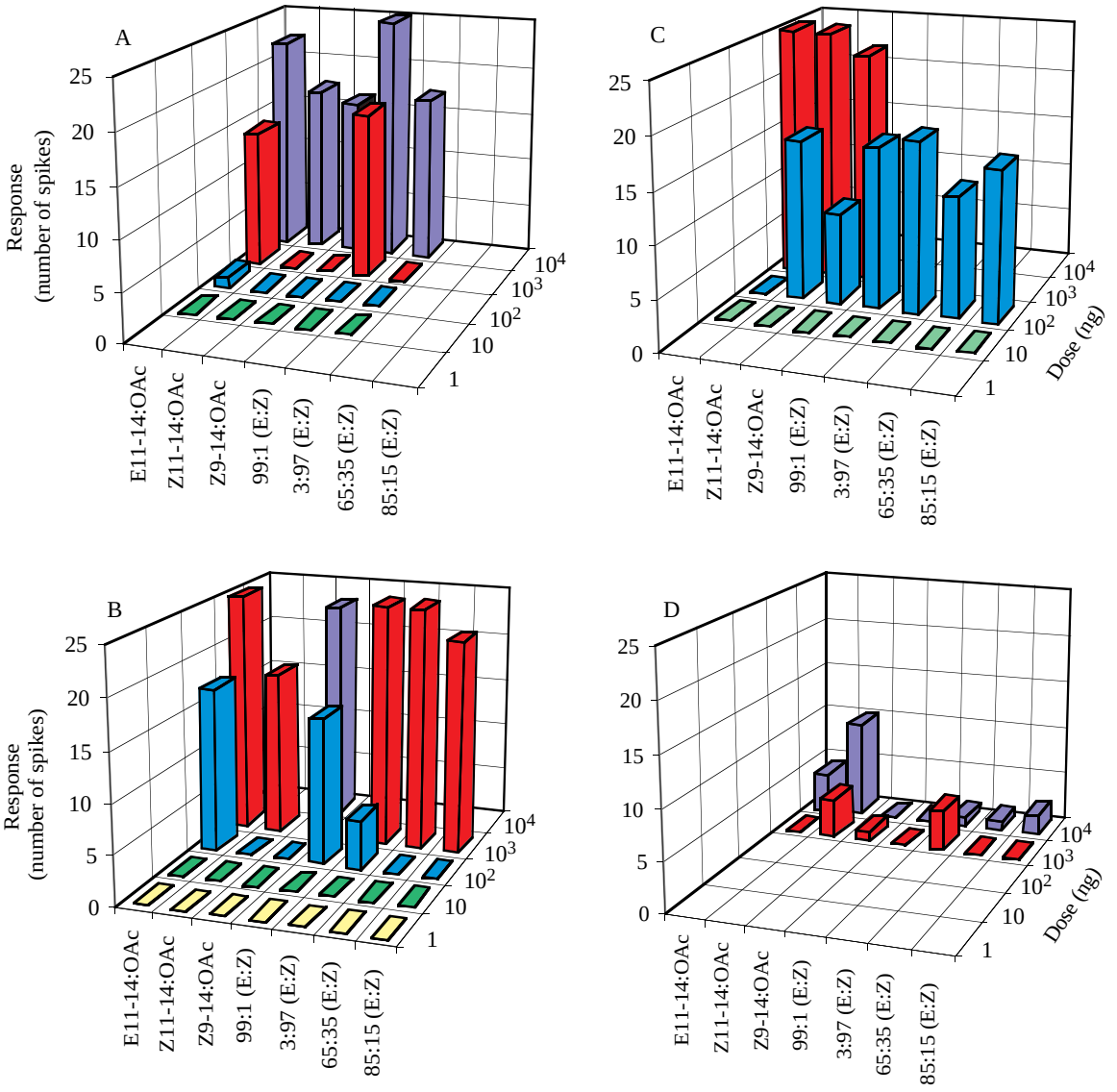

Fig. 7. Histograms of responses of typical E (A,B) and Z (C,D) neurones to different amounts of the different pheromone stimuli in *Ostrinia nubilalis*. (A,C) E-strain males, (B,D) Z-strain males. The number of response net spikes minus blank net spikes is given (see Materials and methods). Empty squares indicate amounts/stimuli that were not tested.

and arborized extensively in all MGC glomeruli (Fig. 12D). The axon was only partially stained. A second projection neurone had a medial cell body, dendritic arborizations within the posterior part of MGC glomerulus *a*, projected through the IACT and had a few axonal branches in the central part of the calyces of the mushroom bodies and more extensive branches in the lateral horn of the protocerebrum (Fig. 12E). This projection neurone responded equally well to E-11-14:OAc and Z11-14:OAc at the same concentrations (Fig. 6D). Another projection neurone had a medial cell body and widely spread dendritic arborizations within MGC glomeruli *a* and *c*. It projected through the IACT and had a few axonal branches in the calyces of the mushroom bodies and extensive arborizations in the inferior lateral protocerebrum (Fig. 12F). This projection neurone was more sensitive to the Z blend than to the other stimuli (Fig. 9D).

Discussion

The present study provides information regarding how central nervous processing of sex pheromones can result in recognition of pheromone isomers and isomer ratios in two strains of *O. nubilalis*. The abundance and sensitivity of antennal lobe neurone types has a tendency towards strain-specific characteristics.

Males of both parental strains have three types of sensilla trichodea on their antennae (sensillum types A, B and C) which contain one, two or three receptor neurones, responding to pheromone stimuli (Hallberg *et al.* 1994). Sensillum type C contains one receptor neurone responding either to the major pheromone component or to the behavioural antagonist. Sensillum type B is innervated by a large-spiking neurone responding to the major pheromone component and a small-

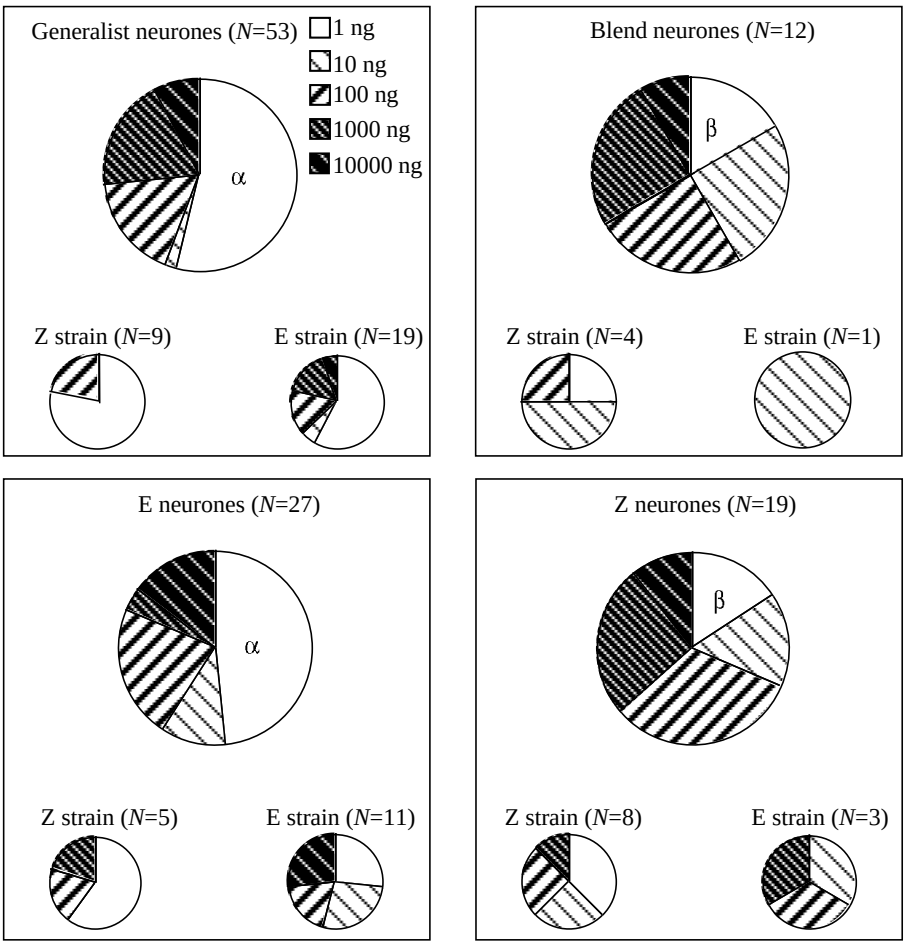

Fig. 8. Sensitivity of the four different response-type neurones, pooled over all male *Ostrinia nubilalis* types (large diagram) and in the E and Z parental strains (smaller diagrams). The percentage of neurones of a particular type with a given threshold is indicated. The sensitivities of neurone types marked with different letters (α, β) are statistically different (see text).

spiking neurone responding to the minor component. Sensillum type A contains two neurones with the same characteristics as type B sensilla and a third neurone responding to the behavioural antagonist (Hansson *et al.* 1987; Hallberg *et al.* 1994; Cossé *et al.* 1995). In hybrids, the two pheromone-component-responding neurones within sensillum type A respond with the same intermediate spike amplitude to the two isomers. Sensilla in paternal backcross males contain receptor neurones with intermediate- and paternal-size spike amplitudes (Hansson *et al.* 1987). A study by Hansson *et al.* (1994*b*) showed that the dendritic diameters of the receptor neurones are correlated with the different spike amplitudes. Cossé *et al.* (1995) found that the spike frequency in response to the pheromone components was correlated with the spike size: receptor neurones responding to the major component exhibited a higher spike frequency than neurones responding to the minor component. Discrimination of the pheromone components as such can thus be achieved at the peripheral level, but more complex discrimination, such as blend detection, is resolved in the central nervous system (Cossé *et al.* 1995).

Discrimination between the two isomers of a single component and discrimination between blends containing different ratios of these isomers are more difficult tasks than discrimination between chemically more distinct components. As receptor neurones in *O. nubilalis* respond to both pheromone isomers at high concentrations (B. S. Hansson, unpublished observation), it is not surprising that antennal lobe neurones show the same response characteristics. The specific responses of receptor neurones and antennal lobe neurones, which occur at low concentrations and often only one order of magnitude below more general responses, show the importance of using pheromone concentrations and ratios that mimic those that the male will encounter naturally. Thus, stimulus amounts seem to be crucial for the recognition of strain-specific pheromones. However, the difference in absolute sensitivity of different antennal lobe neurones allows for specificity over a larger range of stimulus concentrations.

The large number of generalist antennal lobe neurones with high sensitivity could reflect the setting of too high a level for the minimal stimulus in the experiments, although the amounts needed to produce behavioural responses are clearly higher than those used here (C. E. Linn Jr, personal communication). It could be that, by testing lower concentrations, more specificity would have been found. There are, however, a number of less-sensitive and therefore clearly generalist neurones in all of the types of male *O. nubilalis* we tested.

In *O. nubilalis*, blend-specific neurones discriminating

between blends containing different ratios of the two pheromone isomers were found. The types of blend-specific neurones found were to a large extent consistent with the behavioural responses of each type of male. Blend-specific neurones might be a neuronal correlate of the ability of insects to discriminate behaviourally between pheromone ratios of different strains.

Differences in the abundance of neurone types were found in the two pheromone strains of *O. nubilalis*, the hybrids and the paternal backcrosses. In the parental strains, more neurones responded specifically to the appropriate major component isomer than to the minor component. The differences were more pronounced in the E strain than in the Z strain. This is surprising when comparing these results with the behaviour of males in a wind-tunnel. Antennal lobe neurones in E-strain males responded more specifically to their major pheromone component than neurones in Z-strain males, although Z-strain males showed less variability in their behavioural responses than E-strain males (Roelofs *et al.* 1987; C. E. Linn Jr, personal communication). In parental-strain males, more blend-specific neurones responding to their 'own' blend were also found.

Similar results were obtained in a study of two parental strains of *Agrotis segetum* using different ratios of the same sex pheromone components (Wu *et al.* 1996). In two separate populations of *A. segetum*, differences in the abundance of receptor neurones on the male antennae, tuned to different

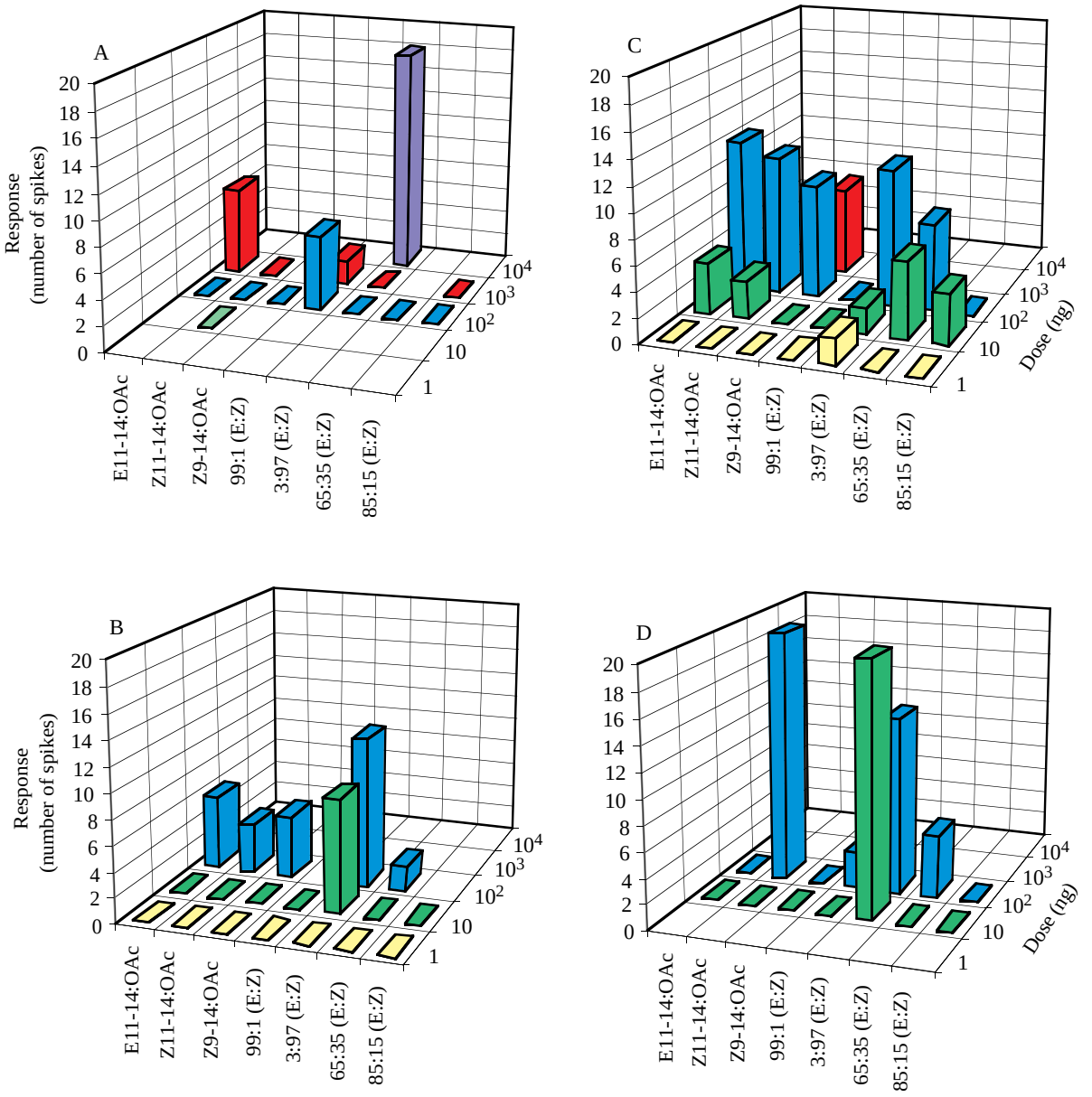

Fig. 9. Histograms of responses of typical blend-specific neurones in Z-strain *Ostrinia nubilalis* males. (A) E-blend-specific neurone; (B,C,D) Z-blend-specific neurones. The neurone in D was characterised morphologically (see Fig. 12F). The number of response net spikes minus blank net spikes is given (see Materials and methods). Empty squares indicate amounts/stimuli that were not tested.

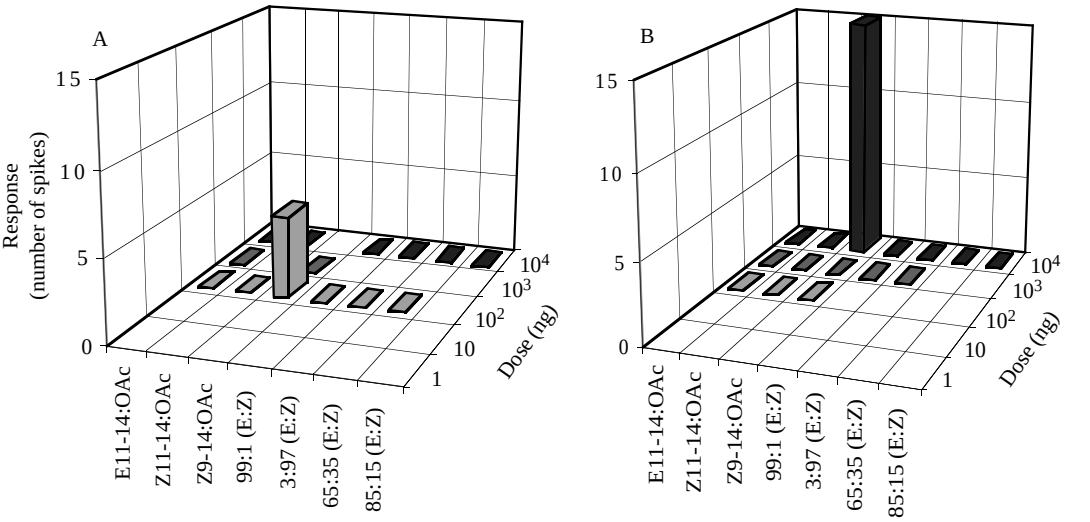

Fig. 10. Histograms of responses of Z9-14:OAc-specific neurones in *Ostrinia nubilalis* with different sensitivity. The neurone in A responds only to 100 ng, but not to 1 µg. The two Z9-14-specific neurones did not respond to any other stimuli. (A) Z×E hybrid male, (B) EZ×Z backcross male. The number of response net spikes minus blank net spikes is given (see Materials and methods). Empty squares indicate amounts/stimuli that were not tested.

pheromone components, correlated with the different ratios produced by the females (Wu *et al.* 1995). In addition, morphological features of antennal lobe interneurones reflected behavioural differences in males of the two populations. In each of the populations (Swedish and Zimbabwean), several antennal lobe neurones responded to their own, strain-specific blends but not to blends of the other strain (Wu *et al.* 1996).

The range of specificity of blend-specific neurones in *O. nubilalis*, however, was different from that of noctuid species. In *O. nubilalis*, blend specificity was only present at low stimulus levels, and single odorants elicited responses in the same neurones when presented in amounts that were just one order of magnitude higher. In *A. segetum* and *Spodoptera littoralis* (Boisd.) males, blend specificity was present in antennal lobe neurones over a larger range of concentrations (Hansson *et al.* 1994*a*; Anton and Hansson, 1995).

In the present study, we describe for the first time the anatomy of the antennal lobe of a male pyralid moth. The general morphology appears to be very similar to that of other families of moths, with a large number of ordinary glomeruli and a smaller number of MGC glomeruli. In *O. nubilalis*, the MGC consists of one large and two smaller glomeruli, an architecture very similar to that of the noctuid moth *S. littoralis* (Ochieng' *et al.* 1995), and the inhomogeneous structure of the *a* glomerulus of the MGC also accords with earlier observations. Towards its caudal aspect, this glomerulus displays a large number of invaginations and folds. This condition has been proposed as being a transitional state, wherein this large MGC glomerulus is in the process of differentiating into several separate glomeruli over evolutionary time (Hansson, 1995).

It has been suggested that the number of MGC compartments is an indicator of the number of sex pheromone components and pheromone attraction antagonists detected by specialised antennal receptor neurones. The most striking example of such a correlation is the cabbage looper, *Trichoplusia ni*, which has six behaviourally active sex pheromone components, one behavioural antagonist and seven MGC compartments (Todd *et al.* 1995). In the European corn borer, two pheromone components and one behavioural antagonist have been shown to be detected by different types of receptor neurones. Thus, the number of MGC compartments mirrors the number of receptor types involved in sex attraction. However, recent work suggests that additional pheromone components may exist in *O. nubilalis* (W. Roelofs, personal communication). Possibly, the highly folded MGC glomerulus *a* represents more than one pheromone component.

The morphology of antennal lobe projection neurones in *O. nubilalis* resembles, to a large extent, the structure of projection neurones in other moths. Dendritic arborizations of pheromone-responding neurones are restricted to the MGC, the cell body is usually situated in the lateral or medial cell group, and the axon projects to the calyces of the mushroom bodies

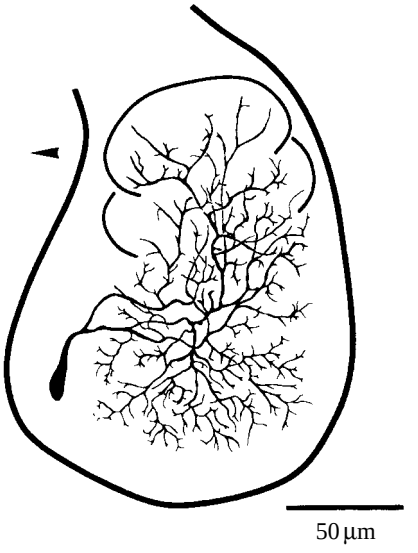

Fig. 11. Reconstruction of a local interneurone in an E-strain *Ostrinia nubilalis* male. The neurone arborizes in a large number of ordinary glomeruli and in all glomeruli of the MGC. Arrowhead points laterally.

Fig. 12. Projection neurones in E- (A,B,C) and Z-strain (D,E,F) *Ostrinia nubilalis* males. Reconstructions are from frontal (A,C,D,E) and dorsal (B,F) sections. The projection neurones show arborizations in different parts of the macroglomerular complex (thin solid lines). The completely stained neurones (B,C,E,F) arborize within the calyces of the mushroom bodies (hatched lines) and extensively in the lateral protocerebrum (LP). Thick solid lines mark the outline of the antennal lobe. Arrowheads point laterally.

and the lateral protocerebrum (Kanzaki and Shibuya, 1992; Hansson, 1995). However, arborizations in the lateral protocerebrum in *O. nubilalis* are quite extensive, and the area covered by arborizations in the lateral protocerebrum appears to be larger than in other moth projection neurones in relation to the size of the brain. These large protocerebral branches probably overlap with protocerebral neurones dealing with other sensory input, which might be a sign of extensive integration between olfactory and other sensory modalities.

The arborization area of pheromone-responding projection neurones within the MGC and the protocerebrum is not clearly correlated with the response characteristics of the neurones in *O. nubilalis*. Although only a few neurones have been described morphologically, a simple correlation between structure and function can be ruled out from these data. Projection neurones with the same response characteristics differed in their arborization areas within the MGC, while projection neurones sharing arborization areas within the MGC or the protocerebrum showed different response characteristics. These data show that the pyralid system might resemble the noctuid system, where no simple structure–function correlation in the dendritic arborization area of projection neurones has been demonstrated (Christensen *et al.* 1989, 1991; Hansson *et al.* 1994*a*; Anton and Hansson, 1995; Wu *et al.* 1996), in contrast to the sphingid moth *Manduca sexta*, where a clear structure–function correlation was found (Hansson *et al.* 1991). The lack of correlation between projection neurone response characteristics and arborization areas in the antennal lobe, in spite of functionally distinct receptor neurone projections in noctuid and pyralid moths, emphasises the importance of local interneurones as mediators between receptor neurones and projection neurones (Christensen *et al.* 1993).

One may hypothesise that differences in the response characteristics of the antennal lobe neurones are controlled by the sex-linked locus determining the behavioural response profiles of males, thus providing a physiological explanation for the observed differences in behaviour. The response characteristics of antennal lobe neurones in F_1 hybrid and parental backcross males of *O. nubilalis* coincide to some

extent with behavioural observations in wind-tunnel experiments. In Z×E hybrid males, the numbers of neurones found for each neurone type resembled those found in the E-strain males but, in addition, two hybrid-blend-specific neurones were found. The presence of hybrid-blend-specific neurones in F_1 males, not occurring in any of the parental-strain males, correlates with behavioural responses to hybrid blends. However, a high proportion of E neurones and E-blend neurones does not accord with the behaviour of the Z×E males in the wind-tunnel. In E×Z hybrids, too few recordings were available to suggest any pattern. Under sex-linked inheritance, the EZ×Z backcross should give all Z-type males among the progeny. Although the proportion of neurones of each neurone type resembles that in the parental Z-strain males, one cannot draw any firm conclusions as there are too few recordings to allow the characterisation of individual males.

The results of this study provide evidence that discrimination of different ratios of pheromone components, even if they are isomers of the same compound, occurs, at least partially, at the level of antennal lobe neurones. The presence of different neurone types in the antennal lobe shows, however, that there are different parallel olfactory pathways integrating information at different levels in the moth brain. Further investigations of neurones at different levels in the olfactory pathway will help to elucidate the mechanisms of pheromone ratio discrimination.

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