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Morphology and Physiology of Neurons Responding to the Nasonov Pheromone in the Antennal Lobe of the Honeybee, *Apis mellifera*

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ABSTRACT—Interneurons in the antennal lobe of the worker honeybee brain were stained with Lucifer Yellow and their responses to the Nasonov pheromone were examined. The antennae were stimulated with a synthetic blend of Nasonov pheromone at 5 dose levels, which elicited both behavioral and electroantennogram responses dose-dependently. Morphologically characterized neurons were categorized into 4 classes: projection neurons with axons in the median antenno-glomerular tract and uniglomerular dendritic arborizations in the antennal lobe, Pm(u); projection neurons with axons in the median lateral antenno-glomerular tract and multiglomerular arborizations, Pml(m); projection neurons with axons in the antennal lobe commissure, Pc(m); local interneurons, L(m). All classes of neurons exhibited changes of spike activity in response to the pheromone blend. The temporal patterns of the responses tended to be correlated with these morphological classes: Pm(u) showed mainly phasic-tonic excitation, and in some cases on-off excitation or inhibition; Pc(m) showed slight excitation; Pml(m) and L(m) showed mainly phasic excitation. The response intensity of most cells increased dose-dependently, and the response pattern of some Pm(u) changed with the dose of the blend. These results suggest that signals induced by the Nasonov pheromone are distributed in multiple morphological classes of antennal lobe neurons, each having various response characteristics. The architecture of the bee antennal lobe relating to Nasonov pheromone processing thus appears to be complex.

INTRODUCTION

It has been well documented that the honeybee (*Apis mellifera* L.) has a great ability to discriminate various and complex odors [27, 37], thus enabling it to express suitable behavior in response to naturally changing circumstances. In addition, the honeybee is a well studied species of social insect for which olfactory cues such as species- and colony-related odors play a major role in social organization [11]. Thus, the olfactory system of the honeybee provides a good model for the study of olfaction in insects.

The Nasonov pheromone from the Nasonov gland of the worker bee is one of the essential odors associated with colony formation and maintenance [11]. Release of the pheromone has been found to occur in several behavioral contexts such as swarm formation [29], return of foragers to the nest [32, 34] and search for food and water sources [11, 13]. In each case, worker bees are attracted to the pheromone source [11]. The pheromone comprises seven components: geraniol, nerol, (E)-citral (geranial), (Z)-citral (neral), geranic acid, nerolic acid, and (E, E)-farnesol [31]. All of them have been chemically categorized as terpenic compounds, and the average amount of each component in the gland of a worker bee has been evaluated [31]. Thus, this pheromone is one of the best known and important odors to which honeybees respond.

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In the honeybee, the physiology of olfactory receptor cells in the antenna and the anatomy of antennal pathways in the brain have been described in some detail [1-4, 10, 12, 17, 18, 23, 28, 37]. It has been reported that the olfactory receptor cells related to sensilla placodea on the antenna respond to the Nasonov pheromone in worker and drone bees [18]. The axons of receptor cells are mainly directed to characteristic structures in the antennal lobe (AL) of the brain, the glomeruli [28, 33]. The glomeruli are thus likely to have important roles in the primary processing of antennal information in the brain. Interneurons innervating the glomeruli have been broadly classified into two types on the basis of morphology: projection and local interneurons [4, 10, 12, 19, 26]. The former have an axon projecting to the protocerebrum from the AL, indicating a role in the mediation of antennal information. The latter have processes restricted to the AL and may have specific roles in signal processing within the AL. It has been reported that both neuronal classes are divided into several morphological subclasses in honeybees and other insects [12, 16, 21]. However, there have been few studies of the responses of these interneurons to honeybee pheromones including the Nasonov pheromone [9, 15, 35].

The present study was designed to search for the AL neurons responsive to the Nasonov pheromone in the worker bee brain. A synthetic blend of the Nasonov pheromone (NPB) was used as a stimulant because the use of synthetic chemicals allows more quantitative analysis. In order to estimate an effective dose range for the stimuli, the dose-response relations at behavioral and electro-antennogram levels were first examined. Using the same range of NPB dose, the response of neurons in the AL was studied in-

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tracellularly. Neurons stained with Lucifer Yellow were divisible into 4 classes based on their morphology. Here, their morphology and response characteristics are described.

MATERIALS AND METHODS

Animals

Apis mellifera L. foraging workers were captured at a hive entrance. They were fed on 50% sucrose solution until satiated, and then anesthetized by chilling for 10–30 min at 4°C. Subsequent experiments were done after a recovery time of more than half an hour.

Stimulation

All the identified components of the Nasonov pheromone [31] and their chemical features are listed in Table 1. (E)- and (Z)-citral, geranic and nerolic acid were purchased from Takasago Corporation, geraniol and nerol were from Tokyo Kasei Kogyo Co., Ltd., and farnesol was from Sigma Chemical Co. The purchased farnesol was a mixture of (E, E), (E, Z), (Z, E) and (Z, Z) isomers.

TABLE 1. Characteristics of components of Nasonov pheromone

Component	Relative proportion*1	Average amount*1 (μg/worker bee)	Molecular structure
Geraniol	100.0	1.8	Terpenic alcohol
Nerol	0.4	0.007	Terpenic alcohol
(E)-Citral	1.1	0.02	Terpenic aldehyde
(Z)-Citral	0.6	0.01	Terpenic aldehyde
Geranic acid	11.1	0.2	Terpenic acid
Nerolic acid	77.8	1.4	Terpenic acid
(E, E)-Farnesol*2	44.4	0.8	Terpenic alcohol

*1 Relative proportions and average amounts of each component are derived from Pickett *et al.* [31].
*2 We used a mixture of four types of isomers in this study.

The blend (NPB) used as the stimulant was prepared by mixing each pure chemical. The dose was expressed in terms of 'gland equivalent' (GE): 1 GE of NPB represented a blend of the amount of each component present naturally in a Nasonov gland (Table 1). Each of 5 dose levels (10^{-1}, 10^{0}, 10^{1}, 10^{2}, and 10^{3} GE) of NPB was dissolved in 100 μl of n-hexane, and pure n-hexane was used as a control solution. Each solution was dropped onto a separate piece of filter paper (1 cm×8 cm) and the solvent evaporated. Each paper was then inserted into a glass pipette. The outlet of the pipette was set at a distance of about 1 cm from the antenna. For behavioral analysis, a 5-sec puff of odorous air was applied to the antennae of the bee by manually pressing the silicone teat of the pipette. For electrophysiological study, each pipette was connected to a motor-driven syringe. By operating the syringe, a 1.5-sec puff of air was applied to the antenna. The velocity of air flow was about 1.8 m/sec at the outlet of the pipette. During these experiments, the air surrounding the preparation was extracted continuously to the outside of the laboratory.

Behavioral analysis

As a preliminary experiment, we observed behavioral responses to stimulation with 10^{2} GE of NPB. A particular form of response behavior was observed in most of the worker bees which were tested (see Results). The dose-response relation of the behavior was examined by stimulation tests using a control and the 5 dose levels of NPB listed above. Each bee was placed individually in a clear 20-ml glass bottle. Prior to the tests, all bees spontaneously showing the behavior were excluded. Each bee was exposed to one of the stimulants and we observed whether or not the bee responded with the behavior. The probability of the occurrence of the behavior for each dose of NPB, indicated by the proportion of bees responding to the test, was plotted on a dose-response curve.

Recording of electroantennogram (EAG) responses to the NPB

An antenna was excised from the head of a worker bee and fixed horizontally in a recording chamber. The proximal and distal ends of the antenna were placed in pools of bee saline [36] and held by soft wax to keep the frontal surface of the flagellum toward the odor source. The electrical potential of the antenna was recorded using 2 blunt glass microelectrodes (resistance: <10 MΩ); one was placed at the distal end as a recording electrode and the other at the proximal end as an indifferent electrode. Signals were monitored with an oscilloscope through an amplifier and stored on FM tape. The amplitude of each response was measured on the charts obtained by playback of the tapes.

Intracellular recording and staining of neurons in the AL

A bee was mounted in an aluminum tube with its neck inserted between 2 small plastic plates fixed to the top end of the tube. The head was then fixed in place using soft wax. The antennae were waxed at their scapes and pedicels to keep the frontal surface of the flagellum directed toward the stimulants. The cuticle of the head capsule was removed along with the compound eyes, the roots of the antennae and the median ocellus. The gland and tracheal tissues were also removed to expose the surface of the AL. To reduce the extent of brain movement, the mouthparts and muscles were carefully removed. The surface of the brain was covered with bee saline during the recording. A Ag-AgCl wire placed under the head capsule with a small piece of filter paper soaked in saline served as an indifferent electrode. The surface of the median portion of the AL was desheathed surgically using a fine tungsten needle, and a glass microelectrode filled with a 4% aqueous solution of Lucifer Yellow CH (resistance: 50–200 MΩ) was inserted into the AL. The stimulus was applied ipsilaterally to the recording side of the antenna after observation of the ongoing spike firings. Signals were monitored and stored using the same set of EAG recordings. For analysis of response properties, the number of spikes per 100 msec was counted sequentially on the charts. The sequential spike numbers during a 5-sec period, including the stimulation period, were recorded as a spike frequency histogram.

After intracellular recording, Lucifer Yellow was injected into the cell iontophoretically by application of a 1–10 nA hyperpolarizing direct current for 1–10 min. The brain was then dissected and fixed in 4% paraformaldehyde. After clearing in methyl benzoate, it was observed under an epifluorescence microscope. The morphology of the cells was reconstructed from color slide photographs or drawings made using a camera lucida.

RESULTS

Behavior and electroantennogram response to the Nasonov pheromone blend.

In order to estimate the effective dose range of NPB, the

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dose-behavior response relationship was examined. Preliminary experiments indicated that stimulation with 10^2 GE of NPB elicited a particular form of behavior in worker bees. This behavior comprised 4 sequential actions: stopping walking, fanning the wings without flight, raising the tip of the abdomen, and exposure of the groove between the 6th and 7th terga where the Nasonov gland opens [25]. The behavior lasted for between a few seconds and a few minutes. The delay between stimulation and onset of the behavior was about 1–2 sec. The dose-response relationship, obtained with 129 bees for each dose level, is shown in Figure 1. The behavior occurred in 27.9% of the control bees and those receiving 10^{-1} GE of NPB. The probability increased greatly from 10^{-1} to 10^2 GE. The maximum was 70.5% at 10^2 GE, and then the probability tended to decrease from 10^2 to 10^3 GE. The increase was significant between the control and 10^0 GE (χ^2-test: $P<0.05$) but non-significant between the control and 10^{-1} GE. Therefore, the threshold level was estimated to lie between 10^{-1} and 10^0 GE.

The effective range of NPB on antennal receptor cells was evaluated by EAG recordings made from 6 antennae, each stimulated with the 5 dose levels of NPB. No change, or only small changes, in potential were observed with control air and 10^{-1}-10^0 GE of NPB (Fig. 2A). However, stimulation with 10^1-10^3 GE clearly elicited EAG responses, which were negative deflections in potential at the distal end of the antenna compared with that at the proximal end during stimulation (Fig. 2A). The average amplitude of the re-

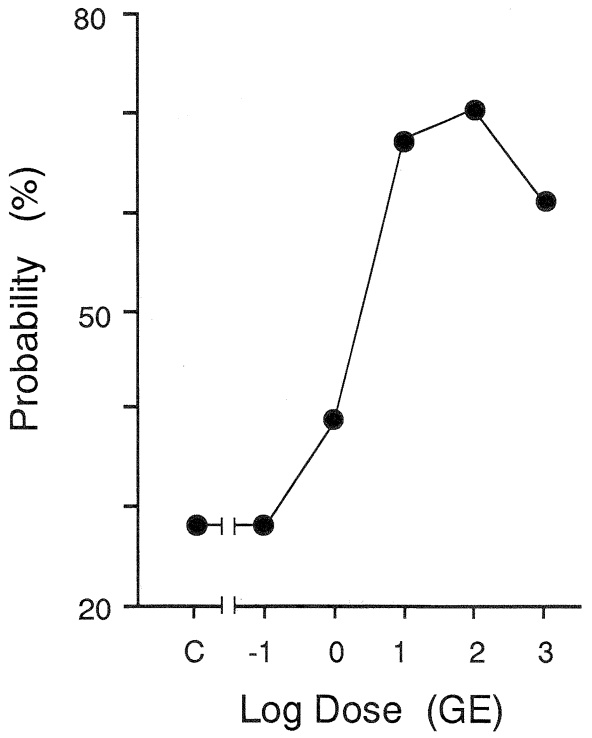

FIG. 1. A dose-response curve for the behavior induced by NPB. Each point indicates the probability of bees responding with a particular form of behavior (see text) among 129 tested bees. Numerals on the abscissa indicate log doses of NPB and C indicates the control stimulus.

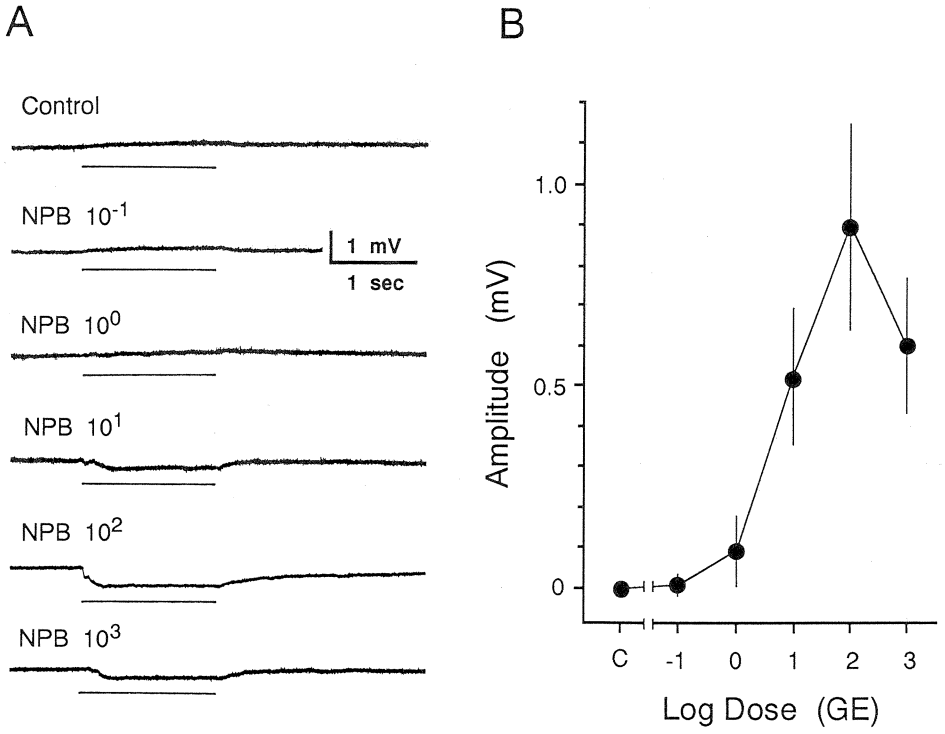

FIG. 2. EAG responses to NPB. (A): A sample recording of EAG responses to NPB. Horizontal lines under each trace indicate the stimulation period. (B): A dose-response curve of EAG responses. Each point indicates the average of 6 recordings, with the standard error represented by the vertical bar.

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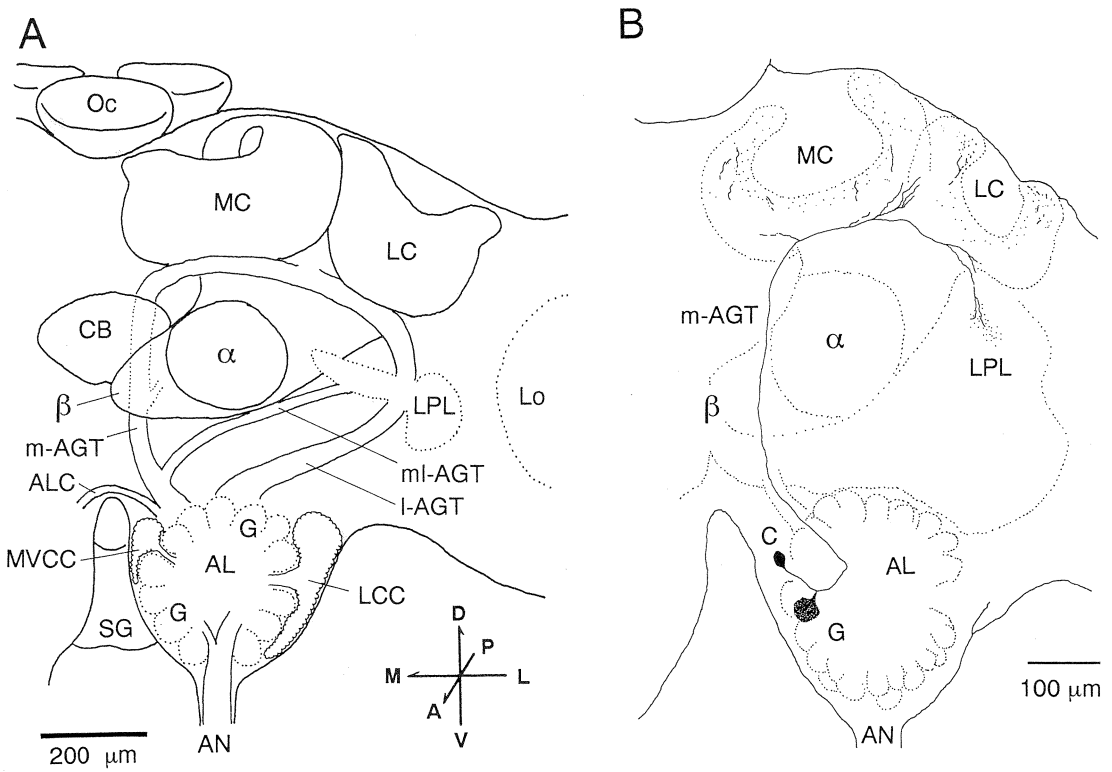

FIG. 3. Anatomy of the worker honeybee brain. (A): Schematic drawing of the brain. A, anterior; AL, antennal lobe; ALC, antennal lobe commissure; AN, antennal nerve; CB, central body; D, dorsal; G, glomerulus; L, lateral; l-AGT, lateral antenno-glomerular tract; LC, lateral calyx; LCC, cell cluster of lateral side; LPL, lateral protocerebral lobe; Lo, lobula; M, medial; m-AGT, median antenno-glomerular tract; MC, medial calyx; ml-AGT, median lateral antenno-glomerular tract; MVCC, cell cluster of medial and ventral side; O, ocellus; P, posterior; SG, suboesophageal ganglion; V, ventral; α, α-lobe of the mushroom body; β, β-lobe of the mushroom body. (B): A drawing of the morphology of a Pm(u) neuron stained with Lucifer Yellow; C indicates a cell body and G indicates a glomerulus innervated by the dendritic arborizations of the neuron. Other abbreviations are the same as those in Figure 3A. A single axon passed through the m-AGT to the protocerebrum and fine terminals were found in the calyces of the MB and the LPL. Responses to NPB were not recorded from this neuron.

sponses increased as the dose was increased from 10^{-1} to 10^{2} GE, coinciding with the behavioral result (Fig. 2B). A decrease in amplitude between 10^{2} and 10^{3} GE was found, as with the behavioral result (Fig. 2B). The increase in amplitude between the control and 10^{0} GE was significant (Wilcoxon signed rank test: $P<0.05$) but non-significant between the control and 10^{-1} GE. Thus, the threshold value was estimated to lie between 10^{-1} and 10^{0} GE, as for the behavioral threshold.

These data indicated that 10^{0} to 10^{3} GE of NPB had a significant effect on both behavior and the antennal receptor cells of worker bees. We therefore used these dose levels for recording the response of interneurons in the AL.

Characterization of neurons responding to the NPB in the antennal lobe

The responses to NPB were recorded from 88 interneurons, 22 of which were successfully stained with Lucifer Yellow, in the median portion of the AL within 280 μm of its surface (Fig. 3A). Morphologically characterized neurons fell into 2 classes: 12 projection neurons with axons projecting into the protocerebrum and uni- or multi-glomerular dendritic arborizations in the AL, and 10 local interneurons with

TABLE 2. Morphological and physiological characterization of interneurons in the AL of the worker bee brain

Morphological type	Response pattern		n*1
	NPB*2	Control*2	
Projection neurons			
Pm(u)	−and++*3	−*4	3
	++and±*3	nr*4	4
Pml(m)	+	+	2
Pc(m)	+'	+'	2
Local interneurons			
L(m)	+	+	8
	±	±	1
Totals			20

++: phasic-tonic exciation, +: phasic excitation, ±: on-off excitation, +': slight excitation, −: inhibition, nr: no response

*1 Numbers of neurons recorded as showing each class of morphology and response.

*2 NPB indicates a synthetic blend of the Nasonov pheromone used as the stimulant. Stimulation with a pure air puff is shown as the Control.

*3 These cells exhibited two types of response pattern according to the dose level of NPB.

*4 Each contained one neuron which was not tested using the control stimulation.

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multiglomerular processes restricted to the AL. Twenty of these neurons exhibited changes of spike activity in response to NPB, and the remainder (one projection and one local interneuron) were not tested using NPB. Most of them (8 projection and 9 local interneurons) showed excitatory responses to NPB; the other 3 neurons showed both excitatory and inhibitory responses (Table 2). The detailed response characteristics differed not only among morphological classes but also among individual cells. The morphological and physiological features of the cells are described in detail below.

Morphology and response characteristics of projection neurons

The 12 projection neurons were subdivisible into 3 classes based on their morphology: Pm(u) (n=8, Fig. 3B); Pml(m) (n=2, Fig. 7A); and Pc(m) (n=2, Fig. 8A). 'Pm', 'Pml' and 'Pc' refer to projection neurons with axons in the median (m) antenno-glomerular tract (AGT), in the median lateral (ml) AGT and in the AL commissure (c), respectively. '(u)' and '(m)' refer to uni- (u) and multi-glomerular (m) arborizations in the AL, respectively.

Pm(u): All neurons identified as Pm(u) had a cell body in the medial and ventral rind of the AL and their dendritic arborizations were mainly confined to a single glomerulus (Fig. 3B). A single axon passed through the m-AGT to the protocerebrum and fine terminals were found in the calyces of the mushroom body (MB) and the lateral protocerebral lobe (LPL) (Fig. 3B). Remarkable differences among individual cells were found in the position of the glomerulus innervated by the dendritic arborizations of each cell, e.g. the cell illustrated in Figure 3B innervated a glomerulus located in the medial portion of the AL, the cell shown in Figure 4A innervated one in the lateral portion, and the cell drawn in Figure 5A innervated a glomerulus in the antero-medial portion.

Pm(u) exhibited remarkable spike responses to NPB. The cell illustrated in Figure 4B was inhibited by 10^0–10^1 GE of NPB as well as by the control air. The inhibition was characterized by suppression of spike activity during stimulation with rebound activity after the cessation of stimulation. In contrast, stimulations with 10^2–10^3 GE elicited phasic-tonic excitation in which the frequency of spikes increased immediately after the start of stimulation, this high-frequency state lasted for the duration of stimulation with a gradual decrease, and the activity was usually suppressed after the cessation of stimulation. In addition, rhythmic bursts of spike activity were found during excitation, although this effect was not observed in other cells. The cell illustrated in Figure 5B showed no response to the control stimulation or to 10^{-1}–10^0 GE, but exhibited phasic-tonic excitations with 10^1–10^2 GE. Stimulation with 10^3 GE of NPB elicited an on-off excitation, characterized by brief increases of spike frequency after both the start and finish of stimulation.

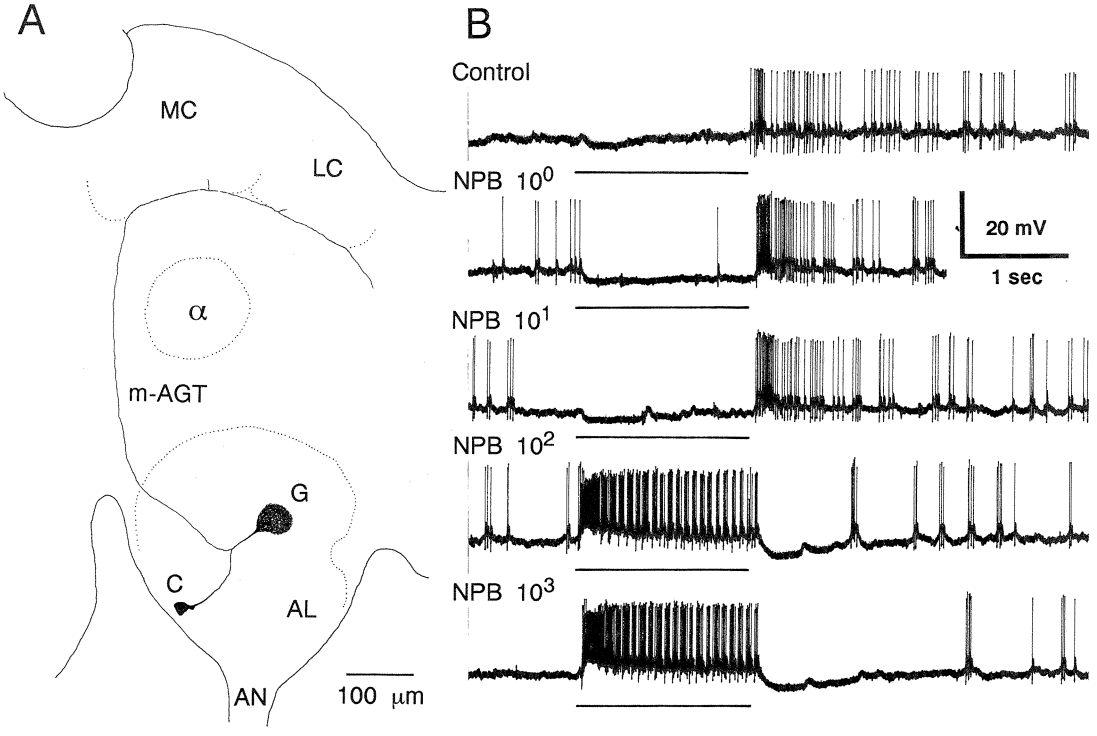

FIG. 4. Morphology and physiology of a Pm(u) neuron. (A): Drawing of a Pm(u). Abbreviations are the same as those in Figure 3A. This cell could not be traced to fine axonal terminals, but showed an axon in the m-AGT and looked similar enough to suggest that it was a Pm(u). (B): Responses of a Pm(u) to stimulation with NPB. Horizontal lines under each trace indicate the stimulation period. This cell showed inhibition of spike frequency during stimulation with the control air puff and 10^0–10^1 GE of NPB, and exhibited phasic-tonic excitation with 10^2–10^3 GE of NPB. In addition, this cell showed rhythmic spike bursts during excitation.

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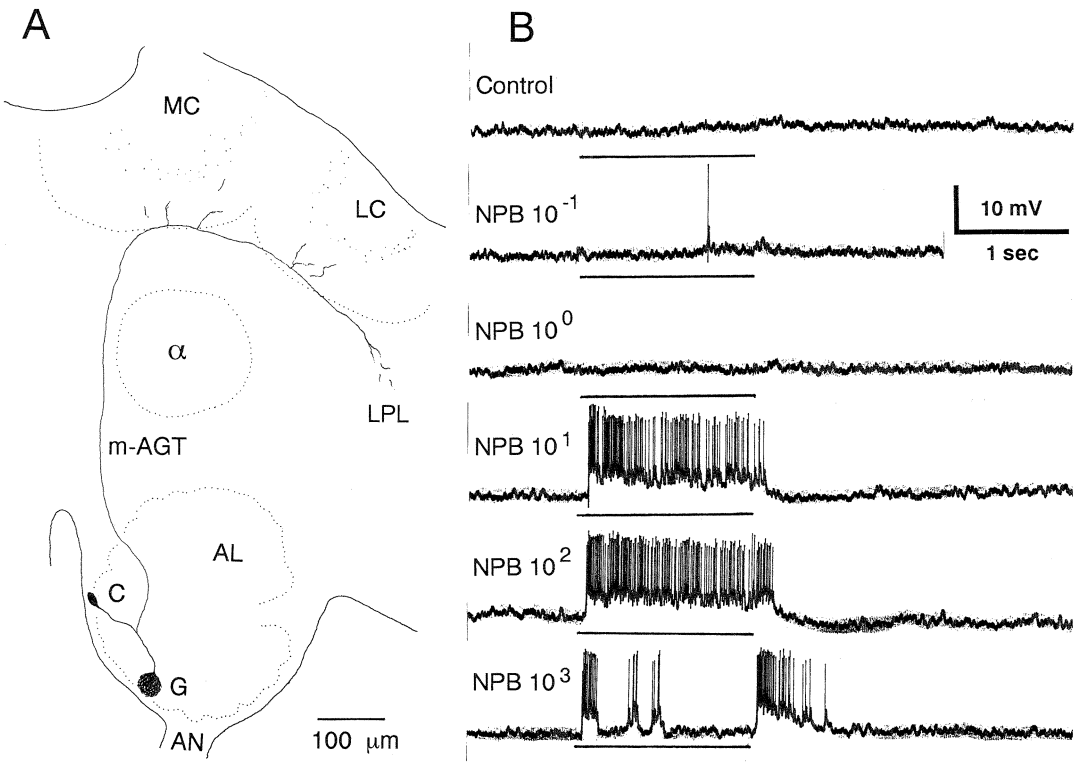

FIG. 5. Morphology and physiology of a Pm(u) neuron. (A): Drawing of a Pm(u). Abbreviations are the same as those in Figure 3A. (B): Responses of a Pm(u) to NPB. Horizontal lines under each trace indicate the stimulation period. Stimulation with the control air puff and 10^{-1}–10^{0} GE of NPB elicited no response. The cell exhibited phasic-tonic excitation with 10^{1}–10^{2} GE NPB, and on-off excitation with 103 GE of NPB.

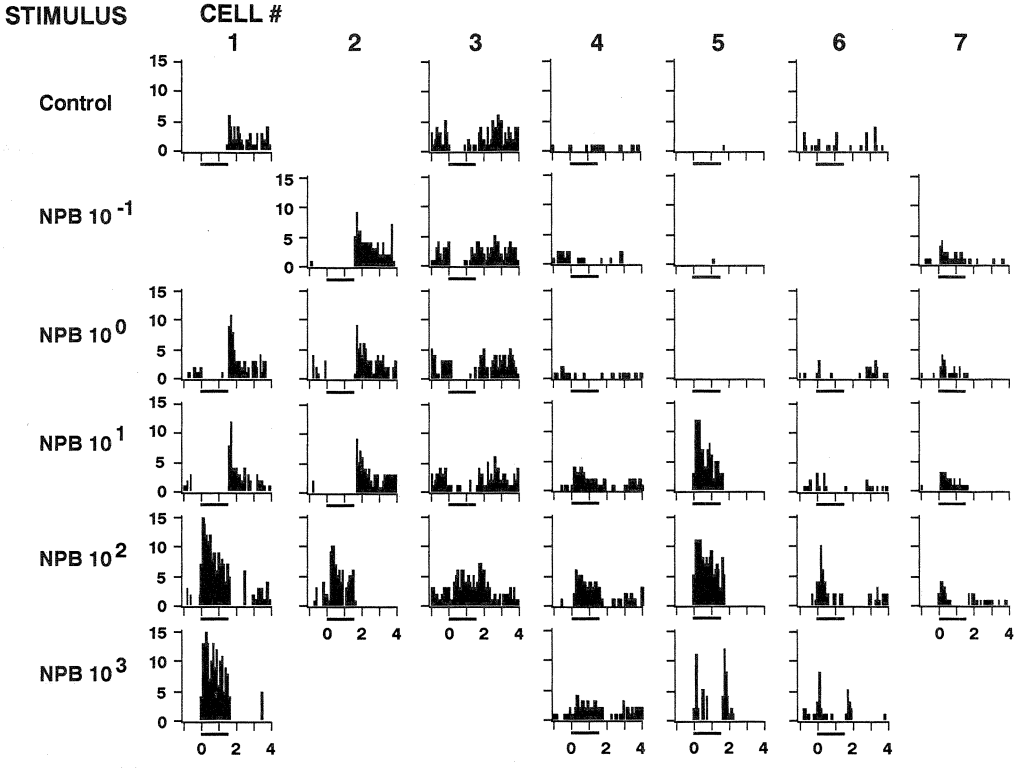

FIG. 6. Responses of 7 Pm(u) neurons to NPB, represented by spike frequency histograms. Each column headed CELL # shows the responses of a Pm(u) to a series of stimulations. CELL #1 is the neuron whose morphology and recordings are illustrated in Figure 4. CELL #5 is the neuron whose morphology and recordings are illustrated in Figure 5. The ordinate of each histogram indicates spike number per 100 msec. Abscissa indicates time (sec), with 0 being the onset of the stimulus. Horizontal lines under each histogram indicate the stimulation period. A blank space indicates that the stimulus was not tested.

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The spike frequency histograms of all responses obtained from 7 Pm(u) are shown in Figure 6. Their background spike activities varied widely from cell to cell (between 0 and 30 Hz). The histograms demonstrated that individual cells exhibited variable patterns and intensities of responses according to the dose level of NPB, but they all exhibited phasic-tonic excitation in response to at least one of the five dose levels of NPB. Cells #1–3 exhibited both inhibition and excitation by NPB, as mentioned above. Cells #4–7 showed only excitatory responses to relatively high dose levels of NPB, but little or no response to the control and lower dose levels of NPB. The threshold differed from one cell to another: cells #4 and #5 were excited by NPB above 10^1 GE, cell #6 only above 10^2 GE, and cell #7 at every dose. Cell #4 showed only phasic-tonic excitations but cells #5–7 showed both phasic-tonic excitation (10^1–10^2 in cell #5, 10^2 in #6, and 10^{-1}-10^1 in #7) and on-off-type excitation (10^3 in cell #5 and #6, and 10^2 in #7). The dose-dependency of spike number during stimulation of cells #4 and #5 was similar to that of the behavior and the EAG.

Pml(m): The soma of the Pml(m) drawn in Figure 7A was located in the anterior and ventral rind of the AL neuropil and numerous dendritic branches extended throughout the AL. The axon passing through the ml-AGT was divided into several branches in the lateral protocerebrum, and terminated mainly around theα-lobe of the MB and at the LPL (Fig. 7A). Numbers of bleb-like varicosities were observed at the LPL and around the α-lobe (Fig. 7A). The 2 Pml(m) neurons exhibited relatively high background activity of between 20 and 30 Hz, and showed slight excitation in response to NPB. These responses were characterized by phasic excitations with only a transient increase of spike frequency after the start of the stimulus (Fig. 7B). The increases of spike frequency induced by stimulation were brief and relatively small, but tended to become larger as the dose of NPB was increased (Fig. 7B).

Pc(m): The 2 Pc(m) had multiglomerular arborizations both ipsi- and contra-lateral to the cell body which was located at the dorsal border between the AL and the protocerebrum (Fig. 8A). The axon entered the contralateral AL through the AL commissure, and then projected to the lateral horn (LH) of the protocerebrum (Fig. 8A). The processes had fine, smooth endings in the AL ipsilateral to the cell body, whereas those in the contralateral AL and the protocerebrum had bleb-like endings (Fig. 8A). No background firings and only slight excitations in response to NPB are seen in Figure 8B. This type of response was characterized by several spikes during stimulation when the stimuli were applied to the antenna ipsilateral to the cell body, and was distinguishable from the phasic-tonic, the phasic, or the on-off excitations mentioned above. Response to contralateral stimulation of the antenna was not measured in this

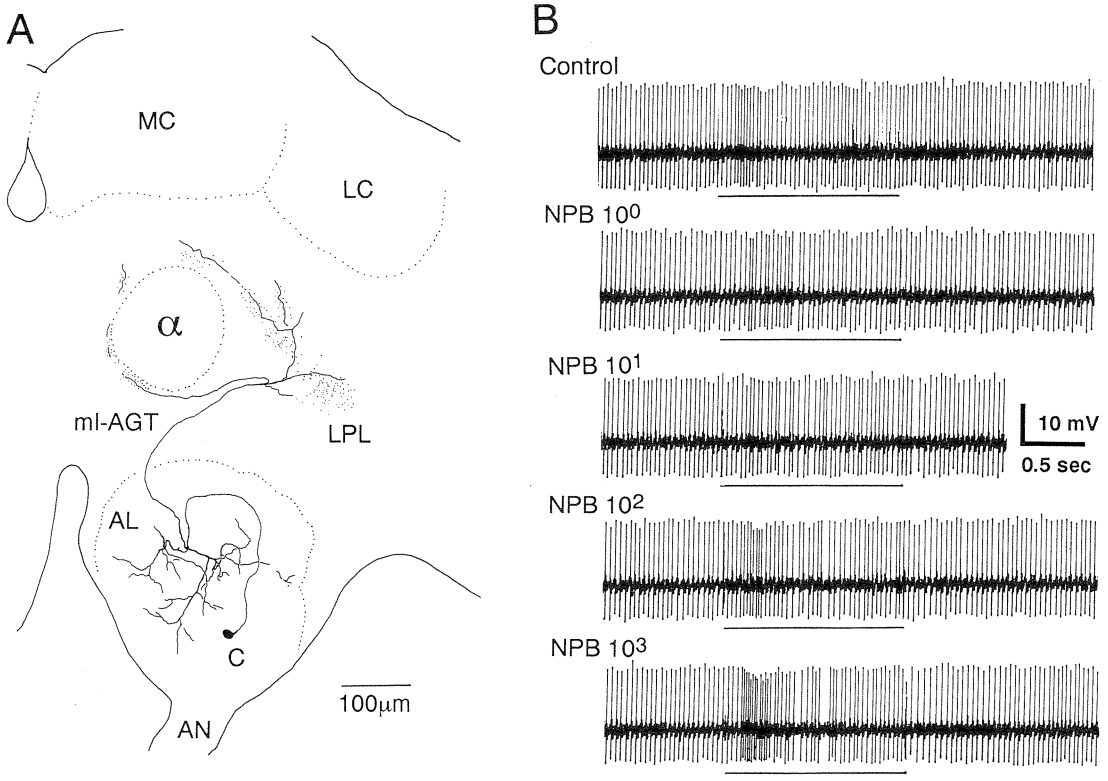

FIG. 7. Morphology and physiology of a Pml(m) neuron. (A): Drawing of a Pml(m). Abbreviations are the same as those in Figure 3A. This cell had multiglomerular arborizations in the AL and an axon projecting into the protocerebrum through the ml-AGT. (B): Responses of a Pml(m) to stimulation with NPB. Horizontal lines under each trace indicate the stimulation period. This neuron showed phasic excitation in response to NPB, characterized by a slight, brief increase of spike frequency after the start of the stimulation.

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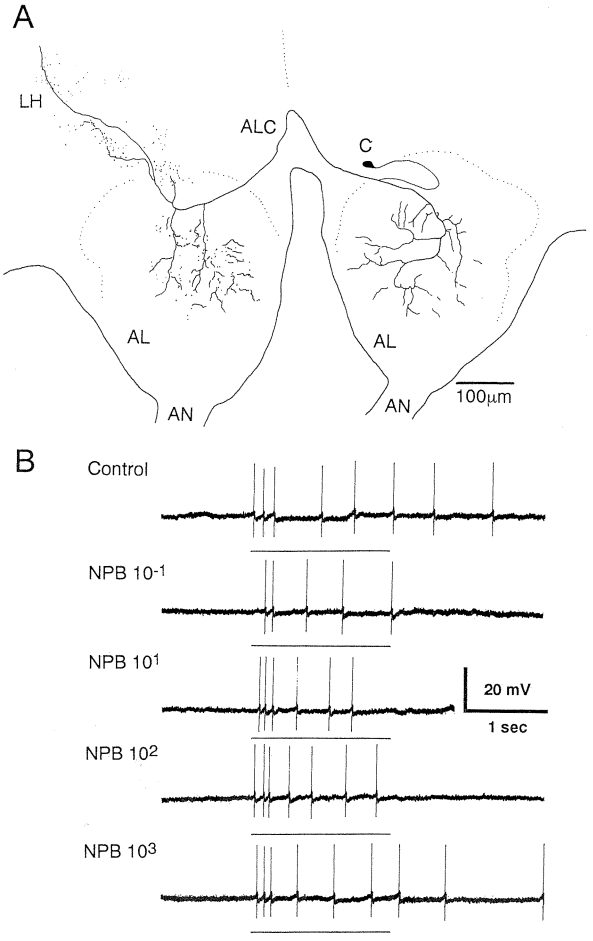

study.

Morphology and response characteristics of local interneurons

Ten neurons were identified as local interneurons, and designated L(m). All of them had a soma located in the lateral cell cluster of the AL (Fig. 9). The detailed morphology varied among individuals: 5 had dense arborizations at one or a few glomeruli in addition to several processes extending to many of the other glomeruli (Fig. 9A); the others did not have such dense arborizations (Fig. 9B).

The 9 L(m) which were stimulated by NPB responded only slightly to each dose. The cell illustrated in Figure 10A showed no background spike activity, and was excited slightly in response to stimulation at all dose levels of NPB as well as the control stimulation. The cell drawn in Figure 10B was also excited in response to antennal stimulation, although it showed a relatively high frequency of background firings. The temporal features of both cells were characterized by a transient brief excitation period after the start of the stimulation, designated as phasic excitation. Such temporal features were similar to those of Pml(m) mentioned above.

FIG. 8. Morphology and physiology of a Pc(m) neuron. (A): Drawing of a Pc(m). The axon of this cell extended into the contralateral AL through the AL commissure (ALC), and then projected into the lateral horn of the contralateral protocerebrum (LH). Other abbreviations are the same as those in Figure 3A. (B): Responses of a Pc(m) to stimulation with NPB. Horizontal lines under each trace indicate the stimulation period. This neuron responded only slightly to NPB. The stimuli were applied to the antenna ipsilateral to the cell body.

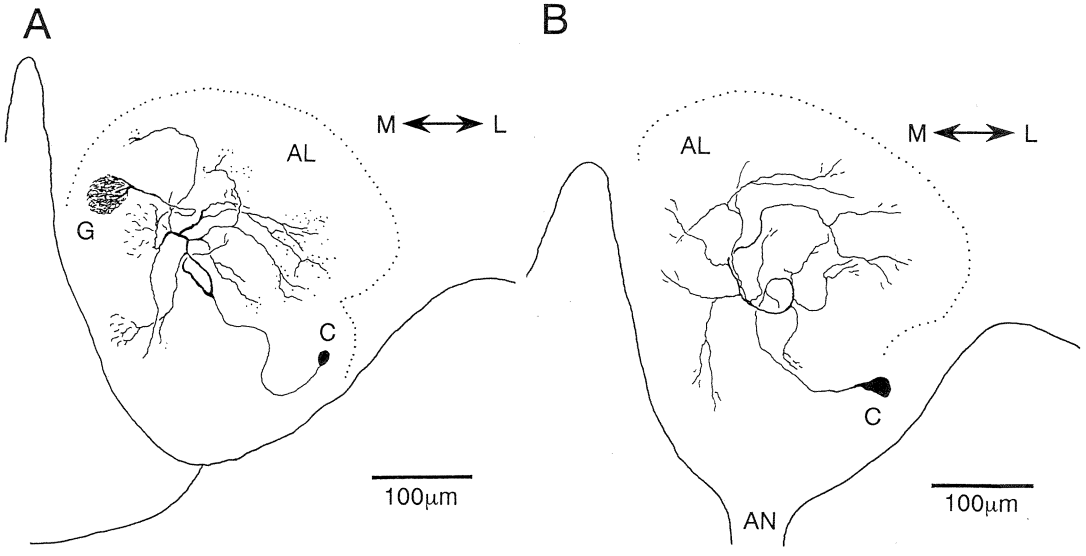

FIG. 9. Morphology of L(m) neurons. (A): Drawing of morphology of an L(m) neuron. Abbreviations are the same as those in Figure 3A. The processes were restricted to the AL and the cell body (C) was located in a cell cluster of the lateral rind. This cell had dense arborizations at a glomerulus (G) in addition to many processes extending to many other glomeruli in the AL. Responses to NPB were not recorded from this neuron. (B): Drawing of an L(m). Abbreviations are the same as those in Figure 3A. This cell had no dense arborizations, but many processes extending to many glomeruli.

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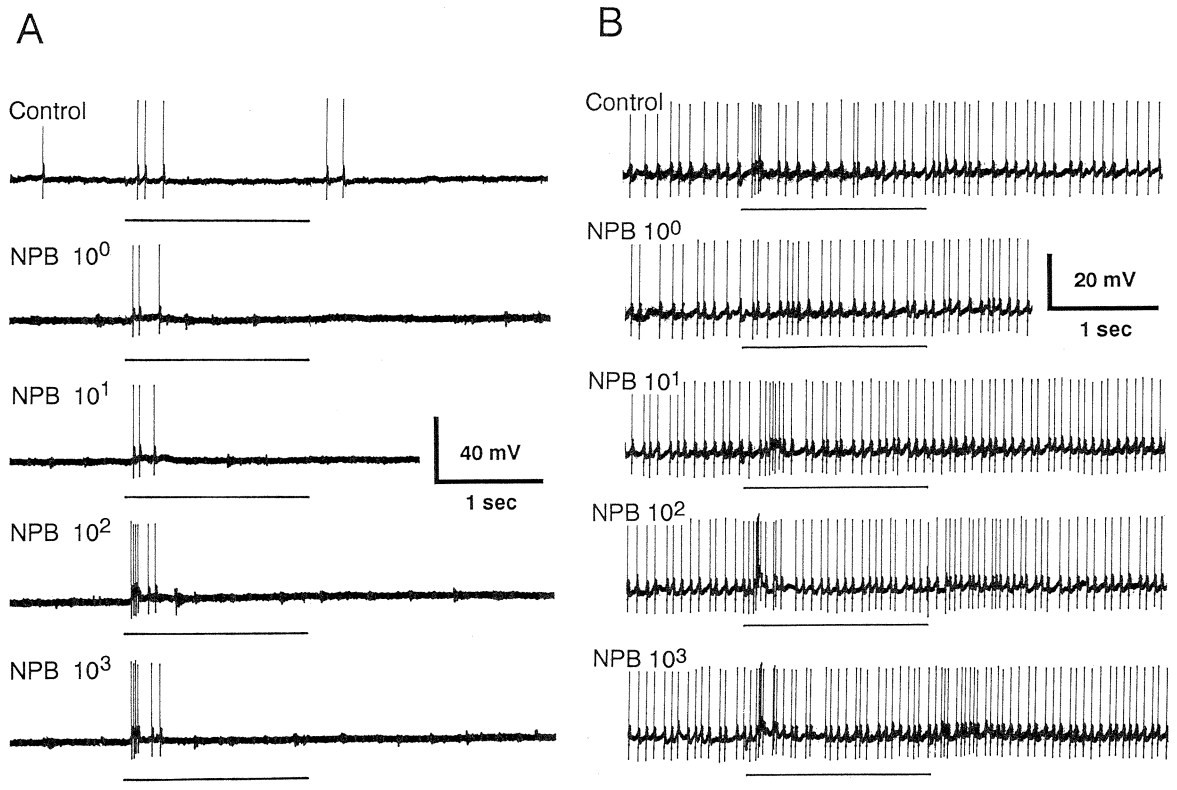

FIG. 10. Sample recordings of responses of 2 L(m) to NPB. Horizontal lines under each trace indicate the stimulation period. (A): Responses of an L(m) to stimulation with 5 dose levels of NPB. This recording was obtained from the neuron illustrated in Figure 9B. This cell showed phasic excitation characterized by transient excitation after the start of stimulation. (B): Responses of another L(m) to NPB. This cell showed relatively high background activity and phasic excitation after the start of stimulation.

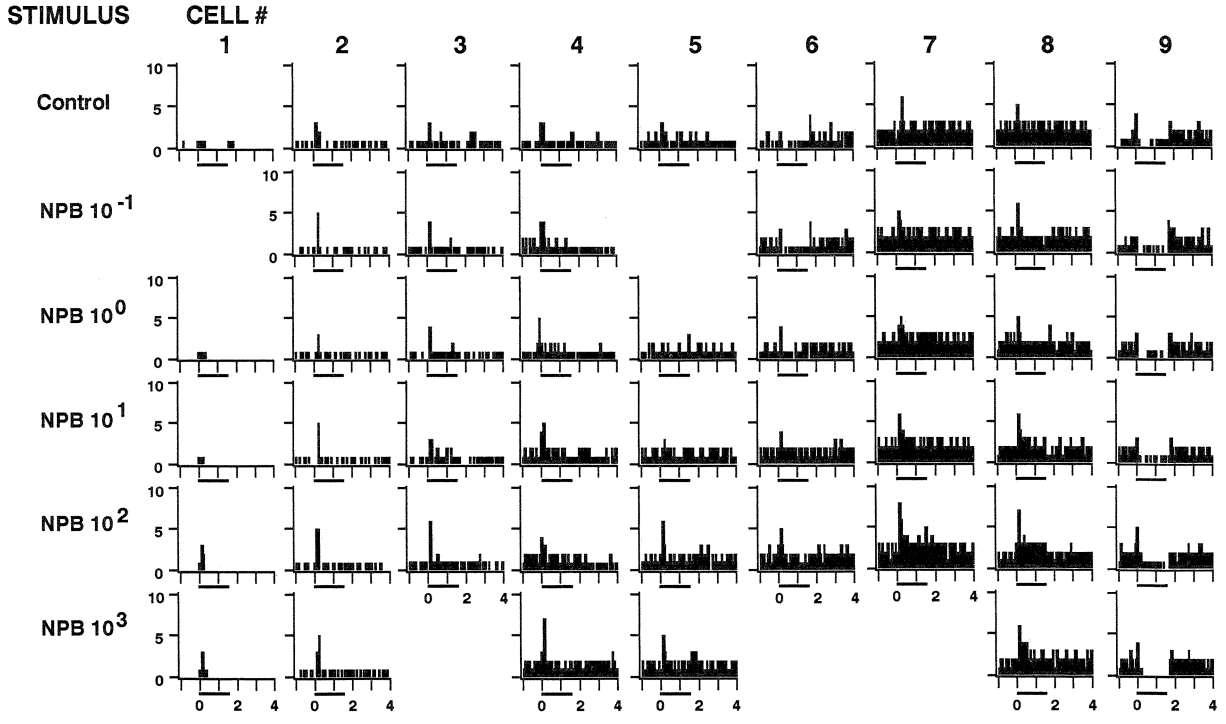

FIG. 11. Responses of 9 L(m) neurons to NPB, shown by spike frequency histograms. Each column marked headed CELL # shows the responses of an L(m) to a series of stimulations. CELL #1 is the same recording as that in Figure 10A, and CELL #5 the same as that in Figure 10B. Ordinate, abscissa and horizontal lines on each histogram have the same meaning as those in Figure 6. A blank space indicates that the stimulus was not tested.

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The spike frequency histograms of all responses obtained from 9 L(m) are shown in Figure 11. The background activity varied widely among individual cells, but was less than 30 Hz. The response of one neuron (cell #9 in Figure 11) was found to show a more complex pattern, characterized by transient excitation after both the start and the finish of stimulation with inhibition during stimulation: this resembled the on-off excitations exhibited by some Pm(u). The histograms demonstrated that the increases of spike frequency induced by stimulation were relatively small, but tended to become larger as the dose of NPB was increased, as with the Pml(m).

DISCUSSION

In this study, responses of honeybee AL interneurons to the Nasonov pheromone were examined for the first time. Most interneurons examined exhibited changes of spike activity in response to the pheromone. It is necessary to consider whether these responses were identical to those elicited by the pheromone naturally emitted from worker bees, since we used only a synthetic blend (NPB) as the stimulant. Our study demonstrated that NPB elicited a particular form of behavior, which has been reported previously as the Nasonov release, etc. [8, 11, 13, 24, 30, 34]. It has been reported that this behavior was elicited by odors derived from excised Nasonov glands, which were thought to be identical to the natural pheromone [8]. Honeybees have also been reported to respond naturally when exposed to dishes containing the same doses of a synthetic blend as those used in our study [30, 38]. Thus, NPB is likely to have almost the same effect on behavior as the natural pheromone. In addition, we found that the dose-response relationship revealed by EAG coincided with that of the behavioral response, suggesting that the sensory neurons in the antenna are adequately stimulated by NPB [39]. Thus, each response of the interneurons examined in this study is likely to be one of the principal signals relating to the Nasonov pheromone processed in the AL of the honeybee brain.

We identified 4 classes of interneuron in the AL based on their morphology, and demonstrated that all them responded to NPB, indicating that the signals derived from the Nasonov pheromone are widely recognized in multiple morphological classes of AL neurons. The morphological characterization accords well with previous work: Pm(u) have been described as uniglomerular output neurons [12, 17] or extrinsic mushroom body neurons [15], Pml(m) as pluriglomerular output neurons [12], and L(m) as local interneurons [9, 10, 12, 17]. The Pc(m) had not been studied previously in honeybees. Fonta *et al.* [12] described bilateral AL neurons with a morphology resembling that of Pc(m). However, unlike the Pc(m), these cells had no axons projecting to the protocerebrum. The detailed morphology of Pc(m) suggests that the signals provided by an antenna are conveyed to the protocerebrum not only ipsilaterally but also contralaterally. On the other hand, we have never obtained any neurons having axons in the lateral AGT, which is one of the major tracts to the protocerebrum from the AL [2, 12, 28]. Thus, our knowledge of the AL neurons involved in the pheromone processing may still be incomplete.

In this study, at least 5 types of temporal patterns in response to NPB were recorded from morphologically characterized neurons in the AL: phasic-tonic, on-off, and phasic types of excitation, a type of inhibition, and slight excitation. Sun *et al.* [35] have reported that 10 types of temporal pattern were recognized among responses of AL interneurons when worker bee antennae were stimulated with 3 kinds of pure chemicals and their mixtures. Our classification of response patterns seems to be included in the previous one [35]. The fact that the type of odor we used as a stimulant was different from that used in the previous study may explain the difference in the number of response types between the two studies. On the other hand, we demonstrated that the temporal patterns tended to be correlated with the morphological classes: Pm(u) showed mainly phasic-tonic excitations, and in some cases on-off excitations or inhibitions; Pc(m) showed only slight excitation; Pml(m) and most of the L(m) showed mainly phasic excitation. According to the temporal features, both the phasic-tonic excitation and the inhibition can be interpreted as suitable for encoding stimulus duration, and the phasic and the on-off excitation may encode the timing of stimulus start or finish. Thus, it is possible that each class of neuron may contribute to information processing of distinct parameters related to the pheromone stimulation, although the parameters conveyed by the Pc(m) cannot be predicted. Furthermore, information processing of such distinct parameters may be related to distinct sites in the protocerebrum because the projection neurons send axons to different sites, according to their neuronal class.

The concentration of pheromone is thought to be one of the most important pieces of information received by honeybees. Pm(u) showed remarkable changes in the temporal pattern of responses and in spike frequency during stimulation, depending on the dose of NPB. However, the other 3 classes of neurons showed invariant temporal patterns at different dose levels of NPB, and exhibited only slight increases in spike number during stimulation as the dose of NPB was increased. Thus, the concentration information may be detected by every class of neuron to some extent, but Pm(u) may be the most suitable cells for mediating such information. Since the dose-response relationships varied widely among individual Pm(u), the Pm(u) population may mediate information about various levels of pheromone concentration at the same time. Interestingly, it has been reported that the Nasonov pheromone induces multiple types of behavior in worker bees [11]. Thus, the variation in the dose-response relationship of Pm(u) may be related to such multiple functions of the pheromone. Some Pm(u) showed a dose-dependency corresponding to that observed in our behavioral study, suggesting that such cells may mediate the concentration information relating to induction of the particular form of behavior described in this paper. However,

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some other cells, which showed thresholds different from that deduced from our behavioral results, may be associated with other kinds of behavior such as attraction to the pheromone source. Further study will be necessary in order to confirm this possibility.

Many of the recorded neurons responded to the control air current—that is, to a mechanosensory stimulus [15, 21]. Typical examples of such bimodal responses were shown by some Pm(u), which exhibited marked inhibition of spike activity with the control stimulus but were strongly excited by NPB (e.g. Fig. 4B). As all stimuli were applied by a puff of air in this study, all NPB stimulations contained a mechanosensory factor. This factor might have had an inhibitory effect on these cells, but the stimulation resulting from higher doses of NPB might have had a sufficient excitatory effect to overcome any such inhibition, and so these cells might exhibit either inhibitory or excitatory responses depending on the dose of NPB. In addition, the rhythmic bursts seen during excitation, as demonstrated in Figure 4, appear to support the possibility that the neuron receives both inhibitory and excitatory synaptic inputs upon stimulation with NPB. These findings suggest that some AL interneurons responsive to the Nasonov pheromone may mediate the bimodal information provided by antennal stimulation [15, 17, 21].

Differences in response characteristics among individual cells are thought to originate from individual differences in synaptic connections to receptor cells and to other interneurons. Previous studies [33, 28] have demonstrated that individual axons of many antennal receptor cells terminate in a single glomerulus. Thus, the interaction between interneurons and receptor cells may be simply observed in the responses of uniglomerular neurons rather than those of multiglomerular neurons. We demonstrated that individual Pm(u) had dendritic arborizations in different positions of a glomerulus, and that their response characteristics varied widely from cell to cell. These facts suggest that numerous axons of the receptor cells responding to the Nasonov pheromone may be widely distributed to many glomeruli in the AL rather than concentrated in a small number of specific glomeruli. On the other hand, our data indicate that many multiglomerular neurons, such as L(m), respond to the NPB. These neurons may be employed in signal processing within the AL by providing inter-glomerular interactions between interneurons. It is possible that such interaction may modify largely the responses of other interneurons even if they are uniglomerular neurons. Currently, however, there is little direct evidence for this in honeybees [9, 12]. For further understanding of the pheromone processing mechanism in the honeybee AL, it will be necessary to examine the interactions between receptor cells and interneurons, and also between interneurons. At the very least, however, these results indicate that the architecture of the bee AL relating to pheromone processing may be complex, as suggested by Sun *et al.* [35].

It is thought that the induction and control of behavior are mediated by a higher center in the brain [7, 15, 20, 22]. Both the antennal receptor and the AL neuron levels showed responses that closely followed the temporal features of the stimulation: most of examined interneurons exhibited spike responses to NPB during stimulation or at the start and/or finish of stimulation; EAG responses were also characterized by a change in potential during stimulation. On the other hand, the duration of the behavior described in this paper varied widely from a second to a few minutes, and the behavior frequently continued after the cessation of stimulation. Thus, the temporal features of the behavioral responses differ from those of the AL interneurons. This suggests that the responses of AL interneurons are not mediated by direct signals which control the motor systems related to the behavior, and that further processing by higher centers is needed for control of these motor activities. The study of such higher centers may serve to further our understanding of olfactory behavior in insects.

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REFERENCES

1 Akers RP, Getz WM (1993) Response of olfactory receptor neurons in honeybees to odorants and their binary mixtures. J Comp Physiol A 173: 169–185

2 Arnold G, Masson C, Budharugsa S (1985) Comparative study of the antennal lobes and their afferent pathway in the worker bee and the drone (*Apis mellifera*). Cell Tissue Res 242: 593–605

3 Boeckh J, Kaissling KE, Schneider D (1965) Insect olfactory receptors. Cold Spring Harbor Symp Quant Biol 30: 263–280

4 Boeckh J, Ernst K-D, Sass H, Waldow U (1984) Anatomical and physiological characteristics of individual neurones in the central antennal pathway of insects. J Insect Physiol 30: 15–26

5 Burrows M, Boeckh J, Esslen J (1982) Physiological and morphological properties of interneurones in the deutocerebrum of male cockroaches which respond to female pheromone. J Comp Physiol 145: 447–457

6 Christensen TA, Hildebrand JG (1987) Male-specific, sex pheromone- selective projection neurons in the antennal lobes of the moth *Manduca sexta*. J Comp Physiol A 160: 553–569

7 Erber J (1987) Response characteristics and after effects of multimodal neurons in the mushroom body area of the honeybee. Physiol Entmol 3: 77–80

8 Ferguson AW, Free JB (1981) Factors determining the release of Nasonov pheromone by honeybees at the hive entrance. Physiol Entmol 6: 15–19

9 Flanagan D, Mercer AR (1989) Morphology and response characteristics of neurons in the deutocerebrum of the brain in the honeybee *Apis mellifera*. J Comp Physiol A 164: 483–494

10 Flanagan D, Mercer AR (1989) An atlas and 3-D reconstruction of the antennal lobes in the worker honeybee, *Apis mellifera* L. (Hymenoptera: Apidae). Int J Insect Morphol Embryol 18: 145–159

11 Free JB (1987) Pheromone of social bees. Chapman and

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Hall, London.

12 Fonta C, Sun X-J, Masson C (1993) Morphology and spatial distribution of bee antennal lobe interneurones responsive to odors. Chemical Senses 18: 101–119

13 Frisch K von (1923) Über die 'Sprache' der Bienen. Zool Jahrb Abt Allg Zool Physiol 40: 1–186

14 Hansson BS, Christensen TA, Hildebrand JG (1991) Functionally distinct subdivisions of the macroglomerular complex in the antennal lobe of the male sphynx moth *Manduca sexta*. J Comp Neurol 312: 264–278

15 Homberg U (1984) Processing of antennal information in extrinsic mushroom body neurons of bee brain. J Comp Physiol 154: 825–836

16 Homberg U, Montague RA, Hildebrand JG (1988) Anatomy of antenno-cerebral pathways in the brain of the sphynx moth *Manduca sexta*. Cell Tissue Res 254: 255–281

17 Itoh T, Yokohari F, Tominaga Y (1991) Response to humidity change of deutocerebral interneurons of the honeybee, *Apis mellifera* L. Naturwissenschaften 78: 320–322

18 Kaissling K-E, Renner M (1968) Antennale Rezeptoren f r Queen Substance und Sterzelduft bei der Honigbiene. Z vergl Physiol 59: 357–361

19 Kanzaki R, Shibuya T (1986) Identification of the deutocerebral neurons responding to the sexual pheromone in the male silkmoth brain. Zool Sci 3: 409–418

20 Kanzaki R, Shibuya T (1986) Descending protocerebral neurons related to the mating dance of the male silkworm moth. Brain Res 377: 378–382

21 Kanzaki R, Arbas EA, Strausfeld NJ, Hildebrand JG (1989) Physiology and morphology of projection neurons in the antennal lobe of the male moth *Manduca sexta*. J Comp Physiol A 165: 427–453

22 Kanzaki R, Shibuya T (1992) Olfactory processing pathways of the insect brain. Zool Sci 9: 241–264

23 Lacher V (1964) Elektrophysiologische Untersuchungen an einzelnen Rezeptoren für Geruch, Kihlendioxyd, Luftfeuchtigkeit und Temperatur auf den Antennen der Arbeitsbiene und der Drone (*Apis mellifica* L.). Z vergl Physiol 48: 587–623

24 Lecomte J (1957) Scent-marking of sources of food by foraging bees. C R Acad Sci Paris 245: 2385–2387

25 McIndoo NE (1914) The scent producing organ of the honeybee. Proc Soc Natl Sci Phil 66: 542–555

26 Matsumoto SG, Hildebrand JG (1981) Olfactory mechanisms in the moth *Manduca sexta*: response characteristics and morphology of central neurons in the antennal lobes. Proc R Soc Lond B 213: 249–277

27 Menzel R, Hammer M, Braun G, Mauelshagen J, Sugawa M (1991) Neurobiology of learning and memory in honeybees. In "The Behaviour and Physiology of Bees" Ed by LJ Goodman, RC Fisher, C·A·B International, Wallingford, pp 323–353

28 Mobbs PG (1982) The brain of the honeybee *Apis mellifera*. I. The connections and spatial organization of the mushroom bodies. Phil Trans R Soc Lond B 298: 309–354

29 Morse RA, Boch R (1971) Pheromone concert in swarming honey bees (Hymenoptera: Apidae). Ann Ent Soc Am 64: 1414–1417

30 Pflumm W, Wilhelm K (1982) Olfactory feedback in the scent marking behaviour of foraging honeybees at the food source? Physiol Entmol 7: 203–207

31 Pickett JA, Williams IH, Martin AP, Smith MC (1980) Nasonov pheromone of the honey bee, *Apis mellifera* L. (Hymenoptera: Apidae). Part I. Chemical characterization. J Chem Ecol 6: 425–434

32 Ribbands CR, Speirs N (1953) The adaptability of the homecoming honeybee. Br J Anim Behav 1: 59–66

33 Rospars JP (1988) Structure and development of the insect antennodeutocerebral system. Int J Insect Morphol Embryol 17: 243–294

34 Sladen FWL (1901) A scent organ in the bee. Br Bee J 29: 1513–1521

35 Sun X-J, Fonta C, Masson C (1993) Odor quality processing by bee antennal lobe interneurones. Chemical Senses 18: 355–377

36 Suzuki H, Tateda H (1974) An electrophysiological study of olfactory interneurons in the brain of the honey-bee. J Insect Physiol 20: 2287–2299

37 Vareschi E (1971) Duftunterscheidung bei der Honigbiene-Einzellzell-Ableitungen und Verhaltensreaktionen. Z vergl Physiol 75: 143–173

38 Williams IH, Pickett JA, Martin AP (1981) The Nasonov pheromone of the honeybee *Apis mellifera* L. (Hymenoptera: Apidae). Part II. Bioassay of the components using foragers. J Chem Ecol 7: 225–237

39 Williams IH, Pickett JA, Martin AP (1982) Nasonov pheromone of the honeybee *Apis mellifera* L. (Hymenoptera, Apidae). Part IV. Comparative electroantennogram responses. J Chem Ecol 8: 567–574

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