Neural plasticity of mushroom body-extrinsic neurons in the honeybee brain

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Summary

Central interneurons exiting the alpha lobe of the mushroom bodies were studied with respect to their plasticity by electrically stimulating their presynaptic inputs, the Kenyon cells. Special attention was given to the analysis of a single, identified neuron, the PE1. Three stimulation protocols were tested: double pulses, tetanus (100 Hz for 1 s), and tetanus paired with intracellular de- or hyper-polarization of the recorded cell. Double-pulse stimulations revealed short-term facilitation and depression, tuning the responses of these interneurons to frequencies in the range of 20–40 Hz. The tetanus may lead to augmentation of responses to test stimuli lasting for several minutes, or to depression followed by augmentation. Associative long-term potentiation (LTP) was induced in the PE1 neuron by pairing a presynaptic tetanus with depolarization. This is the first time that associative LTP has been found in an interneuron of the insect nervous system. These data are discussed in the context of spike tuning in the output of the mushroom body, and the potential role of associative LTP in olfactory learning. It is concluded that the honeybee mushroom body output neurons are likely to contribute to the formation of olfactory memory.

Key words: plasticity, central neurons, insect, honeybee, associative LTP, mushroom body neurons.

Introduction

The mushroom bodies (MB) of the insect brain are higher-order integration centers that are involved in sensory processing, memory storage and the control of motor patterns like walking and stridulation (Huber, 1990; Heisenberg, 2003; Menzel, 2001). The role of the MB in olfactory learning, memory formation and retrieval from memory has been the focus of research since researchers first determined, in bees (Menzel et al., 1974; Erber et al., 1980) and then in *Drosophila* (Heisenberg et al., 1985), that MB are required for the formation of olfactory memory. Manipulating second messenger cascades in *Drosophila* MB using molecular genetic tools revealed that both the acquisition process in olfactory learning and the formation of memory traces can be assigned to the mushroom body's intrinsic neurons, the Kenyon cells (K-cells; Connolly et al., 1996; Waddell et al., 2000; Pascual and Preat, 2001). Interfering with the presynaptic machinery in selected groups of K-cells, it was possible to relate the cellular processes underlying short-term olfactory memory to a sub-compartment of the output regions of the MB, the gamma-lobe, and to document that synaptic transmission from K-cells to extrinsic neurons is not required for acquisition and short-term memory formation. Long-term memory, however, appears to involve other output regions (the alpha-and/or beta lobe), as seen in experiments in which these lobes were missing in both MB, which demonstrated that animals had lower retention 24 h after learning (Pascual and Preat, 2001). The model arising from such studies on olfactory learning and memory processing in *Drosophila* relates the underlying synaptic plasticity to the presynaptic compartments of the MB, the lobes, and assumes that all forms of olfactory learning (aversive and appetitive learning as well as extinction of aversive learning) are confined to the output sites of the MB (for a review, see Heisenberg, 2003).

The data from the honeybee are conceptualized differently. Hammer (1993) identified an unpaired median neuron arising from the subesophageal ganglion (the ventral unpaired median neuron no. 1 of the maxillary neuromere, VUMmx1) whose activity immediately following an odour stimulus is sufficient to implement the reinforcing function of the sucrose stimulus in olfactory reward conditioning. This neuron arborizes in the antennal lobes, the lip regions of the MB calyces and the lateral horns on both sides of the brain, suggesting that its convergence with the olfactory pathway at these three symmetrical sites induces associative plasticity at these sites. Support for the conclusion that release of the putative transmitter of VUMmx1, octopamine (Kreissl et al., 1994), at two of these sites (antennal lobe and MB) is involved in the formation of the memory trace comes from local injections of octopamine into the antennal lobe or MB as a replacement for the reward in an olfactory conditioning experiment (Hammer and Menzel, 1998). It is hypothesized that the olfactory memory trace is distributed between the antennal lobes and the

input sides of the MB, the lip regions of the calyx. Calcium-imaging studies support this conclusion (Faber et al., 1999).

Furthermore, the output site of the bee's MB was also related to olfactory learning in an electrophysiological study by Mauelshagen (1993), who recorded from an identified alpha lobe-extrinsic neuron, the PE1. This neuron arborizes extensively in the peduncle and projects to the lateral horn. Differential conditioning leads first to a reduction (after a single learning trial) and then to an enhancement (in the course of five learning trials) of its response to the conditioning stimulus, CS^+. No changes were observed in its responses to the CS^-, the non-rewarded odour. The associative changes in PE1 may have largely arisen in its synapses with K-cells, thus in the output side of the MB, or upstream of the K-cells. If the PE1 and other alpha lobe-extrinsic neurons (e.g. the recurrent neurons running in the protocerebral–calycal tract; Grünewald, 1999) are involved in associative plasticity, the underlying mechanisms could be expected to differ from those in the lip region of the MB calyces, because the lobes do not receive direct input from the VUMmx1. It is thus concluded that associative plasticity in honeybees may reside in both synaptic regions of the MB, its input (calyx) and its output sites.

In contrast to the mammalian brain, cellular and network mechanisms of neural plasticity in central neurons of the insect brain have not yet been studied with electrophysiological methods in any great detail. The modulatory effect of biogenic amines, in particular octopamine and serotonin, on the excitability and spiking pattern of neurons in the thoracic ganglia were examined with the aim of understanding the release and performance of motor patterns (chapter 6 in Burrows, 1996; Pflüger, 1999). These modulatory effects are usually brief and are not related to learning. Visual interneurons in the bee brain were found to be antagonistically modulated by octopamine (upregulation) and serotonin (downregulation; Kloppenburg and Erber, 1995), but the mechanisms are unknown. Several classes of olfactory interneurons change their response properties when honeybees are trained to an odour (projection neurons: Abel, 1997; PCT neurons, the recurrent neurons from the alpha lobe to the calyx: Grünewald, 1999; the PE1 neuron: Mauelshagen, 1993), but the cellular mechanisms and the synapses involved are unknown. Long-term potentiation was observed in field potentials recorded in the MB of the honeybee, but the underlying mechanisms are unknown (Oleskevich et al., 1997). In particular, it is unknown whether central insect neurons show Hebbian plasticity in the sense that coincidence of pre-post-synaptic activity leads to lasting changes of synaptic transmission and/or neural excitation. This form of plasticity was studied in great detail in hippocampal and cortical neurons of the mammalian brain (Bear et al., 1987; Bear and Malenka, 1994; Fregnac et al., 1994; Markram et al., 1997). It was found that the precise timing of pre- and postsynaptic spikes leaves traces of lasting synaptic plasticity: either long-term potentiation (LTP) or long-term depression (LTD), depending on the timing of pre- and postsynaptic activity, and

such plasticity is considered a substrate of associative learning, on the basis of both theoretical considerations (Bienenstock et al., 1982; Fox et al., 1998) and experimental data (Young et al., 1994; Singer, 1995; Markram et al., 1997; Feldman, 2000).

This study characterizes synaptic transmission from K-cells to alpha lobe-extrinsic neurons with the goal of searching for learning-related plasticity at the output side of the MB. K-cells were electrically stimulated in their somata region, the calyx; alpha lobe-extrinsic neurons were recorded intracellularly and some of them were marked. The recording site was a location at the ventral aspect of the alpha lobe at a depth of 150–250 µm from the front surface of the brain where bundles of rather prominent axons, including the PE1, leave the MB (Mobbs, 1984; Rybak and Menzel, 1993 and 1998; Strausfeld, 2002). Stimulus protocols were applied to examine depression, short-term facilitation, prolonged augmentation and long-term potentiation. All these phenomena were found, and most importantly, when the PE1 was depolarized during presynaptic tetanic stimulation, LTP was induced in the PE1 neuron of some preparations. This form of pairing specific enhancement (associative LTP) was not seen in PE1 recordings of most preparations, suggesting that unknown modulatory conditions were also important. This is the first demonstration of associative LTP in a central insect neuron. Its existence suggests a role in odour coding and/or odour learning, and thus could be related to the associative plasticity seen in PE1 during olfactory conditioning (Mauelshagen, 1993).

Materials and methods
Preparation

Foraging honeybee *Apis mellifera* L. workers were collected from our laboratory's colonies, chilled on ice and fixed in a specially designed chamber by inserting the neck into a slit and fixing the head to a thin plastic sheet with beeswax. Bees were fed to satiation with 40% sucrose solution and stored overnight in a cool, moist, dark box. In preparing for dissection, animals were first chilled on ice, and then cool air (5°C) was blown onto the animal throughout the dissection procedure. The brain was exposed by cutting a window into the head capsule between the ocelli, the compound eyes and the antennae. A container of bee saline (NaCl 137 mmol l^{-1}, KCl 2.7 mmol l^{-1}, Na_2HPO_4 8 mmol l^{-1}, KH_2PO_4 1.8 mmol l^{-1}, sucrose 105.2 mmol l^{-1}, $CaCl_2$ 2.0 mmol l^{-1}, pH 6.7, 400 mOsm) was connected *via* a tube, providing a stream of warm (27°C) saline running behind the head capsule. Thin silk threads were laid on top of the brain; these kept a thin film of saline flowing over the brain. In order to prevent the oesophagus from moving the brain, it was pulled upward and fixed with a sharp needle. Modelling clay was pressed against the abdomen to reduce its pumping effect. The tracheal sack lying on top of the alpha lobe on one side was gently pushed aside, and the other tracheal sacks were not dissected. The neural sheath was cut only at the ventral aspect of one alpha lobe.

Intracellular recording and marking

Glass capillary electrodes were pulled from WPI (Sarasota, FL, USA) glass tubes (1 mm diameter) filled with a filament (type Nr. 1B100F-3) using a Flaming/Brown horizontal puller (P 87, Sutter Instruments, Novato, CA, USA), filled with 1 mol l^{-1} potassium acetate or a dye (Alexa 568 hydrazide, Molecular Probes, Eugene, OR, USA) in 2.5% distilled water or in 0.2 mol l^{-1} potassium acetate; dextrane, tetramethylrhodemine, TRITC, Molecular Probes, in 0.2 mol l^{-1} potassium acetate). The electrodes' resistance ranged from 80 mol l^{-1} (potassium acetate) to 200 mol l^{-1} (dyes). A WPI Intra 767 preamplifier was used together with a conventional electrophysiological set-up (CED Mikro 1401, CED Cambridge Electronic Design, Cambridge, UK) for data acquisition and digitalization, and Spike II software (Version 3.19 CED) for data analysis. Custom-made programs (Spike II tools) allowed us to produce dot plots of spike distributions and to control the timing and properties of the olfactory stimuli and electrical stimulations. Only those recordings in which the baseline was stable were included in our analysis.

The dyes were injected by depolarizing or hyper-polarizing constant currents of a few nA for several minutes. The brains were fixed overnight in 4% formaldehyde, washed in saline, dehydrated in rising alcohol concentrations and cleared in methylsalicylate.

Electrical stimulation

Kenyon cells were stimulated by one or two pairs of wires (nichrome, H.P. Reid, Palm Coast, USA, 5 μm diameter). The distance between the two wires in a pair was approximately 50 μm, thus bridging about a third of the diameter of either the median or the lateral calyx of one MB. In most experiments both the median and the lateral calyces of the right MB were stimulated, and alpha lobe-extrinsic neurons were recorded from this MB. Manipulation of the very soft stimulating wires was facilitated by inserting both pairs of wires into a glass tube (1 mm o.d.) and pulling it together with the wires. Stimulus isolation units (Iso-Flex) were triggered by a Master 8 timer (A.M.P.I., Jerusalem, Israel). Stimulus voltage was set such that a single stimulus elicited 1–4 spikes in the recorded neurons. Stimulus duration was set to 1 ms. The tetanus consisted of 100 stimuli in 1 s.

Intracellular de- and hyper-polarization of the recorded neuron

In some experiments the recorded neuron was de- or hyper-polarized during the application of the tetanus. The depolarizing currents were set to a value that induced spike frequencies in the upper range of the neuron's normal response to natural stimuli (like odours), and differed for different neurons. The same currents were used for hyper-polarization. The range was 0.5–3 nA.

Confocal imaging and reconstruction of neurons

Whole-mounts and vibratome sections (thickness 200 μm) from individual specimens were imaged using a confocal laser scanning microscope (Leica TCS 4D, Leica, Bensheim, Germany) with a Leica HC PL APO 10(/0.4 dry lens or 20× /0.7 immersion lens. The chromophore was excited at 575 nm (Alexa) or 543 nm (TRITC), imaged at 600 nm, and digitized at 8-bit resolution. We used the multiple image-stack acquisition software as required to combine image stacks (3-D-MISA, Zuschratter et al., 1998). Stacks were subsequently combined using custom software or a script running in Amira Version 3.0 (Konrad-Zuse-Zentrum, Berlin; Indeed-Visual Concepts GmbH, Berlin, Germany; TGS Template Graphics Software, Inc. http://www.amiravis.com). The neurons were reconstructed using the AMIRA software (Stalling et al., 2004).

Statistics

'Statistica' (StattSoft, Tulsa, Usa, Version 5.5, 1999 edition, tool: descriptive statistics) was used to calculate analyses of variance (ANOVAs) for repeated measures. Main effects and *post hoc* comparisons for different time intervals are reported in the figure legends.

Results
Material

Plasticity of synaptic transmission between K-cells and alpha lobe-extrinsic neurons was tested by electrical stimulation of K-cells in their somata region, and intracellular recordings of action potentials in extrinsic neurons. Paired-pulse short-term facilitation and depression, tetanus-induced augmentation lasting up to several minutes, and associative long-term potentiation (LTP) were found. Since postsynaptic potentials were usually not seen in our recordings (with the exception of the PE1 neuron), we evaluated the stimulus-induced spikes after setting the stimulus strength to a value that elicited a small number of spikes (1–4) per stimulus. This means that we were not able to differentiate between changes of synaptic transmission and changes of excitability in the recorded and stimulated neurons.

A total of 133 neurons were recorded; 35 were stained. The PE1 neuron was recorded in 54 animals, and seven were marked. The non-marked PE1 neurons could be identified on the basis of their characteristic double and triple spike patterns, and by their large summing EPSPs (Mauelshagen, 1993). Eight neurons (five PE1 neurons) could be recorded long enough (>30 min) to test whether depolarization of the neuron occurred during the application of tetanus-induced associative LTP. Two of these neurons, both PE1 neurons, showed associative LTP.

Responses to sensory stimuli and plasticity

Many neurons did not respond to any sensory stimuli. We tested four different odours, light flashes, mechanical and gustatory stimuli to both the antennae and the proboscis. These findings confirm earlier observations that were usually not included in the publications (Mauelshagen, 1993; Rybak and Menzel, 1998; Grünewald, 1999), because if a neuron did not

respond to sensory stimuli it could not be further characterized. In the present work it was possible to test whether such neurons receive input from K-cells, because we stimulated the K-cells electrically. J. Mauelshagen observed in her studies following published data that up to 2/3 of the neurons that she had qualified as coming from PE1 neurons showed no responses to sensory stimuli (J. Mauelshagen, unpublished observations). In the recordings that led to the paper by Rybak and Menzel (1998), we observed that about half of the neurons lacked responses to sensory stimuli. At that time, J. Mauelshagen and we both interpreted these observations as indications for non-optimal dissection and/or recording conditions, and tried hard to improve them. Initially we had the same attitude in this study, and it was only later in our studies that we also exposed such neurons to our test program. We tested 32 neurons for their responses to sensory stimuli and electrical stimulation of the K-cells. All of them were excited by electrical stimulation. Fifteen of them were classified as PE1 neurons. Ten neurons out of the 32 did not respond to any sensory stimuli; four of them were designated as PE1 neurons. Of the 17 neurons that responded to sensory stimuli ten of them responded only to light stimuli (either On or Off responses, or both), and eight only to odour stimuli. All 32 neurons recorded gave no indication that recording quality was compromised. Action potentials initiated by natural stimuli and by electrical stimuli had the same amplitude and time course.

The 32 neurons were included in our analysis of plasticity. No difference was found between their responses to double-pulse or tetanus protocols and those from neurons that responded to sensory stimuli. A particularly striking example was a PE1 neuron, reported on below, that developed LTP after pairing presynaptic tetanus and postsynaptic depolarization. We therefore conclude that responses of MB-extrinsic neurons to sensory stimuli are not necessary for neural plasticity as we tested them here. This could mean that a considerable proportion of neurons were not excited by natural input, but by the potentially stronger input during our electrical stimulation. Since they developed short-term facilitation (double pulse protocol), mid-term augmentation (tetanus) and LTP (tetanus plus depolarisation), it might be concluded that these neurons were recruited to respond afterwards to lower stimuli in the short-term, mid-term or long-term range. Unfortunately, we did not test them with sensory stimuli after we applied our stimulus protocols.

Short-term neural plasticity
Paired-pulse depression and facilitation

A total of 16 neurons were tested with the paired-pulse protocol; four neurons showed short-term depression and six short-term facilitation. Depression was induced when stimulation voltages were set to values that elicited 3–5 spikes per stimulus in single stimulations. Lower stimulus intensities (e.g. eliciting a single spike) did not induce depression. Fig. 1 shows two representative examples, one from an unidentified alpha lobe-extrinsic neuron (Fig. 1A), and one from a PE1 neuron (Fig. 1B). Time delays of Δt=30–60 ms produced the
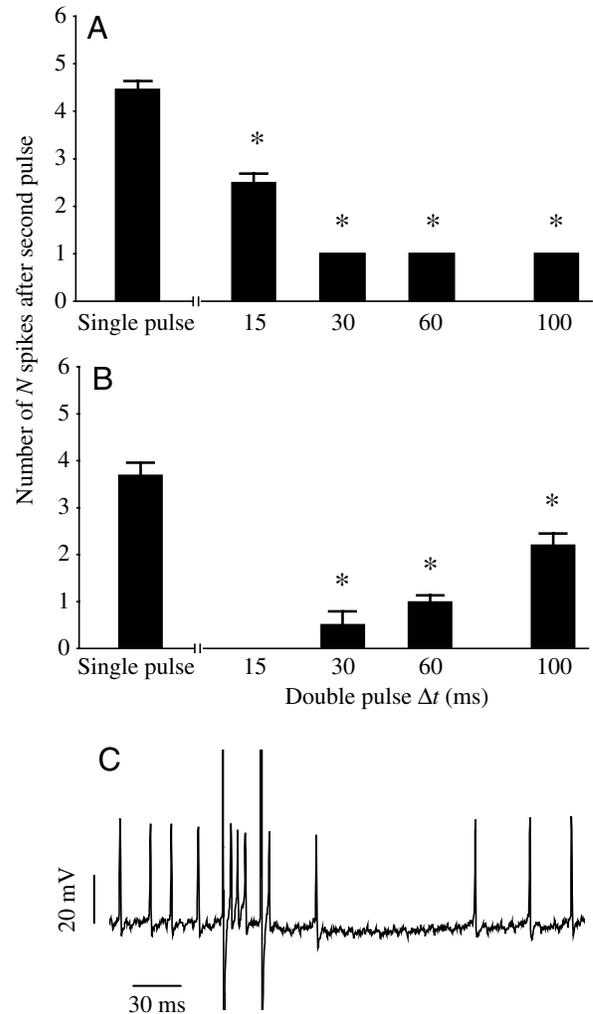

Fig. 1. Two representative examples of paired-pulse depression. (A) An unidentified alpha lobe-extrinsic neuron. All double-pulse values are significantly different from the single pulse value ($P<0.001$, *post hoc* tests, main effect: F=33, d.f.=3, $P<0.001$, N=43, values are means ± S.D.). (B) A PE1 neuron. All values are significantly different from the single-pulse value (*$P<0.001$, *post hoc* tests, main effect: F=55.38, d.f.=4, $P<0.001$, N=43, values are means ± S.D.). (C) Spike train recorded from the neuron in A. Interval between the two pulses = 30 ms. The stimulus artefacts mark the test pulses. The ordinate gives the number of spikes elicited by a single stimulus, and the abscissa plots the interval Δt between the two stimuli in the paired-pulse protocol.

strongest depression. Repetitions (5, interval 20 s) of paired pulses at optimal Δt did not prolong depression times that lasted less than 2 min.

Paired-pulse stimulation could lead to facilitation. In the two cases shown in Fig. 2, stimulus intensity was set rather high, eliciting 3–5 spikes per stimulus, but in most other cases in which we observed paired-pulse facilitation, stimulus intensity was set to lower intensities, e.g. inducing 1–2 spikes to each stimulus, or an occasional spike to the first stimulus and 1–2 spikes to the second stimulus. Optimal time intervals between
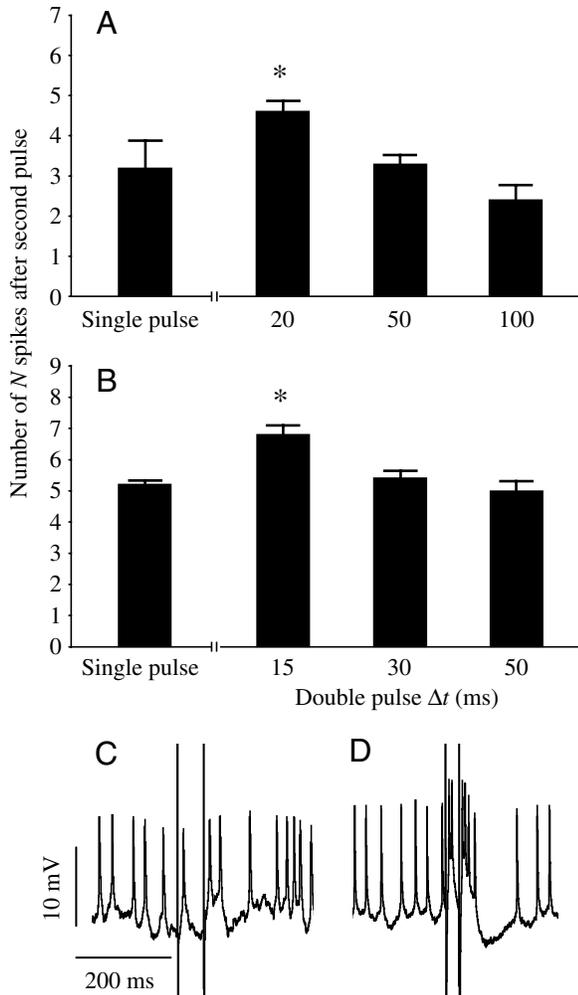
Fig. 2. Two representative examples of paired-pulse facilitation. (A) An unidentified alpha lobe-extrinsic neuron. The value for 20 ms is significantly different from all other values (*$P<0.01$, *post hoc* test, main effect: $F=5.66$, d.f.=3, *$P<0.01$, $N=45$, values are means ± S.D.). (B) A PE1 neuron. The value for 15 ms is significantly different from all other values ($P<0.001$, *post hoc* test, main effect: $F=13.27$, d.f.=3, *$P<0.001$, $N=28$). The ordinate gives the number of spikes elicited by a single stimulus. The abscissa gives the interval (Δt) between the two stimuli in the paired-pulse protocol. (C) Spike trains from the PE1 neuron in B. (D) Spike train from a different PE1 neuron, which was stimulated with higher-pulse currents eliciting more spikes per stimulus. The stimulus artefacts mark the test pulses.

Fig. 3. Cumulative paired-pulse facilitation from an unidentified alpha lobe-extrinsic neuron (A,B) or a PE1 neuron (C). Δt was set to the optimal time interval of the two pulses for the induction of paired-pulse facilitation (30 ms in A, 20 ms in C). Filled circles, spike numbers elicited by the first pulse; open circles, spike numbers to the second pulse. The ordinate gives the number of spikes elicited by a single stimulus, and the abscissa time in s. In B the stimulus artefacts mark the test pulses.

the two stimuli were shorter than for the induction of paired-pulse depression (e.g. 15 and 20 ms, Fig. 2A,B). Fig. 2B shows paired-pulse facilitation in the PE1 neuron. In this experiment the single pulse released five spikes riding on large summed EPSPs, and the second pulse followed by 15 ms elicited larger summed EPSPs and a significant increase to seven action potentials. The recording shown in Fig. 2D comes from a different recording of a PE1 neuron, in which the first pulse elicited two spikes and the second elicited four spikes (after 30 ms). The optimal time window for paired-pulse facilitation was also narrow in this PE1 recording.
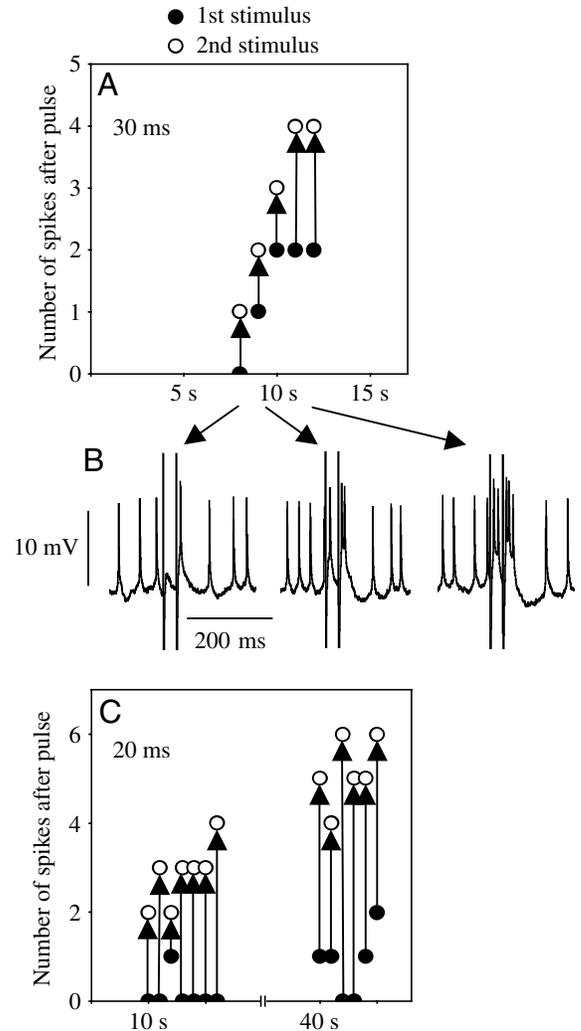
Induction of paired-pulse depression or facilitation was a robust phenomenon for the particular neuron. We did not see a transition from paired-pulse depression to facilitation, or *vice versa*, in the same neuron. Thus it appeared that these forms of short-term plasticity were characteristic for a particular neuron in a particular preparation. In different preparations, however, the PE1 neurons that showed paired-pulse plasticity responded either with paired-pulse depression or facilitation. These differences may result from the stimulation of different subgroups of K-cells or from unknown modulatory effects.

Repetition of paired-pulse stimulation at optimal time intervals ($\Delta t=20–30$ ms) led to cumulating facilitation in three out of seven unidentified alpha lobe-extrinsic neurons and six out of 16 PE1 neurons (Fig. 3). The neuron in Fig. 3A,B

responds with cumulative facilitation both to the first and the second pulse, whereas the PE1 neuron in Fig. 3C facilitates only the response to the second pulse. No such cumulative paired-pulse facilitation was seen for $\Delta t \geq 50$ ms in any neuron. Unfortunately, none of our recordings in which we studied paired-pulse effects lasted longer than 10 min, so we were not able to test whether the accumulation of paired-pulse facilitation would have led to lasting augmentation or LTP.

Short-term plasticity induced by tetanus

We tested 69 neurons with a tetanic stimulation (1 s, 100 Hz); 32 of these neurons were assigned as PE1 neurons. In 42 of the 69 neurons, recordings lasted longer than 10 min. Five different kinds of neural plasticity effects were seen after a single tetanus. (1) Short-term depression (Fig. 4; 4 out of 69 neurons, none was identified as a PE1 neuron). (2) Augmentation lasting up to a few minutes (Fig. 5; 24 out of 69 neurons, 15 of them PE1 neurons). (3) Prolonged depression lasting for several minutes (14 out of 69 neurons, 8 of them were PE1 neurons). Prolonged depression may by followed by augmentation (see below, Fig. 8A,B). (4) No change of
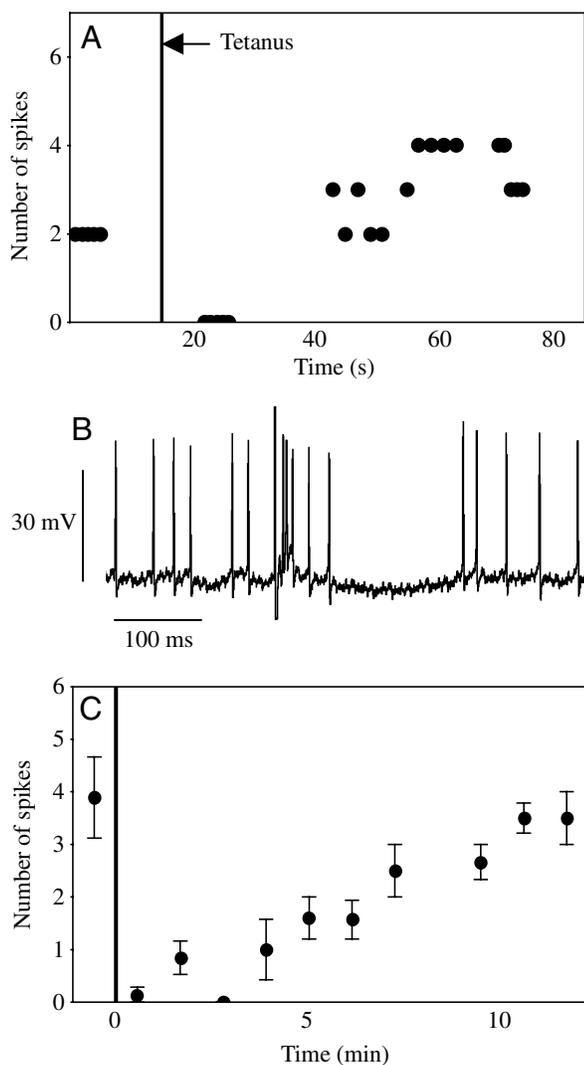

Fig. 4. (A) Tetanus-induced short-term depression followed by lasting augmentation. Depression induced in this unidentified alpha lobe-extrinsic neuron lasted for about 30 s and was followed by augmentation. Similar time courses of depression and augmentation were seen in three other neurons; none was identified as a PE1 neuron. (B) Spike trains of this neuron 70 s after tetanus. The stimulus artefacts mark the test pulses. (C) Average time course of depression in five PE1 neurons. Notice the different time scale in A and C. The ordinates give the number of spikes elicited by a single stimulus (± S.D. in C.) and the abscissa time in s (A) or min (C).
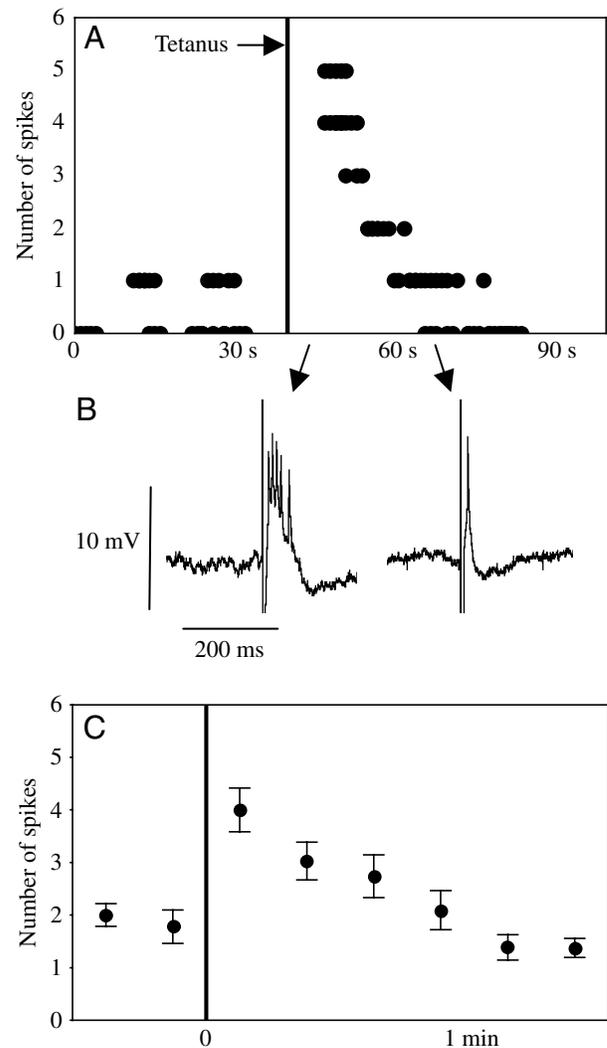
Fig. 5. (A) Tetanus-induced augmentation in an A5 alpha lobe-extrinsic neuron. The neuron was identified by intracellular marking and categorized according to Rybak and Menzel (1993). The augmentation effect was very constant in three consecutive tetani, and the neuron returned to its initial sensitivity within 1 min. Therefore, the responses to test stimuli shortly before and after the three tetani were overlaid in the figure. (B) Spike trains of the neuron in A recorded at two intervals after the first tetanus (arrows). The stimulus artefacts mark the test pulses. (C) Augmentation of 11 PE1 neurons. The ordinates give the number of spikes elicited by a single stimulus (± S.D. C), and the abscissa time in s.

synaptic transmission (29 out of 69 neurons, 11 of them were PE1 neurons).

Depression was frequently seen when rather high stimulus intensities were applied (3–5 spikes per single stimulus, Fig. 4), but lower stimulus intensities (1–2 spikes per single stimulus) could also elicit depression by a tetanus. Depression lasting for less than 1 min was frequently followed by augmentation (Fig. 4A) that may last longer than 10 min (see below), but all these neurons were unidentified alpha lobe-extrinsic neurons, not PE1 neurons. Fig. 4C shows the average responses of five PE1 neurons that underwent depression after tetanus. As seen in these PE1 neurons, depression lasted for several minutes and in these cases no augmentation was seen. Since no PE1 neuron responded with short-term depression and delayed augmentation to tetanus, but rather with longer-lasting depression, we conclude that short-term depression and delayed augmentation is a property of other alpha lobe-extrinsic neurons, not the PE1 neuron.

Tetanus-induced augmentation was a very prominent response property both in PE1 neurons and other alpha lobe-extrinsic neurons (e.g. A5 neurons in Fig. 5A and PE1 neurons in Fig. 5C, according to the notation of Rybak and Menzel, 1993). Augmentation led to stimulus-released spike responses several times higher than before the tetanus (responses in the A5 neuron shown in Fig. 5A were five times higher; responses in PE1 neurons of Fig. 5C doubled). The time course of augmentation was the same in unidentified alpha lobe-extrinsic neurons, in the A5 neuron shown in Fig. 5A,B and in 11 PE1 neurons (Fig. 5C). Reponses returned to pre-tetanus conditions within about 1 min.

Repeated tetani usually did not enhance the respective plasticity effects, depression or augmentation. Two exceptions are shown in Fig. 6. Augmentation is reduced by repeated tetani in the neuron in Fig. 6A,B. This neuron had a tendency to produce spike bursts at intervals of approximately 10 s, and this tendency became more obvious after repeated tetani (see also dot plots in Fig. 6A). Bursting is not rare in alpha lobe-extrinsic neurons, but the bursts usually occur at intervals of 0.3 to 1 s. Spike intervals within the bursts may drop to 10 ms. The neuron shown in Fig. 6C is the only one we observed with
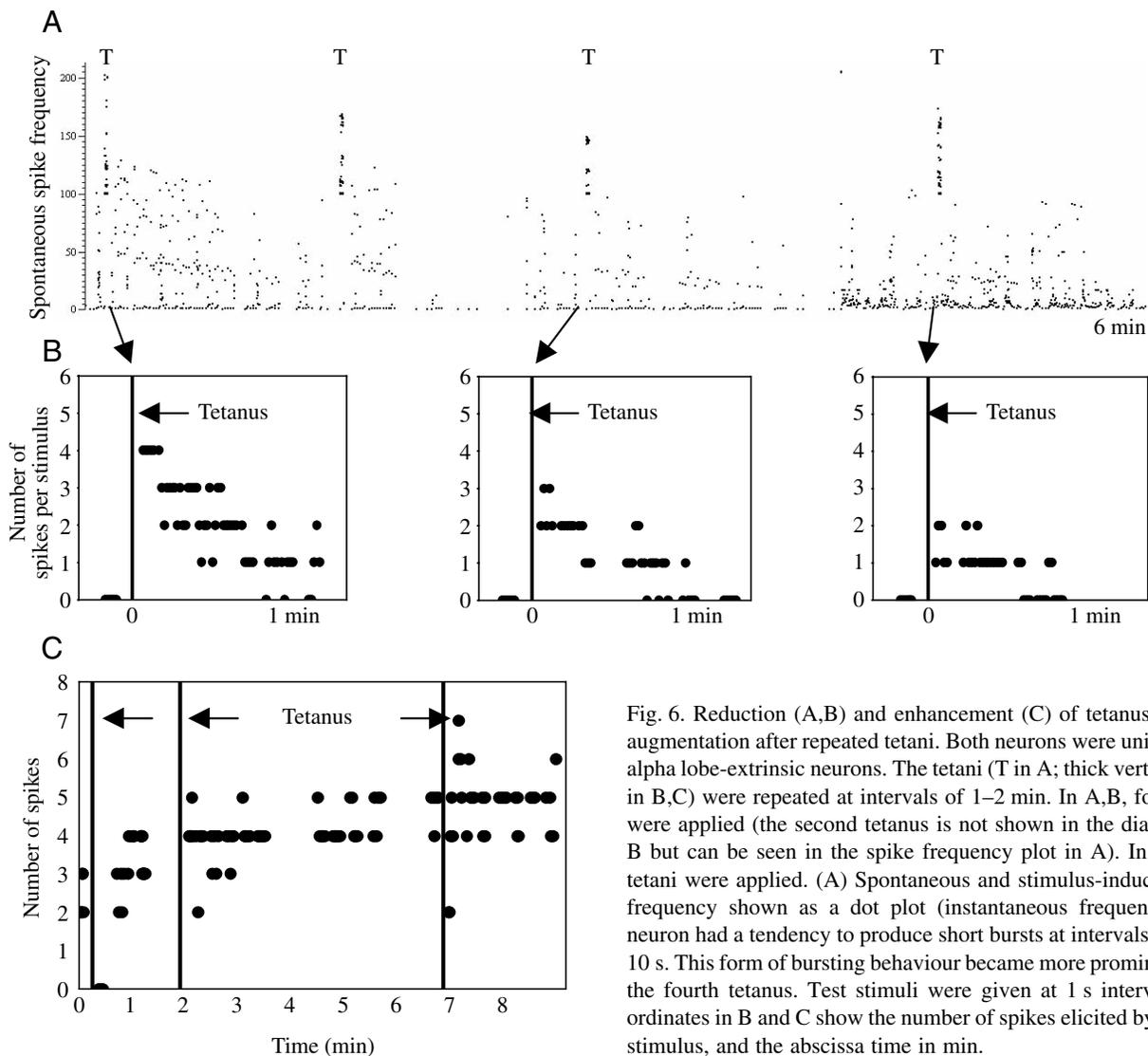

Fig. 6. Reduction (A,B) and enhancement (C) of tetanus-induced augmentation after repeated tetani. Both neurons were unidentified alpha lobe-extrinsic neurons. The tetani (T in A; thick vertical lines in B,C) were repeated at intervals of 1–2 min. In A,B, four tetani were applied (the second tetanus is not shown in the diagrams in B but can be seen in the spike frequency plot in A). In C, three tetani were applied. (A) Spontaneous and stimulus-induced spike frequency shown as a dot plot (instantaneous frequency). The neuron had a tendency to produce short bursts at intervals of about 10 s. This form of bursting behaviour became more prominent after the fourth tetanus. Test stimuli were given at 1 s intervals. The ordinates in B and C show the number of spikes elicited by a single stimulus, and the abscissa time in min.

an enhancement of the augmenting responses after repeated tetani. Three tetani were applied. Depression decreased and augmentation increased. Unfortunately the recording did not last long enough to test whether augmentation may have in fact led to LTP.

We also asked how paired-pulse facilitation and tetanus-induced augmentation may be related. A mechanistic analysis was difficult in our preparation, because we measured spike activity and not synaptic potentials, which excluded the possibility of separating mechanisms leading to changes of synaptic transmission and changes of excitability of the recorded cell. But we could ask whether tetanus-induced augmentation reduces paired-pulse facilitation, leaves it unaltered or enhances it. A reduction of paired-pulse facilitation or even a transition to paired-pulse depression could give us a hint for presynaptic effects comparable to those seen in hippocampal neurons (Staubli et al., 1990; Zalutsky and Nicoll, 1990). There, paired-pulse facilitation is reduced after LTP induction, indicating an increase of release probability of synaptic vesicles during LTP. Three neurons were tested with this protocol; two were PE1 neurons and one an A5-3 neuron according to Rybak and Menzel (1993). Fig. 7A,B shows the
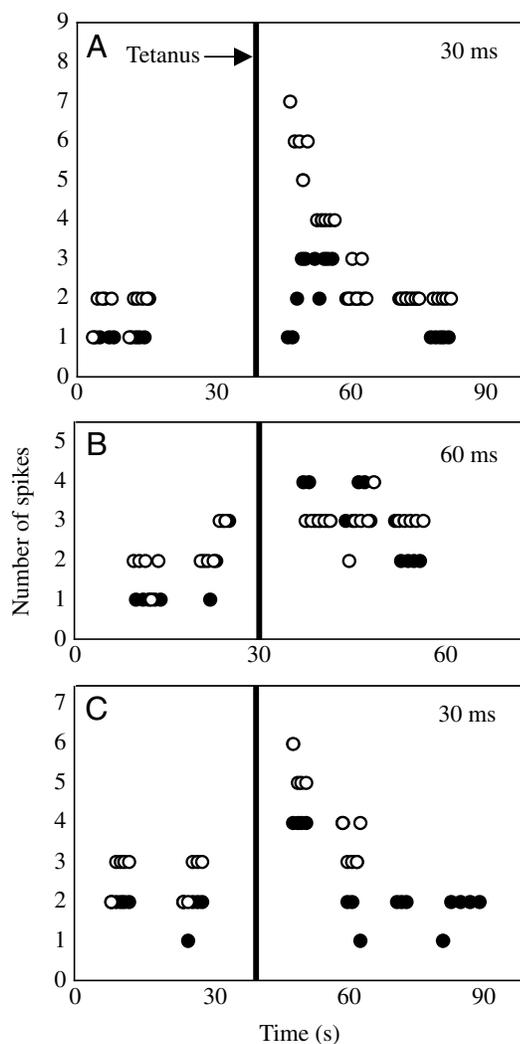
results for the A5-3 neuron, and Fig. 7C those of a PE1 neuron. We combined repetitive paired stimuli (Δt=30 or 60 ms) with one tetanus. Before the tetanus the paired pulses caused a slight facilatory effect with little difference between the repeated paired pulses of 30 ms and 60 ms. Responses to the second pulse facilitated after the tetanus in the Δt=30 ms protocol, but not in the Δt=60 ms protocol. The facilatory effect of the tetanus is also seen for the first stimulus in both protocols (after a very short depression as seen in the 30 ms experiment), but much less strongly than for the second stimulus at intervals of 30 ms. In the PE1 neuron shown in Fig. 7C the facilatory effect is seen in the responses both for the first and the second pulse, and the paired pulse effect does not change after the tetanus. These results indicate that tetanus-induced augmentation does not reduce paired-pulse facilitation, and that both facilatory effects can be additive, depending on the precise setting of the intervals between paired pulses. Interestingly, the response to the second stimulus is strongly enhanced, but the time course of facilitation is not prolonged. Similar results were found for the two PE1 neurons.

Pre- or postsynaptic plasticity?

Each MB consists of two parallel parts, which receive their inputs *via* the two calyces, the median calyx and the lateral calyx (Fig. 8A). All of the alpha-lobe-extrinsic neurons that we marked intracellularly arborized across the peduncle or alpha-lobe and are thus likely to receive inputs from Kenyon cells originating in both the median and lateral calyces. So far we have reported experiments in which most K-cells were stimulated, because we placed a pair of stimulation electrodes into both calyces of one MB. Stimulating the median and the lateral calyx separately provides us with the opportunity to test whether plasticity induced by the tetanus to K-cells of the median calyx is transferred to those of the lateral calyx, and

Fig. 7. Mechanistic relationship between paired-pulse facilitation and tetanus-induced augmentation for (A,B) a A5-3 neuron, a type of neuron MB-extrinsic neuron that was characterized with respect to its branching pattern and the location of its soma by Rybak and Menzel (1993), and (C) a PE1 neuron. (A,B) The A5-3 neuron was first stimulated by repeated paired pulses with pulse intervals of 30 ms, then a tetanus (thick vertical line) was applied and the paired pulses were continued (A). Afterwards the neuron was stimulated with paired pulses with pulse intervals of 60 ms, then a tetanus was applied, followed by a continuation of paired-pulse stimuli (B). Before the tetanus the paired pulses caused a slight facilatory effect with little difference between the repeated paired pulses of 30 ms and 60 ms. Responses to the second pulse facilitated after the tetanus in the Δt=30 ms protocol, but not in the Δt=60 ms protocol. (C) The PE1 neuron was first stimulated with paired pulses at intervals of 30 ms, then tetanus was applied. The facilatory effect of the tetanus is expressed in the responses to both the first and the second pulse, and no change is found between paired pulse facilitation before and after the tetanus. Filled circles, number of spikes for the first stimulus in the paired pulses; open circles, those for the second stimulus. The ordinate in the diagrams gives the number of spikes elicited by a single stimulus, and the abscissa time in s.

vice versa. In the case of a postsynaptic form of plasticity, we would expect the change of plasticity to also be seen by stimuli applied to the K-cells that did not receive the tetanus. If synaptic plasticity is restricted to the tetanized K-cells, we would at least expect a strong presynaptic component. 18 neurons were tested with the protocol of stimulating the K-cells of the median and lateral calyx separately; four showed a side-specific effect (the plasticity effect induced by the tetanus to one calyx was restricted to the stimulus site), and in the other 14 neurons the plasticity effect was transferred to the other side. The three examples shown in Fig. 8 come from four neurons with different forms of synaptic plasticity. The depression seen in the PE1 neuron of Fig. 8B is not restricted to the input side, but augmentation following depression is side-specific, indicating a postsynaptic component in depression and a presynaptic component in augmentation. The short augmentation seen in the examples in Fig. 8C,D is restricted to the tetanized side, supporting the conclusion that augmentation is a side-specific effect, which suggests a presynaptic component.

Long-term neural plasticity
Associative LTP

Besides short-term facilitation and depression, a tetanus applied to K-cells may led to response augmentation lasting for up to a few minutes in most of the alpha lobe-extrinsic neurons.
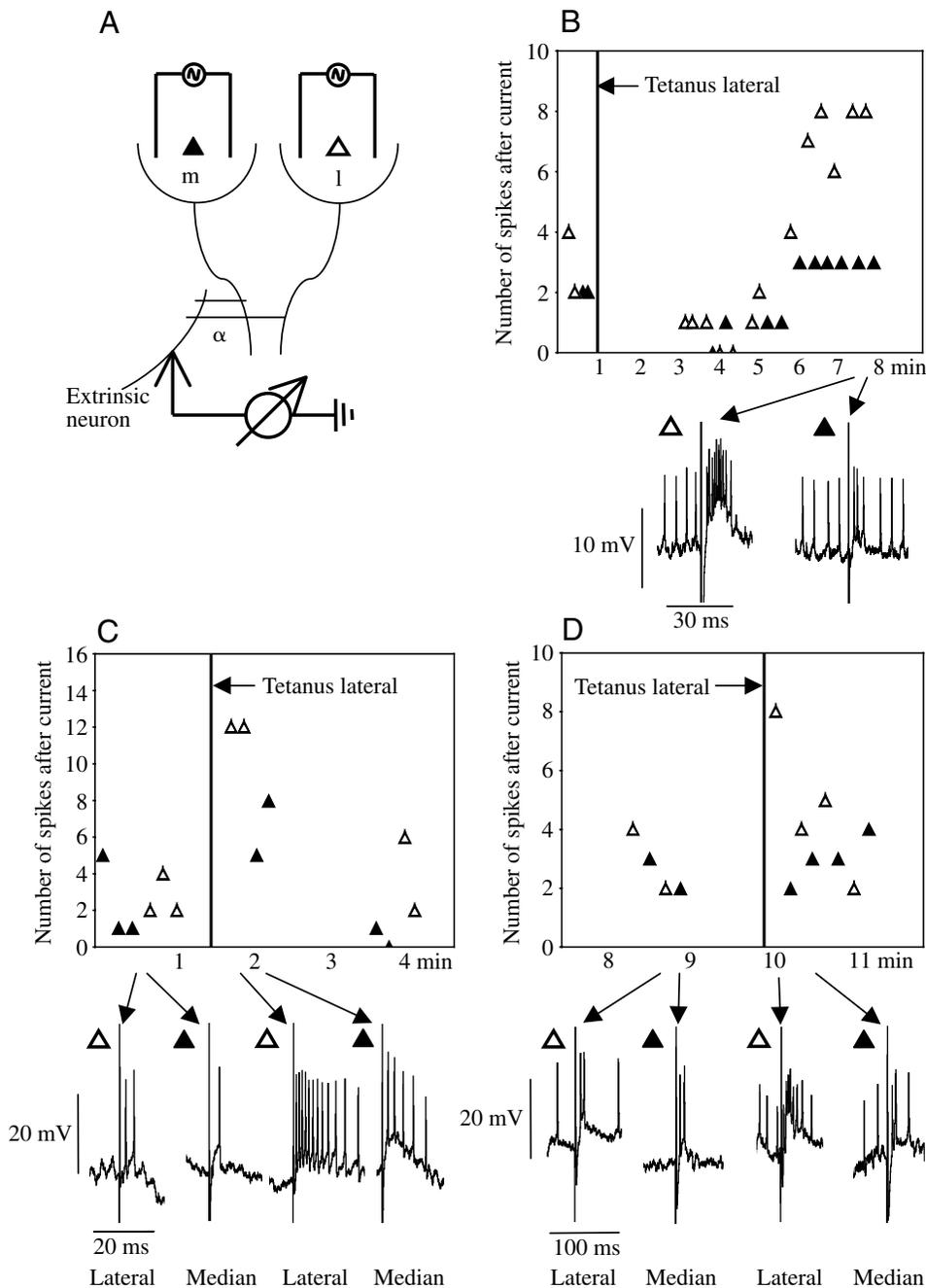

Fig. 8. Site-specificity of tetanus-induced plasticity in alpha lobe-extrinsic neurons. (A) Each MB consists of two halves. One half is composed of K-cells with their somata and dendrites in the median calyx (m), and another half has somata and dendrites in the lateral calyx (l). The axons of the K-cells fuse in the peduncle and send their collaterals into the alpha lobe (α). There the lateral and the median sectors house the axon collaterals of the respective calyces. All alpha lobe-extrinsic neurons that we marked in the course of our study branched across the whole alpha lobe, making it very likely that they receive input from both median and lateral K-cells. (B) This Pe1 neuron responded with depression after tetanus to the lateral calyx. Depression was found for the test stimuli to both the median (filled triangles) and the lateral calyx (open triangles). The delayed augmentation effect, however, was site-specific. Only the input from the tetanized calyx augmented. (C,D) Two examples from unidentified alpha lobe-extrinsic neurons for site-specific facilitation induced by tetanus. Again the tetanus was applied to the lateral calyx, and the responses to test stimuli in the median (filled triangles) and lateral (open triangles) were monitored during successive minutes. The ordinate in the diagrams gives the number of spikes elicited by a single stimulus, and the abscissa time in min. The stimulus artefacts in B, C and D mark the test pulses.

In some neurons the augmented responses were still recorded at the time when we lost the cell (e.g. Figs 6B, 8B), leaving open the possibility that it might have continued for longer periods of time. The PE1 did not differ in this respect. In the PE1, large summing EPSPs were recorded on which the spikes were riding (Figs 2C,D, 3C, 5B, 8B, 9A,B). We therefore evaluated the synaptic potentials as well as the number of

spikes released by a single test stimulus (Fig. 9A,B). The typical response of the PE1 neuron to the tetanus was a first phase of depression followed by an augmentation that reached a maximum after 5–10 min. The depression was particularly obvious in the amplitude of the summed EPSPs of the PE1 neuron in Fig. 9A. (The neuron in Fig. 9B was not tested frequently enough following the tetanus.) Afterwards synaptic
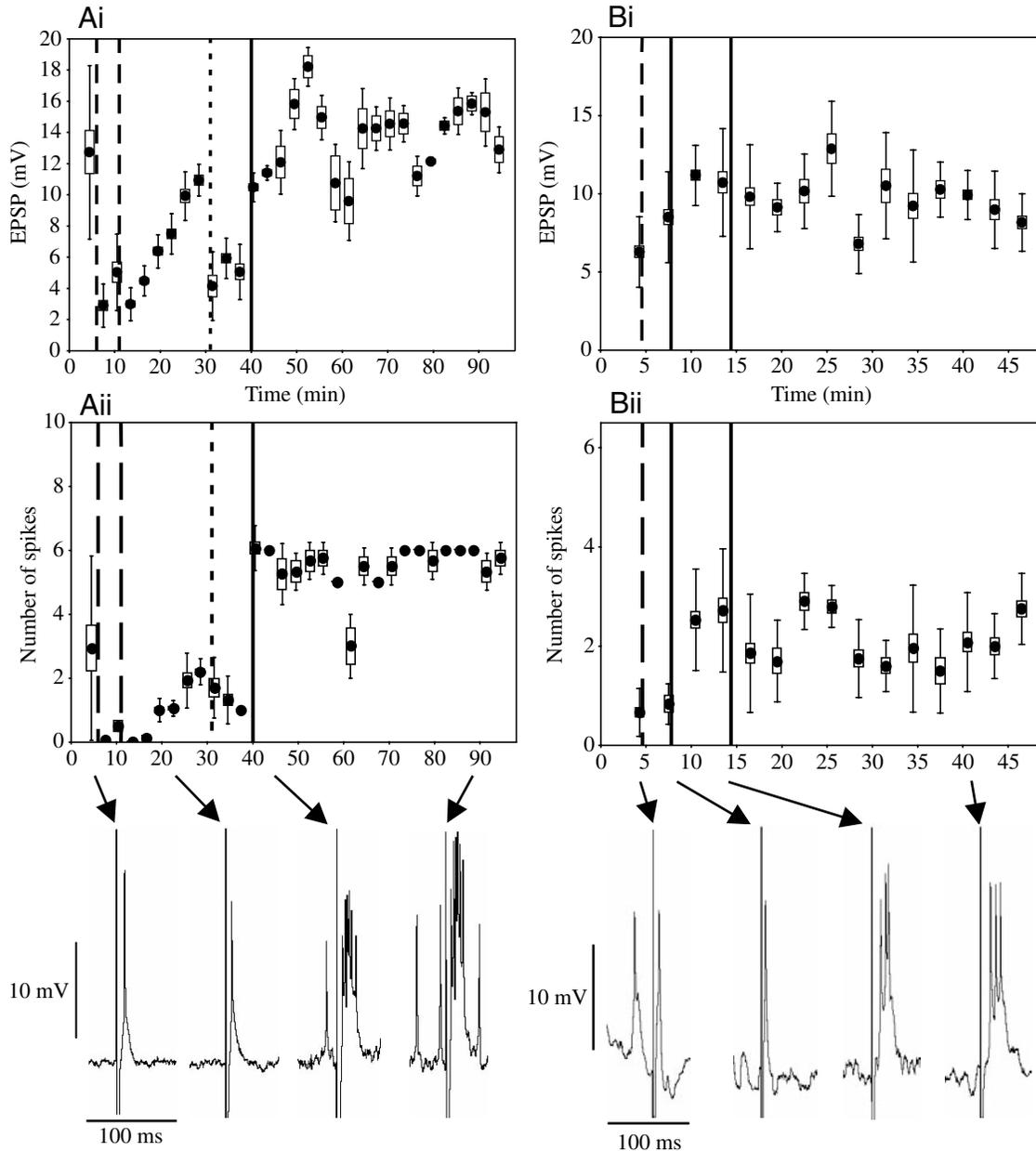

Fig. 9. Long-term potentiation induced in two PE1 neurons (A,B) by pairing tetanus to the K-cells with simultaneously depolarizing the PE1 neuron. The broken lines indicates a tetanus, dotted lines a tetanus paired with hyper-polarization of the PE1 neuron, and bold lines a tetanus paired with depolarization. Test stimuli were applied at intervals of 20 s. The stimulus artefacts mark the test pulses. (Ai,Bi) The results for the amplitude of summed EPSPs in mV. The maximum of the dc component was measured as EPSP amplitude. Eight consecutive test stimuli were averaged. (Aii,Bii) Mean of the number of spikes to eight consecutive test stimuli. Values are means ± s.d.; box, s.e.m. Where only filled circles are shown all eight values were the same. Notice the draught about 20 min after the pairing of the tetanus with depolarization (see text). The ordinate gives the dc amplitude (Ai,Bi) and number of spikes (Aii,Bii) elicited by a single stimulus, and the abscissa time in min (notice the different scales in the two graphs, and the high value of summed EPSPs before the application of a tetanus in the neuron of A).

transmission returned to its pre-tetanus stage, both with respect to the summed EPSP amplitude and the number of spikes triggered by the test stimulus. Hyper-polarizing the PE1 neuron during the tetanus reduced the depression effect as measured with the number of spikes, but not in the EPSP amplitude (Fig. 9A, dotted line).

LTP was induced in PE1 neurons when the tetanus to its input from K-cells was paired with depolarization of the PE1. 15 out of 54 recorded PE1 neurons were tested with this protocol, and could be recorded for at least 15 min. Two neurons showed LTP as measured with the EPSP amplitude and the number of spikes that lasted for the entire duration of recording, 55 min in the case of Fig. 9A, and 35 min in the case of Fig. 9B. In these experiments the test stimuli were presented at longer intervals (20 s) before and after the pairing event. We also measured the latency between the stimulus and the first spike in the two examples shown in Fig. 9. Latency increased transiently after each tetanus from 6.5 to 15 ms on average, and returned to pre-stimulus conditions within a few minutes. No specific changes of latency were seen after pairing depolarization with tetanus, and no further latency shortening was observed.

The PE1 in Fig. 9A was exposed to one tetanus pairing, the PE1 neuron shown in Fig. 9B received two pairings with the aim to test whether potentiation might be enhanced by the second pairing. This was not the case; rather, the second pairing induced a slight reduction of the potentiation effect. Most interestingly, in both cases a draught of potentiation is seen around 20 min after induction (notice the different time scales in Fig. 9Ai,Bi and Aii,Bii). The reduction of potentiation shortly after the second pairing in the neuron in Fig. 9B may, in fact, reflect a draught of potentiation following the first pairing, and the later reduction that of the second pairing.

Associative LTP was found in only two out of 15 neurons that were recorded for longer than 15 min without performing other tests following a pairing of presynaptic tetanus and postsynaptic depolarization. It is unknown why not all PE1 neurons stimulated in this way showed associative LTP. We cannot exclude that different K-cells may have been stimulated in different preparations. Furthermore, since our preparation was not an isolated brain or a slice of the brain, but rather a whole animal with its brain embedded into its normal input and output connections and its modulatory supply, we had no control over the modulatory input. No obvious correlation was found between the induction of associative LTP and the general conditions of the animal as judged by the movement of the antennae or the abdomen, or the quality of the recording (resting potential, stability of resting potential, appearance of summed EPSPs, and duration of recording). Furthermore, associative LTP might develop only over longer period of time, and since we were able to record from the PE1 for longer than 30 min in only five preparations (without performing other stimulus protocols) we cannot exclude the possibility that associative LTP may be a more frequent phenomenon.

Particular attempts were made to test whether LTP induced by pairing tetanic stimuli in one calyx with depolarization of the PE1 is restricted or transferred to stimulus input from the other calyx. Unfortunately, we were unsuccessful in inducing LTP in long-lasting PE1 recordings in which the PE1 could be stimulated by both calyces.

Discussion

The mushroom body and alpha lobe-extrinsic neurons

Central neurons of the insect brain have been intensively studied with respect to sensory coding (Homberg, 1984; Abel et al., 2001; Müller et al., 2002), modulation by biogenic amines (Erber et al., 1993; Kloppenburg and Erber, 1995) and plasticity related to olfactory learning (Mauelshagen, 1993; Hammer, 1997; Grünewald, 1999). No attempts have been made so far to analyze synaptic plasticity, because this requires the combination of intracellular recording and selective activation of particular subsets of neurons. Here we present a first step in this direction. We focused on neurons that exit the alpha lobe of the MB, since they can be expected to serve as an output of the MB, and may also be involved in transmitting the plastic components of the MB. Indeed, two kinds of these neurons, the single identified PE1 neuron and recurrent neurons running in the protocerebral calycal tract, were found to respond to stimuli of different modalities and to change their response properties during olfactory learning (Mauelshagen, 1993; Grünewald, 1999). We stimulated K-cells electrically and monitored intracellular responses in MB-extrinsic neurons. In most cases no synaptic potentials could be recorded. Therefore, we restricted our analysis to the initiation of spikes and evaluated synaptic strength more indirectly by counting the spikes elicited by a single test pulse. Summed EPSPs were visible in the case of the PE1 neuron, which was physiologically identified by the occurrence of large summing EPSPs and double or triple spikes. On rare occasions we also saw summing EPSPs in other neurons (e.g. in Fig. 3B), but the absence of double or triple spikes made the physiological identification of the PE1 neuron rather reliable. Measures of synaptic potentials like amplitude and latency could, therefore, be included only for the PE1 neuron. The latency of excitatory responses to a stimulus in the calyx was in the range <4 ms for all recorded neurons, indicating a direct synaptic input from K-cells and proving that our stimulation electrode excited only the direct inputs to these neurons. This conclusion is supported by anatomical evidence for the PE1 neuron, which was found to be in close contact with K-cells (Rybak and Menzel, 1993 and 1998; Strausfeld, 2002, Brandt et al., 2005), and postsynaptic to these cells (Rybak, 1994). The strength of the test pulses was chosen to elicit a small number of spikes with the aim of uncovering both reduction and enhancement of neural plasticity. Although we tried to mark the recorded cells, we were able to identify only a few neurons because of technical problems (e.g. an injection time that was too short, leading to incomplete marking; multiple neurons marked). In the case of the PE1 neuron we could still relate recordings to this neuron because of its very characteristic spiking pattern

(double and triple spikes, summing EPSPs), which we did not see in other alpha lobe-extrinsic neurons.

We worked with whole unanaesthetized animals, intending to keep the conditions as close as possible to those under which animals learn to associate an odour with sucrose reward (Bitterman et al., 1983; Menzel, 1990; Menzel and Müller, 1996). Rather similar conditions were used when it was shown that intracellular stimulation of a particular modulatory neuron arising from the suboesophageal ganglion, the VUMmx1, could replace the rewarding stimulus in a conditioning experiment (Hammer, 1993). The problem of a whole animal preparation, however, is that the modulatory state of the brain may differ between preparations and between different times of working with the same preparation. It is thus not surprising that we did not find exactly the same forms of plasticity, even in the same neuron (e.g. the PE1) in different preparations (see below).

Special emphasis was given to an identified neuron, the PE1. This neuron exists only once on each side of the brain, responds to a large range of odours and other sensory modalities (Rybak and Menzel, 1998), and changes its responses to a learned odour in an associative way (Mauelshagen, 1993). These associative changes are first a reduction of its responses to the learned odour after a single learning trial, and then, in the course of multiple differential learning trials, an enhancement of the responses. The responses to an odour not paired with sucrose reward in the differential conditioning paradigm did not change. The PE1 can, therefore, be considered as a read-out of learning-related plasticity in the MB of the bee (Menzel, 2001).

Short-term plasticity: paired-pulse facilitation and depression

We applied the paired-pulse protocol to test whether particular neurons show short-term depression or facilitation, whether this form of plasticity is reliably connected with other forms of plasticity, in particular tetanus-induced augmentation, and which pulse intervals induce the strongest effects. Since we could not measure postsynaptic potentials in most neurons, but only spike activity, we could not critically test the underlying mechanisms (Katz and Miledi, 1968; Fisher et al., 1997). We found that alpha lobe-extrinsic neurons have robust paired-pulse depression or facilitation. In no case did we find a transition from depression to facilitation or *vice versa* during the recording, but in different preparations with recordings from the same neuron (PE1), either paired-pulse depression or facilitation or no effect was found (e.g. in five recordings from the PE1 two responded with facilitation, two with depression and one showed no effect). Repetitions of paired pulses frequently led to an enhancement of the facilatory effect (Fig. 3), possibly indicating that synaptic transmission is not restricted by a limited presynaptic pool. For example, paired-pulse facilitation in a sensory-motor synapse of *Aplysia* was found to diminish during repeated double pulses (Jiang and Abrams, 1998), and these data were interpreted to indicate a limited presynaptic pool of releasable vesicles (but other mechanisms, e.g. network properties, are also possible). Our interpretation is supported by the finding that tetanus-induced

augmentation and paired-pulse facilitation are additive rather than competitive (Fig. 7). The latter was found in hippocampal neurons (Staubli et al., 1990; Zalutsky and Nicoll, 1990). Here paired-pulse facilitation is reduced in long-term potentiated synapses, a phenomenon believed to indicate limited presynaptic pools and the necessity to recruit releasable vesicles during LTP induction. However, we have not tested paired-pulse plasticity after LTP induction. Therefore, we can only conclude that augmentation in the minutes range and paired-pulse facilitation appear to address different mechanisms, either at the synapse and/or the excitability of the alpha lobe-extrinsic neurons.

The optimal time windows for paired-pulse facilitation (10–30 ms) and depression (20–60 ms) could be interpreted as an indication for temporal tuning of the network to which these neurons belong. It is conceivable that action potentials arriving at intervals around 20–30 ms (leading to a dominant frequency around 30–50 Hz) are facilitated in some neurons, and spikes arriving at longer intervals (corresponding to <30 Hz) are depressed in other neurons. If these neurons are interconnected in an appropriate way they could sharpen the window for optimal responses and thus serve coincidence detection, because spikes arriving at the edge of a window for coincident detection (here at approximately 40 ms) will be reduced in their ability to fire the postsynaptic cell, a mechanism comparable to that seen in neurons of the chick auditory brain stem (Cook et al., 2003). It has been argued for the locust that the olfactory input to the MB *via* the projection neurons may be phase-locked to MB-related network activity that oscillates at 25–35 Hz (Laurent, 2003). It is tempting to speculate that double-pulse depression, as seen here in alpha lobe-extrinsic neurons of the bee, may be involved in setting up such oscillatory activity, particularly if the inhibitory recurrent neurons running in the protocerebral calycal tract also have properties similar to the alpha lobe-extrinsic neurons recorded here.

Tetanus-induced plasticity

A tetanus induced four different types of neural plasticity: (1) short-term depression, (2) depression lasting for up to 2 min, (3) prolonged depression lasting for several min, and (4) prolonged depression followed by lasting augmentation. An attempt was made to localize these forms of plasticity to the pre- and post-synaptic sites. Since our single neuron markings indicated that the alpha lobe-extrinsic neurons received input across the whole MB, in some of the experiments we stimulated the median and lateral calyces separately, addressing the question whether the particular neural plasticity is restricted to the input from those K-cells that were stimulated with the tetanus. We found that response enhancement (brief and longer-lasting augmentation) is transferred across stimulation sites, but depression is not, indicating that augmentation may be dominated by presynaptic and depression by postsynaptic phenomena. This conclusion is supported by the finding that hyper-polarization of the PE1 neuron during a tetanus reduces depression (Fig. 9A).

Augmentation was also seen in evoked field potentials recorded in the mushroom body of honeybees (Oleskevich et al., 1997). It seems that this study monitored plasticity of the input synapses to the MB neurons from the olfactory projection neurons, and not, as in our study, the output synapses from MB neurons to alpha lobe-extrinsic neurons. Furthermore, augmentation was found for very low stimulus frequencies (0.02–1 Hz). We never saw augmentation to such low stimulus frequencies.

As discussed above, short-term facilitation as seen in paired-pulse experiments appears as an independent and additive component to response augmentation. In experiments where no cumulative enhancement of repeated paired-pulse facilitation was seen a tetanus could induce transient (Fig. 7) cumulative paired-pulse facilitation. Thus, mechanisms leading to response enhancement appear to be located both presynaptically (paired-pulse facilitation) and postsynaptically (augmentation).

Associative long-term potentiation (LTP) in two PE1 neurons

Augmentation of responses to single test stimuli following a tetanus usually lasted only a few minutes, but in some neurons (e.g. Figs 6B, 8B) augmentation had not yet relaxed to pre-tetanus conditions before we either continued with the experiment (marking the neuron) or lost the cell.

Five PE1 recordings were held for longer than 30 min without applying other stimulus protocols, and in two neurons (recorded for 48 and 95 min, respectively) we induced LTP when the PE1 was depolarized during the tetanus. No LTP was found after pairing a tetanus with hyper-polarization or after tetani alone, even when they were repeated. The depolarization of the PE1 or other alpha lobe-extrinsic neurons did not lead to a change in its response to test stimuli. The depolarization coincident with the tetanus was set to a value that caused the PE1 to spike at high frequency (between 50 and 100 Hz), well within the normal range of spiking of the PE1 neuron. The current applied ranged between 0.5–3 nA. Two phenomena are noticeable. Not all PE1 neurons studied with this protocol showed associative LTP, and in both cases where associative LTP was induced, a dip of potentiation is seen around 20 min after the pairing of tetanus and depolarization.

Associative LTP was first found in the CA1 region of the mammalian hippocampus, but is now well-documented for a number of different types of neurons in vertebrates (Bliss and Collingridge, 1993; Abbott and Sejnowski, 1999). Associative LTP has been related on formal grounds to Hebb's learning rule (Hebb, 1949), because it requires coincident activity at the pre- and postsynaptic sides. It is now well-established for cortical neurons that the 'Hebb synapse' may undergo LTP when pre- and postsynaptic spike activities are precisely correlated, and long-term depression (LTD) when these activities are not or anti-correlated (Markram et al., 1997; Sejnowski, 1999). Associative LTP was considered to be restricted to the vertebrate brain until it was found that the sensory-motor synapses in *Aplysia* also enhance synaptic transmission according to Hebb's rule (Roberts and Glanzman,

2003). No such form of lasting enhancement of neural transmission has so far found in insects. The augmentation effect seen in field potentials recorded in the MB of honeybees after low frequency stimulation of the olfactory pathway (Oleskevich et al., 1997) cannot be considered to reflect an associative form of lasting plasticity, because only extracellular field potentials were recorded and the low stimulus frequencies applied could not lead to any form of additive effect.

Glutamatergic (Glu) transmission is found to be an essential component in Hebbian plasticity in the mammalian brain, and in the nervous system of molluscs (Glanzman, 1995). Glu transmission occurs at the neuromuscular junction in insects, and has also been well-studied with respect to plasticity (Zhong and Wu, 1991), but only sparse information exists on central Glu transmission in the insect brain. A small proportion of K-cells label with an antibody against Glu, both in the bee (Bicker et al., 1988; Schürmann et al., 2000) and various other insect brains (Farris and Strausfeld, 2003; Farris et al., 2004, Strausfeld et al., 2003), but the role of Glu transmission is not known. Blocking Glu uptake was found to interfere with the formation of longer-lasting memory, but the mechanisms are not understood (Maleszka et al., 2000). Thus it is not known whether Glu receptors in the insect brain may have a role comparable to the one they play in mammalian and mollusc brains. Since no information exists about receptors for the transmitter(s) of K-cells, our study could only prepare for future investigations on the pharmacology of synaptic transmission in the MB by demonstrating how intracellular recordings from efferent neurons may be used to characterize synaptic transmission in the MB.

The time course of associative LTP showed a significant dip around 20 min after induction in the preparation shown in Fig. 9A. In the recording where we repeated the pairing of tetanus and depolarization (Fig. 9B), a first dip is seen around 12 min after the first pairing and a broader dip begins 20 min after the first and 13 min after the second pairing. It is tempting to relate this phenomenon with the temporal dynamics of memory processing in the bee brain as revealed from behavioural and biochemical studies (Menzel and Müller, 1996; Menzel, 1999). The transition from an early short-term memory to late short-term memory occurs within a few minutes after a single learning trial. In the case of multiple learning trials, several processes are initiated that proceed partially in parallel and partially in series, and for these the time period around half an hour after conditioning is critical (Müller, 2000; Müller and Hildebrandt, 2002). Different signalling cascades are involved. For example, mid-term memory relies on the activation of a soluble NO synthase, and the signalling pathways leading to long-term memory require an enhancement and prolongation of adenylate cyclase activity. It is tempting to speculate that these forms of early memory dynamics may be related to the time course of associative LTP as seen in the two PE1 recordings here. A hint in this direction comes from the studies of Mauelshagen (1993), who observed that multiple learning trials led to stronger responses of the PE1

to the conditioned odour, but that these learning-specific effects lasted for only about 10 min. Mauelshagen worked with isolated heads, not with whole animals, and thus a later phase of neural plasticity may not have developed (possibly due to lack of oxygen supply). The picture arising from these observations portrays the PE1 as a neuron that undergoes associative plasticity and reflects in its responses to the learned odour associative processes that occur in both the upstream neurons (projection neurons to K-cell synapses) and in its synapses with the K-cells.

Structural components of PE1-associative LTP

The structure of the PE1 is well-known (Mauelshagen, 1993; Rybak and Menzel, 1993, Strausfeld, 2002). Reconstructions of two PE1 neurons indicated two input regions characterized by dense spiny structures, one close to its basal stem of dendritic arbours in the ventral part of the alpha lobe, and one at the outer parts of the finger-like protrusions of the dendrites that cross through most other K-cell axons, potentially making synaptic contacts with K-cells from the lip, collar and basal ring (Brandt et al., in press). The multi-sensory response properties of PE1 (Rybak and Menzel, 1998) may, therefore, result from inputs *via* a large number of K-cells. The spatial separation between two input regions may reflect a structural correlate of its associative plasticity, since inputs received by one input region (the finger-like protrusions) may reach the PE1 neuron when it is depolarized *via* the other input region (the K-cells of the ventral part of the alpha lobe; Fig. 10). The K-cells of the ventral alpha lobe include the so-called clawed K-cells (Rybak and Menzel, 1993, Strausfeld, 2002), and these K-cells receive input from the different calyx compartments in the input side of the MB. Thus both input regions of the PE1 appear to receive information from across the MB but in two different structural forms. Further studies on the response properties of these different K-cells will be needed before we understand their specific contributions to the PE1's response properties.

Modulation of neural plasticity

Our approach allowed us to record from the same individually identified neuron in different whole-animal preparations. The PE1 neuron showed different forms of plasticity in different preparations, and thus it is likely that other MB-extrinsic neurons may have also responded differently to our test protocols in different preparations. One may ask why such differences were found, although we recorded from the same individual neuron. Even though we cannot exclude the possibility that we stimulated different subsets of K-cells in different preparations, we consider this rather unlikely, because the pairs of stimulation electrodes (one pair in each

of the two calyces of the MB) were located such that the current must have flown through the somata of practically all K-cells. Behavioural pharmacology in the honeybee has shown that responses to stimuli, learning and memory processing as well as neuron responsiveness can be manipulated by blocking or enhancing modulatory systems (for reviews, see Menzel et al., 1994; Blenau and Erber, 1998; Farooqui et al., 2003). It is thus likely that the animals differed with respect to the status of their modulatory systems, and that this influenced the outcome of the tests. This is particularly noteworthy for the induction of associative LTP in PE1. Only two out of five neurons tested in the same way showed associative LTP. Thus the modulatory systems must be set to a particular condition for the induction of associative LTP, but we do not know what these conditions would be.

A considerable proportion of neurons did not respond to sensory stimuli, but responded to electrical stimuli and showed normal plasticity in all our stimulus protocols (short-term facilitation, mid-term augmentation and associative LTP). It is thus likely that some of the MB-extrinsic neurons are not excited by natural sensory input but might need to be recruited by stronger or particularly organised patterns of input, as is the case in double pulses, in a tetanus or when a tetanus is paired with the neuron's depolarisation. If this were the case, a different set of MB output neurons would code the same input after being exposed to a particular kind of excitatory input. This form of plasticity is of special interest for long-lasting plasticity as seen in associative LTP, because it could reflect a mechanism underlying learning and memory formation.

Conclusion

Electrical stimulation protocols applied to K-cells for the study of synaptic transmission to MB-extrinsic neurons revealed two major results. (1) Short-term facilitation and depression induced by double-pulse protocols are tuned such that spike intervals in the range of 20–30 ms are favoured. This
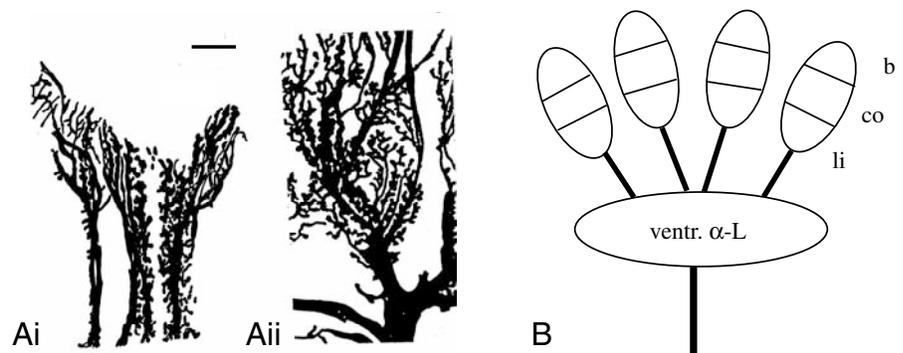

Fig. 10. Structural correlates of the inputs from the K-cells to the PE1 neuron. (A) High resolution camera lucida drawing of the two spiny regions of the PE1 dendrites; (Ai) finger-like spiny terminals of the dendrites in the region deep in the peduncle, (Aii) spiny branches at the base of the PE1 close to the integrating segment of the dendrites. Scale bar, 10 μm. (B) Scheme of the two spiny regions of the PE1 dendrites in the finger-like branches that make contact with K-cell axons from the three calyx regions, lip (li), collar (co), basal ring (br), and those in the region of the ventral alpha lobe (ventr. α-L).

may lead to oscillatory activity in the output of the MB in the frequency range 30–50 Hz. Such oscillations were not seen during intracellular recordings, but may become detectable only in extracellular recordings from multiple units and local field potentials (for the locust, see Perez-Orive et al., 2002, 2004). (2) Associative LTP was induced in the PE1 neuron by pairing a presynaptic tetanus with depolarization. This is the first time that associative LTP has been found in an insect. Viewed against the background of the mushroom bodies' well-documented participation in associative olfactory learning and memory formation, it is therefore possible that the output synapses of the MB in the honeybee may contribute to the formation of a memory trace.

List of abbreviations

CS	conditioning stimulus
Glu	glutamate, glutamatergic
K-cell	Kenyon cell
LTD	long-term depression
LTP	long-term potentiation
MB	mushroom body
PE1	pedunculus-extrinsic neuron number 1
VUMmx1	the ventral unpaired median neuron number 1 of the maxillary neuromere

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