J Comp Physiol A (2001) 187: 825–836
DOI 10.1007/s00359-001-0253-y

ORIGINAL PAPER

Sabine Gauglitz · Hans-Joachim Pflüger

Cholinergic transmission via central synapses in the locust nervous system

Accepted: 2 October 2001 / Published online: 10 November 2001

Abstract In this study we examine the nature of chemical synaptic transmission between identified filiform hair receptors on the prothoracic segment of a locust and the identified postsynaptic projection interneuron (A4I1). The effects of pressure ejected acetylcholine, and various ligands of acetylcholine receptors on the activity of the postsynaptic neuron A4I1, or on wind-elicited responses in A4I1 are reported. It is suggested that the transmitter of the afferent fibers is acetylcholine, and that fast transmission is mediated by nicotinic acetylcholine-receptors. Both nicotine and carbachol act as agonists, whereas *d*-tubocurarine and α-bungarotoxin act as antagonists. The presence of muscarinic acetylcholine receptors was also evident from the modulatory effects of muscarine, oxotremorine and pilocarpine, which were blocked by bath application of atropine. GABA, and its agonists muscimol and *cis*-4-amino-crotonic-acid lead to inhibition of A4I1 responses. This inhibition was prevented by the additional application of picrotoxin. This suggests involvement of a ligand-gated GABA receptor which, most likely, increases chloride conductance. Metabotropic GABA-receptors do not seem to be involved, since baclofene, diazepam and bicuculline ejections had no effects. Glutamate also inhibits wind elicited A4I1 responses. Although attempts were made to further characterize the receptor involved, tested substances such as kainic acid, glycine, CNQX or GDEE had no effect.

Keywords Insects · Mechanosensory systems · Behavioral pharmacology · Acetylcholine · Inhibition

S. Gauglitz · H.-J. Pflüger (✉)
Freie Universität Berlin; Institut für Biologie, Neurobiologie, Königin-Luise-Strasse 28–30, 14195 Berlin, Germany
E-mail: pflueger@neurobiologie.fu-berlin.de
Tel.: +49-30-83854676
Fax: +49-30-83855455

Abbreviations *ACh* acetylcholine · *ACh*-R acetyl choline receptor · *CACA cis*-4-amino-crotonic-acid · *CNS* central nervous system · *GABA* γ-amino-butyric acid · *mACh-R* muscarinic acetylcholine receptor · *nACh-R* nicotinic acetylcholine receptor · *PTX* picrotoxin

Introduction

One of the most important neurotransmitters in the insect central nervous system (CNS) is acetylcholine (ACh) (Sattelle 1985; Breer and Sattelle 1987; Osborne 1996) which in contrast to vertebrates is not involved in neuromuscular transmission but seems to be a principal transmitter of many, if not all, mechanosensory neurons, perhaps with the exception of *Drosophila* where histamine has been proposed as a transmitter candidate (Buchner et al. 1993). For example, cholinergic transmission has been described for the synapses between cercal filiform hairs and giant interneurons in cockroaches (Callec et al. 1971; Shankland et al. 1971; Blagburn and Sattelle 1987) and crickets (Meyer and Reddy 1985; Ferber et al. 2001), between tactile hairs and motor neurons in larval *Manduca sexta* (Trimmer and Weeks 1989), and as a transmitter of the giant fiber pathway of *Drosophila* (Gorczyca and Hall 1984). In addition, the locust wing hinge stretch receptor is believed to release ACh (Leitch and Pitman 1995). Further evidence comes from studies with antibodies raised against choline acetyltransferase (locusts: Lutz and Tyrer 1987, 1988; *Drosophila,* Yasuyama and Salvaterra 1999), which stain peripheral cell bodies of sensory neurons and their central nervous terminals. An antibody raised against nicotinic ACh receptor (ACh-R) protein (Sattelle et al. 1989; Leitch et al. 1993) labeled all major neuropiles in abdominal and thoracic ganglia including the ventral mechanosensory neuropiles, but did not stain the terminal afferent fibers themselves (Sattelle et al. 1989). Similar antibody stains were performed in the locust brain and labeled all

major neuropiles (Vieillemaringe et al. 1987). ACh has also been suggested to be the transmitter between giant and thoracic interneurons in the cockroach central nervous system (Casagrand and Ritzman 1992) and as a transmitter in optical processing pathways of flies (Brotz and Borst 1996; Yasuyama et al. 1995) and locusts (Leitinger and Simmons 2000). In grasshoppers the stridulatory behavior can be released by injection of acetylcholine into special brain neuropiles (Heinrich et al. 1997). It has also been described as the transmitter between descending interneurons and a vasopressin-immunoreactive interneuron (VLPI) in the locust CNS (Baines and Bacon 1994), and between unknown interneurons and neuromodulatory octopaminergic neurons in the cockroach CNS (Grolleau et al. 1996). Furthermore, it was characterized as a transmitter in the mushroom bodies (Bicker 1999; Cayre et al. 1999; Oleskevich 1999), and the antennal lobes (Blake et al. 1993; Homberg et al. 1995). Synaptic cholinergic transmission has even been reported for connections formed between cultured neurons prepared from *Drosophila* wild-type central nervous system (Lee and O'Dowd 1999).

Given this large body of evidence for cholinergic transmission in the insect and other invertebrate nervous systems, the number of behavioral-pharmacological studies is rather small. The behavioral relevance of a transmitter is determined by the effects of either its ionotropic or metabotropic receptor molecules, and it is therefore necessary to find out which specific receptor molecules are involved in a particular functional pathway.

In the present study, the central synapses between filiform prosternal hair receptors and an identified ventral cord interneuron (A4I1, Pflüger 1984) within the locust prothoracic ganglion are examined. The A4I1 interneuron makes output synapses to motor neurons of special wing steering muscles (Burrows and Pflüger 1992), is thought to play a role in adult flight steering, and is thus one of many parallel pathways that exist in the locust flight system (for review see Burrows 1996). The filiform prosternal hair receptors make monosynaptic connections to this interneuron in the prothoracic ganglion (Pflüger and Tautz 1982; Watson and Pflüger 1984), and this is also where its main spike initiation zone is situated. Differences in the efficacies of synaptic connections between individual afferents and the interneuron correlate well with its prosternal position and age of the afferent neuron (Pflüger and Burrows 1990).

The results provide evidence for the cholinergic nature of the afferent-to-interneuron synapses with the existence of both nicotinic and muscarinic ACh-Rs. In addition, we tested the effects of γ-aminobutyric acid (GABA) and glutamate on synaptic transmission, as these were shown by immunohistochemical methods to be present in neurons presynaptic to the afferent terminals (Watson and Pflüger 1994). Both GABA and glutamate have inhibitory effects on transmission between afferents and the A4I1-interneuron.

►

Fig. 1 **a** Experimental set up showing locust ventral side up and the prosternal area (*shaded square*) which is shown on the right side indicating the exposed prothoracic ganglion and the position of the multibarrel electrode. The central axonal paths of two hair receptor cells, and the axon of the left A4I1 interneuron are drawn schematically. *Stippled area within the ganglion* shows the borders of the respective sensory neuropile. **b** A typical A4I1 response recorded extracellularly from the abdominal connective (see hook electrodes in **a**), elicited by short wind puffs to the prosternum. Note good signal-to-noise ratio of the large units which are activated by a wind stimulus to the head, and which have been shown to belong to the A4I1 interneuron (Pflüger 1984), and the much smaller ventilatory units. *V* valve. **c**, **d** The two stimulus regimes that were used in our experiments

Materials and methods

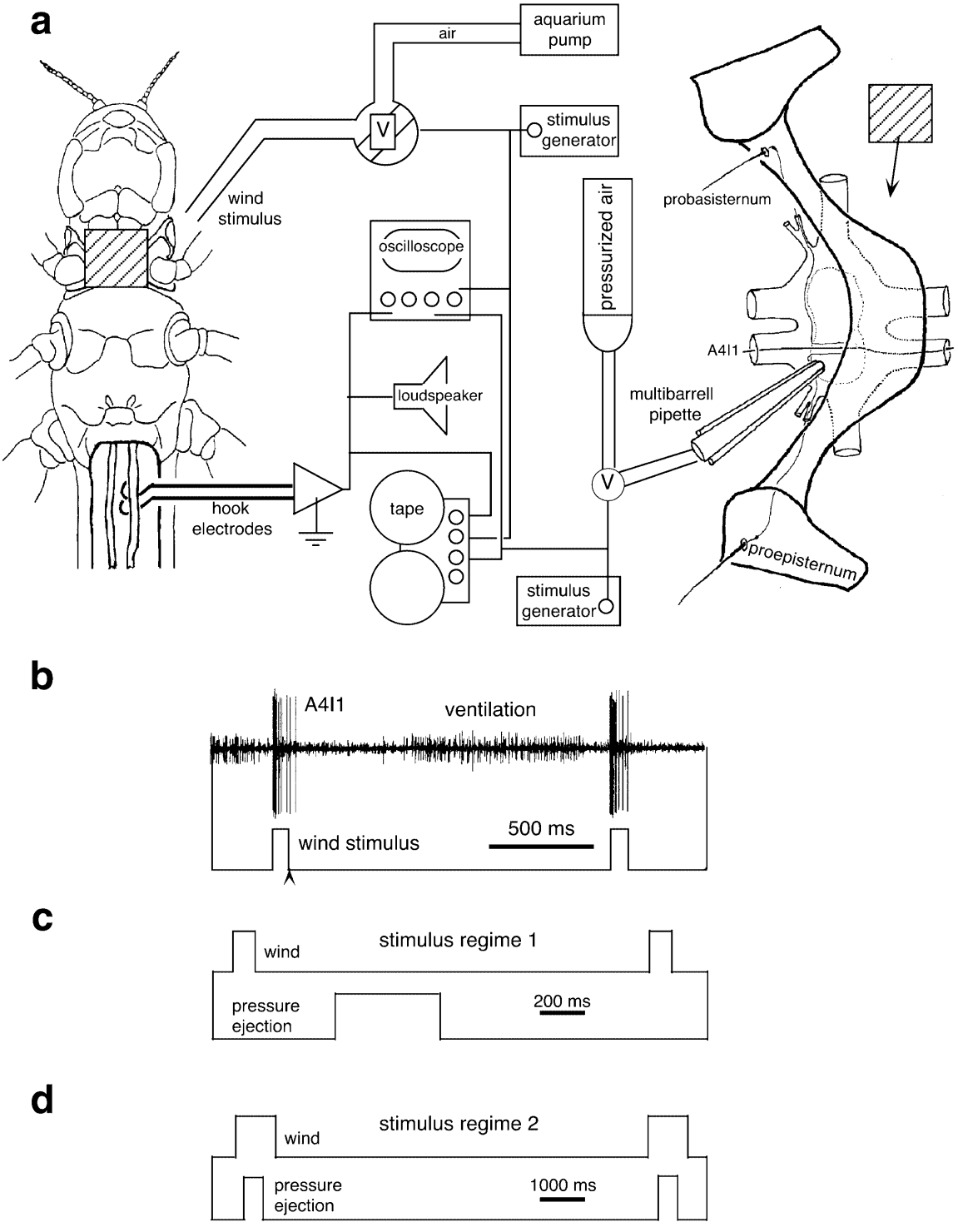
All experiments were performed on adult *Locusta migratoria* from a crowded colony maintained in Berlin. Locusts were dissected from the ventral side to expose the meso- and metathoracic ganglia. The prothoracic sternum and episternum which bear the filiform hair receptors that are part of A4I1's receptive field were left intact. The soft cuticle of the neck membrane anterior to the prothoracic sternum was carefully removed to expose the anterior part of the prothoracic ganglion and the connectives between the prothoracic and suboesophageal ganglion (see Fig. 1). To reduce the neuronal background activity in the connectives, all peripheral nerves of the meso- and metathoracic ganglion were cut, except those belonging to the prothoracic ganglion. In most cases the connectives posterior to the fourth abdominal ganglion were also cut. A wax coated stainless steel platform was carefully placed underneath the prothoracic ganglion to stabilize the preparation. It was constantly perfused with hypotonic saline containing (mmol l^{-1}): NaCl 140, KCl 10, NaH_2PO_4 4, Na_2HPO_4 6, $CaCl_2$ 2, at approx. 2 ml min^{-1} (Clements and May 1974) flowing from a small opening in the steel platform. Flooding of the prothoracic filiform hair receptors by saline was carefully avoided as this prevented normal movements of the hair receptors.

Electrophysiological recordings and sensory stimulation

The activity of the A4I1 interneuron was recorded extracellularly from the connectives between the metathoracic and 4th abdominal ganglia with either bipolar steel hook electrodes or suction electrodes (Fig. 1). The A4I1 neuron was stimulated by directing a defined wind stimulus to the prosternal hair receptors which form its receptive field. The wind stimulus was generated by an aquarium pump (Schego optimal) and a circuit driving a 3-way magnetic valve (Lee; LFAA 120141 8 H). The opening of a polyethylene tube with a diameter of 1 mm was placed at an angle of 45° and at the level of the proepisternum (Fig. 1). In general, the duration of the applied wind stimulus was 100 ms, but was increased to 1000 ms or 2000 ms in some experiments. Stimulus intensity was measured only in relative units, and could be divided in steps from 0 to 8 by controlling the outflow of the aquarium pump.

Pressure microejection into neuropilar areas

Small amounts (0.2–7.1 nl, minimum to maximum) of the tested substances were ejected by pressure (3 kPa cm^{-2}) into the ventral neuropilar area of the prothoracic ganglion where the synapses between the filiform hairs and the A4I1 interneuron are made. Penetration of the multibarrel pipette was eased by softening the ganglionic sheath with pronase (Sigma; P-5147, Type XIV). Pressurized air could be applied to each of four electrodes which were part of a multibarrelled glass micropipette, thus allowing ejection of substances at different concentrations in one experimental



session. The duration of the ejection pulse could be varied from 100 ms to 800 ms, and was increased to 1000 ms to 1200 ms in a few experiments. Variations of the dose of a particular substance were achieved either by varying the duration of the pressure pulse, or by changing the concentration of the ejected substance in the micropipette (Buckingham et al. 1994; Waldrop 1994). In order to avoid desensitization effects, intervals of up to 3 min between consecutive ejections were chosen. After each experiment each micropipette was calibrated by ejecting the test substance into paraffin oil, and the diameter of the droplet was measured using an ocular micrometer. Ejection of a fluorescent substance, e.g., lucifer yellow, into the respective neuropile confirmed that diffusion was confined to the neuropilar borders, at least for the time during which experiments were made.

Experimental procedure, tested substances, data acquisition and analysis

After dissection several wind stimuli were given, and the normal A4I1 response was monitored; then the multibarrel glass micropipette was placed into the respective neuropile of the prothoracic

ganglion, and the A4I1 response was tested again. Only those preparations were used which showed the same A4I1 response before and after electrode placement. The respective substances were then pressure ejected after an interval of 10 min in which the animal was left unstimulated. The following ligands acting on acetylcholine receptors were tested (concentrations in mol l^{-1}): acetylcholine (Sigma; A-6625, 10^{-1} to 10^{-6}), carbachol (Sigma; C-4383, 10^{-2} to 10^{-7}), nicotine (Sigma; N-3876, 10^{-3} to 10^{-7}), *d*-tubocurarine (Sigma; T-2379, 10^{-3} to 10^{-5}), α-bungarotoxin (Sigma; T-3019, 10^{-4} to 10^{-6}), pilocarpine (RBI; P-113, 10^{-3} to 10^{-9}), muscarine (Sigma; M-0406, 10^{-3} to 10^{-8}), oxotremorine (Sigma; O-9126, 10^{-3} to 10^{-8}), atropin (Sigma; A-0257, 10^{-1} to 10^{-4}). For testing GABAergic transmission the following substances were used: GABA (Fluka; 07240, 10^{-1} to 10^{-5}), muscimol (Sigma; M-1523, 10^{-2} to 10^{-6}), *cis*-4-amino-crotonic-acid (CACA) (RBI; A-201, 10^{-1} to 10^{-4}), picrotoxin (PTX) (Sigma; P-1675, 10^{-3} to 10^{-6}), bicuculline (Sigma; B-9130, 10^{-3}), baclofen (RBI; B-020, 10^{-2} to 10^{-4}), diazepam (Synopharm; 10^{-2} to 10^{-3}). Glutamatergic transmission was tested by the following substances: glutamate (Sigma; G-2128, 10^{-1} to 10^{-4}), kainic acid (RBI; G-020, 10^{-2} to 10^{-5}), glutamic acid di-ethyl-ester (GDEE) (RBI; G-101, 10^{-2} to 10^{-3}), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (RBI; FG-9065, 10^{-3}), PTX (Sigma; P-1675, 10^{-3} to 10^{-4}), glycine (Roth; 3908.2, 10^{-2}).

After ejection of a substance into the neuropile, its effects on the A4I1 response was tested. Antagonists and neuromodulatory substances were tested by using two different stimulus regimes: In regime 1, wind puffs lasting for 100 ms were directed to the prosternum at a repetition rate of 0.5 Hz, and the respective substances were pressure ejected between wind puffs approximately 500 ms after a wind stimulus. In regime 2, wind puffs lasting 1 s were applied at a repetition rate of 0.1 Hz and pressure ejections were made during the wind stimulus. The efficacy of this substance was determined by comparing the responses of the A4I1 interneuron to wind stimuli before and after ejection of substances into the respective neuropile. For all experiments controls were performed by measuring the A4I1 responses after pressure ejection of saline or the solvent of a particular substance.

The following parameters were measured: (1) spike frequency, duration, and onset (latency) of the A4I1 response after pressure ejection of an excitatory substance (putative transmitter or agonist); (2) number of action potentials during a wind stimulus, and during inter-stimulus-intervals after ejection of antagonists or neuromodulatory substances; (3) delay between time of pressure ejection of an antagonist and is first noticeable effects (latency); (4) time to recovery of the A4I1 response (full recovery was if the interneuron response to a wind stimulus after the experimental procedure matched that of the initial response before any drug ejection); and (5) in addition, dose-response curves were determined for a few tested substances.

Electrophysiological data were stored on a videotape recorder (Vetter, Rebersburg, Pa., USA), digitized by a CED 1401 plus (Cambridge Electronic Design, Cambridge, UK), and analyzed with the Spike 2 program (CED). For statistical analysis the mean and standard deviations were determined, and in dose-response-curves a sigmoid curve fit was achieved by using Microcal Origin 4.1 (Microcal Software, USA). The same program was also used for testing the significance of differences (Student's *t*-test, $P \leq 0.05$).

Results

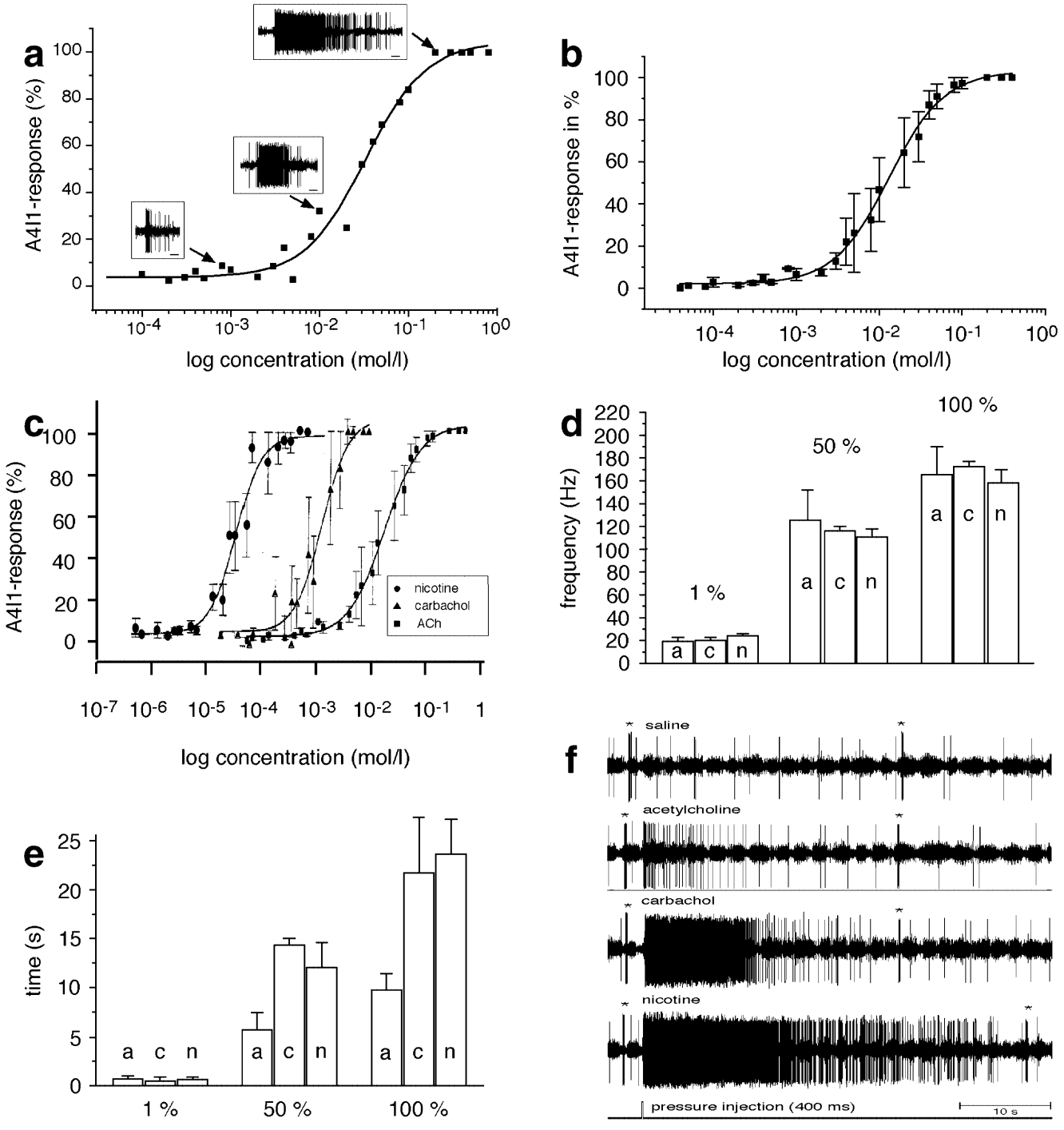
The effects of pressure-ejected acetylcholine, carbachol and nicotine

To test whether the synaptic transmission between the filiform hair receptors and the A4I1 interneuron is cholinergic in nature, acetylcholine (10^{-1} mol l^{-1} to 10^{-6} mol l^{-1}) and its ionotropic receptor agonists carbachol (10^{-2} mol l^{-1} to 10^{-6} mol l^{-1}) and nicotin (10^{-2} mol l^{-1} to 10^{-7} mol l^{-1}), were ejected into the neuropile by pressure pulses lasting from 100 ms to 1.2 s. All three substances excited the A4I1-interneuron. Figure 2a shows representative traces and the dose-response-curve for ACh ejection in one animal, and Fig. 2b shows the mean response for six animals. Nicotine first elicited a response at a concentration of 2×10^{-6} mol l^{-1}, and was thus more active than carbachol (10^{-4} mol l^{-1}). Figure 2c shows a comparison of the dose-response-curves, and reveals different potencies in binding to the ionotropic ACh-R in the order nicotine > carbachol > ACh. In all experiments the delay time between ejection and the first A4I1 response was short and ranged from 10 ms to 46 ms for all three substances tested. When the spike frequency of A4I1 bursts was measured over the 1st second, no differences were found between ACh, carbachol and nicotine (Fig. 2d), and this applied to all tested concentrations (shown are concentrations corresponding to 1%, 50%, or 100% increase in response activity of the dose-response-curve). There were, however, differences with respect to the duration of the elicited A4I1 burst (Fig. 2e). At higher concentration ACh always elicited a shorter burst than carbachol and nicotine. This is also demonstrated in Fig. 2f, in which the three substances were ejected into one individual animal sequentially, each ejection separated by saline washes. The short A4I1 bursts marked by asterisks were elicited by short (100 ms) wind puffs to the prosternum which stimulated the neuron naturally via its filiform hair receptors.

Fig. 2 Dose-response curves of acetylcholine (ACh) ejected into the respective neuropile and the evoked A4I1 response: **a** data from one individual animal, and **b** pooled data ($n = 6$, mean with standard deviation). *Insets* in **a** show original recordings of A4I1 responses at concentrations indicated by arrows. **c** A comparison between dose-response-curves of ejected ACh, nicotine and carbachol (mean values with standard deviations). **d** Comparison of A4I1 spike frequency within the 1st second of a response after ejection of acetylcholine (*a*), carbachol (*c*) and nicotine (*n*) at different concentrations (1%, 50%, and 100% of the dose-response curve ($n = 5$, mean and standard deviation). **e** Duration of A4I1 reponse after ejection of acetylcholine, carbachol and nicotine at different concentrations (see dose-response curves; $n = 5$, mean and standard deviation). **f** The responses of the A4I1 interneuron to pressure-ejected (stimulus duration 400 ms) saline (control), acetylcholine, carbachol and nicotine into the neuropile (all at concentrations 1×10^{-4} mol l^{-1}). *Bursts marked with asterisks* were elicited by 100-ms-long wind puffs to prosternum

The effects of ejected *d*-tubocurarine and α-bungarotoxin

The previous results suggested fast cholinergic transmission between filiform hair receptors and the A4I1 interneuron. We therefore tested *d*-tubocurarin and α-bungarotoxin as blockers of nicotinic ACh-Rs (nAChRs). In these experiments stimulus regime 1 (Fig. 1) was applied. As shown in Fig. 3a, b, both substances effectively blocked A4I1's response to wind puffs, but with



different sensitivities (Fig. 3b). The first effect of blocking was usually observed within 3–5 s after ejection. This block lasted for several minutes, and even after washing for 120 min with saline only 10–20% of the initial A4I1 response recovered. *d*-Tubocurarine also abolished any response of A4I1 elicited either by pressure ejection of nicotine, or by natural wind stimulation. Hence, we conclude that the prosternal filiform hair afferents use ACh as their transmitter, and that the fast responses in the postsynaptic A4I1 interneuron are mediated by nACh-R.

The effects of the muscarinic ACh-R agonists muscarine, oxotremorine, and pilocarpine

To test whether metabotropic ACh-Rs are also involved in these synapses, the effects of ejecting muscarine, oxotremorine and pilocarpine, all agonists of muscarinic ACh-R (mACh-R) in vertebrates, were studied during wind-elicited A4I1 bursts (stimulus regime 2, Fig. 1). All three substances had similar effects and increased the number of spikes in A4I1 bursts elicited by 1-s-long wind stimuli of the same intensity. The first noticeable effect

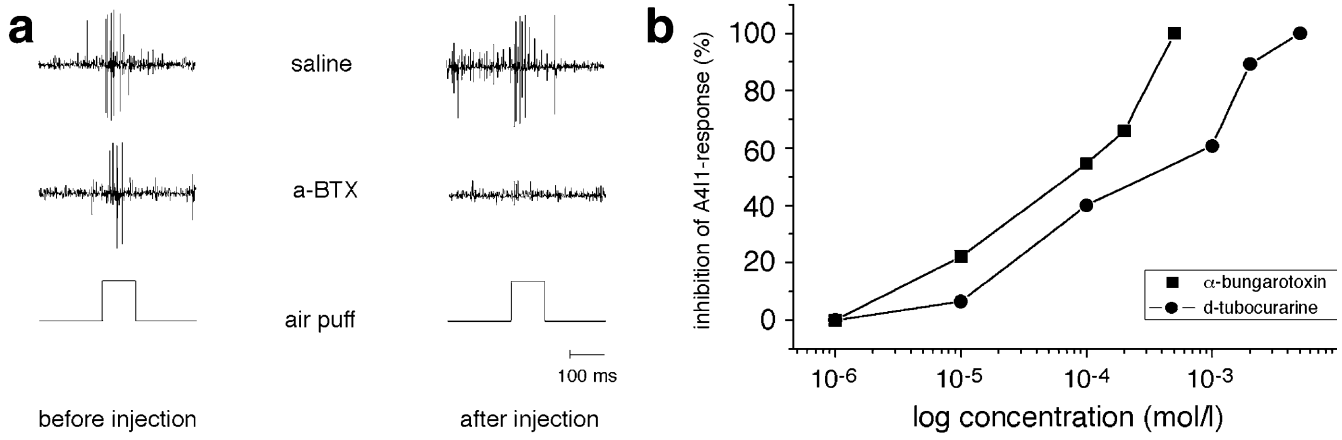


Fig. 3 **a** Inhibition of a wind-elicited A4I1 response by ejection of α-bungarotoxin (*middle traces*). As a control saline ejection has no effect on the A4I1 response (*upper traces*). *Lower traces* show the wind-puff to the prosternum to elicit the A4I1 response. **b** Percentage of inhibition of A4I1 response depending on the concentration of ejected α-bungarotoxin or *d*-tubocurarine ($n = 5$, mean values, standard deviations not shown)

of muscarine started after 3 ± 2 s ($n = 6$), reached a maximum after 29 ± 12 s, continued for as long as 114 s, and then gradually decreased until the initial unaffected condition was reached at 221 ± 55 s. The first effects of pilocarpine, in contrast to those of muscarine and oxotremorine, could only be detected after 13 ± 2 s ($n = 5$) which is significantly different from the other two substances tested ($P \leq 0.05$). No differences were detected between the three substances when they reached maximal efficacy between 20 s and 30 s. Figure 4a shows the percentage increase in the A4I1 responses in relation to the concentration of ejected muscarine in an individual animal, and Fig. 4b shows the pooled data for muscarine ($n = 6$), oxotremorine ($n = 5$), and pilocarpine ($n = 5$). The graph reveals different potencies of these substances in binding to the mACh-R in the order pilocarpine > oxotremorine > muscarine. From these results we conclude that not only nACh-R are present in this system but also mACh-R, which may exert additional, as yet unknown, modulatory effects.

Effects of the mACh-R antagonist atropine

The previously demonstrated increase in the A4I1 response after ejection of mACh-R agonists should be abolished by ejection of the mACh-R antagonist atropine. First, it was tested whether atropine ejection alone has an inhibitory effect on A4I1 bursts elicited by natural wind stimulation, and therefore stimulus regime 1 (Fig. 1) was applied and atropine ejected. Figure 4c shows that atropine has a concentration-dependent inhibitory effect on synaptic transmission, which can be observed approximately 3–6 s after ejection of atropine. From these results we conclude that in this system atropine not only affects mACh-R but also nACh-R (see Sattelle 1980). In a second series of experiments we applied stimulus regime 1 (Fig. 1), ejected muscarine (3×10^{-5} mol l^{-1}) first, then observed the increase in A4I1's response, followed by ejection of atropine (5×10^{-4} mol l^{-1} or 5×10^{-3} mol l^{-1}). The lower concentration of atropine caused a reduction of A4I1's response to the control value which corresponds to the A4I1 response after saline ejection, whereas the higher concentration even reduced the response below the control value (approx. 20% inhibition below the control value; Fig. 4d).

Effects of GABA, muscimol, CACA, PTX, and bicuculline

Immunocytochemical studies by Watson and Pflüger (1994) revealed that the axon terminals of filiform hair afferents receive many input synapses which stain with an antibody to GABA. Therefore, the effect of GABA ejection on the A4I1 response elicited by natural wind stimulation was tested. Figure 5a shows that GABA has an inhibitory effect, with a short delay of 18 ms, which is concentration dependent (Fig. 5b).

Both muscimol, an agonist of ionotropic GABA-receptors, and CACA, an agonist of $GABA_c$ receptors, have similar inhibitory effects with a fast onset after ejection (muscimol,19 ms; CACA, 16 ms). The dose-response curves in comparison to that of GABA are shown in Fig. 5b (for muscimol only one individual animal is shown), and reveal the following order of potencies in binding to GABA-receptors: muscimol > GABA > CACA.

A selective blocker of chloride channels is PTX. It was tested, therefore, whether PTX ejection could abolish the inhibitory effects caused by previously ejected GABA, muscimol or CACA. In these experiments the A4I1 response was elicited by natural wind puffs, and the inhibitory substance (GABA, muscimol, or CACA) was ejected first according to stimulus regime 1 (Fig. 1). After the inhibitory effects were observed, PTX was ejected subsequently and the A4I1 response to a natural wind stimulus tested again 1 min after ejection of PTX. Figure 5c shows that PTX removes the

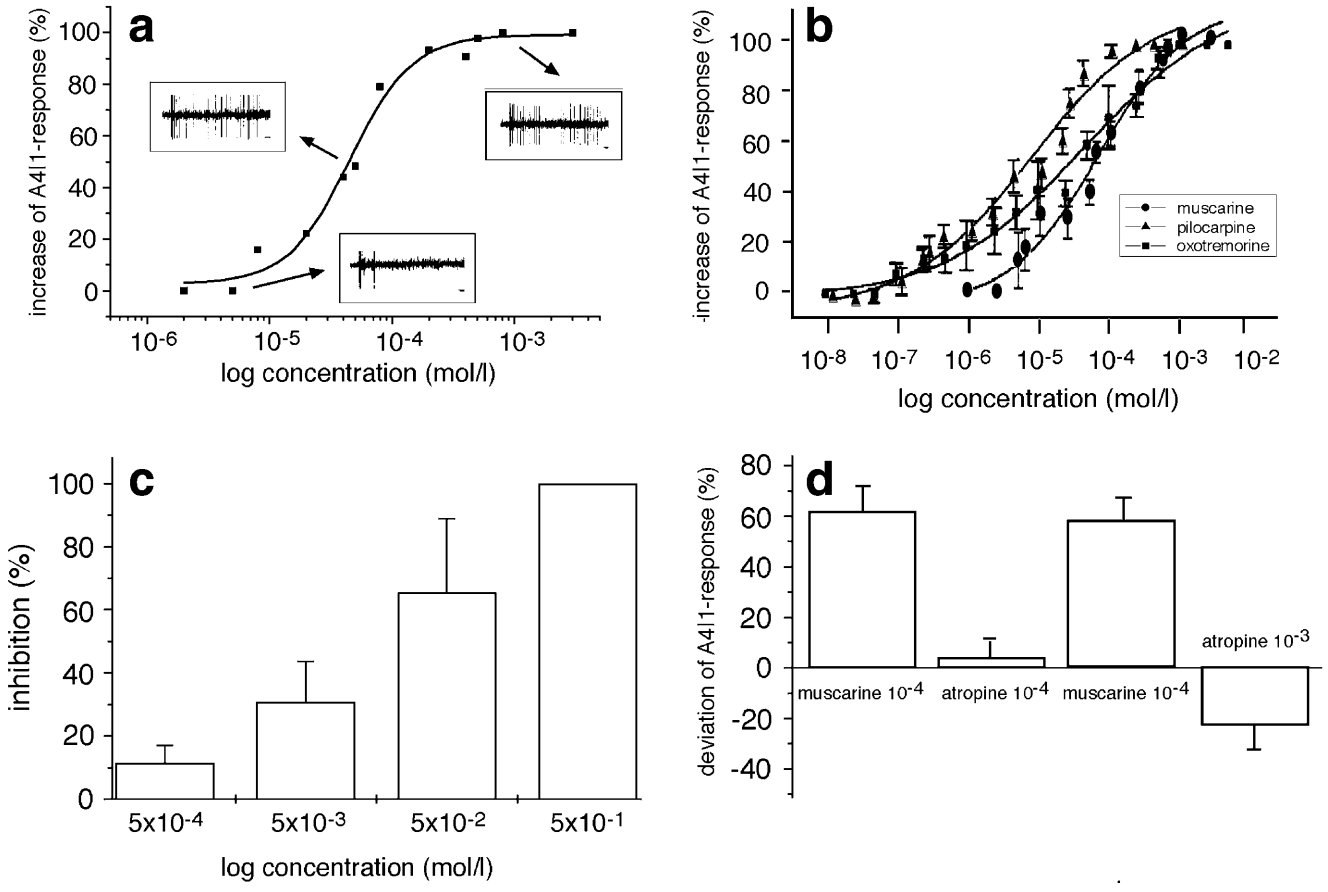


Fig. 4 **a** Increase in A4I1 response, elicited by a 1-s-long wind stimulus of the same intensity, as a result of ejecting muscarine into the neuropile. Dose-response curve of one individual animal. *Insets* show original recordings at concentrations indicated by arrows. **b** A comparison between dose-response curves of the muscarinic ACh receptor (mACh-R) agonists muscarine ($n=6$), pilocarpine ($n=5$) and oxotremorine ($n=5$, mean values and standard deviations). **c** Inhibition of wind-elicited A4I1 responses after ejecting different concentrations of atropine (mean values and standard deviations). **d** Muscarine-mediated increases in A4I1 responses and their inhibition after ejection of atropine into the neuropile (mean values and standard deviations)

inhibitory effects of GABA itself and the agonists muscimol and CACA. This suggests that the ionotropic GABA receptor in this system may be associated with a ligand-gated chloride sensitive ion channel. Bicuculline, an antagonist of the vertebrate $GABA_A$ receptor, however, had no effect (concentration 1×10^{-3} mol l^{-1} to 8×10^{-3} mol l^{-1}).

To test whether inhibition by GABA plays a role in shaping the normal A4I1 response to a wind stimulus, PTX was ejected into the neuropile and A4I1 bursts were elicited by wind puffs (stimulus regime 1, Fig. 1). Figure 5d shows that ejection of PTX into the neuropile causes an increase of the A4I1 response, for example the number of spikes increases. This suggests that presynaptic inhibitory mechanisms, for example lateral inhibition, may be involved in shaping the normal A4I1 response.

Baclofen, a metabotropic GABA-receptor antagonist, had no effect on wind-elicited A4I1 responses. In addition, diazepam, a modulator of ionotropic GABA receptors via allosteric binding, did not have any effects on the inhibition of A4I1 responses mediated by previously ejected GABA.

Effects of glutamate and its agonists

Glutamate immunoreactive profiles have also been identified as presynaptic elements to the axon terminals of prosternal hair afferents in previous immunohistochemical studies (Watson and Pflüger 1994). Therefore, we tested the effects of glutamate ejection. Preliminary studies revealed a fast inhibition of the wind-elicited A4I1 response (Fig. 6a) which was complete after 23 ms in all experiments, and which was also dose dependent (Fig. 6b).

For further characterization of the putative glutamate receptor the following agonists were ejected: (1) kainic acid, as excitatory agonist of ionotropic glutamate receptors, and (2) glycine, as an agonist of the vertebrate *N*-methyl-D-aspartate (NMDA) receptor. Neither had any significant effect on wind-elicited A4I1 responses. Unlike the GABA-mediated inhibition, the inhibitory effect of glutamate was not abolished in the presence of the Cl^- ion channel blocker PTX. Furthermore, ionotropic glutamate receptor antagonists such

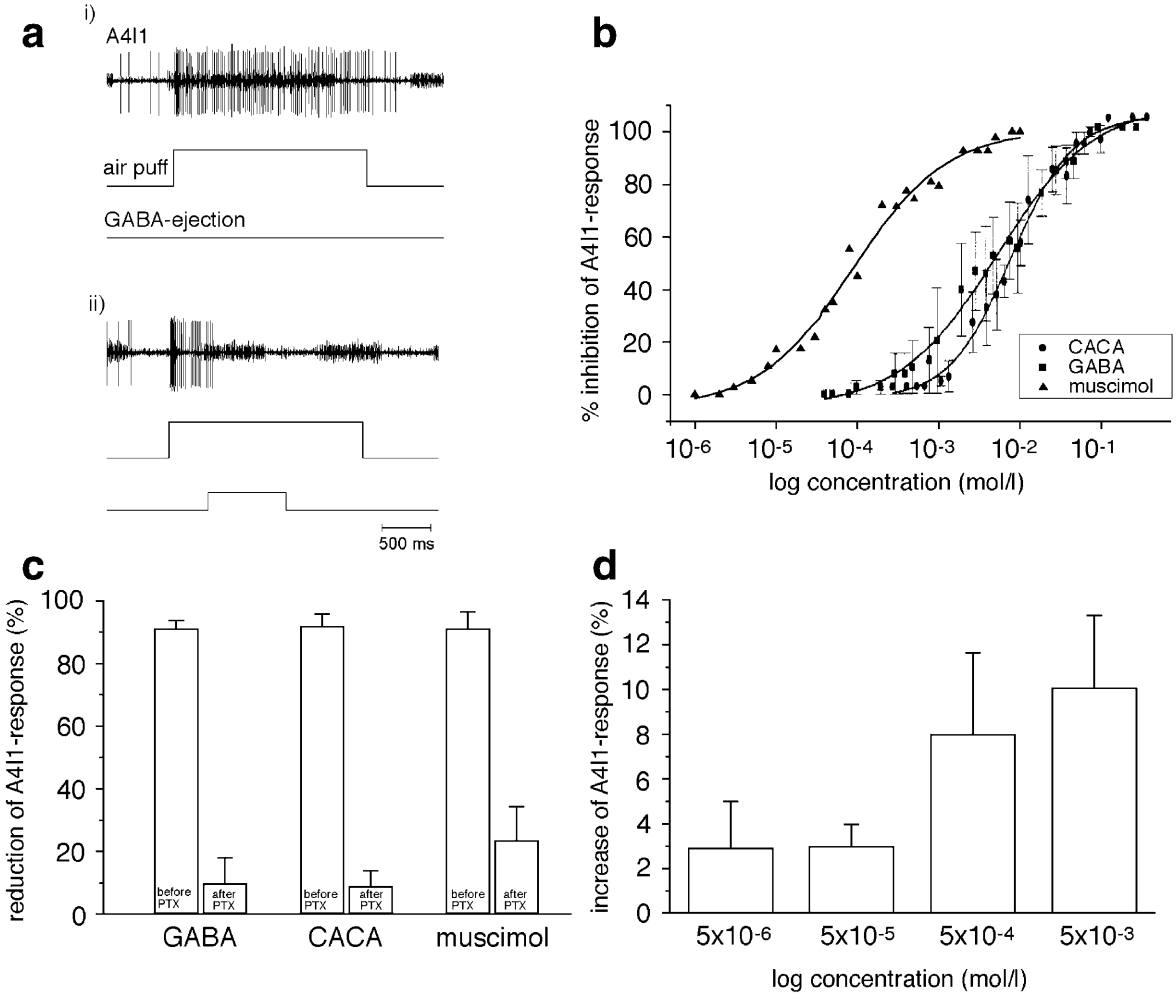


Fig. 5 **a** Inhibition of the wind-elicited A4I1 response (*panel i*) during ejection of GABA into the neuropile (*panel ii*). *Upper traces*: A4I1 response; *middle traces*: wind stimulus (air puff); *lower traces*: indication of GABA ejection. **b** Comparison between dose-response curves (percentage of inhibition versus concentration) of γ-aminobutyric acid (GABA), *cis*-4-amino-crotonic-acid (CACA) and muscimol ($n = 5$, mean values and standard deviations, not shown for muscimol). **c** Effects of picrotoxin (PTX) on GABA-, CACA-, or muscimol-mediated inhibitory effects (reduction of A4I1 responses). **d** Increase of wind-elicited A4I1 responses (100 ms long, repetition rate 0.5 Hz) after ejecting picrotoxin into the neuropile (mean values with standard deviations)

as GDEE and CNQX also failed to block the observed inhibitory glutamate response (Fig. 6c).

Discussion

ACh as transmitter

The excitation of the A4I1 neuron after pressure ejection of ACh and its agonists carbachol and nicotine suggests that this fast response is mediated by nACh-Rs, and hence is similar to fast excitatory responses observed in many other insect (see Heinrich et al. 1997) and crayfish (Ushizawa et al. 1996) cholinergic synapses. Compared to ACh, the effects of the nACh-R agonists carbachol and nicotine are longer lasting. This might be explained by the fast turnover of the natural agonist, ACh, by ACh-esterase.

Further support for the existence of nACh-Rs in this system is that both antagonists of nACh-Rs, *d*-tubocurarine and α-bungarotoxin, block the fast A4I1 response, with α-bungarotoxin being the more potent antagonist. Our results are in agreement with those described for insect nACh-Rs (Breer and Sattelle 1987; Sattelle et al. 1983), and this also holds for the effects of carbachol, a vertebrate agonist for muscarinic ACh-Rs, which acts as an agonist of nACh-Rs in insects (David and Sattelle 1984; Tareilus et al. 1990; Gorczyca et al. 1991). Binding studies on nACh-Rs also reveal that nicotine is more potent than ACh, and that α-bungarotoxin has better binding than *d*-tubocurarine (Breer and Sattelle 1987; Trimmer and Weeks 1989).

In addition to nicotinic ACh-Rs, those of the muscarinic type (mACh-Rs), which are associated with intracellular signaling cascades and therefore have slower modulatory effects, are also found in this and other insect systems (Knipper and Breer 1989; Bai and Sattelle 1994; Heinrich et al. 1997). Muscarine itself, as well as

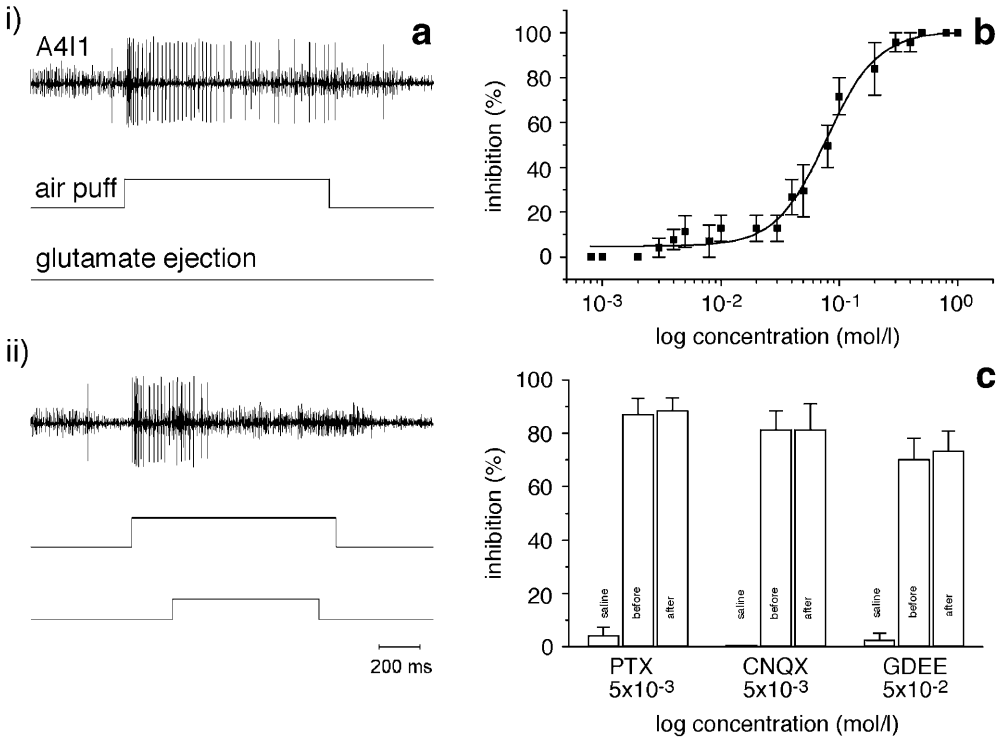


Fig. 6 **a** Inhibition of the wind-elicited A4I1 response (*panel i*) during ejection of glutamate into the neuropile (*panel ii*). *Upper traces*: A4I1 response; *middle traces*: wind stimulus (air puff); *lower traces*: indication of glutamate ejection. **b** Dose-response curve of inhibitory effects of glutamate ($n = 6$, mean and standard deviation). **c** Percentage of glutamate-mediated inhibition before and after ejection of PTX, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and glutamic acid di-ethyl-ester (GDEE). *First columns* show ejections of saline as controls which do not inhibit the A4I1 responses

the mACh-R agonists oxotremorine and pilocarpine have excitatory effects on wind elicited responses of A4I1. The onset of this modulatory response is slow (within 10 s), with muscarine and oxotremorine being significantly faster than pilocarpine. In addition, the modulatory effects are relatively long lasting (up to 3 min), and can be blocked by ejecting the mACh-R antagonist, atropine. Furthermore, there is some evidence that atropine at higher concentrations can also interact with the nACh-R in insects. The normal A4I1 response is inhibited in a dose-dependent way if atropine is ejected into the respective neuropile. This is in agreement with other studies on insects (Sattelle 1980; David and Sattelle 1984; Trimmer and Weeks 1989). ACh-Rs of the muscarinic type have been described in cockroaches, flies, bees and locusts (Bai and Sattelle 1994). Studies on a postsynaptic mACh-R on a giant interneuron in the cockroach show that ejection of muscarine leads to a slow depolarization lasting for over 10 s which can be blocked by subsequent atropine ejection (Le Corronc and Hue 1993). Similarly, in grasshoppers the stridulatory behavior released by muscarine has a longer latency and a longer duration (Heinrich et al. 1997). In our system, ejection of the mACh-agonists, muscarine, oxotremorine and pilocarpine, also leads to an increase in the wind elicited A4I1 response. We could not determine whether the mACh-Rs are situated postsynaptically, on the dendrites of the A4I1 interneurone, or presynaptically on the terminals of the sensory afferents, but both sites have been localized in other systems (Trimmer and Weeks 1989, 1993; Parker and Newland 1995).

GABA as an inhibitor of the A4I1 response

Ultrastructural studies by Watson and Pflüger (1994) show that the terminals of the afferent fibers receive input synapses that are immunoreactive against GABA. This may suggest inhibitory actions, for example autoregulation, between sensory receptors similar to those described for other mechanosensory systems in insects (Burrows and Laurent 1993). Therefore, we tested the action of GABA and some of its receptor agonists in this system. GABA itself, the $GABA_A$-receptor agonist muscimol, and the $GABA_C$-receptor agonist CACA, have fast inhibitory actions, suggesting that it is mediated by a ligand-gated ionotropic GABA receptor. Baclofen, a $GABA_B$-receptor agonist, has no effect over 5 min, suggesting that only ionotropic and no metabotropic GABA receptors are involved in this system. Similar results were obtained in *Manduca sexta* (Waldrop 1994). Both muscimol and CACA cause inhibition of locust muscle fibers (Scott and Duce 1987; Murphy and Wann 1988).

Interestingly, in *Manduca* a specific antagonist of the $GABA_A$ receptor, bicuculline, was ineffective in most experiments, although in some experiments a reduction of the inhibitory action was noticed (Waldrop 1994). Similar results were obtained for a giant interneuron in cockroaches (Buckingham et al. 1994), which suggests an effect of bicuculline on cholinergic but not on GABA-receptors. This has been confirmed in studies on isolated unidentified locust neurons (Benson 1988). As in other studies (Anthony et al. 1993) we could not detect any effect when ejecting bicuculline into the neuropile. In

contrast to other studies in insects, diazepam (as a benzodiazepine agonist) has no modulatory effect on the inhibition mediated by GABA ejection (Robinson et al. 1986; Ozoe et al. 1989). In support of the existence of an ionotropic GABA receptor are the results on the ejection of PTX, a blocker of chloride channels. PTX effectively blocks the inhibition mediated by GABA, muscimol or CACA. Similar results were obtained in cockroach motor neurons where PTX blocks any inhibitory responses caused by GABA ejection (David and Sattelle 1984).

Glutamate has inhibitory action

In addition to GABAergic profiles, the ultrastructural studies also revealed a similar number of fibers presynaptic to the afferent terminals which stained with a glutamate antibody (Watson and Pflüger 1994). Glutamate is known to be the principal excitatory transmitter of peripheral neuromuscular synapses in insects (Usherwood 1967), and is also an excitatory transmitter of the insect central nervous system (Sombati and Hoyle 1984; Parker 1994). In contrast to this, glutamate had exclusive inhibitory effects on the A4I1 response. This is in agreement with previous studies by Dubas (1990, 1991) which revealed a dual action of glutamate within the CNS of insects because ejection of glutamate into locust neuropiles either excited or inhibited flight motor neurons. Perhaps a direct inhibitory effect of glutamate is due to the activation of K^+-ion channels as in molluscs (King and Carpenter 1989; Kehoe 1994), or due to recently identified glutamate-gated Cl^- channels in *Drosophila* (Cully et al. 1996). Although rendered unlikely due to its fast action within 23 ms, which is in a similar range than the action of GABA, we cannot exclude the possibility that glutamate may activate inhibitory neurons first which then block the A4I1 response. In the filiform hair-A4I1 system, ejection of kainic acid, an agonist of AMPA/kainate receptors, had no effect, thus suggesting a different receptor identity than the corresponding vertebrate type. Furthermore, a potentiating effect on glutamate action by additional ejection of glycine, which would be expected in the case of a NMDA type of glutamate receptor, was not observed. Ejections of antagonists such as CNQX or GDEE also had no effects. Therefore, the identity of the type of glutamate receptor present in this system remains unclear, although it is known that glutamate receptors of both the AMPA/kainate and NMDA type exist in insects (Castle et al. 1984), and recently genes of novel glutamate receptor subunits have been characterized in *Drosophila* (Völkner et al. 2000).

Functional significance

This system is one of the few in which the behavioral significance of some substances acting on identified synaptic connections can be tested. The extent to which the efficacy of a mechanosensory synapse can be modified is surprising, and the full implication of this is still not fully understood. The presence of mACh-Rs suggests long-lasting modulation, e.g., an increase of the efficacy of synaptic transmission. Both GABA and glutamate revealed their inhibitory action, although we cannot distinguish between pre- or postsynaptic effects in our experiments. That such inhibitory interactions play a role in normal synaptic transmission is supported by the effects of PTX ejection which increase wind-elicited A4I1 responses. Thus, it is very likely that there exist inhibitory interactions between sensory receptors of filiform hair receptors, perhaps similar to the automatic gain control mechanism of chordotonal afferents (Burrows and Laurent 1993). Glutamate in this system has an exclusively inhibitory action, suggesting further modulatory capacity, perhaps with respect to the duration of inhibition, or with respect to different inhibitory pathways used in different behaviors. Unfortunately, all attempts to further classify the glutamate receptors involved in this system failed. Inhibitory interactions may play a prominent role in this system: (1) the filiform hair receptors and the A4I1 interneuron express an enormous sensitivity to wind stimuli of very low intensity (Pflüger and Tautz 1982), and (2) the convergence of many of such sensitive receptors onto one postsynaptic neuron requires special mechanisms to prevent saturation of the interneuron, or permanent function at its upper limit.

Acknowledgements The support of the DFG (Forschergruppe "Lernen, Gedächtnis und Neuromodulation bei Arthropoden", S. G., and currently SFB 515, project B6, H.-J.P.) is gratefully acknowledged. We also thank Paul Stevenson, Leipzig, for numerous discussions, Peter Kloppenburg, Ithaca, Richard B. Levine, Tucson, and Gerd Bicker, Hannover, for critically reading the manuscript, and Markus Miertschink and Dr. Rüdiger Gauglitz for help with data analysis and programming. We also thank the two anonymous referees for their helpful comments.

References

Anthony NM, Harrison JB, Sattelle DB (1993) GABA receptor molecules of insects. In: Pichon Y (ed) Comparative molecular neurobiology. Birkhäuser, Basel, pp 172–209

Bai D, Sattelle DB (1994) Muscarinic acetylcholine receptors on an identified motor neurone in the cockroach, *Periplaneta americana*. Neurosci Lett 175:161–165

Baines RA, Bacon JP (1994) Pharmacological analysis of the cholinergic input to the locust VPLI neuron from an extraocular photoreceptor system. J Neurophysiol 72:2864–2874

Benson JA (1988) Bicuculline blocks the response to acetylcholine and nicotine but not to muscarine or GABA in isolated insect neuronal somata. Brain Res 458:45–71

Bicker G (1999) Histochemistry of classical neurotransmitters in antennal lobes and mushroom bodies of the honeybee. Microsc Res Tech 45:174–183

Blagburn M, Sattelle DB (1987) Presynaptic depolarisation mediates presynaptic inhibition at a synapse between an identified mechanosensory neurone and giant interneurone 3 in the first instar cockroach *Periplaneta americana*. J Exp Biol 127:135–157

Blake AD, Anthony NM, Chen HH, Harrison JB, Nathanson NM, Sattelle DB (1993) Drosophila nervous system muscarinic acet-

ylcholine receptor: transient functional expression and localization by immunocytochemistry. Mol Pharmacol 44:716–724
Breer H, Sattelle DB (1987) Molecular properties and functions of insect acetylcholine receptor. J Insect Physiol 33:771–790
Brotz TM, Borst A (1996) Cholinergic and GABAergic receptors on fly tangential cells and their role in visual motion detection. J Neurophysiol 76:1786–1799
Buchner E, Buchner S, Burg MG, Hofbauer A, Pak WL, Pollack I (1993) Histamine is a major mechanosensory transmitter in *Drosophila melanogaster*. Cell Tissue Res 273:119–125
Buckingham SD, Hue B, Sattelle DB (1994) Actions of bicuculline on cell body and neuropilar membranes of identified insect neurones. J Exp Biol 186:235–244
Burrows M (1996) The neurobiology of an insect brain. Oxford University Press, Oxford
Burrows M, Laurent G (1993) Synaptic potentials in the central terminals of locust proprioceptive afferents generated by other afferents of the same sense organ. J Neurophysiol 13:808–819
Burrows M, Pflüger H-J (1992) Output connections of a wind-sensitive interneurone with motor neurones innervating flight steering muscles in the locust. J Comp Physiol A 171:437–446
Callec JJ, Guillet JC, Pichon Y, Boistel J (1971) Further studies on synaptic transmission in insects. II. Relations between sensory information and its synaptic integration at the level of a single giant axon in the cockroach. J Exp Biol 55:123–149
Casagrand JL, Ritzmann RE (1992) Evidence that synaptic transmission between giant interneurons and identified thoracic interneurons in the cockroach is cholinergic. J Neurobiol 23: 627–643
Castle F, Evans RH, Kirkpatrick JNP (1984) A comparison of the effect of kainate and some related amino acids on locomotor activity in cockroaches and electrical activity recorded from locust ventral nerve cord. Comp Biochem Physiol 77C:399–402
Cayre M, Buckingham SD, Yagodin S, Sattelle DB (1999) Cultured insect mushroom body neurons express functional receptors for acetylcholine, GABA, glutamate, octopamine, and dopamine. J Neurophysiol 81:1–14
Clements AN, May TE (1974) Studies on locust neuromuscular physiology in relation to glutamic acid. J Exp Biol 60:673–705
Cully DF, Paress PS, Liu KK, Schaeffer JM, Arena JP (1996) Identification of a *Drosophila melanogaster* glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. J Biol Chem 271:20187–20191
David JA, Sattelle DB (1984) Actions of cholinergic pharmacological agents on the cell body membrane of the fast coxal depressor motoneurone of the cockroach (*Periplaneta americana*). J Exp Biol 108:119–136
Dubas F (1990) Inhibitory effect of L-glutamate on the neuropile arborisation of flight motoneurones in locusts. J Exp Biol 148:501–508
Dubas F (1991) Actions of putative amino-acid transmitters on the neuropile arborisations of locust flight motoneurones. J Exp Biol 155:337–356
Ferber M, Hörner M, Cepok S, Gnatzy WJ (2001) Digger wasp versus cricket: mechanisms underlying the total paralysis caused by the predator's venom. J Neurobiol 47:207–222
Gorczyca MG, Hall JC (1984) Identification of a cholinergic synapse in the giant fiber pathway of *Drosophila* using conditional mutations of acetylcholine synthesis. J Neurogenet 1:289–313
Gorczyca MG, Budnik V, White K, Wu CF (1991) Dual muscarinic and nicotinic action on a motor program in *Drosophila*. J Neurobiol 22:391–404
Grolleau F, Lapied B, Buckingham SD, Mason WT, Sattelle DB (1996) Nicotine increases $(Ca^{2+})i$ and regulates electrical activity in insect neurosecretory cells (DUM neurons) via an acetylcholine receptor with 'mixed' nicotinic-muscarinic pharmacology. Neurosci Lett. 220:142–146
Heinrich R, Hedwig B, Elsner N (1997) Cholinergic activation of stridulatory behavior in the grasshopper *Omocestus viridulus* (L). J Exp Biol 200:1327–1337
Homberg U, Hoskins SG, Hildebrand JG (1995) Distribution of acetylcholinesterase activity in the deutocerebrum of the sphinx moth *Manduca sexta*. Cell Tissue Res 279:249–259
Kehoe J (1994) Glutamate activates K^+ conductance increase in *Aplysia* neurons that appears to be independent of G-proteins. Neuron 13:691–702
King WM, Carpenter DO (1989) Voltage-clamp characterization of Cl^- conductance gated by GABA and L-glutamate in single neurons of *Aplysia*. J Neurophysiol 61:892–899
Knipper M, Breer H (1989) Subtypes of muscarinic receptors in insect nervous system. Comp Biochem Physiol 90C:275–280
Le Corronc H, Hue B (1993) Pharmacological and electrophysiological characterization of a postsynaptic muscarinic receptor in the central nervous system of the cockroach. J Exp Biol 181:257–278
Lee D, O'Dowd DK (1999) Fast excitatory synaptic transmission mediated by nicotinic acetylcholine receptors in *Drosophila* neurons. J Neurosci 19:5311–5321
Leitch B, Pitman RM (1995), Modulation of transmitter release from the terminals of the locust wing stretch receptor neuron by muscarinic antagonists. J Neurobiol 28:455–464
Leitch B, Watkins BL, Burrows M (1993) Distribution of acetylcholine receptors in the central nervous system of adult locusts. J Comp Neurol 334:47–58
Leitinger G, Simmons PJ (2000) Cytochemical evidence that acetylcholine is a neurotransmitter of neurons that make excitatory and inhibitory outputs in the locust ocellar visual system. J Comp Neurol 416:345–355
Lutz EM, Tyrer NM (1987) Immunohistochemical localization of choline acetyltransferase in the central nervous system of the locust. Brain Res 407:173–179
Lutz EM, Tyrer NM (1988) Immunohistochemical localization of serotonin and choline acetyltransferase in sensory neurones of the locust. J Comp Neurol 267:335–342
Meyer MR, Reddy GR (1985) Muscarinic and nicotinic cholinergic binding sites in the terminal abdominal ganglion of the cricket (*Acheta domesticus*). J Neurochem 45:1101–1112
Murphy VF, Wann KT (1988) The action of GABA receptor agonists and antagonists on muscle membrane conductance in *Schistocerca gregaria*. Br J Pharmacol 95:713–722
Oleskevich S (1999) Cholinergic synaptic transmission in insect mushroom bodies in vitro. J Neurophysiol 82:1091–1096
Osborne RH (1996) Insect neurotransmission: neurotransmitters and their receptors. Pharmacol Ther 69:117–142
Ozoe Y, Fukuda K, Mochida K, Nakamura T (1989) Actions of benzodiazepines on the housefly. 3. In vitro binding of [3H]Ro5-4864 responding to GABA receptor ligands. Comp Biochem Physiol 93C:193–199
Parker D (1994) Glutamatergic transmission between antagonistic motor neurones in the locust. J Comp Physiol A 175:737–748
Parker D, Newland PL (1995) Cholinergic synaptic transmission between proprioceptive afferents and a hind leg motor neurone in the locust. J Neurophysiol 73:586–594
Pflüger H-J (1984) The large fourth abdominal intersegmental interneurone: a new type of wind-sensitive ventral cord interneuron in locusts. J Comp Neurol 222:343–357
Pflüger H-J, Burrows M (1990) Synaptic connections of different strength between wind-sensitive hairs and an identified projection interneuron in the locust. Eur J Neurosci 2:1040–1050
Pflüger H-J, Tautz J (1982) Air movement sensitive hairs and interneurons *in Locusta migratoria*. J Comp Physiol A 145:369–380
Robinson T, MacAllan D, Lunt GG, Battersby M (1986) γ-Aminobutyric acid receptor complex of insect CNS: characterisation of a benzodiazepine binding site. J Neurochem 47: 1955–1962
Sattelle DB (1980) Acetylcholine receptors of insects. Adv Insect Physiol 15:215–315
Sattelle DB (1985) Acetylcholine receptors. In: Kerkut GA, Gilbert GI (eds) Comprehensive insect physiology, biochemi-

stry and pharmacology, vol 11. Pergamon Press, New York, pp 395–434
Sattelle DB, Harrow ID, Hue B, Pelhate M, Gepner JI, Hall LM (1983) α-Bungarotoxin blocks excitatory synaptic transmission between cercal sensory neurones and giant interneurone 2 of the cockroach *Periplaneta americana*. J Exp Biol 107:473–489
Sattelle DB, Madler U, Heilgenberg H, Breer H (1989) Immunocytochemical localization of nicotinic acetylcholine receptors in the terminal abdominal ganglion of the cockroach (*Periplaneta americana*). Proc R Soc Lond Ser B 238:189–192
Scott RH, Duce IR (1987) Pharmacology of GABA receptors on skeletal muscle fibers of the locust (*Schistocerca gregaria*). Comp Biochem Physiol 86C:305–311
Shankland DL, Rose JA, Donniger C (1971) The cholinergic nature of the cercal nerve-giant fiber synapse in the sixth abdominal ganglion of the American cockroach, *Periplaneta americana* (L.). J Neurobiol 3:247–262
Sombati S, Hoyle G (1984) Glutamatergic central nervous transmission in locusts. J Neurobiol 15:507–516
Tareilus E, Hanke W, Breer H (1990) Neuronal acetylcholine receptor channels from insects: a comparative electrophysiological study. J Comp Physiol A 167:521–526
Trimmer BA, Weeks JC (1989) Effects of nicotinic and muscarinic agents on an identified motoneurone and its direct afferent inputs in larval *Manduca sexta*. J Exp Biol 144:303–377
Trimmer BA, Weeks JC (1993) Muscarinic acetylcholine receptors modulate the excitability of an identified insect motoneuron. J Neurophysiol 69:1821–1836
Usherwood PNR (1967) Insect neuromuscular mechanisms. Am Zool 7:553–582
Ushizawa T, Nagayama T, Takahata M (1996) Cholinergic transmission at mechanosensory afferents in the crayfish terminal abdominal ganglion. J Comp Physiol A 179:1–13
Vieillemaringe J, Souan ML, Grandier-Vazeilles X, Geffard M (1987) Immunocytochemical localization of acetylcholine receptors in locust brain using auto-anti-idiotypic acetylcholine antibodies. Neurosci Lett 79:59–64
Völkner M, Lenz-Böhme B, Betz H, Schmitt B (2000) Novel CNS glutamate receptor subunit genes of *Drosophila melanogaster*. J Neurochem 75:1791–1799
Waldrop B (1994) Physiological and pharmacological properties of responses to GABA and ACh by abdominal motor neurons in *Manduca sexta*. J Comp Physiol A 775–785
Watson AHD, Pflüger H-J (1984) The ultrastructure of prosternal sensory hair afferents within the locust central nervous system. Neuroscience 11:269–279
Watson AHD, Pflüger H-J (1994) Distribution of input synapses from processes exhibiting GABA- or glutamate-like immunoreactivity onto terminals of prosternal filiform afferents in the locust. J Comp Neurol 343:617–629
Yasuyama K, Salvaterra PM (1999) Localization of choline acetyltransferase-expressing neurons in *Drosophila* nervous system. Microsc Res Tech 45:65–79
Yasuyama K, Kitamoto T, Salvaterra PM (1995) Localization of choline acetyltransferase-expressing neurons in the larval visual system of *Drosophila melanogaster*. Cell Tissue Res 282:193–202