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CHOLINERGIC ACTIVATION OF STRIDULATORY BEHAVIOUR IN THE GRASSHOPPER *OMOCESTUS VIRIDULUS* (L.)

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Summary

When acetylcholine (ACh) and its agonists are injected into neuropile regions of the protocerebrum and the suboesophageal ganglion of male and female grasshoppers of the species *Omocestus viridulus* (L.), they elicit stridulation in a pattern no different from that of natural song. Stridulation can even be evoked in mated females which normally do not sing. By choosing suitable ACh agonists, nicotinic and muscarinic ACh receptors can be activated selectively. Activation of nicotinic ACh receptors produces individual song sequences with rapid onset; the stridulation induced by activation of the muscarinic ACh receptors begins after a longer latency, increases slowly in intensity and is maintained for many minutes. The sites within the cephalic ganglia where song can be initiated pharmacologically coincide with regions in which descending stridulatory command neurones arborize.

Key words: grasshopper, *Omocestus viridulus*, stridulation, brain, neuropharmacology, acetylcholine.

Introduction

An understanding of the processes within the central nervous system (CNS) that control stridulatory behaviour in orthopterans has long been sought by neuroethologists (reviewed by Elsner, 1994). The use of classic neurobiological methods, such as electrical stimulation of the brain and histological analysis of the stimulated neuropile regions (reviewed by Huber, 1964), has since been complemented by recordings from single, behaviourally relevant brain neurones that can be stained for subsequent histological identification (Hedwig, 1994, 1995). More recently, techniques of pharmacological stimulation have also been adopted to study the neuronal control of stridulation in gomphocerine grasshoppers, after it was shown that the application of neuroactive substances in various invertebrates initiates simple motor activities or the underlying patterns of central nervous excitation. For example, this has now been demonstrated for insect flight (Sombati and Hoyle, 1984; Claassen and Kammer, 1986; Stevenson and Kutsch, 1986), for swimming in leeches (Willard, 1981) and for walking in grasshoppers (Ryckebusch and Laurent, 1993; Büschges *et al.* 1995). A role for acetylcholine agonists was shown by Otto (1978), who found that stridulation could be induced by injecting them into crickets.

Ocker *et al.* (1995) were the first to induce behavioural responses in gomphocerine grasshoppers by pharmacological stimulation of the brain. They injected acetylcholine (ACh) into central neuropile regions in the protocerebrum of male *Omocestus viridulus* and *Chorthippus mollis*, as a result of which the animals performed song sequences. However, these experiments left unanswered the question of whether the cephalic system for the control of stridulation is activated by nicotinic or muscarinic receptors. It is also conceivable that both are involved, since nicotinic and muscarinic acetylcholine receptors have been pharmacologically identified in the central nervous system of insects (Breer and Sattelle, 1987; Gundelfinger, 1992; Hannan and Hall, 1993).

Nicotinic receptors are present at high density throughout the insect CNS, as seen in cockroaches (Bai *et al.* 1992), in grasshoppers (Goodman and Spitzer, 1980) and in flies (Lane *et al.* 1982), and participate in rapid synaptic transmission. The muscarinic ACh receptors are approximately one-tenth as numerous and tend to be concentrated on somata and in distinct neuropile regions, as shown in cockroaches (Bai and Satelle, 1994; LeCorronc and Hue, 1993), in grasshoppers (Knipper and Breer, 1988) and in flies (Gorczyca *et al.* 1991). They are coupled to intracellular signalling pathways by way of G-proteins and can have a modulatory action on synaptic transmission mechanisms (Bai *et al.* 1992; LeCorronc and Hue, 1993; Parker and Newland, 1995).

The aim of the present study was to activate the two cholinergic receptor types in the cephalic control system for grasshopper stridulation *selectively* so that their individual contributions to stridulatory behaviour with respect to latency, duration or song type could be analysed. Another question of interest was whether singing could be elicited pharmacologically in females as well as in males, since the

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hormonal control of stridulation in the two sexes is known to be different. Unlike males, the females sing only in the virginal state or after having been separated from males for at least 3 weeks. Furthermore, singing activity depends on the egg-deposition cycle (Loher, 1966; Loher and Huber, 1966; Hartmann *et al.* 1994).

Each of these problems was approached experimentally by injecting various cholinergic agonists into the cephalic ganglia of males and females of the gomphocerine grasshopper species *Omocestus viridulus* (L).

Materials and methods

Animals

Omocestus viridulus (L.) were caught as imagines or subadult larvae in the vicinity of Göttingen. Males and females were kept separately in the laboratory for a maximum of 3 weeks.

Preparation

For the pharmacological stimulation experiments, the grasshoppers were waxed to a holder by the pronotum and the head was fixed to the thorax to prevent movements relative to one another. The frontal part of the head capsule was opened with a splinter of razor blade to expose the ventral surface of the brain. The rest of the animal was kept intact and it was able to move all its appendages freely, particularly its hindlegs. For injections into the suboesophageal ganglion, the mouthparts also had to be removed to allow access to the ganglion.

Injection of drugs

The neuroactive substances (obtained from Sigma-Aldrich) were usually dissolved in grasshopper saline (Clements and May, 1974) to give concentrations of 10^{-4} mol l^{-1}. These were injected into central nervous neuropile regions through glass microelectrodes using a pressure-injection device (WPI, model 820). Before the experiment, the ends of the electrodes were broken under visual control to produce a tip diameter of approximately 10–15 μm. The pressure and pulse duration delivered by the apparatus were adjusted so that approximately 1 nl of the substance was applied per injection; this was confirmed by measuring the volume of a droplet injected into Vaseline. Control experiments with saline or with specific antagonists and the marking of the injection site with fluorescent latex particles (Molecular Probes, type L-5081, diameter 1 μm) were performed using double-barrelled electrodes. With these electrodes, various substances could be applied to the tissue at the same site, simultaneously or sequentially. All experiments were carried out at a temperature of 25–28 °C.

Histology

The injection sites were visualized by fixing the cephalic ganglia of the experimental animals and of some control animals with paraformaldehyde. After dehydration, these were embedded in polyester wax (1 g of 1-hexadecanol plus 99 g of polyethyleneglycol-400 distearate). Histological sections 12 μm thick were prepared using a microtome (Reichert Jung, Biocut 1130). The tissue of the control animals was stained with haematoxilin. To preserve the fluorescence of the latex particles, these sections were enclosed in Karion F (Merck) after they had been stained with Methylene Blue. The preparations were examined under a microscope (Leitz Dialux 20) and photographed.

Recording the stridulatory movements

The stridulatory movements of the grasshoppers were recorded by glueing a piece of reflecting foil (Scotchlite 3M, type 7610) approximately 2 mm in diameter to the distal part of the femur of each of the two hindlegs and monitoring the up-and-down movements of these using opto-electronic cameras equipped with position-sensitive photodiodes (von Helversen and Elsner, 1977). One camera monitored the left hindleg and another the right. Each measurement system provided a voltage signal proportional to the leg movement. In addition to the recordings during the pharmacological experiments, natural stridulatory movements were recorded while intact animals were moving freely in an arena.

Data processing

The signals recorded on magnetic tape with an AM/FM recorder (Racal Store 4 DS) were A/D-converted (Data Translation DT 2821 F-8DI) and stored as data files. The sampling rate for recording the stridulatory movements and the injection pulses was 4 kHz. Subsequent evaluation and construction of histograms were accomplished using the software package Neurolab (Hedwig and Knepper, 1992).

Results

A summary of the number of experiments carried out, the pharmacological agents used and the main features of the behavioural responses observed is given in Table 1.

Pharmacological and local specificity of song induction

Control experiments with saline

Acetylcholine and its agonists did not induce stridulatory behaviour when applied by way of the surrounding haemolymph. To be effective, they had to be injected into selected regions of the neuropile of cephalic ganglia containing the arborizations of stridulatory command neurones (Fig. 1A,B). This raised the possibility that the cause of stridulation was a mechanical stimulation of command fibres by the pressure pulse rather than a pharmacological action. It is well known that song can be initiated by stimulating the orthopteran brain mechanically with fine needles (Huber, 1952, 1955*a*,*b*). Indeed, in our experiments, brief stridulation was occasionally observed when the drug-filled microelectrode first penetrated the brain or its position was changed. To examine this possibility, control experiments using saline injections were carried out using double-barrelled microelectrodes. A site in the central

Table 1. *A summary of the effects of pharmacological agents in eliciting stridulation in the grasshopper* Omocestus viridulus

Omocestus viridulus	Substance injected	Number of animals/ experiments	Delay of onset (s)	Maximal duration of stridulation	Behavioural performance
Male	Saline	6/25	–	–	1
	Acetylcholine	12/40	0.25–0.60	50 s	2, 6
	Nicotine	5/18	0.24–0.54	1.5 min	3, 6
	Muscarine	8/23	3.60–6.10	2.5 min	4, 7, 8
	Pilocarpine	5/12	3.00–6.20	60 min	5, 7, 8
Female	Acetylcholine	2/9	0.60–1.60	20 s	2, 6
	Nicotine	3/12	0.60–1.40	30 s	3, 6
	Muscarine	3/16	5.30–7.10	2 min	4, 7, 8

(1) No stridulatory movements.
(2) One song sequences of natural duration per stimulus.
(3) One or two song sequences of natural duration per stimulus.
(4) Up to three song sequences of natural duration per stimulus.
(5) Up to 90 song sequences of natural duration per stimulus.
(6) Natural increase in movement amplitude at the beginning of each sequence.
(7) Slow increase in movement amplitude at the beginning of the first sequences.
(8) Song sequences preceded by a short series of stridulatory movements of increasing duration.

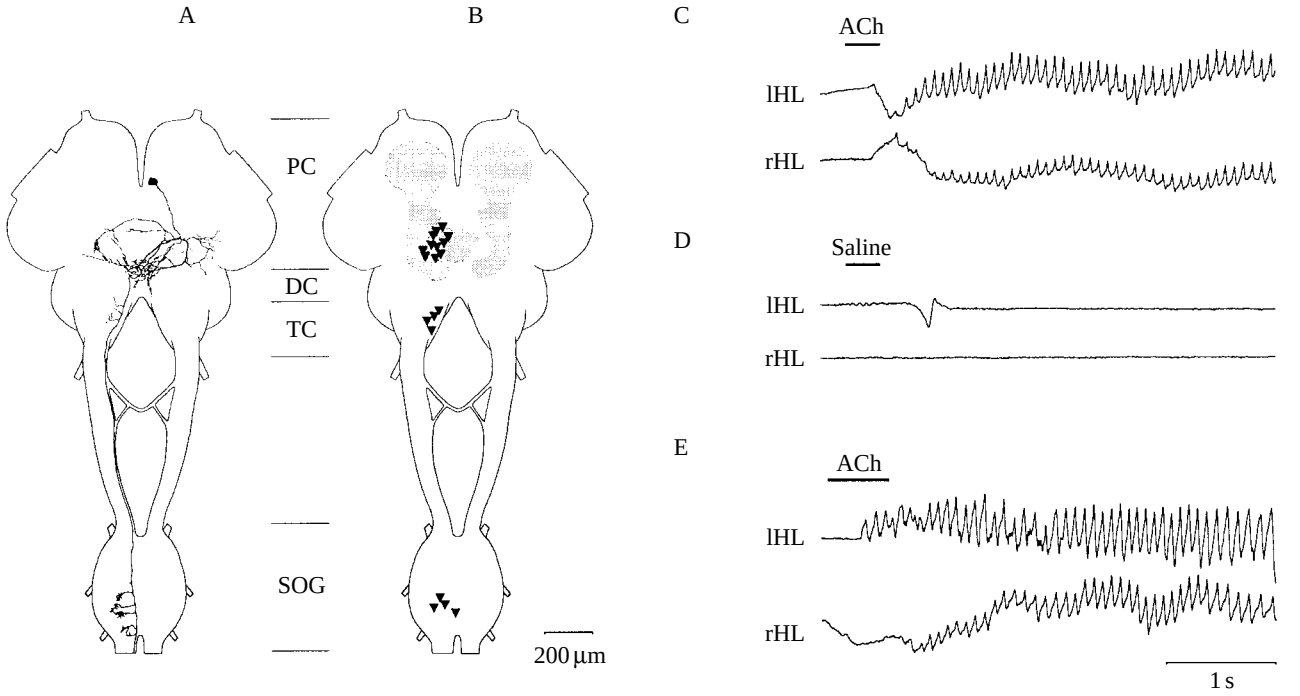

Fig. 1. (A) Structure of a stridulatory command neurone of *Omocestus viridulus* within the brain and suboesophageal ganglion (SOG). (B) Locations within the central nervous system where stridulatory behaviour could be elicited by microinjection of cholinergic agonists. The positions of the central body complex and mushroom bodies are shaded. PC, protocerebrum; DC, deutocerebrum; TC, tritocerebrum. (C) Microinjection of 10^{-4} mol l^{-1} acetylcholine (ACh) into the protocerebrum elicits regular stridulatory movements. (D) Microinjection of saline solution at the same site as in C has no effect. (E) Microinjection of 10^{-4} mol l^{-1} ACh into the posterior part of the SOG elicits stridulation. lHL and rHL, stridulatory up-and-down movements of the left and the right hindleg.

neuropile of the protocerebrum was found where the injection of acetylcholine through one barrel of the electrode reliably and repeatedly elicited species-specific stridulatory movements (Fig. 1C). Saline was then injected at the same site through the second barrel using an identical pressure pulse. In most cases, there was no discernible reaction; when a response was observed, it was no more than a single brief twitch of one hindleg (e.g. Fig. 1D). Hence, it is unlikely that the pressure pulses alone triggered stridulation by mechanically stimulating the neuropile.

Application of drugs in the supraoesophageal ganglion

Acetycholine and ACh agonists were only effective in inducing stridulation when injected into particular areas. The response was most reliably obtained by injection into the central neuropile of the protocerebrum (Fig. 1B,C). Even a slight change of position of the electrode in the neuropile resulted in the stimulation eliciting either no response or merely a short period of unspecific, uncoordinated leg or antennal movements.

In 10 animals, the injection site that elicited song was localized very precisely in the region of the medial protocerebrum. The large diameter of the electrode tip made it possible to mark the position of the tip by ejecting fluorescent latex particles from the electrode along with the drug. In the subsequent histological examination of transverse sections, the brain areas in which stridulation was particularly reliably elicited proved to be concentrated posterior and dorsal to the central body and at its ventral and lateral boundary. The command fibres for stridulation identified by Hedwig (1994, 1995) pass through this region (Fig. 1A,B).

In several preparations, stridulation was also induced by injecting acetylcholine into medio-dorsal regions of the tritocerebrum where, as shown in Fig. 1B, the descending command neurones also send collaterals into this neuropile region.

Application of drugs in the suboesophageal ganglion

Injection of acetylcholine into the posterior region of the suboesophageal ganglion, but not into other areas, elicited audible sequences of 'ordinary' stridulation (Fig. 1E) lasting approximately 10–15 s. This region contains arborizations of the command fibres descending from the brain and neurones of the suboesophageal ganglion that pass to the metathoracic ganglion, where they have an excitatory influence on the pattern generators (Hedwig, 1986; Lins and Elsner, 1995*a*,*b*). These suboesophageal ganglion sites differed from those in the brain (see below) in that stimulation here induced only the movement pattern of ordinary stridulation (calling song and the main part of the courtship song) and not the hindleg shaking or the subsequent precopulatory movements that occur at the end of courtship (see below).

Pharmacological stimulation in the posterior region of the suboesophageal ganglion induced hindleg stridulatory movements even after transection of the circumoesophageal connectives between the brain and the suboesophageal ganglion.

Stridulation elicited pharmacologically in males by injection of ACh and ACh agonists

Natural stridulation

To evaluate the pharmacologically elicited song patterns, a few remarks about the natural songs of *O. viridulus* are needed. The ordinary stridulation of male *O. viridulus* consists of sequences of approximately 10–30 s in duration (Fig. 2A), separated from one another by pauses lasting approximately 15–20 s. Each sequence is subdivided into subunits

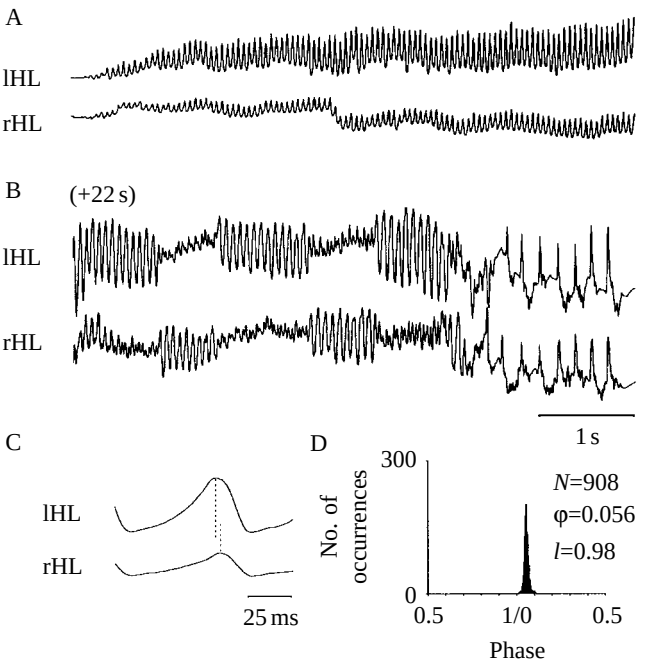

Fig. 2. (A,B) A sequence of natural stridulation of a male *Omocestus viridulus*. There are three different movement patterns which are produced sequentially in a complete courtship sequence: ordinary stridulation (A), hindleg shaking (B, left) and precopulatory movements (B, right). (C) One movement cycle of ordinary stridulation at a higher time resolution. Dotted lines refer to the coordination of the upper reversal points of hindleg movements. (D) Phase histogram of the upper reversal points of the right hindleg with respect to the cycle of the left hindleg. lHL and rHL, stridulatory up-and-down movements of the left and right hindleg; *N*, total number of occurrences; φ, mean phase value; *l*, mean phase vector, which is a measure of the scatter of the phase values. The closer *l* is to 1, the smaller is the scatter.

approximately 70 ms long (at 30–35 °C), each of which comprises a single up-and-down movement of the hindlegs (Fig. 2C). The two legs move with different amplitudes and with a slight, but strictly maintained, phase shift. This ranges in different individuals from φ=0.03 to φ=0.07, where φ is the phase (Fig. 2D). The movement patterns can be exchanged between the legs from one sequence to the next or, rarely, within a sequence (Elsner, 1974).

During courtship in the presence of a female, the sequences of ordinary stridulation are lengthened to a duration of approximately 30–50 s. The number of such sequences performed by the male before trying to copulate depends largely on the behaviour of the female. The last song sequences preceding an attempt at copulation are extended by two characteristic movement patterns not previously executed (Fig. 2B). First, the normal stridulation gives way to a pattern in which each hindleg alternates between a series of large- and small-amplitude strokes, each series lasting approximately 800 ms; the patterns of the two hindlegs are 180 ° out of phase with one another. Jacobs (1953) called this part of the behaviour *Schenkelschütteln* (hindleg shaking). This pattern is continued for 4–5 s, after which the hindlegs make 10–20

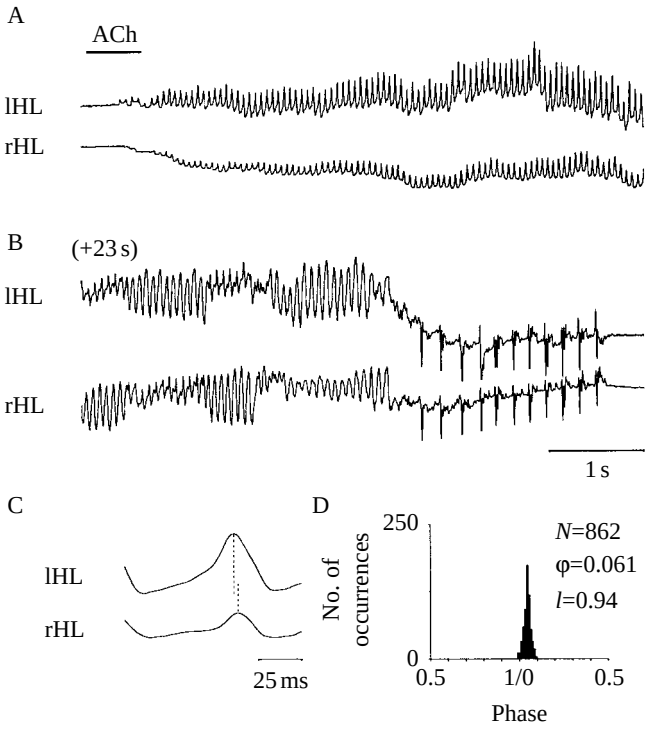

Fig. 3. (A,B) A sequence of courtship song elicited by microinjection of 10^{-4} mol l^{-1} acetylcholine (ACh) into the protocerebrum with the beginning of ordinary stridulation (A), hindleg shaking (B, left) and precopulatory movements (B, right). (C) One movement cycle of ordinary stridulation at a higher resolution. Dotted lines refer to the upper reversal points of hindleg movements. (D) Phase histogram of the upper reversal points of the right hindleg with respect to the cycle of the left hindleg. lHl and rHL, stridulatory up-and-down movements of the left and right hindleg; *N*, total number of occurrences; φ, mean phase value; *l*, mean phase vector (see Fig. 2).

rapid, synchronous, large-amplitude up-and-down movements that produce sharp sounds. These introduce an attempted copulation and accompany a successful attempt and are therefore termed precopulatory movements.

Injection of acetylcholine

To test the effects of pharmacological agents, a microelectrode was inserted into the dorsal median neuropile region of the protocerebrum and was carefully guided down into the tissue. As it was advanced, brief pharmacological stimuli were applied to check whether stridulatory behaviour could be induced. When a suitable site in the neuropile was found, each injection of acetylcholine (approximately 1 nl; 10^{-4} mol l^{-1}) elicited a single song sequence of ordinary stridulation (Fig. 3A). Taking its duration of 30–45 s into account, this was interpreted as the first part of the courtship song (Fig. 3A). In a few cases, the ordinary stridulation was followed by hindleg shaking and precopulatory movements (Fig. 3B). The song sequences elicited by acetylcholine began approximately 0.5 s after injection. The movement patterns, which consisted of normal stridulation, hindleg shaking and precopulatory jerking, did not differ from those of naturally singing animals. For example, the two hindlegs were moved up and down with different amplitudes approximately 12–14 times per second, the upward movements lasting distinctly longer than the downstrokes. The movements were slightly phase-shifted, and the leg stridulating with the larger amplitude reached its upper reversal point first (cf. Figs 2C,D and 3C,D). We could discern no relationship between the side of the brain in which the drug was injected and the distribution of the movement patterns between the two hindlegs. In some cases, the legs changed their roles after a further injection of acetylcholine. Changes in acetylcholine concentration had no effect on the performance or the duration of the induced stridulatory sequences, and ordinary stridulation could be induced by acetylcholine at a concentration as low as 10^{-6} mol l^{-1}.

Injection of nicotine

The injection of approximately 1 nl of nicotine (10^{-4} mol l^{-1}) elicited stridulation with the same latency as that occurring when acetylcholine was applied (Fig. 4A). The movement patterns of both hindlegs corresponded to those of naturally singing animals. Each injection usually induced one stridulatory sequence, but in rare cases two were generated. The time course of the song sequence and the execution of the various movement elements were no different from those produced under the influence of acetylcholine. However, after 2–3 nicotine injections, the hindlegs became immobile, possibly because nicotine is not broken down by ACh esterase, and its concentration at the injection site remains high for some time. Singing could not then be elicited for 10–15 min.

Injection of nicotine and d*-tubocurarine*

It is possible that nicotine might have induced singing by a nonspecific action on muscarinic receptors. As a check on the specificity of the action of nicotine, we applied an antagonist selective for the nicotinic receptors, *d*-tubocurarine. This plant alkaloid has a higher affinity than nicotine for the nicotinic ACh receptor and blocks its binding sites without opening the cation channel, so that the receptor cannot be activated by nicotine. In this experiment, a double-barrelled electrode was used to deliver first nicotine and then a mixture of nicotine and *d*-tubocurarine to the same site in the protocerebrum (Fig. 4B). Whereas each nicotine injection induced stridulation lasting 1–2 min, there was no response when the mixture with *d*-tubocurarine was injected. This indicates that, when nicotine was used alone, it was indeed selectively activating nicotinic ACh receptors.

Injection of muscarine

Unlike acetylcholine or nicotine, muscarine elicited stridulatory movements only after a delay of approximately 5 s. The movements were initially very small and were often interrupted (Fig. 5A). After approximately 25 s, a first complete song sequence of normal duration was performed, usually followed by 1–2 sequences of similar duration. These responses did not differ from natural singing with respect to

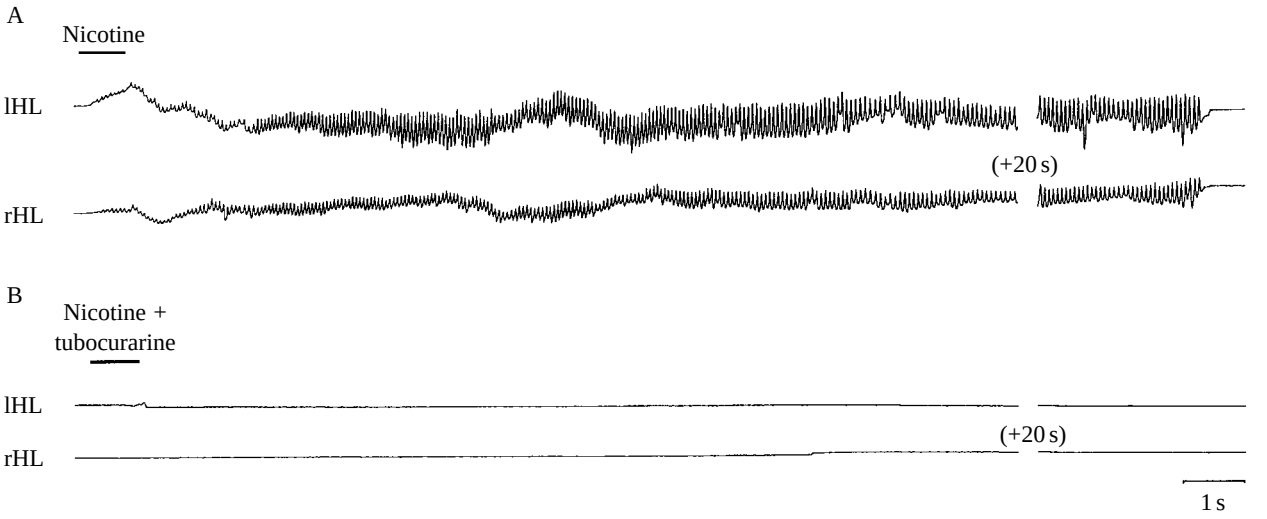

Fig. 4. (A) Microinjection of 10^{-4} mol l^{-1} nicotine elicits ordinary stridulation. (B) Microinjection of a mixture of nicotine and its antagonist *d*-tubocurarine (10^{-4} mol l^{-1} each) at the same site within the protocerebrum fails to evoke stridulatory behaviour. lHl and rHL, stridulatory up-and-down movements of the left and right hindleg.

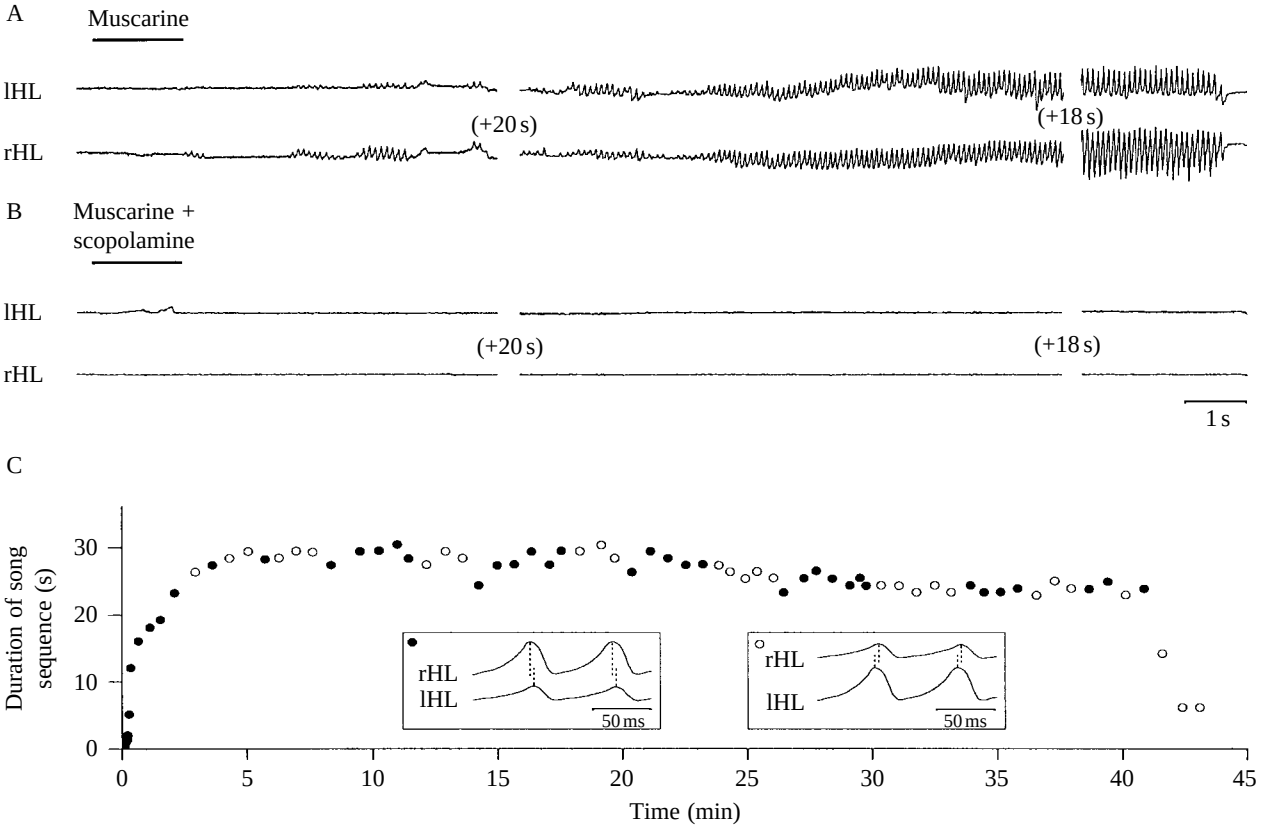

Fig. 5. (A) Microinjection of 10^{-4} mol l^{-1} muscarine elicits stridulation with slower onset characteristics than does nicotine or acetylcholine (ACh). (B) Injection of a mixture of muscarine and its antagonist scopolamine (10^{-4} mol l^{-1} each) at the same site within the neuropile does not induce stridulation. (C) Microinjection of 10^{-4} mol l^{-1} pilocarpine elicits long-lasting stridulatory activity. The animal performs several dozen song sequences. The movement patterns of the hindlegs and their coordination change several times. Open circles indicate song sequences with the left hindleg producing the large-amplitude pattern, and filled circles indicate sequences with the right hindleg producing the large-amplitude pattern, as shown in the insets. lHL and rHL, stridulatory up-and-down movements of the left and right hindleg.

the movement pattern and the coordination of the legs. After approximately 2–3 min, stridulatory activity came to an end. However, it was resumed immediately after a new injection of muscarine, and the amplitude increased more rapidly than it did the first time. In contrast to the experiments with nicotine, the hindlegs did not become immobile even after muscarine had been administered several times.

Muscarine injection usually induced song sequences that included only ordinary stridulation, thus corresponding to the calling song or the main part of the courtship song. In a few cases, however, sequences were elicited that began with ordinary stridulation and then proceeded to hindleg shaking and precopulatory movements, so that they corresponded to the complete stridulatory sequences in courtship.

Injection of muscarine and scopolamine

Scopolamine is a competitive antagonist that binds specifically to muscarinic receptors of a great variety of subtypes. When injected together with muscarine (10^{-4} mol l^{-1} each) through a double-barrelled electrode, scopolamine prevented the induction of singing by muscarine (Fig. 5B) and also interrupted song sequences previously induced by muscarine, with a latency of only approximately 1 s (not shown in Fig. 5). After injection of the antagonist, it was necessary to wait 2–3 min before singing could again be elicited by muscarine injection. These results indicate that the stridulation system can be activated by excitation of muscarinic receptors alone.

Injection of pilocarpine

This muscarinic cholinergic agonist has a high affinity for muscarinic ACh receptors and is probably inactivated only slowly in the nervous system. Pilocarpine injection (10^{-4} mol l^{-1}) elicited prolonged stridulation in a pattern no different from that of naturally singing animals. As in the case of muscarine injections, the singing activity built up very gradually, with the first song sequences lasting only a few seconds, and it often took 2–3 min after the injection for the sequences to reach the natural duration of 20–30 s. Unlike muscarine, a single injection of pilocarpine sufficed to induce stridulatory activity lasting for up to an hour, during which time the animals produced many individual song sequences (Fig. 5C). Once the stridulation had reached full intensity, the sequence duration was 20–30 s and the pauses between sequences were 10–15 s long. As in naturally singing animals, the movement pattern and the coordination of the two legs changed at irregular intervals (Fig. 5C) between sequences. In most cases, the singing activity consisted of ordinary stridulation from beginning to end. Sometimes, however, the animals also exhibited hindleg shaking and precopulatory movements. Immediately after a long period of stridulatory activity had concluded, a new series of song sequences could be elicited by pilocarpine injection.

It is particularly remarkable that pilocarpine induced not only more song sequences than the other ACh agonists but also induced long-lasting stridulatory behaviour that was

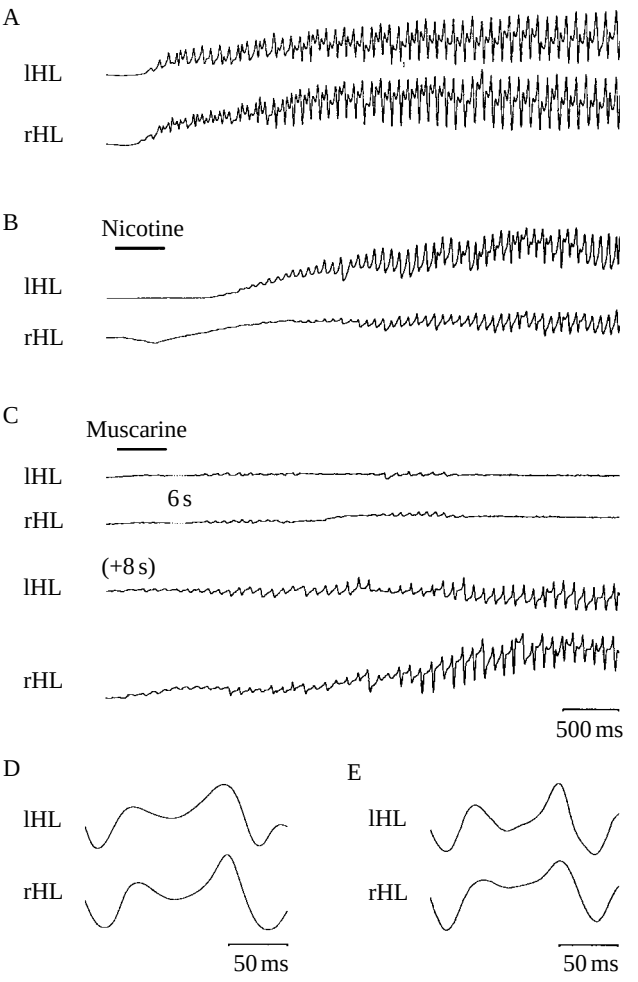

Fig. 6. Stridulatory movement patterns of *Omocestus viridulus* females. (A) Natural stridulation of a freely moving female. (B,C) Stridulation elicited by microinjection of 10^{-4} mol l^{-1} nicotine (B) and 10^{-4} mol l^{-1} muscarine (C) into the protocerebrum. (D,E) One movement cycle of natural stridulation (D) and one elicited by muscarine (E) at a higher time resolution. lHL and rHL, stridulatory up-and-down movements of the left and right hindleg.

subdivided into phases of stridulation with pauses inserted between the individual sequences, as in natural singing. This finding suggests that pilocarpine did not activate the descending command neurones directly but acted on the networks presynaptic to the command neurones.

Pharmacological induction of song in females by injection of ACh agonists

Natural songs

Virginal females and females kept apart from males for a few weeks often respond with songs of their own while a male is singing. The sequences of the female songs are relatively short, lasting only approximately 5–10 s. The stridulatory movements of females resemble those of males in their basic pattern but are more variable. In a given individual, it is possible to observe all the transitional forms between a simple up-and-down movement (as in males) and a cycle with two

pronounced peaks (Fig. 6A,D). In contrast to the stridulatory movements of males, both hindlegs of the females oscillate with approximately the same amplitude and one does not precede the other in any regular way.

The effects of injection of acetylcholine, nicotine or muscarine

The typical song produced by female *O. viridulus* in response to male stridulation can also be elicited by microinjection of these drugs. The effective injection sites are the same as those in the males. It is noteworthy that both virginal and mated females are equally susceptible to the pharmacological induction of stridulation. As in the males, microinjection of acetylcholine and nicotine (Fig. 6B) caused one or two stridulation sequences to be started approximately 1 s after stimulus onset, whereas muscarine elicited singing with a latency of 5–7 s. In the latter case, as in males, the performance was associated with a slow increase in both the amplitude of the movement and the duration of the sequences (Fig. 6C). These hindleg movement patterns (Fig. 6E) did not differ from those of naturally singing females, if their variability is taken into account (Fig. 6D).

Discussion

Behavioural specificity of pharmacological stimulation

Injection of acetylcholine and its agonists into specific regions of the cephalic ganglia of male and female *O. viridulus* induced stridulation indistinguishable from that of naturally singing grasshoppers. Two aspects of the results are especially significant.

First, in the male, these neuropharmacological agents did not just elicit ordinary stridulation, i.e. the calling song and the main part of the courtship song, but often induced complex behavioural sequences including ordinary stridulation, hindleg shaking and precopulatory movements, arranged in a sequence typical of natural courtship behaviour. Each of these three parts of courtship behaviour is controlled by specific command neurones (Hedwig, 1995; Hedwig and Heinrich, 1997). The orderly, sequential arrangement of the three movement patterns in response to pharmacological stimulation indicates that the descending command neurones for these types of behaviour are being activated one after another in a well-coordinated way.

Second, the induced stridulatory activity was maintained for a long time, particularly after injection of the muscarinic agonist pilocarpine. Since prolonged singing has been observed to follow mechanical stimulation of the brain in crickets (Huber, 1955*a*), pharmacological stimulation would initially have been expected to produce sustained activation of the associated neuronal structures. In fact, however, the injection of pilocarpine did not result in continuous stridulation. Instead, there was an orderly temporal alternation of stridulatory sequences and pauses in activity, corresponding to the grasshopper's natural behaviour. The same applies to the cases in which only one of the three parts of the courtship song – ordinary stridulation, hindleg shaking or precopulatory sounds – was elicited. Here, too, the sequences were separated from one another by pauses.

In view of these two findings, it seems justifiable to conclude that, at least in some experiments, pharmacological stimulation activated neuropile structures in the protocerebrum that are presynaptic to the descending neurones and that coordinate and control the timing of command neurone activity. Unfortunately, in these particular experiments, the stimulus sites could not be localized.

With regard to the control of behaviour, it is also significant that the rivalry song typical of the species has never been induced in *O. viridulus* by pharmacological or electrical stimulation. This song, produced by rival males, lasts 1–1.5 s, and its stridulatory movements are performed with full amplitude and force from the very beginning. It may be that the vigorous movements of the rivalry song are governed by different cephalic control mechanisms from those involved in the slow, gradually increasing ordinary stridulation of the calling and courtship songs. A greater involvement of sensory inputs, such as visual and auditory stimuli associated with the presence of another male, is possibly also significant here.

In the gomphocerine species *Gomphocerus rufus*, *Euthystira brachyptera*, *Chorthippus biguttulus* and probably also in *Omocestus viridulus*, the readiness of a female to sing in response to the male's song is under strong hormonal control, e.g. by the juvenile hormone (Loher, 1966; Loher and Huber, 1966; Hartmann *et al.* 1994). However, the manner in which hormonally controlled mechanisms interact with the neuronal control of female stridulation at the cellular level is at present entirely unknown. It can, however, be stated that these hormonal mechanisms must be sought at a higher functional level than the cholinergic control elements. This is shown by the finding that nicotinic and muscarinic ACh agonists induced stridulation in females regardless of whether the females were still virginal, and hence ready to sing, or whether they had already mated, in which state they do not normally sing. The implication is that all neuronal structures of the song-controlling system downstream of the site of pharmacological stimulation are not subject to hormonal control. The hormonal mechanisms are presumably situated in the neuropile regions presynaptic to the descending command neurones for stridulation.

Site-specificity of pharmacological stimulation

The efficacy of pharmacological stimulation in the cephalic ganglia should be considered against the background of the neuronal organization of stridulatory behaviour. The neuronal networks that generate stridulatory rhythms and activate motoneurones are in the metathoracic ganglion complex, rather than the brain, and are controlled by descending command neurones originating in the protocerebrum and, in some cases, also by neurones originating in the suboesophageal ganglion (Hedwig, 1986, 1994; Lins and Elsner, 1995*a*,*b*). Thus, pharmacological stimuli do not affect the thoracic pattern-generating networks directly, but act by way of higher-level neurones. This situation is comparable to the initiation of

pleopod movements in crustaceans by descending command neurones (Acevedo *et al.* 1994) and is quite distinct from that found in experiments involving direct pharmacological stimulation of the pattern generators underlying flight (Sombati and Hoyle, 1984) or walking (Ryckebush and Laurent, 1993; Büschges *et al.* 1995), where the central nervous excitation pattern ('fictive behaviour') cannot be elicited unless the animal has been deafferented.

The neuropile sites in the *O. viridulus* protocerebrum which initiated stridulation after injection of cholinergic substances are within the regions of arborization of the stridulatory command neurones, dorsal to the posterior part of the central body. In this region, the descending command neurones form a dense neuropile comprising branches with a dendritic appearance, some of which extend into the lateral protocerebrum (Hedwig, 1994, 1995). It may be that the injected ACh and ACh agonists activate receptors on these dendrites, or they may act on neurones presynaptic to the command fibres, to which the activation is then relayed. In the suboesophageal ganglion, there is also a relationship between the arborization region of the command neurones and the sites of effective stimulation. Here, however, it seems possible that the activated units are, in addition or perhaps even exclusively, neurones involved in stridulation that descend from the suboesophageal to the metathoracic ganglion (Lins and Elsner, 1995*a*,*b*).

At present nothing is known about how the animals would react to pharmacological stimulation of the central body or the mushroom bodies. As Huber (1955*a*,*b*, 1964) showed by stimulating the brain mechanically and electrically, these two prominent neuropile structures in the protocerebrum play a crucial role in the control of stridulatory behaviour in orthopterans and, therefore, these structures are especially interesting targets for future stimulation experiments.

Specificity of ACh receptor activation

By stimulating with either nicotine or muscarine, each of the receptor groups in protocerebral neuropile structures was activated selectively. That this was a genuine selectivity was corroborated by control experiments with antagonists (scopolamine and *d*-tubocurarine). Thus, the existence of both cholinergic receptor types in the cephalic control system for stridulation has been confirmed and it has been shown that each type is individually capable of eliciting stridulatory behaviour when stimulated with its specific agonist.

Activation of the nicotinic ACh receptors always induced a behavioural response with a short latency (approximately 0.5 s), and the response usually consisted of only one song sequence. In contrast, activation of the muscarinic receptors always resulted in gradually developing song sequences, with stridulatory behaviour continuing for many minutes. The differences between the behavioural responses are clearly consistent with the dynamics of the reactions at the cellular level that are mediated by the two receptor types. Whereas nicotinic receptors are predominantly involved in rapid synaptic transmission, muscarinic receptors are coupled to intracellular signalling pathways by way of G-proteins. The responses they mediate are slower (Bai *et al.* 1992) and can serve to modulate synaptic transmission (LeCorronc and Hue, 1993; Parker and Newland, 1995).

Concluding remarks on the neuropharmacological cephalic control of stridulatory behaviour

The effectiveness of acetylcholine and its agonists in inducing stridulatory behaviour when injected into specific cephalic neuropiles indicates that ACh is the excitatory transmitter in the cephalic control system for stridulation in gomphocerine grasshoppers. The differential dynamics of the stridulatory responses to nicotinic and to muscarinic stimulation suggests that the induction of an individual song sequence is started by activation of the nicotinic ACh receptors. At this early stage, muscarinic ACh receptors probably contribute little to the overall excitation of the system, because they must first initiate a chain of signals by way of G-proteins. However, once they begin to have an effect, excitation is more prolonged and there may well be a steady increase in the level of excitation. This accords with the natural pattern of behaviour in which several sequences of ordinary stridulation are generated one after another and, in the case of courtship song in the presence of a female, are concluded by the addition of other movement patterns. Thus, the level of excitation of the control system may be modulated by pathways using muscarinic transmitters. Intense and persistent sensory stimuli in particular, such as those received from a female, could activate the muscarinic system by causing the release of acetylcholine and thus elevate the excitatory state of specific neurones involved in the control of courtship. Such effects of sensory stimuli have been documented in experiments on *Manduca sexta* (Trimmer and Weeks, 1993; Trimmer, 1995). Moreover, Baines and Bacon (1994) demonstrated the existence of both cholinergic ACh receptor subtypes on one interneurone which mediates the state of arousal throughout the central nervous system. The nicotinic ACh receptors generate fast excitatory postsynaptic potentials leading to action potentials, while the muscarinic ACh receptors evoke a sustained depolarization and lower the spike-initiation threshold.

Muscarinic ACh receptors may also be located on the presynaptic side, where they modulate ACh release and may, thus, enhance the excitation of postsynaptic neurones (Knipper and Breer, 1988; LeCorronc *et al.* 1991). With the form of stimulation we used, however, such effects could not be detected.

In the present paper, we hope to have shown that the application of neuropharmacological stimulation techniques is a valuable extension of traditional neurophysiological and neuroanatomical methods. Our results indicate several important areas for future work. (i) The molecular receptors on the neurones of the cephalic stridulation control system must be investigated. (ii) The actions of nicotinic and muscarinic ACh agonists should be analysed in different gomphocerine grasshopper species, characterized by marked differences in

the species-specific song pattern. (iii) Finally, it should be kept in mind that the cephalic control of singing operates not only by excitation but also, as Huber (1955*a*, 1964) has shown, by inhibition of certain brain areas. The transmitter systems that play a role here need to be clarified. A strategy directed towards both the cellular and the comparative systemic level offers considerable promise for advancing our understanding of the cephalic control of stridulatory behaviour.

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