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Identified descending brain neurons control different stridulatory motor patterns in an acridid grasshopper

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Abstract During courtship sequences male grasshoppers of the species *Omocestus viridulus* successively perform with their hindlegs three different stridulatory movement patterns: ordinary stridulation, hindleg shaking and precopulatory movements. Microinjection of acetylcholine into protocerebral neuropil regions can either elicit complete courtship sequences or evoke one of the three motor patterns. Intracellular recordings and stainings revealed three types of descending brain neurons: B-DC-3, B-DC-4 and B-DC-5. All three types of interneurons have a medial axon position in the connectives. They cross the midline of the protocerebrum and exhibit a profuse arborization pattern within the medial dorsal protocerebral neuropil. Stimulation of each type of interneuron specifically elicits one particular motor pattern of courtship behaviour. Courtship of the grasshopper *O. viridulus* may therefore be controlled by successive activation of these descending brain neurons.

Key words Grasshopper · Stridulation · Brain neurons · Motor patterns · Pharmacological stimulation

Introduction

Major topics in neurobiology of insect motor pattern generation are the analysis of the neuronal networks underlying different behaviours, the role of sensory feedback and modulatory aspects of pattern generation. Comparatively little attention is paid to the central control and coordination of different motor patterns, although this is essential to produce complex behavioural sequences. The concept of command neurons (Wiersma and Ikeda 1964; Kupfermann and Weiss 1978) describes the activation of pattern-generating networks: higher neuronal centers are thought to put pattern-generators into action through the tonic activity of single command neurons or systems of functionally uniform command elements.

In crickets and grasshoppers the pattern-generating networks for stridulatory wing or leg movements are located in the thoracic ganglia. Since stimulation of particular brain regions elicits the behaviour (Huber 1960, 1964; Otto 1971; Wadepuhl 1983; Hedwig 1986), descending fibre systems must transmit the commands for stridulation from the brain to the thoracic ganglia. Evidence for such neurons came from stimulation experiments of fibre bundles within the neck connectives (Otto 1971; Bentley 1977). Meanwhile in grasshoppers (Hedwig 1994) and crickets (Hedwig 1996), single descending brain neurons have been identified, which can elicit stridulation and meet the standards established for their characterization as command neurons.

Acridid grasshoppers of the species *Omocestus viridulus* use different sound patterns for acoustic communication. Especially during courtship behaviour, they stridulate three different sound patterns with different underlying hindleg movements. The aim of the experiments was to analyse how these different stridulatory motor patterns are set into action by the brain.

Materials and methods

Animals

Specimen of the acridid grasshopper *Omocestus viridulus* were collected in biotopes in Germany (Solling and Rhön) and were kept for several weeks in the laboratory. Experiments were performed at 25–30 °C. A total of 177 male grasshoppers were used for electrophysiological recordings and 82 in pharmacological experiments.

Preparation

The grasshoppers were glued with their pronotum to a specially designed instrument. The front legs were fixed and the middle legs and hindlegs remained free to move (Hedwig 1986). Stridulatory movements of the hindlegs were recorded with an optoelectronic device (Helversen and Elsner 1977).

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The head capsule was opened frontally to expose the brain. The antennae remained intact during pharmacological experiments, whereas they were removed for recordings from neurons. During intracellular recordings the brain had to be stabilized mechanically. For this purpose a small platform was positioned under the brain and a ring-like instrument was gently pressed on the frontal surface of the protocerebrum.

Pharmacological stimulation

The microinjection of neuroactive substances was carried out with a pneumatic pico-pump (WPI model 820). Glass microelectrodes were broken to a tip diameter of about 10 μm. They were filled with the neuroactive substance (10^{-3} mol l^{-1}) diluted in saline (Usherwood and Grundfest 1965). A pressure of 210 kPa was used for injection. The system was calibrated with a methylene blue solution injected into vaseline. It was set to deliver approximately 1 nl when injections into the brain were performed.

Intracellular recordings

Neuropil recordings from interneurons were obtained in the mediolateral protocerebrum. This region was chosen due to the arborization pattern of the B-DC-3 interneurons. Thick-walled glass capillaries (Hilgenberg, Malsfeld Germany, AD 1.0 mm, ID 0.5 mm) were pulled to microelectrodes (David Kopf, Model 700C). The tip of the electrode was filled with 5% aqueous lucifer yellow CH (Sigma) and the shaft with 1% LiCl. The electrodes had resistances of 100–140 Ohm. Neuron activity was recorded in an AC-coupled mode. This was done to prevent any loss of data during stimulation of interneurons in case of unbalanced electrode resistance. The neurons were named according to Hedwig's system (1986). Briefly, a B-DC neuron is a brain (B) neuron with an axon descending (D) contralateral (C) to the soma. Neurons of this type were numbered in order of their description. In the text ipsilateral and contralateral are used with respect to the soma position of a neuron.

Data recording

Data were recorded directly on disk. An A/D board (Data Translation DT2821-G-8DI) and the 'Turbolab' software (Bressner Technology, Gröbenzell, Germany) were used for data sampling. All channels were sampled with a rate of 10 kHz and with an amplitude resolution of 12 bit. Backups of the data files were permanently stored using a magneto-optical disc drive (Sony SMO 520). The software NEUROLAB (Hedwig and Knepper 1992, Knepper and Hedwig 1996) was used to display, print and analyse the recordings.

Results

In the following section the acoustic behaviour of the grasshopper *Omocestus viridulus* will be described. Thereafter the pharmacological, neurophysiological and morphological evidence for the specific control of courtship behaviour by descending brain neurons will be presented.

Courtship behaviour of the grasshopper *Omocestus viridulus*

During sound production acridid grasshoppers rhythmically rub their hindlegs with up-and-down movements against the elytra. Depending on the behavioural context, males of the grasshopper *Omocestus viridulus* may produce at least nine different types of acoustic signals with specific movement patterns of the hindlegs (Faber 1953; Jacobs 1953). The duration of these signals ranges from less than a second during defensive behaviour to song sequences lasting more than a minute during stridulation in front of a female.

Early observations of the behaviour of *O. viridulus* distinguished an ordinary (or calling) song and a courtship song (Faber 1953; Jacobs 1953). However, based on the analysis of the hindleg movement patterns we found that calling song stridulation and the first section of courtship stridulation are identical. Therefore, in *O. viridulus* the motor pattern of ordinary stridulation is used during calling song stridulation and also during courtship behaviour. During courtship in front of a female the male stridulatory behaviour is clearly divided into three sections: *ordinary stridulation*, *hindleg shaking* and *precopulatory movements* (Fig. 1A, B). Ordinary stridulation is a sequence of rather simple stridulatory up-down movement of the hindlegs (Elsner 1974). A single up-down movement lasts between 55 and 90 ms depending on temperature. Several hundreds of up-down movements are linked to one sequence which may last longer than 100 s. The movements start with small amplitude and increase during the first seconds of stri-

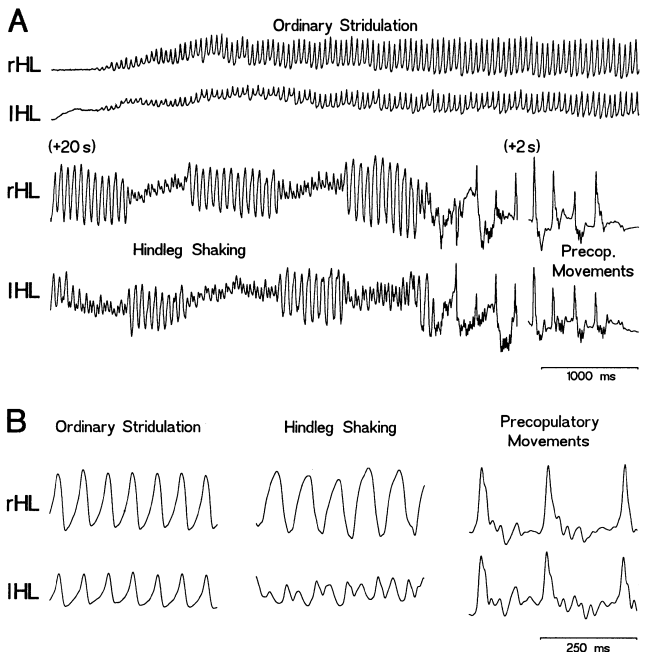

Fig. 1 A Stridulatory movement patterns during a courtship sequence of *Omocestus viridulus*. Beginning of the sequence with ordinary stridulation (*top two traces*) and end of the sequence with hindleg shaking and precopulatory movements (*lower two traces*). Between the *upper* and *lower* part the recording is a break of 20 s. The recording was obtained in an unrestrained freely moving male. **B** Sections of ordinary stridulation, hindleg shaking and precopulatory movements given at a higher resolution. *rHL* and *lHL* movement of the *right* and *left* hindleg

dulation (Fig. 1A, upper two traces). Both hindlegs perform different movement patterns with a specific phase relationship (Fig. 1A, B left) (Elsner 1974).

After ordinary stridulation hindleg movements either stop for about 300 ms or the male continues with a different movement pattern called hindleg shaking (Fig. 1A, lower two traces; Fig, 1B middle). Hindleg shaking lasts 3–5 s and a movement pattern alternating between the two hindlegs is performed: one of the hindlegs moves with a large amplitude and the other with a distinctly smaller amplitude. However, both hindlegs repeatedly change their pattern about every 800 ms. Compared to ordinary stridulation the movement pattern during hindleg shaking is clearly different. The large amplitude movements are more round at the upper reversal point and the small amplitude movements are performed with two peaks per movement cycle. The repetition rate of the up-down movements is about 13 Hz and is lower than during ordinary stridulation which is 17 Hz in this example.

During courtship, the male gets more and more excited and eventually will try to mount the female. Excitation of the male leads to precopulatory stridulatory hindleg movements, which produce short acoustic signals of high pitch. In general precopulatory movements directly follow the sequence of hindleg shaking without any interruption. However, the precopulatory movements may also be produced independently from ordinary stridulation and hindleg shaking. Sometimes males sitting close to a courting couple may join the courting male in the production of precopulatory sounds. The typical elements of this motor pattern are rapid up-down movements of large amplitude which are performed by both hindlegs simultaneously. Every large up-down movement is followed by some irregular oscillations of low amplitude (Fig. 1A lower right, Fig. 1B right). The duration of this motor sequence is quite variable and may last for 2–10 s. The repetition rate of the large up-down movements is only 6–8 Hz.

Thus, during courtship behaviour of *O. viridulus* three types of stridulatory leg movements are generated which differ regarding their pattern and repetition rate. It may be assumed that each pattern of stridulatory leg movements is based on a different motor program. The motor programs are activated in a stereotyped and coordinated manner. Although ordinary stridulation and precopulatory movements may be performed separately by the males, hindleg shaking only rarely occurs without preceding ordinary stridulation.

Pharmacological elicitation of courtship behaviour

In acridid grasshoppers stridulation can be elicited by pharmacological stimulation of protocerebral brain areas (Ocker et al. 1995). Injection of acetylcholine (ACh) into dorsal medial regions of the protocerebrum elicits long lasting ordinary stridulation in *O. viridulus* and other species (Heinrich et al. 1995).

In a continuation of the experiments performed by Ocker et al. (1995) we injected ACh into the neuropil regions of tethered *O. viridulus*. In control experiments we used double-barrel electrodes pulled to a single tip. The chambers were filled with ACh or saline respectively. At certain positions injection of ACh reliably elicited the behaviour, whereas injection of saline, at the same spot did not. Thus, we conclude that stridulation was in fact elicited by the specific effect of the neurotransmitter and was not due to an unspecific mechanical stimulation of neuropil tissue.

At most locations within the protocerebrum test injections of ACh did not elicit any stridulatory behaviour at all. However, when the electrode was impaled into regions in which descending command neurons for stridulation arborize (Hedwig 1994), certain "hot spots" could be found at which injection of ACh would repeatedly elicit stridulation. Stridulation then started within 500 ms after the pressure pulse used to eject the neurotransmitter. Four different types of stridulatory responses could be obtained subsequent to the injection of ACh.

In the first case (n = 10) injections of ACh repeatedly elicited complete sequences of courtship behaviour (Fig. 2). The behaviour started with low amplitude ordinary stridulation. The amplitude of the leg movements then gradually increased and ordinary stridulation lasted for 30–50 s. Thereafter, the movement pattern changed to a movement pattern being identical with the stridulatory movements during hindleg shaking. The sequence of hindleg shaking continued for 3.7 s in the example presented. One of the hindlegs performed a large amplitude up-down movement, whereas the other stridulated with a small amplitude. Both hindlegs changed their movement pattern after 750–1000 ms. The behaviour ended with a sequence of precopulatory hindleg movements lasting for 2.3 s. Since the temperature during the pharmacological experiments was lower than during the behavioural recordings in freely moving animals, the repetition rates of the leg movements in all cases were somewhat lower: ordinary stridulation

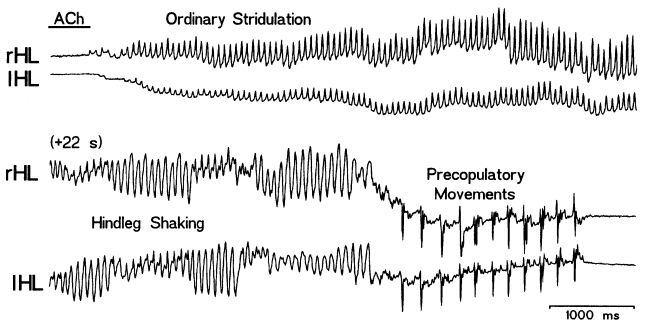

Fig. 2 Beginning (*top two traces*) and end (*lower two traces*) of a courtship sequence of *O. viridulus* elicited by microinjection of ACh into the protocerebrum. The pressure pulse used to eject the neurotransmitter is indicated by a *horizontal bar*. Interruption of the recording between beginning and end for 22 s. ACh pressure pulse for microinjection

14.5 Hz, hindleg shaking 11.5 Hz, precopulatory movements 5–6 Hz.

Pharmacological separation of courtship motor programs

In the three other cases microinjection of ACh into the brain elicited only a specific part of courtship behaviour: ordinary stridulation, hindleg shaking or precopulatory movements could be evoked independently from each other (Fig. 3). Ordinary stridulation ($n = 45$) started with low amplitude leg movements. It continued with increasing amplitude for about 30 s and then stopped (Fig. 3A). Hindleg shaking ($n = 4$) began with the hindlegs being slowly risen into singing position and then continued just as during normal behaviour with the hindlegs changing their movement pattern repeatedly (Fig. 3B). The sequence then ended with some irregular movements which in a similar way can occur at the transition between hindleg shaking and precopulatory movements (see Figs. 1, 2). When precopulatory movements ($n = 11$) were elicited, they started with low amplitude (Fig. 3C). During the sequence, the movements gradually increased until the normal amplitude was reached. The effect of pharmacological stimulation was very specific regarding the position of the electrode. At the actual position of the electrode only one part of courtship behaviour could be elicited. Moving the electrode by a few μm ceased the effectivity of the pharmacological stimulus in all cases.

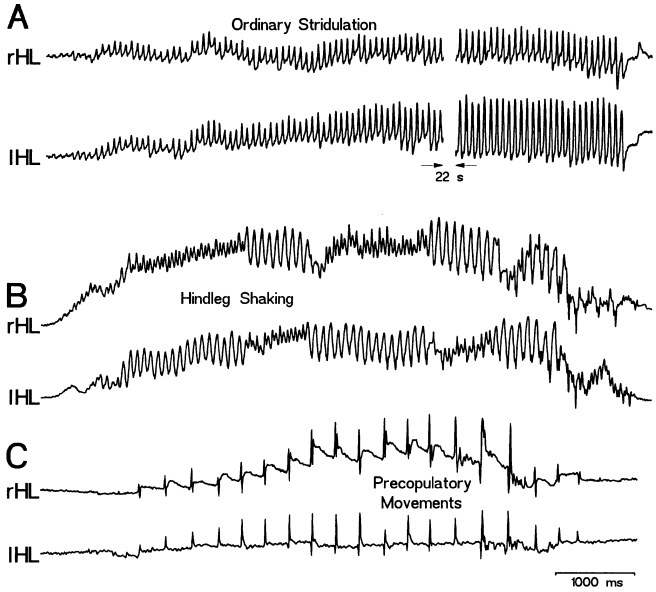

Fig. 3A–C Selective activation of courtship motor programs by microinjection of ACh into the protocerebrum of *O. viridulus*. **A** elicitation of ordinary stridulation; **B** activation of hindleg shaking; **C** elicitation of precopulatory movements. *rHL* and *lHL* movement of the *right* and *left* hindleg

Neurophysiological evidence for differential control of courtship motor patterns

The pharmacological experiments indicate that the brain specifically controls the different stridulatory motor patterns. In acridid grasshoppers, stridulatory behaviour can also be elicited by local and superficial electrical stimulation of the brain (Huber 1955, 1964, Wadepuhl 1983, Hedwig 1986). In *O. viridulus* electrical stimulation preferentially releases ordinary stridulation. However, leg movements resembling hindleg shaking or precopulatory movements may also be elicited at times (Hedwig 1985; B. Hedwig, unpublished results). These results are consistent with the pharmacological stimulation experiments.

Stimulation experiments of this kind, however, cannot reveal the structural and physiological nature of the neuronal control mechanisms of behaviour. To analyse this mechanism we had to choose a direct neurophysiological approach using microelectrodes for intracellular stimulation, recording and even staining of relevant brain neurons within the central neuropil of the protocerebrum. In the experiments the injury discharge of a neuron following penetration of the membrane was a very helpful indicator for its functional relevance. When certain neurons were penetrated, the animal would immediately start a specific motor activity. When the spike activity decreased, then the behaviour also vanished. Additionally each impaled neuron was tested by intracellular current injection whether it could reliably elicit a behavioural response. In each case the activity of an interneuron was always strictly coupled to a specific stridulatory motor program. In none of the neurons eliciting stridulation so far a specific response to sensory stimulation was found.

Elicitation of ordinary stridulation by interneuron B-DC-3

Stridulatory leg movements of ordinary stridulation could be elicited in 65 males by intracellular stimulation of a single interneuron of the type B-DC-3. When the interneuron was penetrated by the microelectrode it sometimes generated a spike response with a discharge rate of about 120 AP s^{-1}. The animal then almost instantaneously started to stridulate with both hindlegs a movement pattern which was identical to ordinary stridulation. The discharge rate of the neuron could gradually be reduced with a hyperpolarizing current (Fig. 4A). When the discharge rate dropped below 50–60 AP s^{-1}, the stridulatory leg movements stopped. This indicates that the discharge of the recorded neuron caused the stridulatory movements as opposed to any other neuronal activity.

The effect of the activity of B-DC-3 on the behaviour was also tested by intracellular current injection in resting males (Fig. 4B). Ordinary stridulation could be elicited by depolarizing current injection. A pulse of 5 nA

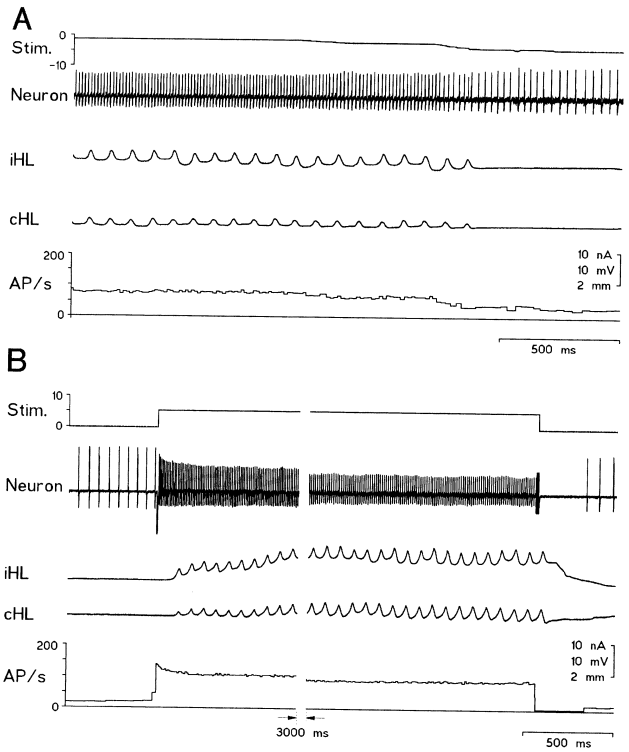

Fig. 4A, B Ordinary stridulation of *O. viridulus* elicited by activation of interneuron B-DC-3: **A** penetration of the interneuron evoked a discharge rate of 80–120 AP s^{-1} and elicited ordinary stridulation. Hyperpolarization of the interneuron decreased the spike rate. Stridulation stopped when the discharge rate dropped below 50–60 AP s^{-1}; **B** intracellular depolarization of a single B-DC-3 interneuron elicited ordinary stridulation in a resting grasshopper. The hindlegs started ordinary stridulation about 300 ms after stimulus onset. The motor pattern stopped with the end of the stimulus. *Stim* intracellularly applied current; *iHL*, *cHL* ipsilateral and contralateral hindleg; *AP/s* discharge rate of the neuron

was injected for about 5 s. It elicited an initial discharge rate of the interneuron of 140 AP s^{-1} which gradually decreased to 120–130 AP s^{-1} within 200 ms. After 150 ms the grasshopper started to perform ordinary stridulation and continued to do so until the end of the stimulus. When the depolarizing current was switched off the spike rate immediately dropped and stridulation ceased within 200 ms.

In similar experiments it could be shown that ordinary stridulation strictly follows the activity of interneuron B-DC-3. If pulselike depolarizations of the neuron were used, then bouts of stridulatory leg movements were elicited in correspondence with the activity bursts of the neuron (Hedwig 1994, 1995).

Elicitation of hindleg shaking by interneuron B-DC-4

In a total of eight experiments interneuron B-DC-4 was intracellularly recorded and stimulated. At the moment of penetration of the neuron a discharge rate of 130 AP s^{-1} was elicited. At the same time the animal started to produce hindleg movements being identical with the pattern of hindleg shaking. One of the legs produced large amplitude almost sinusoidal leg movements with a cycle duration of about 10 Hz. At the same time the other hindleg generated low amplitude movements with two small peaks in every cycle (Fig. 5A). The movement pattern of both legs changed every 1200 ms; then, the hindleg, which had generated the large amplitude movement pattern, produced the small amplitude movement and vice versa. Hyperpolarizing current injection gradually reduced the discharge rate of the interneuron. The motor pattern started to become irregular at about 90 AP s^{-1} and hindleg shaking was no longer maintained, when the discharge rate dropped below 60 AP s^{-1}.

In resting animals it was tested whether activity of the B-DC-4 neuron was sufficient to evoke hindleg shaking. From a constant hyperpolarization the interneuron was slightly depolarized for 9000 ms. The current pulse elicited a phasic-tonic spike discharge. An initial peak of 160 AP s^{-1} was followed by a discharge rate which gradually declined to 100 AP s^{-1} during about 4 s. About 160 ms after the stimulus the animal started to lift

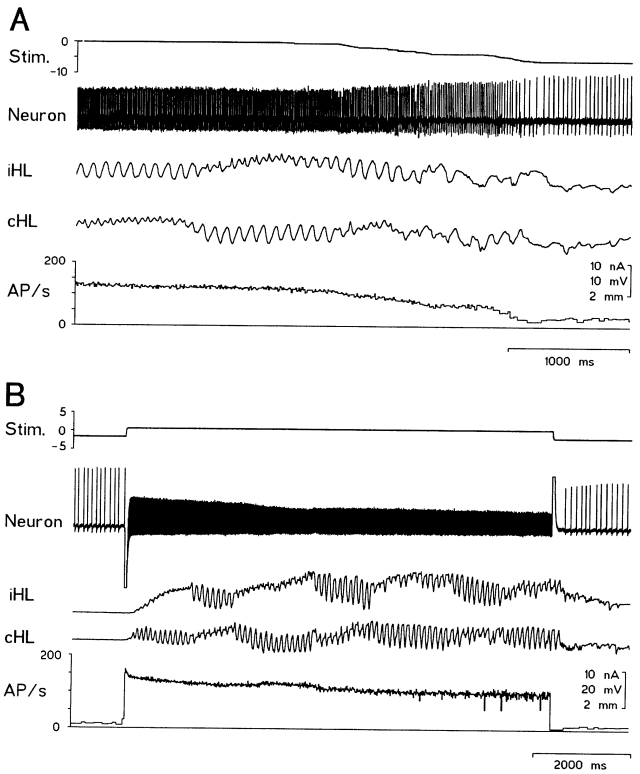

Fig. 5A, B Hindleg shaking of *O. viridulus* elicited by activation of interneuron B-DC-4. **A** penetration of the interneuron evoked a discharge rate of 130 AP s^{-1} and activated hindleg shaking. Hyperpolarization of the interneuron decreased the spike rate. The movement pattern of hindleg shaking stopped when the activity of the neuron dropped below about 60 AP s^{-1}; **B** intracellular depolarization of a single B-DC-4 interneuron in a resting grasshopper elicited hindleg shaking with several typical changes of the movement pattern. The motor pattern stopped within 200 ms after the end of the stimulus. *Stim* intracellularly applied current; *iHL*, *cHL* ipsilateral and contralateral hindleg; *AP/s* discharge rate of the neuron

its hindlegs. The contralateral hindleg began to produce the large amplitude movement of hindleg shaking and the ipsilateral leg generated the low-amplitude movement. The coordination of the movement pattern, however, was not constant. After 1200 ms the legs changed their pattern as during normal behaviour and the animal continued to do so seven times. Hindleg shaking ceased about 200 ms after the end of the evoked interneuron activity. Thus, interneuron B-DC-4 is sufficient to elicit the motor pattern of hindleg shaking.

Elicitation of precopulatory movements by interneuron B-DC-5

In seven grasshoppers the interneuron B-DC-5 could be recorded and stimulated. Similar to the preceeding examples, penetration of the interneuron elicited a discharge rate of about 130 AP s^{-1} (Fig. 6A). Simultaneously with the enhanced activity of the interneuron, the grasshopper started to produce precopulatory movements. These were intensely performed with the contralateral hindleg, whereas the ipsilateral hindleg made only minor movements in synchrony with the opposite leg. A gradually increasing hyperpolarization of the interneuron decreased its spike rate to 10–20 AP s^{-1}. When the discharge rate fell below about 80 AP s^{-1} the animal stopped the precopulatory movements.

During the experiment the neuron was tonically active with 45 AP s^{-1}. However, the hindleg remained in a resting position and no movements occurred. Depolarization of the interneuron for 2.5 s elicited a discharge rate of about 120 AP s^{-1}. As a consequence of stimulation the animal started to perform precopulatory movements (Fig. 6B). Both hindlegs produced synchronous rapid up-down-movements as during natural precopulatory behaviour. The behaviour stopped with the end of the high discharge activity of the neuron. Following the depolarization only one more movement cycle ensued. As a consequence activity of the interneuron B-DC-5 obviously is sufficient to evoke precopulatory behaviour.

Structure of descending stridulatory brain interneurons

The structure of the interneurons B-DC-3, B-DC-4 and B-DC-5 was revealed by intracellular injection of lucifer yellow (Figs. 7, 8). All three interneurons have certain structural features in common. They share a very medial position of the axon within the connectives and there are some axonal side branches within the tritocerebrum. The axon crosses the midline of the protocerebrum in a ra-

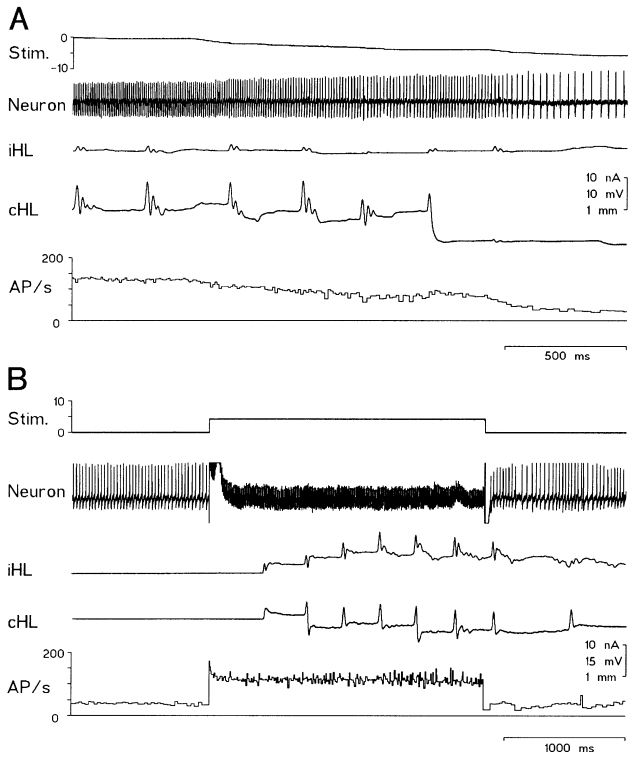

Fig. 6A, B Precopulatory movements of *O. viridulus* elicited by interneuron B-DC-5: **A** following the impalement a discharge rate of about 130 AP s^{-1} was evoked in the interneuron. The activity of the interneuron was accompanied by precopulatory hindleg movements. The spike rate of the interneuron was decreased by hyperpolarization. The precopulatory movements stopped when the activity of the neuron decreased below about 80 AP s^{-1}. **B** intracellular depolarization of a single B-DC-5 interneuron in a resting grasshopper elicited precopulatory movements. The motor pattern gradually stopped after the end of the stimulus. *Stim* intracellularly applied current; *iHL, cHL* ipsilateral and contralateral hindleg; *AP/s* discharge rate of the neuron

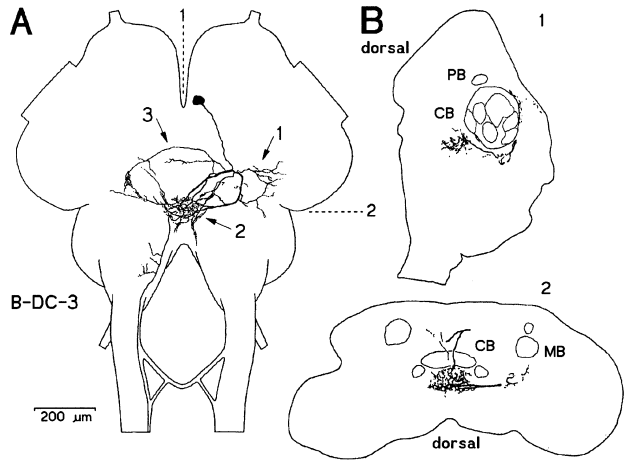

Fig. 7 **A** Arborization pattern of interneuron B-DC-3 (ordinary stridulation) in the brain of *O. viridulus* as seen from a dorsal view. The axon occupies a very medial position in the connective. Note the profuse branching pattern within the dorsal medial neuropil of the protocerebrum; **B** sagittal (*top*) and transversal (*bottom*) sections of the brain. The position of the sections are indicated in the brain outline (*left*). The axon crosses the midline ventral to the central body. The profuse neuronal arborizations are localized dorsal posterior from the central body complex. *PB* protocerebral bridge, *CB* central body. In each drawing three consecutive sections of 20 µm have been superimposed. *Arrows* are explained in the text

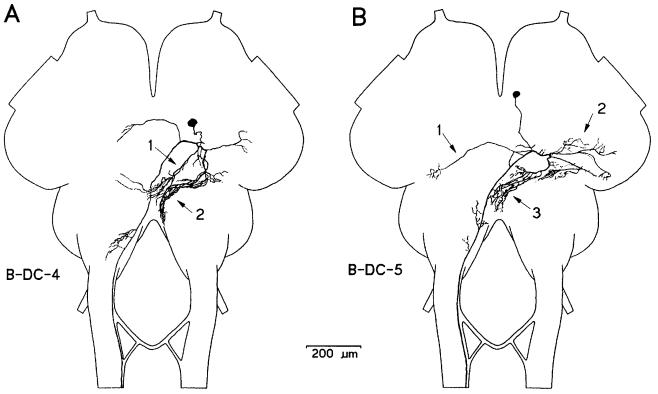

Fig. 8A, B Arborization pattern of interneuron B-DC-4 (hindleg shaking) and interneuron B-DC-5 (precopulatory movements) in the brain of *O. viridulus.* **A** the soma of the B-DC-4 interneuron has a very dorsal position. The arborizations within the dorsal neuropil cross the midline however they are more profuse at the ipsilateral side, where they turn to the tritocerebrum; **B** interneuron B-DC-5 exhibits an anterior soma position. There is also a profuse arborization at the medial dorsal protocerebrum. Side branches project also into the lateral protocerebrum. *Arrows* are explained in the text

ther ventral position. The neurite then takes a sharp turn and runs to the dorsal medial neuropil. At the beginning of this curve all three types of interneurons send off one branch to the contralateral hemisphere of the protocerebrum. Also within this curve a thin primary neurite is sent off to the soma. Within the dorsal medial neuropil all three neurons have profuse arborization patterns of fine branches with a smooth appearance which may be typical for dendrites. Besides these common features, however, there are also specific structural characteristics of each of the three interneurons.

Structure and arborization pattern of interneuron B-DC-3 eliciting ordinary stridulation

Interneuron B-DC-3 was stained 24 times (Fig. 7). The soma position of this interneuron is close to the midline and near the inner border of the protocerebral lobe. The soma has a diameter of 20–30 µm. Branches with a smooth appearance project into the lateral protocerebral neuropil (Fig. 7A, arrow 1). As a most prominent feature there is a profuse branching pattern of the neurites within the medial dorsal neuropil (Fig. 7A, arrow 2). At this location, some of the branches cross the midline and terminate close to an arborization (arrow 3), which crosses the midline more anteriorly. Other small arborizations project posteriorly to the inner region of the tritocerebrum. The axon runs at least to the mesothoracic ganglion. However, so far there are no complete stainings of the thoracic arborizations of the interneuron. Sometimes successive stainings within one experiment demonstrated the existence of at least two sibling B-DC-3 interneurons in each half of the brain. The sibling neurons had identical structures. The soma, axon and all arborizations of the siblings were lying tightly adjacent. This closely corresponds to descriptions of sibling neurons in the auditory pathway of locusts (Römer and Marquart 1984).

Histological sections of the brains were performed to analyse the spatial relationship of the B-DC-3 interneurons to prominent neuropil structures within the brain, especially the central body complex and the mushroom bodies. Sagittal sections performed at the midline (Fig. 7B1) clearly demonstrate that the neurons do not arborize within the central body complex. As the other adjacent sections show, the axon runs at the ventral border of the central body complex and the surrounding neuropil. This path corresponds to the protocerebral commissure *ventral XI* in the study of Boyan et al. (1993). The neurite then follows the border between the neuropil and the central body on its way posterior. The profuse arborizations of the neuron are positioned posterior and dorsal to the central body complex. This is also demonstrated in the transverse sections (Fig. 7B2). The evaluation of histological sections of the brains did not provide any evidence for arborizations of the B-DC-3 interneuron within the mushroom bodies.

Structure of interneuron B-DC-4 eliciting hindleg shaking

Interneuron B-DC-4 was stained seven times. A particular feature of interneuron B-DC-4 is the absolute dorsal position of the soma (Fig. 8A) which has a diameter of 15–20 µm. From the soma a thin neurite runs almost vertically to the axon. Those branches which project to the dorsal medial protocerebrum exhibit a slightly different arborization pattern compared to the branches of interneuron B-DC-3. One branch reaches the dorsal medial neuropil with some arborizations projecting contralaterally (Fig. 8A, arrow 1). The branches coming from the lateral protocerebrum do not cross the midline. Moreover they turn posteriorly and run in the direction of the tritocerebrum (Fig. 8A, arrow 2). The stainings indicate that there are also two sibling B-DC-4 interneurons in each hemisphere of the protocerebrum.

Structure of interneuron B-DC-5 eliciting precopulatory movements

So far interneuron B-DC-5 could be stained three times. It has a very medial soma position at the inner part of the protocerebral lobe similar to that of interneuron B-DC-3. The soma diameter appeared to be relatively small and was only about 15 µm. The lateral branches of this interneuron (Fig. 8B, arrow 1, 2) extended much more laterally than in B-DC-3 and B-DC-4. The branches projecting to the dorsal medial neuropil ended closely after the midline (Fig. 8B, arrow 3). They neither entered the contralateral hemisphere as far as the branches of B-DC-3 nor did they turn posteriorly as the branches of B-DC-4.

Discussion

Normal and elicited behaviour

During courtship behaviour freely moving male *Omocestus viridulus* display complex stridulatory hindleg movements and sound patterns in front of females. The motor pattern of ordinary stridulation, hindleg shaking and precopulatory movements can also be elicited in tethered grasshoppers when protocerebral neuropil regions are stimulated pharmacologically or when certain descending interneurons are sufficiently depolarized. The close similarity between the natural and the elicited hindleg movement patterns indicates that identical neuromuscular motor programs are generated in both cases. As a consequence, the brain of *O. viridulus* not only determines the beginning and end of courtship behaviour, it also activates the thoracic pattern generating networks in such a way that the species specific motor sequences of courtship behaviour are produced in a well ordered manner.

During stridulation the males take up a certain posture with the head and antennae raised and the abdomen in touch with the ground. The tethered grasshoppers were not completely free to adopt a certain body posture. However, even tethered animals with lowered antennae and a rather relaxed body posture changed their posture immediately into singing position when stridulation was elicited by neuronal or pharmacological stimulation. For this reason we do not think that additional fibre systems have to be activated in order to produce both the appropriate motor pattern and associated body posture for stridulation.

Concepts of motor pattern control

An influential idea in invertebrate neurobiology is the concept of command neurons controlling pattern generating systems by tonic activity (Wiersma and Ikeda 1964, Kupfermann and Weiss 1978). The concept implies a strict hierarchical organization of the neuronal network controlling behaviour. It is based on experiments in crustaceans in which stimulation of single interneurons can elicit complex rhythmic motor activity driving appendages (Wiersma and Ikeda 1964; Bowerman and Larimer 1974) or at least a recognizable part of a behaviour (Davis and Kennedy 1972). Therefore, command neurons may act alone or as command elements which must operate in concert to achieve a certain behaviour. The concept received considerable criticism emerging from the analysis of insect walking, where a distributed and parallel organized network seems to be realized. Walking is seen as the consequence of the interaction between recommendations from higher centers, pattern generating networks and sensory feedback (Kien 1983).

In contrast, stridulatory behaviour of acridid grasshoppers seems to be strictly hierarchically organized. Previous brain stimulation experiments have shown the specific control function of the protocerebrum over the thoracic stridulatory network (Huber 1955, 1964, Wadepuhl 1983, Hedwig 1986). Male *O. viridulus* will start stridulation whenever an electrical stimulus is applied to a specific region of the brain. Furthermore, acute sensory feedback in grasshoppers is only of minor importance for the generation of the motor pattern (Elsner and Huber 1969, Elsner 1973). Even after autotomy of both hindlegs the animals will continue to produce the species specific motor pattern. However, a change in left-right coordination of the motor activity will occur within several weeks (Elsner and Hirth 1978).

The results of pharmacological and intracellular stimulation experiments shown here are consistent with a hierarchical organization of stridulatory behaviour. The data emphazise the control function of the brain. During courtship of *O. viridulus* the brain drives the thoracic network by the activity of different descending fibres to produce the appropriate movement patterns. In case of each stridulatory movement pattern only a single descending neuron is sufficient to elicit the behaviour. Without exception every descending stridulatory interneuron elicited one specific stridulatory motor program and never any other movement. Therefore, it seems likely that the successive coordinated timing of the descending interneuron activity is a sufficient way to produce the correct sequence of courtship behaviour in *O. viridulus*. At least as far as the generation of the courtship movement patterns is concerned there is no reason to assume the simultaneous activation of a parallel organized fibre system. However, this does not exclude that the descending stridulatory interneurons are synaptically coupled in a way to support the generation of the behaviour.

In almost all cases of single cell stimulation experiments the question about the activity of the cells under natural conditions cannot be answered. Neither do we know how the descending stridulatory brain neurons are activated during normal courtship behaviour. With the current techniques it seems impossible to record their activity pattern in freely moving animals. However, a possibility to check the activity of the different interneurons during courtship behaviour may arise by a combination of pharmacological stimulation with intracellular recordings. Simultaneous pharmacological activation of complete courtship behaviour in combination with intracellular recordings of the descending interneurons may allow study of the way the descending interneurons are activated during the performance of stridulatory behaviour.

Pharmacological stimulation

Microinjection of ACh in protocerebral neuropil structures can elicit stridulation in different species of acridid grasshoppers (Ocker et al. 1995) and also in crickets (Otto 1978). ACh can be regarded as a widespread

transmitter in the insect brain (Breer 1987) and there are first results of the distribution of ACh receptor proteins within the cephalic neuropil regions of *Drosophila* (Blake et al. 1993).

Depending on the location of microinjection ACh elicited either the complete courtship behaviour or separated stridulatory motor patterns of the behaviour. The pharmacological separation of the different motor patterns corresponds to the specific activation of these patterns by the different descending brain neurons. Histological sections show a considerable overlap of the neuronal arborization patterns and the sites of microinjections (R. Heinrich, unpublished data). We therefore assume that the specific activation of the stridulatory motor patterns was due to direct pharmacological stimulation of the individual descending stridulatory brain neurons. This explanation, however, is not sufficient for the pharmacological release of complete courtship sequences. In this case, we propose an activation of those neuropil structures which initiate stridulatory behaviour. These structures then activate the descending neurons in a coordinated manner and control the motor sequences. In any case the experiments implicate an expression of ACh receptor proteins within the membranes of the descending neurons and/or in the membranes of those structures which are presynaptic to the descending interneurons. Meanwhile the cholinergic and pharmacological specialities of these neuropil structures have been characterized in more detail (Heinrich et al. 1995). Corresponding to focal electrical brain stimulation (Huber 1960, Wadepuhl 1983) the neuropil structures presynaptic to the descending neurons may be the mushroom bodies or the central body. However, histological evidence of the microinjection sites leading to complete courtship sequences is missing so far.

Arborization pattern of the neurons within the brain

All three types of interneurons show a similar course of the axon. They especially exhibit a profuse arborization pattern of smooth dendritic branches within the dorsal medial neuropil of the protocerebrum. The branches of all three types of neurons cross or end very close to the midline and the bilateral neurons must have a considerable degree of overlap in this region. In this specific area of neuropil within the dorsal medial protocerebrum information regarding the control of stridulatory behaviour appears to be processed. At this location the synaptic connections between local brain neurons involved in stridulation and the descending neurons may take place. There is good reason to assume that the identification of the neurons being presynaptic to the descending interneurons will directly lead to those brain structures which control stridulatory behavior.

The analysis of the neuronal control mechanisms for stridulation in the brain of acridid grasshoppers may contribute to a further understanding of the insect brain. With the knowledge about the arborization pattern of the descending neurons prominent stridulatory neuropil locations within the protocerebrum could be identified. The histological sections of the B-DC-3 arborizations within the protocerebrum do not give any evidence for direct projections to the mushroom bodies or to the central body complex. If these neuropil structures are relevant for stridulation, interconnecting local neurons have to be imposed. Identification of the local brain neurons which are presynaptic to the descending stridulation neurons may be necessary to understand the protocerebral control of stridulation. The profuse arborization pattern of the descending neurons may be a reasonable prerequisite to allow the application of suitable tracer techniques.

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References

Bentley DR (1977) Control of cricket song patterns by descending interneurons. J Comp Physiol A 116: 19–38

Blake AD, Anthony NM, Chen HH, Harrison JB, Nathanson NM, Sattelle DB (1993) *Drosophila* nervous system muscarinic acetylcholine receptor: transient functional expression and localization by immunocytochemistry. Mol Pharmacol 44: 716–724

Bowerman RF, Larimer JL (1974) Command fibres in the circumoesophageal connectives of crayfish II Phasic fibres. J Exp Biol 60: 119–134

Boyan G, Williams L, Meier T (1993) Organization of the commissural fibres in the adult brain of the locust. J Comp Neurol 332: 358–377

Breer H (1987) Neurochemical aspects of cholinergic synapses in the insect brain. In: Gupta AP (ed) Arthropod brain - its evolution development structure and functions. Wiley New York pp 416–437

Davis WJ, Kennedy D (1972) Command interneurons controlling swimmeret movements in the lobster. I. Types of effects on motoneurons. J Neurophysiol 35: 1–12

Elsner N (1973) The central nervous control of courtship behaviour in the grasshopper *Gomphocerippus rufus* L. (Orthoptera: Acrididae). In: Salanki (ed) Symp Invert Neurobiology, Tihany Hungary Hung Acad Sci, pp 261–287

Elsner N (1974) Neuroethology of sound production in gomphocerine grasshoppers (Orthoptera: Acrididae). I. Song patterns and stridulatory movements. J Comp Physiol A 88: 67–102

Elsner N, Huber F (1969) Die Organisation des Werbegesanges der Heuschrecke *Gomphocerippus rufus* L. in Abhängigkeit von zentralen und peripheren Bedingungen. Z Vergl Physiol 65: 389–423

Elsner N, Hirth C (1978) Short- and long-term control of motor coordination in a stridulating grasshopper. Naturwissenschaften 65: 160

Faber A (1953) Laut- und Gebärdensprache bei Insekten: Orthoptera (Geradflügler) Teil 1. Mitt Mus Naturk, Stuttgart 287: 1–198

Hedwig B (1985) Untersuchungen zur Kontrolle des Feldheuschreckengesangs durch intersegmentale Neurone. Dissertation Universität Göttingen

Hedwig B (1986) On the role in stridulation of plurisegmental interneurons of the acridid grasshopper *Omocestus viridulus* L. I. Anatomy and physiology of descending cephalothoracic interneurons. J Comp Physiol A 158: 413–427

Hedwig B (1994) A cephalothoracic command system controls stridulation in the acridid grasshopper *Omocestus viridulus* L. J Neurophysiol 72: 2015–2025

Hedwig B (1995) Kontrolle des Stridulationsverhaltens von Feldheuschrecken durch deszendierende Hirnneurone. Verh Dtsch Zool Ges 88.2: 181–190

Hedwig B (1996) A descending brain neuron elicits stridulation in the cricket *Gryllus bimaculatus* (de Geer). Naturwissenschaften, 83: 428–429

Hedwig B, Knepper M (1992) NEUROLAB, a comprehensive program for the analysis of neurophysiological and behavioural data. J Neurosci Methods 45: 135–148

Heinrich R, Elsner N (1995) Pharmacological elicitation of complex behaviour in gomphocerine grasshoppers. In: N Elsner, R Menzel (eds) Göttingen Neurobiology Report Thieme Stuttgart pp: 279–279

Helversen O von, Elsner N (1977) The stridulatory movements of grasshoppers recorded with an opto-electronic device. J Comp Physiol A 122: 53–64

Huber F (1955) Über die Funktion der Pilzkörper (Corpora pedunculata) beim Gesang der Keulenschrecke *Gomphocerus rufus* L. (Acrididae). Naturwissenschaften 42: 566–567

Huber F (1960) Untersuchungen über die Funktion des Zentralnervensystems und insbesondere des Gehirnes bei der Fortbewegung und der Lauterzeugung der Grillen. Z Vergl Physiol 44: 60–132

Huber F (1964) The role of the central nervous system in orthoptera during the co-ordination and control of stridulation. In: RG Busnel (ed) Acoustic behaviour of animals. Elsevier, Amsterdam London New York, pp 440–487

Jacobs W (1953) Verhaltensbiologische Studien an Feldheuschrecken. Beiheft 1 zur Zeitschrift für Tierpsychologie. Parey, Berlin Hamburg, pp 1–228

Kien J (1983) The initiation and maintenance of walking in the locust: an alternative to the command concept. Proc R Soc London Ser B 219: 137–174

Knepper M, Hedwig B (1996) Structure and function of NEUROLAB, a PC-program for the processing of neurobiological data. Comput Meth Programs Biomed (in press)

Kupfermann I, Weiss KR (1978) The command neuron concept. Behav Brain Sciences 1: 3–39

Ocker WG, Hedwig B, Elsner N (1995) Application of putative neurotransmitters elicits and modulates stridulation in two species of acridid grasshoppers. J Exp Biol 198: 1701–1710

Otto D (1971) Untersuchungen zur zentralnervösen Kontrolle der Lauterzeugung von Grillen. Z Vergl Physiologie 74: 227–271

Otto D (1978) Änderung von Gesangsparametern bei der Grille (*Gryllus campestris*) nach Injektion von Pharmaka ins Gehirn. Verh Dtsch Zool Ges, pp 245–245

Römer H and Marquart V (1984) Morphology and physiology of auditory interneurons in the metathoracic ganglion of the locust. J Comp Physiol A 155: 249–262

Usherwood P, Grundfest H (1965) Peripheral inhibition in insect skeletal muscle of insects. J Neurophysiol 28: 497–518

Wadepuhl M (1983) Control of grasshopper singing behavior by the brain: responses to electrical stimulation. Z Tierpsychol 63: 173–200

Wiersma CAG, Ikeda K (1964) Interneurons commanding swimmeret movements in the crayfish, *Procambarus clarki* (Girard). Comp Biochem Phyiol 12: 509–525