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Brain regions for sound processing and song release in a small grasshopper

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ABSTRACT

We investigated brain regions – mostly neuropils – that process auditory information relevant for the initiation of response songs of female grasshoppers *Chorthippus biguttulus* during bidirectional intraspecific acoustic communication. Male-female acoustic duets in the species *Ch. biguttulus* require the perception of sounds, their recognition as a species- and gender-specific signal and the initiation of commands that activate thoracic pattern generating circuits to drive the sound-producing stridulatory movements of the hind legs. To study sensory-to-motor processing during acoustic communication we used multielectrodes that allowed simultaneous recordings of acoustically stimulated electrical activity from several ascending auditory interneurons or local brain neurons and subsequent electrical stimulation of the recording site. Auditory activity was detected in the lateral protocerebrum (where most of the described ascending auditory interneurons terminate), in the superior medial protocerebrum and in the central complex, that has previously been implicated in the control of sound production. Neural responses to behaviorally attractive sound stimuli showed no or only poor correlation with behavioral responses. Current injections into the lateral protocerebrum, the central complex and the deuto-/tritocerebrum (close to the cerebro-cervical fascicles), but not into the superior medial protocerebrum, elicited species-typical stridulation with high success rate. Latencies and numbers of phrases produced by electrical stimulation were different between these brain regions. Our results indicate three brain regions (likely neuropils) where auditory activity can be detected with two of these regions being potentially involved in song initiation.

1. Introduction

Mammals, birds and insects use acoustic signals with variations in carrier frequency, amplitude, and temporal patterns for acoustic communication. Species- and context-specificity of these communication signals are mainly based on the temporal structure of songs arising from combinations and repetitions of stereotypical elements (Gerhardt and Huber, 2002). Insect songs are important for species and gender recognition, localization of reproductive partners, assessment of genetic quality and physiological state and for establishment of female reproductive readiness (Bradbury and Vehrencamp, 1998; Klappert and Reinhold, 2003; Wirmer et al., 2010; Stange and Ronacher, 2012). Among insects, the acoustic behavior of grasshoppers has attracted the interest of neuroethologists for many years (Elsner, 1974) and an especially large body of data on the mechanisms of song production and song recognition has been collected for the species *Chorthippus biguttulus* (see below). Grasshoppers produce species- and context-specific sound patterns by rhythmically rubbing stridulatory files on the inner sides of their hind legs against a cuticular vein of the forewings (von Helversen and von Helversen, 1997). The neuro-muscular activity patterns underlying stridulatory movements are generated by rhythm generating neural circuits in the meso- and metathoracic ganglia (Hedwig, 1986). These networks consist of two hemi-ganglionic pattern generators, each driving one hind leg (Ronacher, 1989; Heinrich and Elsner, 1997), that are connected via local bilaterally arborizing interneurons and thus produce coordinated phase coupled hind leg movements (Hedwig, 1992). The decision about when to sing and which song pattern to produce is made by central brain neuropils that activate descending cephalo-thoracic command neurons which connect to the respective pattern generating thoracic networks (Hedwig, 1994; Hedwig and Heinrich, 1997). Circumstantial evidence indicates that stridulatory command neurons receive direct or indirect excitation from output neurons of the central complex, a midline-spanning set of interconnected neuropils that integrate visual, olfactory, acoustic and other mechanosensory information in grasshoppers and other insects (Homberg, 1985, 2008; Hoffmann et al., 2007; Martin et al., 2015). Pharmacological and other studies suggested that the central complex integrates sensory information that favors or disfavors a particular reaction and information reflecting internal physiological states (reviewed by Fiore et al., 2015). Thereby one appropriate behavior is

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selected for execution while other (competing) behaviors are inhibited at the same time (Heinrich et al., 1997; Popov et al., 2005; Wessnitzer and Webb, 2006; Kunst et al., 2011).

Calling songs stimulate behavioral responses in conspecific receivers in many species. These behaviors typically include a phonotactic approach towards a potential partner for mating or, as in receptive females of the grasshopper *Chorthippus biguttulus*, a response song that may initiate a duet between a phonotactically approaching male and a female (von Helversen and von Helversen, 1997). Only signals whose message (species, gender, and context) and possibly direction can be assessed during sensory processing will initiate appropriate responses. Acoustic signals are perceived by auditory receptor neurons with different best frequencies that synapse on auditory interneurons in thoracic primary auditory neuropils (Römer et al., 1988). Local thoracic neurons process auditory information to enhance directional cues, extract some characteristics of the acoustic signal and synapse on ascending auditory neurons that relay the information to the brain (Stumpner et al., 1991; Clemens et al., 2011; Hedwig and Stumpner, 2016). In grasshoppers, these ascending interneurons terminate in the lateral protocerebrum (Eichendorf and Kalmring, 1980; Boyan, 1983). It is believed that at least part of the neural circuits for acoustic pattern recognition are located in this brain region (Bauer and von Helversen, 1987) but individual neurons contributing to this process have so far not been identified. Appropriate reactions to species-specific acoustic signals require neural connections between pattern recognition circuits and decision making brain centers that integrate diverse sensory information and select particular actions from a repertoire of potential behaviors. The central complex functions as a premotor center implicated with the regulation of flight, walking, climbing over barriers, respiration and sound production in various insect species (Huber, 1960; Heinrich et al., 2001; Strauss, 2002; Homberg, 2004; Martin et al., 2015). It has been demonstrated in *Ch. biguttulus*, that columnar neurons of the central complex receive courtship song-stimulated cholinergic excitation that initiates response singing (Hoffmann et al., 2007; Heinrich et al., 2012). Although Hedwig, (2001) was able to initiate stridulation by intracellular stimulation of a brain neuron that connected regions in the lateral and central protocerebrum, tracer injections into the lateral protocerebral neuropile failed to reveal direct connections to central brain areas (Hoffmann et al., 2007). Thus, the processes that relay sensory processing to appropriate motor responses are only poorly understood.

We have recently introduced multielectrode recordings that allowed simultaneous extracellular registration of electrical activity from up to five ascending auditory interneurons or up to two local auditory neurons in the lateral protocerebral neuropil of *Ch. biguttulus* (Bhavsar et al., 2015a). In order to gain additional information about auditory sensory-to-motor processing we used this method to explore brain regions that show auditory responses and to initiate stridulation by electrically stimulating these areas. We detected auditory neural activity in three brain regions, which likely represent neuropils, namely the lateral protocerebrum, the superior medial protocerebrum and the central complex. Electrical stimulation in two of those regions, the lateral protocerebrum and the central complex, elicited stridulation with high success rate. Stridulation could also be stimulated in the deuto-/tritocerebral regions that are passaged by ascending auditory neurons and descending stridulatory command neurons within cerebro-cervical fascicles. Differences – especially in the duration of stridulation - indicate that the route of activating the command system for stridulation may have been different between these stimulated brain regions.

2. Materials and methods

2.1. Animals

Experiments were performed with caught or first generation lab-reared adult female grasshoppers of the species *Chorthippus biguttulus* (Linnaeus, 1758). The animals were collected from meadows in Göttingen (Germany) or its vicinity between July and October. They were maintained in the laboratory and allowed to lay eggs into containers filled with vermiculite (Deutsche Vermiculite Dämmstoff – Sprockhövel, Germany). The collected eggs were kept at 4 °C for at least 2 months. The nymphs hatched after ~1 week at 26 °C and they were raised to adulthood on wheat and supplemental food for crickets (Nekton – Pforzheim, Germany).

2.2. Animal preparation

Grasshoppers were fixed dorsal side up to a holder using wax. The brain was exposed by opening the head capsule between the compound eyes, the ocelli, and the antennal sockets. The exposed brain was supported by a steel spoon to reduce movements. Tracheae were moved aside from the insertion site and the ganglionic sheath of the brain was carefully opened using extra fine forceps (Dumont – Switzerland) to facilitate the penetration of the electrode. The whole head capsule was filled with locust saline (Pearson and Robertson, 1981).

2.3. Acoustic stimulation

Experiments were performed at room temperature (22–26 °C) in a Faraday cage lined on the inside with sound absorbing pyramidal foam (absorbs at least 50% above 500 Hz; Fritz Max Weiser Schaumstoffe – Bochum, Germany). Grasshoppers were acoustically stimulated by two loudspeakers (D21/2, Dynaudio – Rosengarten, Germany) situated laterally at a distance of 35 cm from the grasshopper. Different auditory stimuli were created in Spike2 7.10: a 5 kHz sine wave (duration: 25 ms, 2 ms rise and fall time), a broadband white noise stimulus (bandwidth 0.5–40 kHz, duration: 100 ms, 2 ms rise and fall time) and various combinations of white noise-filled song models with different combinations of syllable duration, pause duration and gap duration. Using continuous signals, sound pressure levels were adjusted to 75 dB SPL with a calibrated microphone (Type 4133 with grid on) connected to a measuring amplifier (type 2602, Brüel & Kjær – Nærum, Denmark) positioned at the location of the experimental animal and directed towards the speaker. Sound intensities are given in dB SPL (Sound pressure level) re $2 \times 10^{-5}\,\mathrm{N\,m^{-2}}$. The output signal was band-pass filtered between 5 and 60 kHz to reduce high-frequency distortions resulting from digital to analog conversion. All stimuli were stored digitally and presented by Spike2 7.10 with a DA conversion rate of 100 kHz (Power Mk II, CED – Cambridge, UK).

2.4. Recording of auditory activity

Auditory activity in the brain of *Ch. biguttulus* was recorded from the deuto-/tritocerebrum close to the medial-cervical fascicles, the lateral protocerebrum, the central complex and the superior medial protocerebrum. For recording, multielectrodes made from three to four tungsten wires (12 µm diameter, 30–40 kΩ resistance) were used as previously described (Bhavsar et al., 2015a). The multielectrode was attached to an electrode holder that was connected to the head stage (NPI Electronic Instruments – Tamm, Germany). A silver wire (25 µm diameter, Goodfellow – Huntingdon, UK) was placed in one eye of the animal as a reference electrode which was connected to the reference pin of the head stage. The output of the head stage was connected to a differential multichannel amplifier (DPA-2FL, NPI Electronic Instruments – Tamm, Germany). The signal was amplified 2000 times, band pass filtered (300–5000 Hz) and then fed to an interface (Power Mk II, CED – Cambridge, UK) for data acquisition. Data were recorded with a sampling rate of 25,000 Hz and stored digitally with the software Spike2 7.10 (CED – Cambridge, UK). The software enabled monitoring of each channel and allowed separate settings for filtering, offset and single channel magnification. For acoustic stimulation during experiments, different stimuli were combined to stimulus programs which were saved as 'configuration files' in Spike2 7.10.

Search-program. In order to locate regions with auditory neuronal activity, search stimuli (5 kHz sine wave (25 ms) and broadband white noise (100 ms)) were repeated at 1 s intervals. To facilitate placement of the multielectrode in a neuropil with auditory activity, an audio monitor (AUDIS-01D/16 NPI Electronic Instruments– Tamm) was used.

Temporal pattern program. In order to test neuronal responses to artificial songs with variations of temporal patterns, song models with variable syllable durations (40 ms, 60 ms, 80 ms and 100 ms), pause durations (7.5 ms, 10 ms, 15 ms, 20 ms, 25 ms and 40 ms) and gap durations (2 ms and 5 ms) were presented during the recordings. The temporal patterns were generated using broadband white noise (with 2 ms ramp at the beginning and at the end), were presented at 75 dB SPL in a pseudo- randomized order and were separated by 1 s inter stimulus intervals. Two temporal programs were made. Each stimulus series was repeated 10 times. To standardize the effect of adaptation, a broadband white noise pulse (1 s, 75 dB SPL) was presented in the beginning.

2.5. *Data analysis*

2.5.1. *Offline spike sorting*

Spike sorting was done as described in Bhavsar et al., 2015a. Since spike sorting is sensitive to misclassification (Harris et al., 2000; Joshua et al., 2007; Quiroga, 2007), special care was applied to the classification of spikes. As a first step, each channel was subjected to a finite impulse response filter (known as "smoothing" algorithm) with a time constant of 100 µs (comparable with a low-pass filter of 10 kHz) and "DC remove" with a time constant of 3.2 ms which leads to offset adjustment (comparable to a high-pass filter of 312.5 Hz). The Spike2 function 'Analyze as a tetrode' was used for sorting spike forms. Since the spike sorting of tetrodes assumes the existence of four channels, one of the three channels was copied and all four channels were analyzed as a tetrode if only three wires were used. The threshold for spike detection was set to ± 3 times the standard deviation (SD) of the mean signal amplitude from a 10 s recording without acoustic stimulation at the beginning of the experiment (Brill et al., 2013). The time window for the template formation was set from 0.4 ms before to 1 ms after either a positive or a negative peak. The sorted units were clustered by applying the clustering dialogue of Spike2. A cluster in the principle component analysis display represents all spikes whose shapes are similar and similarity decreases with increasing distance to the center of the cluster. Borders of individual clusters were defined as 3.5 times the Mahalanobis distance around the center of gravity (Wölfel and Ekenel, 2005; Brill et al., 2013).

2.5.2. *Syllable-pause and gap tuning*

Male *Ch. biguttulus* produce phrases of 2–6 s duration that consist of repetitions of approximately 80 ms long syllables (series of typically three up- and down strokes of the hind legs, each generating an audible pulse) separated by pauses (von Helversen, 1972; Elsner, 1974). Attractiveness of male songs for females is critically determined by the relation of syllable to pause duration and decreases with increasing duration of gaps between pulses (von Helversen and von Helversen, 1997). Syllable-pause and gap tuning plots of recorded neural activity were compared with data from previous behavioral studies. Since behavioral data published were typically obtained from studies at 35 °C, while neurophysiological experiments were performed at room temperature, we calculated behavioral data from data at 35 °C (von Helversen and von Helversen, 1975, Fig. 6b) by multiplying all pauses by 1.7. This value has been described to scale male singing as well as female recognition for a temperature change by 10 degrees (von Helversen, 1972, Figs. 4–6, 15 and text). The resulting values compare well to response curves published for single females (von Helversen, 1972; Stumpner et al., 1991). Neuronal data were plotted by calculating the mean spike count per stimulus (of 10 syllables) against the pause duration. Behavior data representing response song probabilities of females to stimulation with male song models were obtained from von Helversen, 1972 (syllable-pause tuning) and Ronacher and Stumpner, 1988 (gap dependence). These calculations were done in Excel 2010.

2.6. *Electrical stimulation of the auditory neuropils*

After recording auditory activity, extracellular electrical stimuli were applied to the recording site in order to elicit stridulation. Electrical current was delivered between one of the tungsten wires and the reference wire to stimulate the neurons in the vicinity of the tip. To this end a stimulus isolation unit (iSO-STIM 01 M, NPI Electronic Instruments, Tamm) was used. Current pulses (3 ms current followed by 3 ms pause) of 50 µA were applied for at least 6 s. For comparison of stridulation duration, only stridulations with at least 7.6 s duration were compared and only stridulation within these 7.6 s was evaluated. To detect stridulatory hind leg movements a custom made position detector was installed on the left side of the grasshopper (von Helversen and Elsner, 1977) and a small reflector (Scotchlight 3 M, type7610) was attached to the hind leg on the same side. Light emitted from the position detector was reflected to a photo sensor. The up- and down-strokes of the leg were converted to a proportional voltage signal, amplified and sent to the data acquisition system (Power Mk II, CED – Cambridge, UK). Leg movements were recorded with a sampling rate of 4000 Hz and stored digitally with the software Spike2 7.10. Exemplary sound recordings with a small electret electrical microphone (Conrad Electronic, type TLK-M29, sensitive in the audio and near ultrasound range) placed near the stridulating hindleg confirmed that the leg movements actually produced sounds.

2.7. *Marking the recording/stimulation sites*

The position of the multielectrode during recording and electrical stimulation was marked by electrocoagulation of adjacent tissue (Strube-Bloss et al., 2011). After a successful experiment, current (100 µA) was passed between a tungsten wire and the reference wire for 5 min. After retraction of the multielectrode the brain was extracted from the head and fixed in paraformaldehyde (4% in phosphate buffered saline) for 2 h. For subsequent analysis, the brain was embedded in albumin-gelatine (Crane and Goldman, 1979), fixed in 4% PFA overnight and sectioned horizontally (with respect to the neural axis) into 30 µm slices with a vibrating blade microtome (VT1000 s Leica – Wetzlar, Germany). The sections were transferred to a microscope slide, enclosed under a cover slip using DABCO (Carl Roth – Karlsruhe, Germany) as a medium and viewed with an epi-fluorescence microscope (Axioscope, Zeiss – Jena, Germany).

3. Results

3.1. *Recording auditory activity in the brain of Ch. biguttulus*

Auditory activity of presumably local brain neurons was detected in the lateral protocerebrum (LP), the superior medial protocerebrum (SMP) and the central complex (CX). In order to discriminate the auditory activity of local brain neurons from ascending neurons, a latency criterion was implemented. Since local brain neurons are directly or via interjacent interneurons post-synaptic to the auditory ascending interneurons in the brain (Eichendorf and Kalmring, 1980; Kutzki, 2012), they should fire with longer spike latencies to the auditory stimulus than ascending neurons. The latency of local brain neurons (LP, SMP and CX) was significantly longer (on average by about 4 ms) than the latency of ascending neurons (recorded from the deuto-/tritocerebrum (DT)) as shown in Fig. 1. The largest range of latencies was observed in the central complex (14.3 to 25.3 ms). A

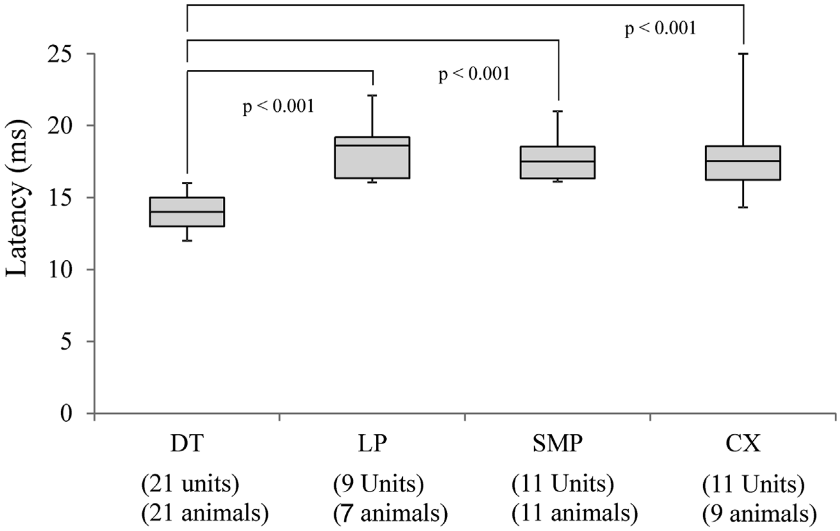

Fig. 1. Latency to the first spike of acoustically stimulated activity recorded in four different regions of the brain. Latency of auditory activity recorded from deuto-/tritocerebrum (DT, 21 units in 21 animals) was compared with lateral protocerebrum (LP, 9 units in 7 animals), superior medial protocerebrum (SMP, 11 units in 11 animals) and central complex (CX, 11 units in 9 animals). Mann-Whitney *U* test followed by a Holm-Bonferroni correction indicated that the latency of auditory activity recorded from deuto-/tritocerebrum was statistically different from the lateral protocerebrum, superior medial protocerebrum and central complex. Boxes contain the central 50% of values with the black line representing the median value and whiskers include the ranges of the upper and lower 25% of values.

statistically significant difference between the latency of auditory activity recorded from DT vs LP ($p = 0.000149$), DT vs SMP ($p = 0.000018$) and DT vs CX ($p = 0.000046$) was found, while latencies in LP, SMP and CX were not different. The asymmetric distribution in LP is caused by latencies that can roughly be sorted into three groups (16.0 to 17.5 ms, 4 units; 18.5 to 20.0 ms, 4 units; above 22 ms, 1 unit), which might represent different classes of neurons. In the other regions there was a more continuous distribution of latencies.

Responses to acoustic stimuli with different syllable-pause patterns and gap durations were recorded and quantified by calculating average spike numbers per stimulus (of 10 syllables) per syllable and plotting them against pause and gap durations. Neuronal data were compared with the behavioral data (for syllable-pause-pattern calculated from von Helversen and von Helversen, 1975, for gap duration from Ronacher and Stumpner, 1988). In Fig. 2A exemplary responses to stimuli composed from 80 ms syllables and varied pauses are displayed. Some units show a more or less consistent increase in response with increasing pause duration (e.g. unit 5 in LP, unit 8 in CX) while in SMP several neurons show an increased response only at longer pause durations. However, only one of these SMP-neurons shows a similar dependency at 40 and 60 ms syllables, but not at 100 ms syllables (data not shown). Clearly, no single neuron was "tuned" like the behavior and response peaks were not consistently seen – neither at 80 ms nor at other tested syllable durations (data not shown). The majority of neurons generated sparse spiking and inconsistent responses with syllable pause patterns. Due to their phasic response, several of the units showed a regularly patterned activity coupled to syllable onsets with longer pause durations – usually detectable, however, only when pooling several repetitions. These units typically were the same units that showed a more or less gradual increase in spike count with pause duration.

For gap detection, only one unit in the SMP (unit 5 in Fig. 2B) fired more intensely at stimuli, which are less behaviorally attractive, while others (like unit 8 in the central complex, Fig. 2B) showed relatively weak correspondences.

3.2. Electrical stimulation of auditory neuropils and descending pathways

After recording the auditory activity, current was applied to the recording sites. For successfully eliciting stridulation (criteria for genuine stridulation are described below) current pulses of at least 50 μA were necessary. Higher current amplitudes applied to the same sites did not principally alter the stridulatory response. At electrode positions where 50 μA stimuli did not elicit stridulation, higher current amplitudes were also not successful. Stridulation with movement patterns, that either resembled the species-typical sonorous stridulation (Fig. 3) or at least contained typical elements of stridulation (hind leg movements related to pulses and syllables) were often combined with slower up and down movements. Stridulation could be elicited reliably whenever it was possible to record also auditory activity with good signal-to-noise-ratio. In LP this was the case in 7 of 9 preparations (two with disputable leg movements) and in CX in 8 of 10 preparations (two with disputable leg movements) at the same position where auditory activity was recorded. Stridulation could not be elicited by stimulating auditory responsive areas in the SMP (11 preparations tested). In 11 preparations, multielectrodes were inserted into DT regions (close to the course of the cerebro-cervical fascicles) in order to directly stimulate descending stridulatory command neurons. Here, stridulation was elicited in at least 10 cases, one was debatable due to unusually fast rhythms. Auditory activity that was detected in this region had short latencies (12–14 ms) and most likely originated from axons of ascending auditory neurons that pass this brain region in close proximity to the descending command neurons.

Fig. 3 shows an example of typical stridulation elicited by electrically stimulating the deuto-/tritocerebrum of a *Ch. biguttulus* female. Stimulation of descending pathways in the tritocerebrum nearly instantly induced stridulatory hind leg movements. Here, as it is typical for natural songs of this species, stridulatory activity was structured into several phrases that were separated by longer pauses. Durations of individual phrases and pauses were quite variable and the typical phrase – pause pattern could not unequivocally be detected in all stridulations. Phrases comprised repetitive movements of the hind legs producing pulses (see Fig. 3) that were grouped in syllables (50–120 ms duration; marked in Fig. 3). A typical syllable was produced by two to five up-and-down movements with periods mostly in the range between 20 and 45 ms. Pattern and regularity of stridulatory hind leg movements including numbers of up-and-down movements per repetitive unit (syllable) were in general independent of the stimulation site (Fig. 4A), although the highest numbers of rather regular songs was elicited in DT. It was not unusual to see much larger movement amplitudes at syllable onset and syllable end than within syllables

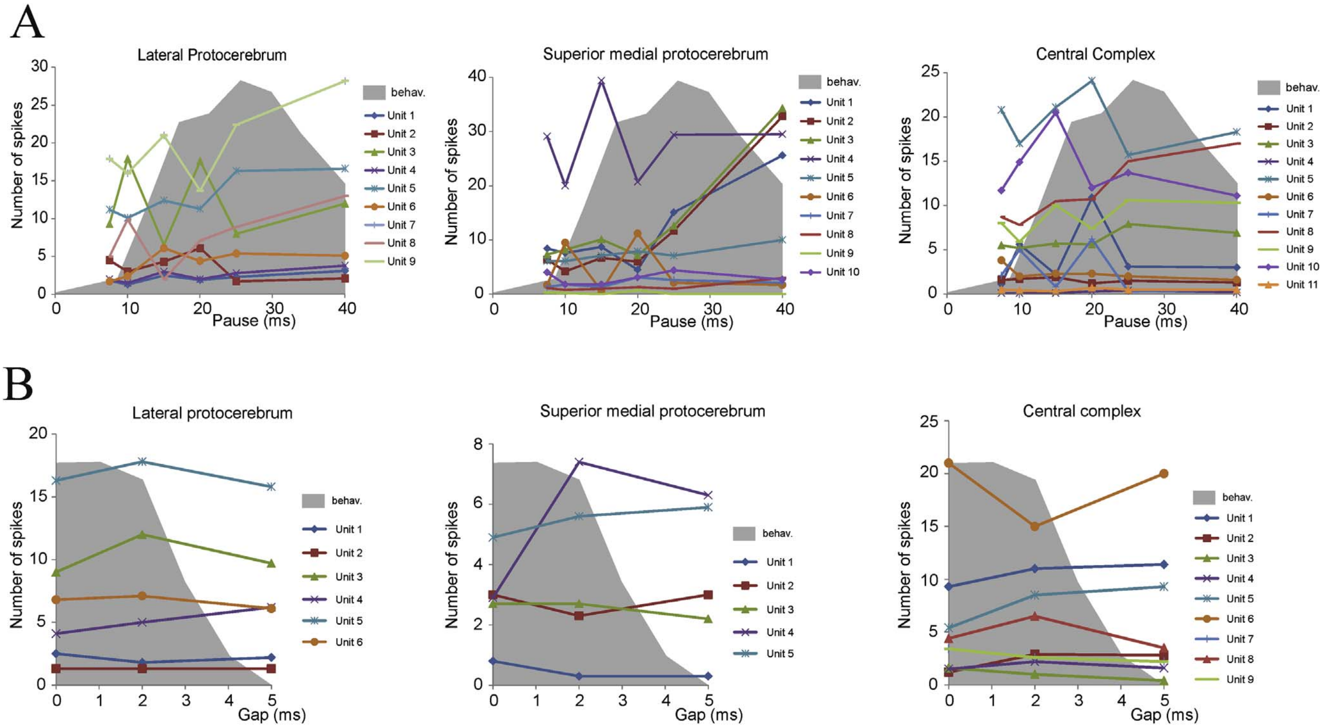

Fig. 2. Syllable/pause and gap tuning of auditory responses. (A) Plots show the neuronal responses of spike-sorted local brain units recorded from lateral protocerebrum (8 units in 7 animals), superior medial protocerebrum (10 units in 10 animals) and central complex (11 units in 9 animals) to male grasshopper model songs with 80 ms syllable duration and variable pauses. The average number of spikes from 10 repetitions of each stimulus is plotted against the pause durations. Behavior data (response singing; shaded area) were calculated after data from von Helversen and von Helversen (1975) – see methods. (B) Plots show the neuronal responses of spike-sorted local brain units recorded from lateral protocerebrum (6 units in 5 animals), superior medial protocerebrum (5 units in 5 animals) and central complex (9 units in 9 animals) to male grasshopper model songs with 80/15 ms syllable/pause durations and variable gaps. The average number of spikes from 10 repetitions is plotted against the pause durations. Behavior data (response singing) were obtained from Ronacher and Stumpner (1988).

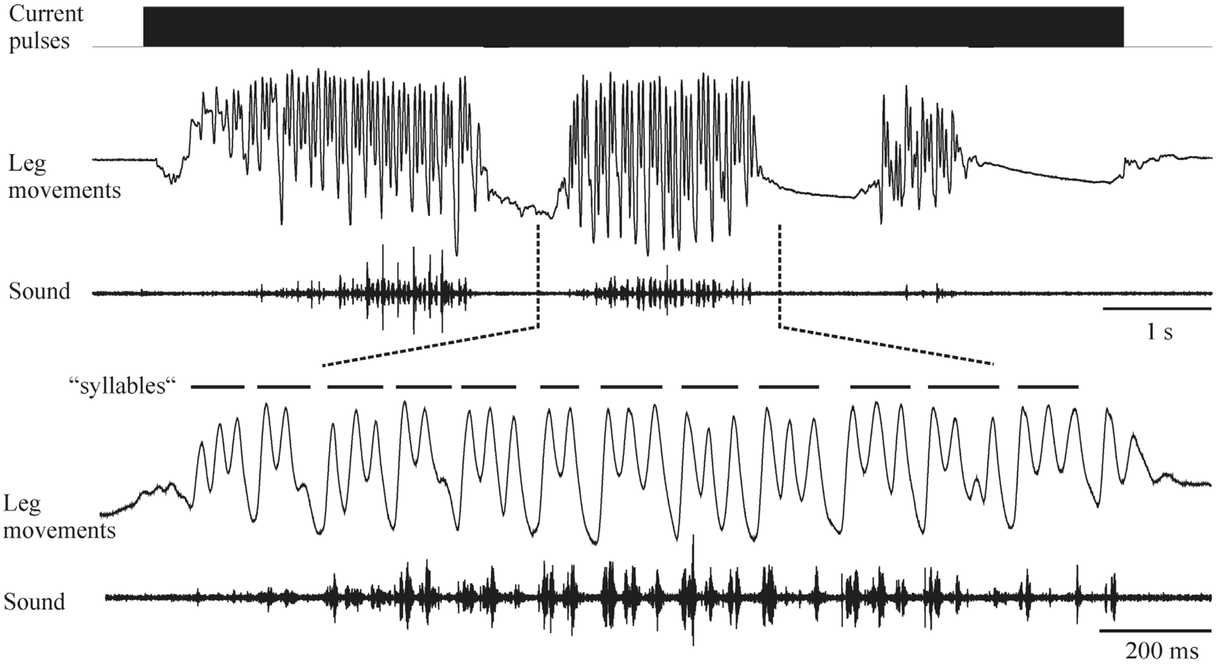

Fig. 3. Electrical stimulation in the deuto-/tritocerebrum of a female *Ch. biguttulus* releases stridulation. Current pulses were injected in the deuto-/tritocerebrum, close to the course of the cerebro-cervical fascicles that include the axons of stridulatory command neurons and leg movements grouped into three phrases were recorded using position detectors. One phrase displayed with extended timescale shows the typical structure of stridulatory hind leg movements consisting of syllables (indicated by black bars above the trace) and pauses. The typical pulsed sound of female songs is shown as well.

(e.g. Fig. 4A, DT, LP) and then only such larger movements may have produced sound. Irregularities could have resulted in part from co-activation of other leg movements that superimposed on stridulation. These movements, however, were much slower than stridulation (see Fig. 4B LP). We defined hind leg movements as stridulation, if regular repetitions of up-and-down movements with periods between 20 and 50 ms occurred and at least occasional larger movements delimiting "syllables" were seen as well (see Fig. 4A,B, extended portions; for

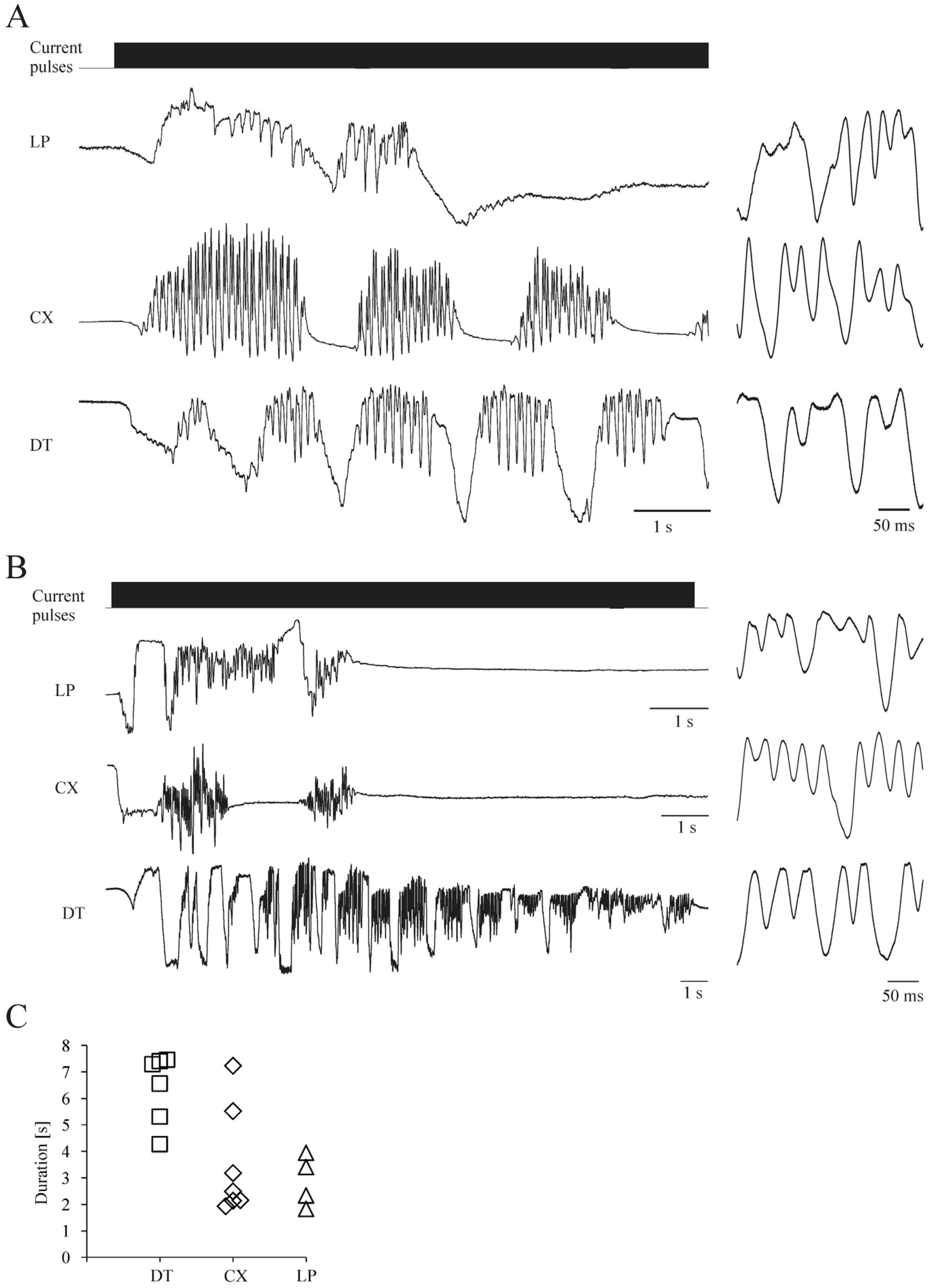

Fig. 4. Electrical stimulation of stridulation in brain regions that receive auditory information. (A) Current pulses into the lateral protocerebrum (LP), the central complex (CX) and the deuto-/tritocerebrum (DT) elicit stridulatory hind leg movements that are similar to the natural stridulation of *Ch. biguttulus* females. Complete responses to equivalent durations of stimuli (left) and part of the stridulatory hind leg movements at higher temporal resolution (right). (B) Further examples of current evoked stridulation with patterns that look less species-typical. Either there are slow movements superimposed (LP) or few syllables with many pulses are produced (CX) or there is nearly continuous stridulation throughout the stimulation with only short pauses (DT). Note the different timescales for the complete songs. The enlarged portions on the right show the syllable-pulse-patterns (identical time scale). (C) Sum of stridulatory movements elicited by current stimuli of 7.6 s duration in 6 (DT), 7 (CX) and 4 (LP) preparations.

peculiarities of female stridulation as opposed to male stridulation see discussion). As the movements on an extended time scale on the right of Fig. 4A demonstrate, hind leg movements stimulated at all three locations (LP, CX and DT) could clearly be identified as stridulation. Continuous electrical stimulation elicited songs that were separated in phrases. In 8 of 13 songs, which could be measured and had more than one phrase, phrase durations decreased as is typical for normal singing (Fig. 4A). In other experiments phrase duration varied irregularly or could not be determined unequivocally due to irregularities at the beginning or end of a stridulatory phase.

The latency between stimulus onset and start of stridulation differed between less than 100 ms and about 1 s. Average latency was shortest in DT (344 ms, n = 6, range 150 to 450 ms) and longest in CX (489 ms, n = 7, range 60 to 990 ms), but determining exact latency was not possible in all cases. If the electrical stimulus ended while the animal was still stridulating, leg movements immediately stopped (e.g. Fig. 4B, DT). This was similar at all stimulation sites. During electrical stimulation in the tritocerebrum, grasshoppers continuously generated up to 7 phrases throughout the entire stimulation time. Sometimes, obvious phrase pauses were entirely absent or much shorter than one second

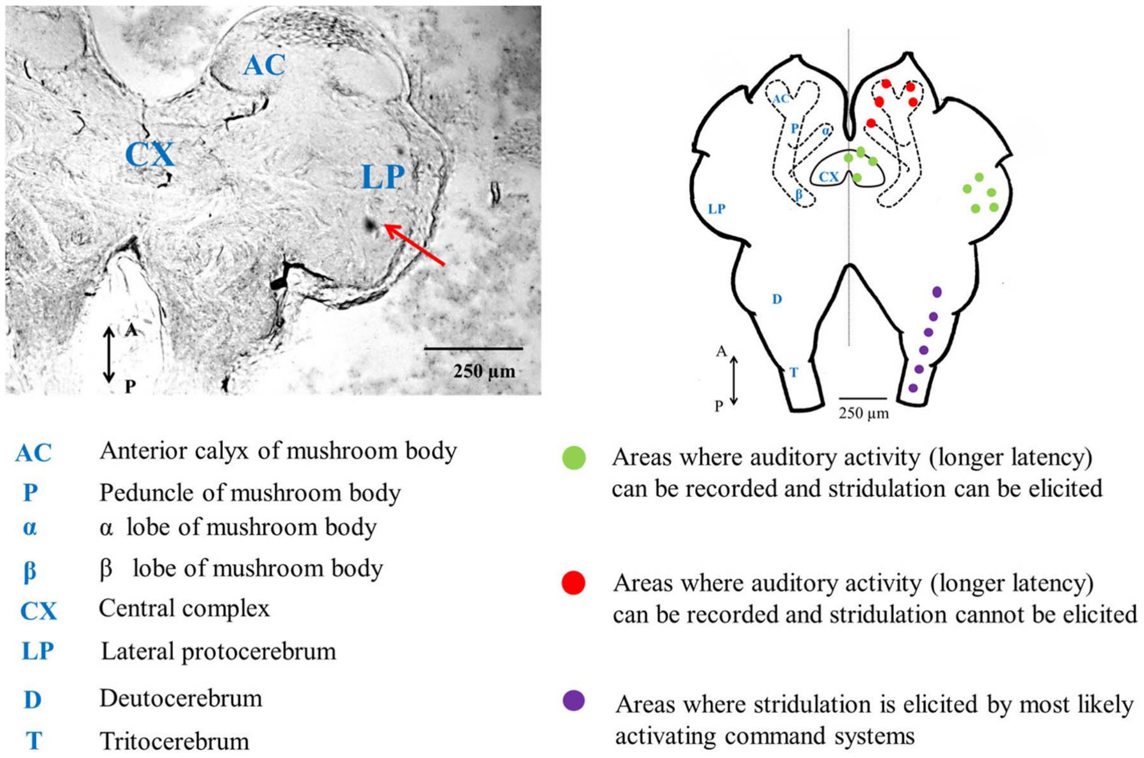

Fig. 5. Localization of recording and stimulation sites in the brain of *Ch. biguttulus* females. Left: A recording/stimulation site in the lateral protocerebrum that was labeled by electrocoagulation (highlighted by a red arrow). Right: Schematic of the brain (without optic lobes) with marked sites where acoustically-stimulated neural activity was recorded and/or stridulation was induced by current injection. The area of the mushroom body (shown as dotted lines) was not visible in the original electrocoagulation sections and so it is approximated by comparing the sections with immunofluorescence sections. Exact locations may be above or below the α-lobe of the mushroom body. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4B, LP and DT). In contrast, stridulatory activity during electrical stimulation in the lateral protocerebrum and the central complex usually stopped after 4–6 s despite an ongoing longer stimulus. Therefore, overall duration of stridulation (sum of entire stridulatory activity) was in general longer in DT than in CX and especially LP (Fig. 4). Repetitions of electric stimulation at a particular stimulation site after pauses of few seconds elicited stridulatory responses that were either nearly identical or showed at least very similar course of movement patterns and activity periods.

3.3. Localization of recording and stimulation sites

After recording and/or stimulation, the position of the tip of the multielectrode within the brain tissue was marked by passing DC-current (100 µA) for 5 min between one of the tungsten wires and the reference wire (Bhavsar et al., 2015b) or between two tungsten wires. This procedure coagulates the brain tissue and generates a black spot in the immediate vicinity of the electrode (Fig. 5 - left). Fig. 5 provides a summary of locations where auditory activity was recorded and stridulation could be elicited. The red dots (Fig. 5 - right) show locations in the superior medial protocerebrum, at which auditory activity was detected, but consistently no stridulation could be elicited. Green dots represent sites in the lateral protocerebrum and the central complex where auditory activity was recorded and stridulation could be elicited by 50 µA current injection.

4. Discussion

In acoustically communicating grasshoppers, a receptive female recognizes a conspecific male's song and if motivated may produce a response song (Hedwig, 2001; Heinrich et al., 2012; Ronacher, 2014). Auditory information is initially relayed to the third thoracic ganglion complex, but neural circuits that perform the final steps of song recognition are obviously situated in the brain (Bauer and von Helversen, 1987). Therefore, one would expect to find brain neuropils, in which "higher order" brain neurons selectively respond to the same stimuli that trigger specific behavior (for crickets see for example Schöneich et al., 2015). On the other hand, in grasshoppers, the neuronal network that suffices to generate the excitation pattern for species-specific song production is located in the metathoracic ganglion. It is controlled, however, by command neurons descending from the brain (Hedwig, 1994; Hedwig and Heinrich, 1997). These command neurons are likely to be activated by higher order neuronal networks located in the brain (Hedwig, 2001). So far, only a few local brain neurons have been described, that may be involved in song processing (Kutzki, 2012). Here, we identified brain regions which likely represent neuropils not only processing auditory information, but potentially being also involved in the initiation of stridulation - a specific motoric behavior of female *Ch. biguttulus* typically only occurring in response to a particular stimulus (attractive song).

4.1. Recording auditory activity in the brain of grasshoppers

Most described ascending auditory interneurons originate from the metathoracic ganglion (Stumpner and Ronacher, 1991), enter one hemisphere of the brain and terminate in the lateral protocerebrum (Eichendorf and Kalmring, 1980; Hedwig, 1986; Kutzki, 2012). We recorded auditory activity in various brain regions, especially in the lateral protocerebrum, the superior medial protocerebrum and the central complex. Latencies of responses detected in these neuropils on average were about 4 ms longer than the response latencies of the ascending interneurons recorded in the deuto-/tritocerebrum (Bhavsar et al., 2015a), suggesting that they may receive excitation from the ascending neurons, either directly or indirectly. Interestingly, however, average latencies of local neurons in the LP, in the SMP and in the CX were similar. Therefore, such different neuropils may not represent, as one might assume, consecutive stations of auditory processing, but rather be locations of parallel processing, as has been suggested earlier for the processing of sound pattern and sound direction (von Helversen, 1984; Stumpner and Ronacher, 1994). No direct evidence supporting this idea can be found in our data, however. Additionally, one has to bear in mind that first spike latencies may be considerably longer than EPSP-latencies – especially in brain neurons (e.g. Ostrowski and Stumpner, 2010; Schöneich et al., 2015). Therefore, huge differences in latencies as observed in the CX or apparent groups of latencies as seen in the LP may, but need not necessarily indicate different levels of processing.

The majority of auditory responsive units responded with relatively few spikes at stimulus onset. There were no units showing a preference for conspecific syllable-pause-patterns as found in the behavior. However, some phasic syllable onset-coupling has been seen, as is typical also for several ascending neurons (Stumpner et al., 1991). Interestingly, in the SMP some neurons responded rather abruptly much better to patterns with long pauses, which might be interpreted as a detection of behaviorally ineffective syllable pause combinations due to too long pauses. Combining excitation of a neuron like AN12 (Stumpner et al., 1991) and inhibition by these neurons might lead to an optimum type response as is found in behavior. All in all, responses were rather variable and any preferences did not occur consistently, when variations of pause durations were compared at different syllable durations. This raises the question about interindividual and intraindividual neuronal variability. It has been described for the auditory system of grasshoppers that response variability increases with level of processing (Vogel et al., 2005). Given the sparse responses typical for brain neurons (Schildberger, 1984; Ostrowski and Stumpner, 2010; Schöneich et al., 2015), it may not be too surprising that the units presented here – which clearly aren't feature detector units - are not very reliable in their spiking activity. By our extracellular recordings and without morphological criteria it was not possible to identify the same units in different individuals and therefore judge interindividual variability. It can be stated though, that a sparse, more or less phasic response type is typical for the vast majority of units recorded in the grasshopper brain.

Although single units were recorded that showed weaker responses to syllables with gaps (which decreases their attractiveness to elicit a response song), their discrimination was less strict than the discrimination already achieved by the AN4 neuron ascending from the thorax to the brain (Ronacher and Stumpner, 1988). Therefore, no "feature detector neurons" being more selective than metathoracic neurons were found in the brain neuropils. One reason for this could be that extracellular electrodes, like the multielectrodes used in our study, preferably detect signals from neurons with larger axons in the vicinity of the tip while small neurons impact extracellular electrical fields weakly (Bhavsar et al., 2015b). Since feature detector neurons – especially those not involved in predator avoidance behavior - may be small neurons they might be underrepresented in such recordings.

4.2. Electrical stimulation in insect brains

Induction of specific motoric behaviors by extracellular electrical stimulation in the brain has previously been described for several insects. For example, Wadepuhl (1983) used sharpened metal wires to stimulate courtship behavior in the grasshopper *Gomphocerus rufus* and Hedwig, (1986) stimulated stridulation in the grasshopper *Omocestus viridulus* by current injection through suction electrodes attached to the surface of particular brain regions. Bender et al., (2010) used multielectrodes that enabled both the recording of neuronal activity and the stimulation of neurons in the central complex of the cockroach *Blaberus discoidalis* (see also Martin et al., 2015). They found a correlation of neuronal activity with stepping patterns, speed and directional changes during walking and were able to modify these patterns by electrical stimulation. Using electrodes for both extracellular recording and stimulation provides the opportunity to stimulate neurons in neuropils that have been demonstrated to be concerned with the processing of particular information in the same individual. Placing the stimulation electrode very close to the neurons whose stimulated activity trigger a particular motoric response goes along with the requirement of only small current amplitudes for neural activation, that don't induce damage of neural tissues. However, the range of current spread from the stimulation electrode through the tissue, and hence the numbers of activated neurons, is difficult to assess (Wadepuhl, 1983).

4.2.1. Electrical stimulation of auditory brain neuropils in the grasshopper *Ch. biguttulus*

Here, we used multielectrodes (Bhavsar et al., 2015a) and induced stridulation by extracellular current application in brain regions – likely neuropils - that receive auditory information. The patterns of stridulatory hind leg movements induced by electrical stimulation were quite variable but similar in the LP, the CX and the DT, even though often more regular in DT (Fig. 4A). A similarity of movements is not surprising since thoracic pattern generators are regulated by descending command neurons which, either directly or indirectly, must have been activated by electrical stimulation at all three sites. Stimulation remained consistently unsuccessful in the SMP (Fig. 5, area with red dots). One interpretation for this finding might be that auditory information which is relevant for song pattern recognition and the generation of appropriate behavioral responses is most likely relayed from the LP to the CX and the command systems. Another interpretation might be that stimulation in LP and CX activates command neurons indirectly through additional (auditory or non-auditory) neurons, while such neurons are not activated by stimulation in the SMP. However, from the neuronal responses there is no indication that neurons in the SMP might be less relevant for song processing than neurons in other brain neuropils. A further interpretation, of course, also might be that command neurons are directly activated at all three sites (DT, LP, CX). Given the described arborizations of command neurons in the grasshopper *Omocestus viridulus* (Hedwig and Heinrich, 1997) or a local brain neuron involved in eliciting stridulation in *Ch. biguttulus* (Hedwig, 2001) direct activation cannot be excluded. Also, command neurons do not project into the SMP. However, whereas stimulation in the DT mostly elicited stridulation (continuous or in phrases) throughout the entire duration of the electrical stimulus, this was typically not the case in the other two regions indicating that the activated brain neurons were not (or at least not always) the same when stimulating the different regions. In accordance with this, the longest (measurable) latency in DT was 450 ms, in CX 990 ms and in LP 1070 ms. On the other hand, short latencies were found in all three regions and, therefore, some of the stimulations in LP and CX might have activated the command system more directly than others.

Electrically stimulated stridulatory activity was frequently subdivided into phrases separated by pauses, which corresponds to natural songs of this species. Natural songs of *Ch. biguttulus* females have been reported to contain phrases of 1–1.5 s (von Helversen and von Helversen, 1975) and pharmacological stimulation of the central body elicited phrases of 1.5–2 s (Weinrich et al., 2008). The average phrase duration in our experiments was well within this range, but also longer and shorter phrases occurred. One should have in mind, though, that at lower temperatures as in the experiments presented here stridulation is slower (von Helversen, 1972) and that *Ch. biguttulus* females generally produce songs with lower accuracy than males, with respect to general temporal structure and precision of movement and sound patterns (von Helversen and von Helversen, 1997). Nevertheless, about sixty percent of the evaluated songs showed a decreasing duration of subsequent phrases, which is typically observed also in naturally singing *Ch. biguttulus*. Similar observations as with electrical stimulation were made, when central complex neuropils were stimulated with cholinergic agonists (Wenzel et al., 2002; Weinrich et al., 2008). The phrase-pause structure of *Ch. biguttulus* likely is generated by neurons located downstream of the command neurons, which is consistent with the fact that described command neurons in grasshoppers are tonically active without modulation in the stridulatory rhythm (e.g. Hedwig, 2001) and with the observation that activation of stridulation by picrotoxin application to the brain does not abolish the phrase and pause patterns of the songs (Heinrich et al., 1998). Therefore, it is surprising that in several experiments, especially when stimulating DT, a continuous stridulation was elicited (Fig. 4B). This stridulation contained syllables and pulses or mainly pulses. Perhaps, different command systems may have been activated leading to conflicting commands in the thorax.

In the deuto-/tritocerebrum, stimulation of stridulation was effective at the same positions of the multielectrode where auditory neuronal activity – likely from ascending neurons - was recorded. Likely, electrical stimulation induced stridulation by direct activation of the descending pathways rather than by activating ascending auditory neurons. This is supported - among others - by the ongoing activation of stridulatory sequences during the whole current application in the tritocerebrum. The lateral protocerebrum receives excitation from ascending auditory interneurons that are also potentially activated by the electrical stimulus. However, electrical stimulation of the lateral protocerebrum stimulated shorter stridulation. This may suggest that here output neurons of the LP and not command neurons may have been electrically stimulated, even though command neurons extend arborizations into lateral regions of the protocerebrum (Hedwig and Heinrich, 1997). Also electrical stimulation in the CX stimulated stridulation at sites, where auditory activity with good signal-to-noise ratio was recorded. The central complex has previously been described as a pre-motor center for stridulation and other behaviors and stridulation in *Ch. biguttulus* and other grasshoppers can be stimulated by injection of drugs mediating neuronal excitation or disinhibition (Heinrich et al., 1998, 2001; Weinrich et al., 2008). Since cholinergic receptor-expressing columnar output neurons receive auditory excitation and sufficiently strong activation of these neurons can initiate stridulation, it is not unlikely that electrical stimulation – at least in some preparations (see above) - activated one or several central complex neurons of this type. Processing of auditory information in the SMP has not previously been reported and stridulation could not be elicited in this brain region. However, one study described successful electrical stimulation of stridulation in the mushroom bodies of another grasshopper species (*Gomphocerus rufus*, Wadepuhl (1983)). The inability to elicit singing from the anterior brain in *Ch. biguttulus* fosters the interpretation that successful stimulation from other neuropils was not just due to a broad spread of excitatory currents throughout large portions of the entire brain. This is also supported by the facts, that (i) at all sites the same minimal current was effective to elicit stridulation – apparently, this current was necessary to bring neurons in the vicinity of the electrode to sufficiently high firing rates – and (ii) an increase of current strength above threshold did not alter its effect, indicating that stronger currents do not reach neuropils far away from the electrode.

5. Outlook

Using a newly developed multielectrode stimulation technique, the present study reveals a brief insight into the location of brain neuropils and potential flow of information related to the song recognition in acoustically communicating grasshoppers. The results presented here indicate that the stridulation in *Ch. biguttulus* is controlled by premotor auditory neuropils located in the central complex and lateral protocerebrum. These neuropils have to be studied further with intracellular electrodes to learn more about processing, since the relevant neurons, especially if small, will not be effectively picked up by the extracellular multielectrodes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2017.03.006.

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