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Possibilities offered by implantable miniaturized cuff-electrodes for insect neurophysiology

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ABSTRACT

Recent advances in microsystems technology led to a miniaturization of cuff-electrodes, which suggests these electrodes not just for long-term neuronal recordings in mammalians, but also in medium-sized insects. In this study we investigated the possibilities offered by cuff-electrodes for neuroethology using insects as a model organism. The implantation in the neck of a tropical bushcricket resulted in high quality extracellular nerve recordings of different units responding to various acoustic, vibratory, optical and mechanical stimuli. In addition, multi-unit nerve activity related to leg movements was recorded in insects walking on a trackball. A drawback of bi-polar nerve recordings obtained during tethered flight was overlay of nerve activity with large amplitude muscle potentials. Interestingly, cuff-electrode recordings were robust to withstand walking and flight activity so that good quality nerve recordings were possible even three days after electrode implantation. Recording multi-unit nerve activity in intact insects required an elaborate spike sorting algorithm in order to discriminate neuronal units responding to external stimuli from background activity. In future, a combination of miniaturized cuff-electrodes and light-weight amplifiers equipped with a wireless transmitter will allow the investigation of neuronal processes underlying natural behavior in freely moving insects. By this means cuff-electrodes may contribute to the development of realistic neuronal models simulating neuronal processes underlying natural insect behavior, such like mate choice and predator avoidance.

1. Introduction

Neuronal processes underlying behavior tackles neuroscientists since half a decade [1,2]. In many studies insects are used as model organisms because of their rather primitive neuronal architecture and stereotyped behavior in response to external cues, such like mating displays. This research already contributed to the development of computational neuronal networks that explain the neuronal processes underlying insect locomotion and flight. However, neuronal response as well as neuronal network activity may change according to the behavior context [3,4], which makes it necessary to study neuronal activity in naturally behaving insects. Therefore, neuronal activity should be recorded when insects are free to walk or to fly. In an early study of Stout [5] suction electrodes were used to record from the cervical connectives of a cricket while the animal was walking. However, due to technical problems related to the additional weight load arising from the electrode and the syringe, this technique has not found acceptance among scientists. Instead thin copper, tungsten or silver electrodes were later used for the recording of extracellular neuronal signals in insects [e.g. 6,7]. Extracellular multi-unit nerve recordings from the ventral nerve cord of a freely moving and flying insect were made by [5,8]. Some electrode wires were implemented in a clip electrode in order to prevent artifacts arising from a weak contact of the electrode and the nerve [9–11]. These electrodes may be regarded as precursors of modern miniaturized cuff-electrodes.

In contrast to extracellular electrodes, intracellular microelectrodes are used to record from identified single units with the drawback of a limited radius of action of the insect under study (see summary in [12]). Insects on a trackball overcome this problem in an elegant way, while simultaneously allowing researchers the recording of single neuronal units during natural behavior, such like phonotaxis in the context of mate choice [13]. Surprisingly, some researchers managed intracellular recordings even during tethered flight (a summary is given in [14]). Nevertheless, extracellular recordings are technically less demanding and allow the recording of neuronal activity for long periods of time. After recording, the identification of certain neuronal units

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in a multi-unit recording is often possible by means of spike shape, but also response properties like response latency, spike rate adaptation and habituation. In future a combination with a light-weight amplifier connected to a wireless transmitter system and implantable electrodes offer the possibility to study extracellular neuronal activity of different neuronal units while the insect is free to walk or to fly.

Takeuchi and Shimoyama [15] fabricated two different types of implantable electrodes and successfully performed recordings from the ventral nerve cord of cockroaches. However, only recently the fabrication of flexible electrodes based on polymer substrate and metallization layers was realized [16,17]. This technique is based on modern microsystems technology and allows the fabrication of implantable electrodes with all necessary characteristics in a precise and flexible manner [18,19]. Flexible multichannel cuff-electrodes offered new possibilities for selective stimulation [20] of small peripheral nerves in rats as well as for recording neuronal activity in humans [21]. Nowadays this kind of electrode is frequently used in many research applications, and is even transferred in chronic implants in human clinical trials because of high biocompatibility and an easy way of implantation [22].

Miniaturized flexible cuff-electrodes offer the possibility for long-term neuronal recordings in intact insects. In this study, we investigated the stability and performance of neuronal recordings obtained with such cuff-electrodes after implantation in the neck of a medium-sized tropical bushcricket (*Mecopoda elongata*). Extracellular neuronal activity was recorded during acoustic, vibratory, optical and mechanical stimulations. Except for acoustic stimulations, signals were not calibrated because the intention of this study was to explore the usefulness of this kind of electrode for experiments in which different signal modalities are used. In this study the action range of insects was limited by the length of the wire connecting the junction part of the electrode with the amplifier. Extracellular discharges were sorted according to spike shape using a commercially-available spike sorting tool or a custom-written spike sorting algorithm. Additionally, the frequency response and electric characteristics of cuff-electrodes and the amplifier was measured in order to estimate the degradation of spike shape caused by the recording system.

2. Materials and methods

2.1. Miniaturized cuff-electrode

In this study flexible micromachined tripolar cuff-electrodes [19,23] of small size were modified in order to record neuronal activity in insects. Cuff dimensions were: 5.7 mm length, 4.14 mm width, 10 μm thickness (Fig. 1C). The cuff electrode was manufactured in a microsystem process in which platinum as interconnect and electrode material was sandwiched between two layers of polyimide that served as substrate and electrical insulation. Three electrodes with a rectangular geometrical area of 0.94 mm^2 (0.2 mm × 4.7 mm) each were linearly arranged at a distance of 1.5 mm. To generate active recording sites, the top polyimide layer was opened in the area described above. The electrode surface consisted either of platinum or the surface was increased through electrochemical deposition of platinum black, a nanostructured enhancement of the platinum surface improving signal acquisition capability and biocompatibility [17]. A more detailed fabrication procedure for the production of the electrode is described in [23,24]. The planar electrode was brought into a

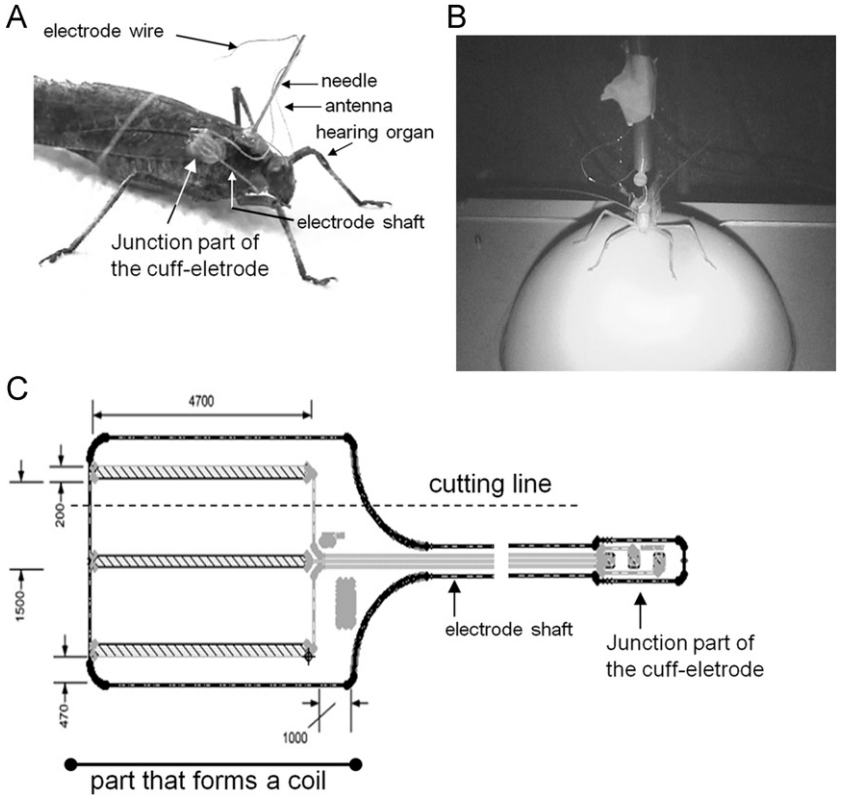

Fig. 1. Cuff-electrode and its application. (A) A tropical bushcricket (*Mecopoda elongata*) wearing an implanted cuff-electrode and a needle that allows fixating this insect in a holder for nerve recordings during spontaneous walking on a trackball (shown in B). The wires connecting the junction part with the amplifier were twisted around the needle. (C) Planar view of a flexible tripolar spiral cuff-electrode. The electrode was trimmed to two channels before implantation (indicated by a dotted line). Dimensions in C are given in μm.

cuff shape by tempering the planar electrode with a diameter of 0.8 mm. In order to reduce the size of the cuff to fit the dimension of the neck of *M. elongata*, a tropical insect of about 3 cm body length, one electrode was removed from the original design as indicated by a dotted line in Fig. 1C.

Each cuff-electrode system was characterized in its electrical impedance characteristics and frequency response before its application [25]. Using impedance spectroscopy characteristic values like electrode capacity and line resistance have been measured [26]. This also includes linearity of the amplitude and phase of the electrodes in the desired frequency band that corresponds to the frequency composition of extracellular neuronal signals (500 Hz–5 kHz). Since main frequency components of neural signals can be expected in the range of 1 kHz, the impedance of all electrodes were set to the lowest possible value at this frequency. In addition, the electrode was fabricated to exhibit a linear behavior with respect to gain and a low phase angle in the frequency range of extracellular signals in order to avoid degradation of signal shape. Electrode impedance and phase angle was measured in a frequency range between 10–10^6 Hz using a sinus of 10 mV amplitude in Ringer's solution (Merck, Germany).

2.2. Amplifier

For extracellular neuronal recordings a recently described amplifier was used [27]. Amplification of this amplifier was fixed to a factor 1000. A band-pass filter between 250 and 5000 Hz delineates the frequency response characteristics with a high common mode rejection ratio of 107 dB at 200 Hz. For verification of the stated capabilities, the amplifier was characterized in order to make sure that its response behavior is suitable for recording neural signals picked up by a cuff electrode (see Fig. 2B). Like the electrodes, the transfer characteristics of the amplifier should not change the original shape of the extracellular nerve signal much.

2.3. Experiment setup and animal preparation

Since electrode implantation is more difficult in small insects, we decided to use *Mecopoda elongata*, a tropical bushcricket species with a body length of about 3 cm (excluding wings), in this study. These insects belong to the family of tettigoniidae and were originally collected in the tropical rainforest of Malaysia. All individuals used in cuff-electrode experiments were taken from an established breeding culture housed in the Zoology Department in Graz.

Animals were anesthetized with chlor-ethylene gas for 1 min and then fixated with Patafix® clay (UHU®). The insect was positioned ventral side up (with legs restrained) and a longitudinal incision was made in the ventral cuticle of the neck. After exposing one connective, a trimmed cuff-electrode with only two channels was brought beneath the connective. The spiral cuff was opened with the help of a blunt insertion tool and then the electrode wrapped itself around the connective. A prevention of microbial infections was achieved by performing all manipulations under semi-sterile conditions. The incision was closed with the remaining cuticle and covered with a droplet of petroleum jelly. A droplet of melted dental wax as put on the jelly so that two ventral processes of the prothorax kept the electrode in position after solidifying. Finally, the junction part of the electrode was fixated with bee wax on the lateral side of the wing basis (Fig. 1A). An indifferent copper wire electrode was inserted in the abdomen and fixed with super glue. Amplifying electrode signals against this electrode yielded a much better signal-to-noise ratio compared to a differential amplification of both cuff-electrodes against each other. Therefore, the electrode with the

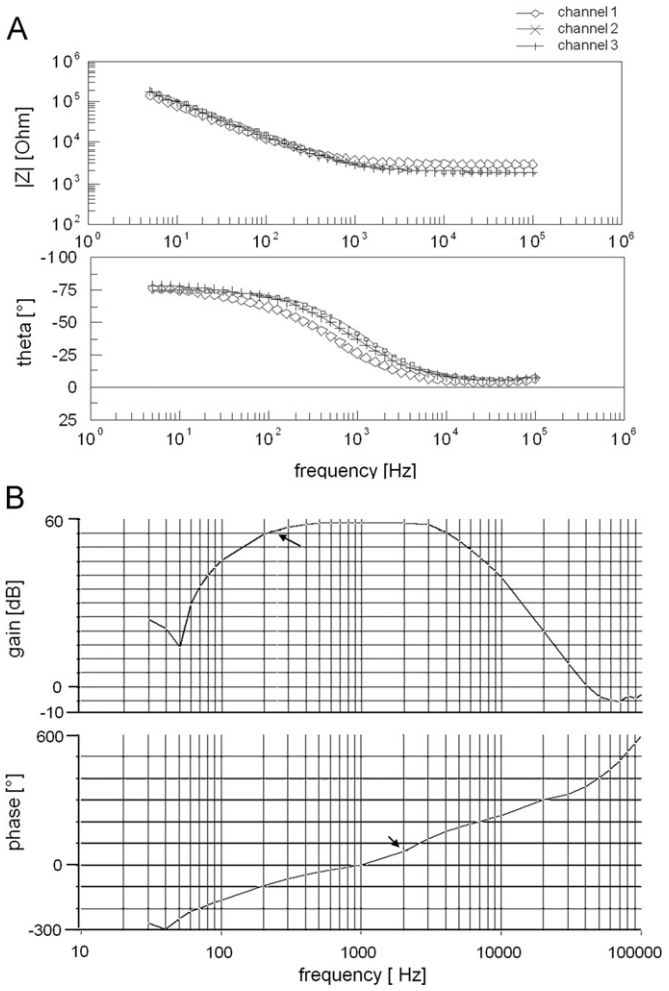

Fig. 2. Impedance spectroscopy of electrodes and electric properties of the amplifier system. (A) Magnitude (upper graph) and phase (lower graph) of the impedance of a cuff electrode; each curve represents one of the three electrodes. Note that the variation between different electrodes is very low and the impedance is nearly linear in the range above 1 kHz. (B) The amplification characteristics (upper graph) and the phase behavior (lower graph) of the amplifier system. The lower 3 dB threshold in the upper graph is found at 250 Hz (arrow) and the upper 3 dB threshold was found at 3.5 kHz. In the lower graph the arrow indicates the phase of 80° at 2 kHz.

best signal-to-noise ratio was selected and amplified against the abdominal reference electrode after the insect was freed from Patafix®.

Amplified electrode signals were recorded by means of an A/D board using a sampling rate of 44.1 kHz (Power 1401, CED, Cambridge). All experiments were performed at an ambient temperature of 22 °C in a dark room with walls equipped with acoustic foam. The amplitude of extracellular nerve signals picked up by the cuff-electrode exhibited amplitudes in the range of 50–200 μV. Since this amplitude showed some variability from preparation to preparation, we decided to label the amplitude of nerve recordings in some of the figures as arbitrary units (a.u.).

2.4. Insects walking on a trackball

Studying neuronal activity during walking was achieved by allowing insects to walk on a track ball floating on air. In order to center insects on top of the track ball, a steel needle was mounted on the dorsal side of the pronotum using super glue. Then the insect was fixated with this needle in a holder above the track ball (Fig. 1B). In order to keep locomotion on the trackball as simple as

possible, individuals which already lost both hindlegs were selected for this experiment. Such insects showed normal four leg locomotion.

The behavior of the animal was recorded by means of an infrared sensitive camera (Nightshot® technology, Sony Corp. Japan) in complete darkness. One audio channel of this camera recorded the amplified nervous activity (see Section 2.2) using a sampling rate of 44.1 kHz and 16 bit resolution. This method allowed a synchronization of video data with neuronal activity.

2.5. Spike sorting algorithm

Multi-unit nerve recordings deserve an elaborate spike sorting in order to isolate certain neuronal units. Digital filtering of nerve recordings and spike sorting of multi-unit recordings was performed in Spike2 (Version 5.2, Cambridge Electronic Design). After neuronal recordings passed a band-pass filter with cut-off frequencies at 500 Hz and 5000 Hz, the amplitude threshold for automatic spike template generation was manually adjusted and stimulus associated units were extracted from neuronal background according to their shape. A new "wavemark template" was automatically generated after the detection of at least 8 similar spikes with a frequency of occurrence of at least 1 out of 50 spikes. A minimum length of 60 points was necessary for template generation. In addition to the spike sorting performed in Spike2, multi-unit neuronal activity elicited during playback of a conspecific signal (acoustic chirp of a male) were sorted by application of a highly sophisticated spike sorting algorithm that was programmed after [28,29].

3. Results

3.1. Characterization of electrodes and amplifier

Fig. 2A shows results obtained by electrical impedance spectroscopy applied to cuff-electrodes in a three-electrode setup with amplitude of the sine wave of 10 mV. In Ringer's solution the impedance was 3 kΩ with a phase angle of 40° at 1 kHz. A typical high-pass behavior of the electrode can be seen in the desired frequency range between 0.5 and 5 kHz in which the resistive part of the electrode was about 3 kΩ—the so called access resistance of the Ringer's solution and the electrode and almost a linear change of the phase were found. The characterization of amplifier properties proved the ability to record neural signals in the range of 0.25 to 5 kHz with a linear amplification of 55 dB and a 0° phase shift at 1 kHz. A linear phase shift in the desired frequency range of 0.25 to 5 kHz was also found in the electronic properties of the amplifier (Fig. 2B). Although the original signal was amplified linearly, a minimal distortion of different frequencies occurred due to the use of a Butterworth filter with low-pass properties in the amplifier design. However, with a 0° phase shift at 1 kHz the influence is expected to be marginal, since the frequency composition of extracellular action potentials can be found in a frequency range of 800–1.8 kHz. Both, the electric properties of the electrode as well as amplifier properties guarantee the conservation of the original shape of extracellular neuronal signals. This is very important for a proper performance of spike sorting algorithms clustering spikes of different neuronal units according to their shape.

3.2. General remarks about cuff-electrode recordings

The behavior of insects (*Mecopoda elongata*) observed after cuff-electrode implantation was not much different from the behavior observed before implantation. This means that insects were able to walk, eat, fly (tethered) and sing (observed in only one male) after cuff implantation. Chronic implantations between the suboesophagial and thoracic ganglion resulted in high-quality nerve recordings in all 13 individuals used in this study. Importantly, unipolar amplification against an abdominal reference electrode yielded much better signal-to-noise ratios compared to nerve recordings in which both cuff-electrodes were amplified against each other (bipolar). After sorting spikes according to their shape, it was possible to map the response to single neuronal units. The electrode was successfully reused several times after removing dental wax and carefully rinsing it with 70% isopropanol.

3.3. Spontaneous walking

First, neuronal background activity was studied while the insect was standing still on a trackball (Fig. 1B). In this situation a multi-unit response of about 60 Hz was observed by taking all units into account exceeding half maximum spike amplitude (Fig. 3, top trace). Single ipsilateral (right) as well as contralateral leg movements of insects walking on a track ball were accompanied by characteristic nervous discharges. Especially the lift of legs and the beginning of the stance phase increased the neuronal activity of certain units (see color code in the examples given in Fig. 3). Neuronal activity was found to be especially high when two legs simultaneously touched the trackball.

3.4. Mechanical stimulation

Touching one antenna of the insect with a grounded pencil resulted in a strong neuronal response (Fig. 4A). Large amplitude units contributing to this discharge may be analogous to the large amplitude descending mechanosensory interneurons identified in cockroaches [11,30,31]. A wind puff towards the head of the insect evoked a massive neuronal discharge in the nerve recording (Fig. 4B). This is most likely the response of descending mechanosensory interneurons (DMIs) [11,30].

Soft vibrations applied to the track ball, on which the insect was standing, evoked a strong neuronal response (Fig. 4C). It was possible to sort units generating small spike amplitudes from units characterized by large spike amplitudes. A candidate of the latter is most likely TN-1, a multi-modal neuron responding to high-frequent sound signals as well as to vibratory signals (e.g. [32]).

3.5. Light stimulus

Opening as well as shutting a light source (light bulb) elicited a burst of neuronal discharge consisting of large amplitude units (Fig. 5A). A shadow quickly moving across insect's eyes also evoked extracellular discharges (Fig. 5B). Some of these units responding to sudden changes of light conditions are most likely analogous to descending movement detector neurons known to respond to on-off stimulations and certain object movements in acridids (grasshoppers) [33]. One of these units receives its input via a spike-transmitting electrical synapse [34] from the lobular giant movement detector (LGMD) [35], presumably to arouse the animal, or trigger startle behavior, such as a jump (e.g. [36]).

3.6. Motion stimulus

More recent work has shown that DCMD (descending contralateral movement detector) responds to objects expanding in the visual field (looming stimulus) and therefore suggests this neuron to play an important role in crash-avoidance of flying locusts (e.g. [37–39]). In *M. elongata* homologous units were excited by an object swiftly approaching the insect in the frontal field (diameter = 8 cm). This looming stimulus evoked DCMD discharges characterized by considerable firing rate adaptation (Fig. 5C). In one individual, two

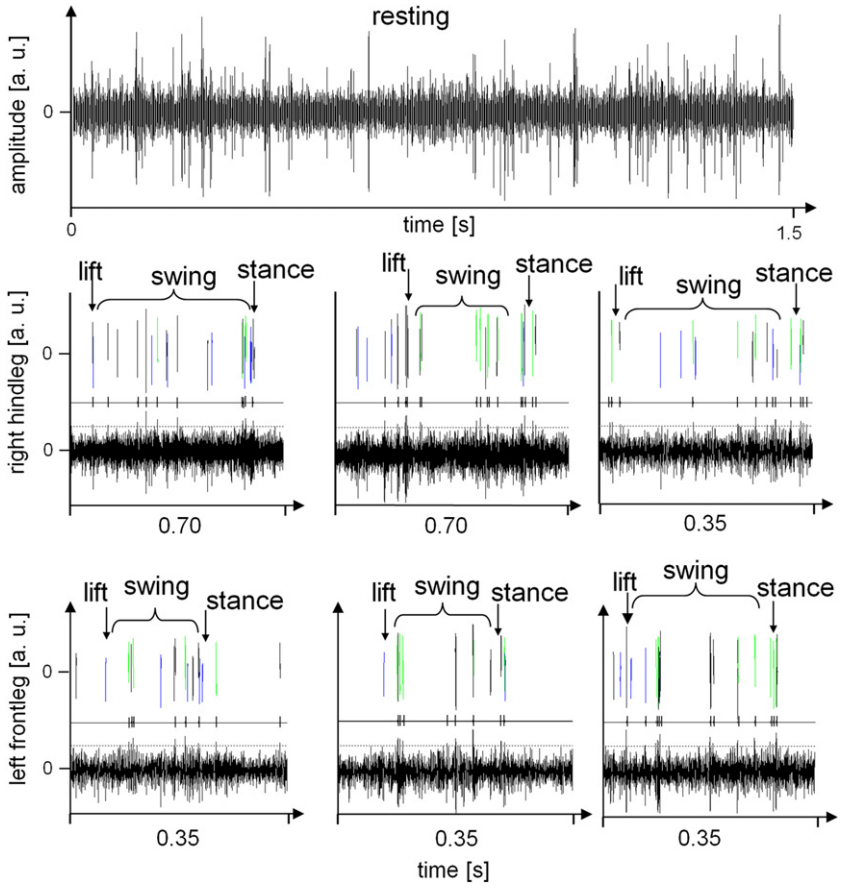

Fig. 3. Neuronal recording in a walking insect. Neuronal activity observed during resting (upper trace) and during single leg movements (6 panels below) of an insect walking on a trackball. Lower traces show original nerve recordings and ticks in the middle indicate spike events that exceeded a manually defined amplitude threshold (dashed horizontal line). Suprathreshold extracellular discharges were sorted according to their shapes. Large amplitude discharges assigned to the same cluster were plotted in the same color code (upper trace).

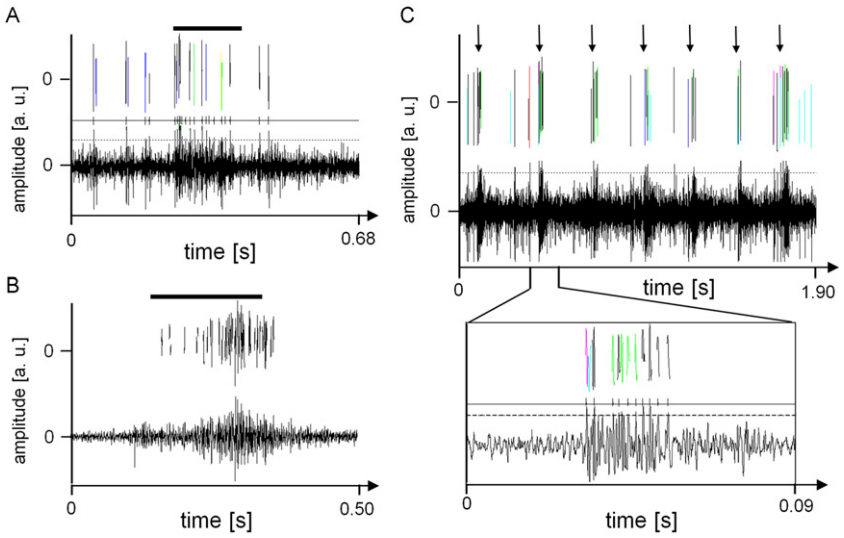

Fig. 4. Response to mechanical stimulations. (A) Neuronal activity evoked by touching the right (ipsilateral) antenna of the insect by means of a grounded pencil. The period of contact is indicated by a horizontal bar. (B) The response to a wind puff directed against the head of the insect. (C) Nervous response to repeated mechanical stimulation (arrows) inducing vibrations of the trackball on which the insect was standing. Stimulus-associated units exceeding a threshold (dotted line) were sorted according to their shape. The color code in upper traces of A and C corresponds to extracellular discharges that were assigned to the same unit.

neuronal units fired in respond to a looming stimulus in close succession. This was obvious by averaging the wave-form of large amplitude units revealing a smaller unit that precedes DCMD spiking by only 0.7 ms (horizontal bar in Fig. 5D).

3.7. Sound stimulations

Rubbing fingers against each other generates ultrasound, a stimulus that was used to assess the quality of neuronal recording

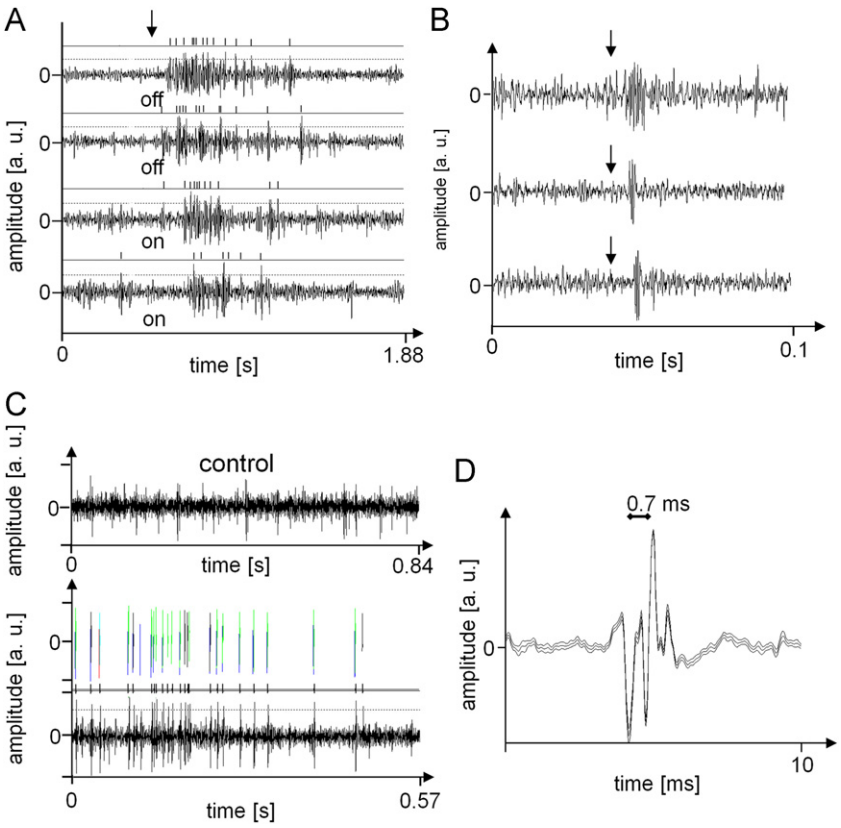

Fig. 5. Response to visual stimulations. (A) Four examples showing the nervous response to sudden changes of light conditions. The arrow indicates the time when the shutter either opened the light path (on) or blocked it (off). Spike events exceeding a manually defined amplitude threshold (dotted horizontal lines) are indicated as ticks in upper traces. (B) Three examples of nerve responses to a shadow that swiftly moved across the eyes of a bright-adapted insect. (C) The nervous response to an object (diameter=8 cm) quickly approaching the insect in the frontal field (Control is shown in the upper trace). Large amplitude spikes exceeding a threshold (dotted line) are shown as ticks and sorted discharge waveforms. (D) The waveform average of 46 large amplitude neuronal responses (probably DCMD). Envelop lines indicate positive and negative SD. Not that in this insect preparation a second unit lacking a positive flank led the large unit by 0.7 ms (bar).

during electrode implantation. The neuronal response to a repetitive presentation of this stimulus is shown in Fig. 6A. Spike sorting revealed a unit generating large spike amplitudes, likely TN-1 (T-fiber) [32]. This unit reliably responds to ultrasound pulses in a phasic manner (upper trace in Fig. 6A) [40]. Therefore, it was suggested as a "bat detector" neuron in some bushcricket families [41].

Repetitive playback of an echolocation signal recorded from the tropical bat *Saccopteryx bilineata* resulted in a strong excitation of TN-1. However, some habituation is clearly visible at a signal period of 50 ms (Fig. 6B, SPL=64 dB, carrier frequency=45 kHz). White noise double pulses as well as conspecific chirps broadcast at 80 dB SPL resulted in a response of different acoustically responding neurons even **three** days after electrode implantation. Stimulus-related neuronal activity obtained during such playback experiments is shown as peri-stimulus-time-histogram and raster plot in Figs. 6C and D. Due to strong habituation of some acoustically responding neurons, the first of two brief white noise pulses, each 100 ms in duration, resulted in a stronger response.

In another insect, the application of a sophisticated spike sorting algorithm programmed after Pouzat et al. [28] and Rutishauser et al. [29] allowed the successful sorting of a multi-unit response to a repetitive playback of a conspecific signal (see methods). This algorithm was able to cluster eight different neuronal units, whereby one (most likely TN-1) perfectly copied the syllable structure of conspecific signals (green dots in Fig. 6E).

3.8. Flight activity

Neuronal recordings from the cervical connective revealed a massive discharge right at the onset of a tethered flight period (Fig. 7A). During flight, large amplitude discharges regularly occurred at ~17 Hz in synchrony with wing beats (Fig. 7B). At the end of a tethered flight period, insects sometimes showed sequences of rhythmic wing muscle activity without obvious wing movements. Recordings from the cervical connective during fictive flight periods revealed repetitive large amplitude discharges occurring at a rate of about 12 Hz (Fig. 7C). The duration of large amplitude discharges during tethered flight and fictive flight exceeded the duration of extracellular spikes by far (6.8 ms, Fig. 7D). This result strongly suggests that cuff-electrodes picked up potentials originating from rhythmic wing muscle activity. These discharges effectively overlaid all neuronal activity in the cuff-electrode recording since common mode rejection of muscle signal artifacts in neural recordings is only effective in a tripolar electrode arrangement.

4. Discussion

The importance of recording neuronal activity from intact, freely behaving insects was demonstrated in several studies. For example the response of an auditory interneuron to a conspecific signal in crickets depends on the phase of leg movement during

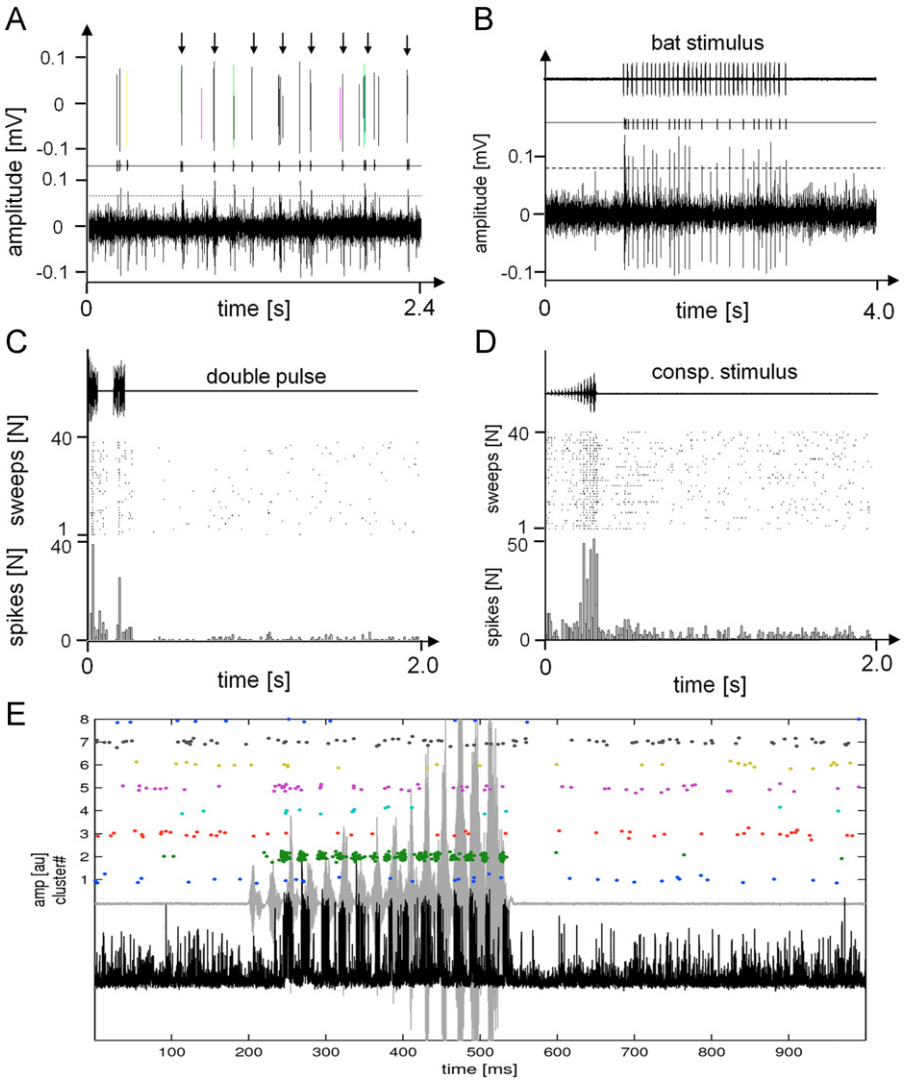

Fig. 6. Response to acoustic signals. (A) Repetitive presentation of ultrasound signals (arrows) evoked the response of large amplitude units, most likely TN-1. The original recording is shown in the lower trace in A. Spikes exceeding a manually-defined threshold (dotted line) were sorted and similar units were plotted in the same color in the upper trace. (B) Neuronal response to the repetitive presentation of an echolocation signal of the tropical bat *Saccopteryx bilineata* (45 ms interval period, 64 dB SPL) exhibiting a carrier frequency of 45 kHz. Ticks in the middle trace in A and B represent units that exceeded a manually defined amplitude threshold (dotted horizontal line). (C) The response of different units to white noise double pulses (100 ms pulse duration, 80 dB SPL) shown as PSTH and as raster plot (37 stimulus sweeps, binsize = 10 ms). (D) Multi-unit response to a conspecific signal broadcast at a SPL of 80 dB shown as PSTH and as raster plot (40 stimulus sweeps, binsize: 10 ms). Note that results shown in C and D were obtained three days after electrode implantation. (E) Result of spike sorting performed after [30,35]. Different spike clusters are shown in different colors. Gray: conspecific sound signal (chirp), Black: the length of extracellular spikes.

walking [3]. Furthermore, the phonotaxis score of female bush-crickets was different between walking and flight activity (*Tettigonia viridissima*) [4]. These examples demonstrate that sensory systems may change their properties in different behavioral contexts and even between different phases of leg movements. The necessity to record from intact insects was also demonstrated in a recently published study, in which moths were found to actively increase the frequency tuning of their ears after stimulation with loud bat cries [42], an adaptive effect, which was absent after decapitation.

The intention of the current study was to rule out possibilities offered by implantable cuff-electrodes for neuroethology with the aim of studying long-term neuronal activity in intact insects. As expected from experiments in which this type of cuff-electrode was implanted in mammalians, miniaturized cuff-electrodes allowed long-term recording of the activity of various neuronal units in insects as well. Neuronal units responded to acoustic, visual, vibratory and mechanical stimulations. Additionally, it was possible to study neuronal activity correlated with leg movements observed during spontaneous walking. However, most likely electromyogram (EMG) signals effectively overlaid all neuronal activity during flight. This is likely a consequence of amplifying against an abdominal copper electrode, which strongly improved signal-to-noise ratio in neuronal recordings, but at the same time amplified EMG signals generated during flight. Therefore, the position of the reference electrode needs to be changed for a proper neuronal recording during flight. Cuff-electrodes with adequate length for the insect model and tripolar electrode arrangement would be the optimum to suppress the overlaying "common mode" signals from muscles in combination with a tripolar recording amplifier [20].

Cuff-electrodes implanted in the neck of intact insects picked up the activity of various neuronal units either ascending to the brain or descending from it. Therefore, this method offers the possibility to study neuronal activity about sensory information conveyed to the brain and descending neuronal activity in naturally behaving insects. In contrast to single unit recordings performed with highly selective tungsten electrodes or microelectrodes, extracellular

recordings of the neuronal activity of whole nerves in intact insects resulted in spike trains that are the sum of the activity of various neuronal units. This makes it difficult to isolate those units responding to external stimulation and units with an activity that is correlated with behavior, such like leg movements. In this case spike sorting is necessary for the study of single neuronal activity. In addition, source recognition algorithms might help to trace back the origin of the signals within the nerve [43]. Acoustic playback experiments performed in this study demonstrated that by use of a sophisticated spike sorting algorithm it is possible to isolate units responding to external stimuli even in the presence of high background neuronal activity. Successful spike sorting was facilitated by electrical properties of the electrodes and the signal amplifier, which preserved the shape of extracellular discharges belonging to different neuronal units.

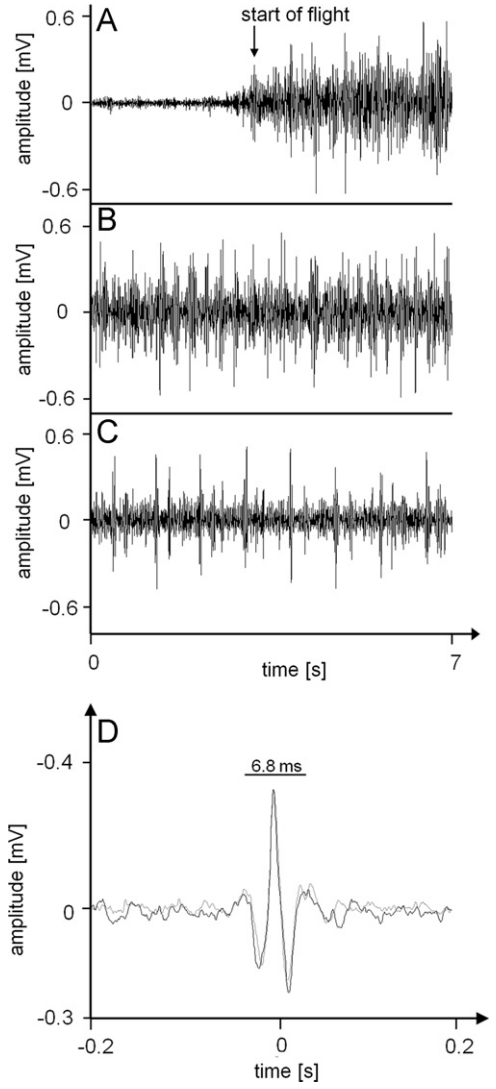

Fig. 7. Recordings obtained during brief periods of flight activity. Cuff-electrode recordings of an insect initiating tethered flight activity (see arrow in A), during tethered flight (B) and during fictive flight (only wing muscle activity) (C). Large amplitude discharges in B were found to be in synchrony with wing movements. (D) Waveform average of 75 large amplitude discharges obtained during tethered flight activity (black line) and during fictive light gray line. All data were obtained from the same insect.

Sorting spikes in extracellular spike trains can be improved by cluster analysis that is based on principal components of the spike wave-form. However, large amplitude discharges may mask small amplitude neuronal units overlapping in time. This was the case in flying insects where EMG signals completely masked all neuronal activity. Not as dramatic, different neuronal units overlapping in time lose their original shape, which makes it difficult for any spike sorting algorithm to identify certain units. The problem is that overlapping spikes are not sufficiently described by the first two principle component scores [44]. Luckily, there are several subtraction methods available for removing overlaps originating from one neuron [45–47]. By application of an appropriate subtraction method prior to spike sorting, the detection of the activity of certain neuronal units could be strongly improved.

Although flexible multi-electrodes were recently used to record potentials from different muscles in a walking insect [18,48], this kind of electrode was never used to record the neuronal activity in intact, freely behaving insects. Miniaturized cuff-electrodes were easy to implant (see also [49]) and due to their electric properties and high biocompatibility allowed the study of neuronal activity even for long periods of time. A problem of cuff-electrodes may be a motion-induced artifact originating from a weak contact of electrodes and the nerve. Thus, the size of the cuff-electrode has to match the dimension of the nerve cord. Some properties of different electrode types used for the recording of neuronal activity in insects are summarized in Table 1. Although certain electrodes are more selective compared to cuff-electrodes, the behavior of insects is strongly restrict.

The maximum range an insect is allowed to move or fly is limited by the length of electrode wires. Therefore, a wireless system that transmits amplified neuronal signals to a receiver will improve the action radius of an insect dramatically (see [50,51]). However up to now most devices are too heavy for insects like *M. elongata*. However, a recently developed lightweight radio-telemetry device (0.25 g including battery) allowed the transmission of muscle potentials recorded by a wire electrode implanted in cockroaches [52], in free-flying large moths [53] and in the wandering spider (*Cupiennius salei*) [54] up to a distance of about 10 m.

5. Conclusions

Miniaturized cuff-electrodes implanted in the neck of insects allowed long-term recording of the activity of various neuronal units responding to acoustic, visual, vibratory and mechanical stimuli. These results suggest this type of electrode to be applicable

Table 1
Properties of electrodes commonly used for the recording of neuronal signals in insects.

Electrode type	Application	Resistance	Problems	Selectivity	Natural behavior	Recording time
Cuff-electrodes	Easy	~3 kΩ	Loose contact, squeezing nerves	Almost whole nerve	Possible	Several days
Hook electrodes (steel or tungsten)	Easy	kΩ	Loose contact, drying-out of nerves	Several units	Possible	Several hours
Suction electrodes	Easy	~	Heavy	Whole nerve	Strongly restricted	Several hours
Sharpened tungsten electrodes	Difficult	~1 Mega-Ω	Injury of neurons	Quasi single cell	Strongly restricted	Several hours
Glass microelectrode	Difficult	~20 MegaΩ	Breakable, single use only	Single cell	Restricted to a treadmill	<1 h

for the study of neuronal encoding of stimuli from the external world in normally behaving insects. However, intrinsic neuronal activity in a naturally behaving insect is high and was found to be correlated with certain phases of leg movements. This makes it necessary to identify neuronal units responding to external stimuli by means of an elaborate spike sorting algorithm. In combination with a wireless transmitter system this cuff-electrode can be used to unravel neuronal processes underlying a receiver's decision in natural mate choice situations or predator avoidance behavior. This approach is advantageous for many questions compared to controlled lab situations, which oversimplify the situation for a receiver regarding changing background noise and light conditions. Recordings of neuronal activity in intact insects likely contribute to the development of bio-inspired neuronal models that are robust to withstand the stimulus situation that a receiver is confronted with in his natural habitat. In a further step such models may provide the basis for the development of naturally behaving insect robots.

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