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# Multielectrode recordings from auditory neurons in the brain of a small grasshopper

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## HIGHLIGHTS

- Small grasshopper auditory brain neurons were recorded with multielectrodes.
- Signal to noise ratio was higher with 12 μm tungsten compared to 15 μm copper wires.
- Stable recordings were achieved for 30 minutes and more.
- Spike sorting and collision analysis discriminated up to 5 units per recording.
- Unit identification by comparing extracellular to intracellular data is difficult.

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## ABSTRACT

*Background:* Grasshoppers have been used as a model system to study the neuronal basis of insect acoustic behavior. Auditory neurons have been described from intracellular recordings. The growing interest to study population activity of neurons has been satisfied so far with artificially combining data from different individuals.
*New method:* We for the first time used multielectrode recordings from a small grasshopper brain. We used three 12 μm tungsten wires (combined in a multielectrode) to record from local brain neurons and from a population of auditory neurons entering the brain from the thorax. Spikes of the recorded units were separated by sorting algorithms and spike collision analysis.
*Results:* The tungsten wires enabled stable recordings with high signal to noise ratio. Due to the tight temporal coupling of auditory activity to the stimulus spike collisions were frequent and collision analysis retrieved 10–15% of additional spikes. Marking the electrode position was possible using a fluorescent dye or electrocoagulation with high current. Physiological identification of units described from intracellular recordings was hard to achieve.
*Comparison with existing methods:* 12 μm tungsten wires gave a better signal to noise ratio than 15 μm copper wires previously used in recordings from bees' brains. Recording the population activity of auditory neurons in one individual prevents interindividual and trial-to-trial variability which otherwise reduce the validity of the analysis. Double intracellular recordings have quite low success rate and therefore are rarely achieved and their stability is much lower than that of multielectrode recordings which allows sampling of data for 30 min or more.



## 1. Introduction

Neuroethology aims at understanding the neuronal basis of animal behavior. Invertebrates have been chosen for many neuroethological studies, since individual neurons can be identified and experiments can be designed for testing the contribution of these neurons to behavior (Comer and Robertson, 2001). In many cases electrophysiological recordings cannot be performed in behaving animals, but neuronal response properties recorded from immobilized animals can be compared to behavioral data (e.g. Roeder, 1998). The identified neuron approach often allows extensive comparison across species (e.g. Yager and Svenson, 2008). However, understanding elaborated behaviors as for example recognition of complex acoustic signals like the species- and situation specific songs of grasshoppers cannot be achieved by analysis of single neuron physiology (e.g. Clemens et al., 2011). Methods to analyze the activity of simultaneously recorded neurons to understand complex behaviors have been established in vertebrate

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research (Nguyen et al., 2009; Gao et al., 2012). Therefore, also in invertebrate research considering groups or populations of neurons instead of single neurons has increasingly gained attention during the last years (Laurent, 2002; Clemens et al., 2011; Campbell et al., 2013). Recording the activity of several neurons at a time, however, is hard to achieve in small animals as many insects are. Instead, activity recorded from single units in several individuals or in one individual successively is widely used to analyze their potential combined activity (Kostarakos and Hedwig, 2012; Meckenhäuser et al., 2014). In order to analyze neural information encoded in the activity of neuronal populations, it would be more appropriate to record activity of several neurons at the same time in the same individual. Not too many studies have achieved this, e.g. for analysis of cockroach antennal functions with regard to locomotion (Ritzmann et al., 2008; Guo and Ritzmann, 2013) or for studies in bee (Brill et al., 2013; Duer et al., 2015) or locust olfactory systems (Saha et al., 2013; Aldworth and Stopfer, 2015). We adopted the method of recording with more than one wire (usually four in a tetrode) from olfactory pathways in the honey bee brain (Brill et al., 2013) to study auditory processing in a small grasshopper. Here we present the adaptations we had to make to solve specific problems that come along with studying the auditory system.

## 2. Materials and methods

### 2.1. Animals

Adult female grasshoppers of the species *Chorthippus biguttulus* (Linnaeus, 1758) were used in all experiments. 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 Nektar – Pforzheim, Germany).

### 2.2. Animal preparation

In order to minimize the movement of the animal, the legs and wings were removed and the animal was fixed with its dorsal side up onto a holder using wax. The brain was exposed by opening the head capsule between the compound eyes, the ocelli, and the antennal sockets. Tracheas were moved aside at the insertion site before electrode placement. The exposed brain was supported by a steel spoon to reduce movements. The ganglionic sheath of the brain was carefully removed 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. Multielectrode design and electrophysiology setup

A multielectrode is used to record multiunit activity from the nervous tissue (Recce and O'Keefe, 1989). The design of the multielectrode was adopted from previous studies on insects (Okada et al., 1999, 2007; Strube-Bloss et al., 2011; Brill et al., 2013, 2014). The multielectrode consisted of either three insulated copper wires (15 μm diameters, Electrisola – Escholzmatt, Switzerland) or three insulated tungsten wires (12 μm diameters, Goodfellow – Huntingdon, UK). The wires were twisted and joined together using heated (~70 °C) dental wax and then glued to a glass capillary which was fixed on a small plexiglas plate. The impedance of multielectrode wires was measured using NanoZ (Neuralynx – Bozeman, USA). The impedance at 1 kHz was 30–40 kΩ for tungsten wires and 300–400 kΩ for copper wires. In case of high impedance (>100 kΩ), the charge capacity of the multielectrode was increased by passing bipolar, constant current square waves to each wire of the multielectrode using NanoZ. The electrode 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 view.

### 2.4. Acoustic stimulation

Experiments were performed in a Faraday cage lined on the inside with sound absorbing pyramidal foam (at least 50% above 500 Hz; Fritz Max Weiser Schaumstoffe – Bochum, Germany). The preparation was acoustically stimulated by two loud speakers (D21/2, Dynaudio – Rosengarten, Germany) situated laterally at a distance of 35 cm from the grasshopper. For the experiment, different auditory stimuli (5 kHz sine wave (duration: 25 ms, 2 ms rise and fall time), 20 kHz sine wave (duration: 25 ms, 2 ms rise and fall time), broadband white noise stimulus (bandwidth 0.5–40 kHz, duration: 100 ms, 2 ms rise and fall time)) were created in Spike2 7.10. Sound pressure levels were calibrated using a continuous signal with a Brüel & Kjær microphone (Type 4133 – Nærum, Denmark) positioned at the location of the experimental animal and directed toward the speaker, grid on, and a Brüel & Kjær measuring amplifier (type 2602). Sound intensities are given in dB SPL (Sound pressure level) re $2 \times 10^{-5}$ N m$^{-2}$. The microphone has been calibrated using a calibrator (Brüel & Kjær type 4230). The signal was then band pass filtered between 5 kHz and 60 kHz to reduce the high frequency distortion 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) during experiments.

In order to detect auditory neuronal activity at the start of the experiment, search stimuli (5 kHz sine wave (25 ms) and broadband white noise (100 ms)) were repeated at 1 s intervals. Activity was considered auditory if spike rates changed during the stimulus with a latency of at least 13–15 ms following stimulus onset. During the search program an audio monitor (AUDIS-01D/16 NPI Electronic Instruments – Tamm, Germany) was used. For obtaining intensity response characteristics, 5 kHz sine wave (duration: 25 ms) and 20 kHz sine wave (duration: 25 ms) stimuli were presented between 50 and 90 dB SPL, increasing in 10 dB steps while the broadband white noise stimuli were delivered between 30 and 90 dB SPL, increasing in 10 dB steps. The various sound amplitudes were achieved by using a digital attenuator (CS3310 Cirrus Logic – Austin, USA) which was controlled by a script (produced by Phillip Jähde, Göttingen) in Spike2. Stimuli were separated by 1 s interstimulus intervals and repeated 10 times at each sound pressure level.

### 2.5. Marking the recording locations

Multiunit recordings have been obtained from ascending auditory neurons and local auditory neurons in the brain of the grasshopper *Ch. biguttulus* at room temperature (22–26 °C). Almost all described ascending interneurons originate from the