Surface electrodes record and label brain neurons in insects

Konstantinos Kostarakos* and Berthold Hedwig**

*Institute for Zoology, Karl Franzens-University, Graz, Austria
**Department of Zoology, University of Cambridge, Cambridge, UK

Correspondence to: B. Hedwig: Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, United Kingdom. E-mail: bh202@cam.ac.uk.

Abstract

We used suction electrodes to reliable record the activity of identified ascending auditory interneurons from the anterior surface of the brain in crickets. Electrodes were gently attached to the neurolem covering the projection area of the ascending interneurons and the ring-like auditory neuropil in the protocerebrum. The specificity and selectivity of the recordings were determined by the precise electrode location, which could easily be changed without causing damage to the tissue. Different non-auditory fibres were recorded at other spots of the brain surface; stable recordings lasted for several hours. The same electrodes were used to deliver fluorescent tracers into the nervous system by means of electrophoresis. This allowed us to retrograde label the recorded auditory neurons, and to reveal their cell body and dendritic structure in the first thoracic ganglion. By adjusting the amount of dye injected, we specifically stained the ring-like auditory neuropil in the brain, demonstrating the clusters of cell bodies contributing to it. Our data provide a proof of principle that surface electrodes are a versatile tool to analyse neural processing in small brains of invertebrates.

New and Noteworthy

We show that surface suction electrodes can be used to monitor the activity of auditory neurons in the cricket brain. They also allow delivering electrophoretically a fluorescent tracer to label the structure of the recorded neurons and the local neuropil to which the electrode was attached. This new extracellular recording and labelling technique is a versatile and useful method to explore neural processing in invertebrate sensory and motor systems.

Keywords: suction electrodes, single cell recordings. auditory neurons, brain, electrophoretic staining

Introduction

Suction electrodes are a well-established method to record the activity of peripheral nerves (Stout 1971, Stout and Huber 1972, Land et al. 2001) or to apply currents for electrical brain stimulation (Hedwig 1986, 1992, Johnson et al. 2007). As gentle low pressure is applied to the inner volume of the electrode, its tip is attached to the surface of a nerve or the cut end of a nerve is sucked into its opening for stable long term extracellular recordings. Recently it has been shown that such electrodes can also be used to deliver fluorescent tracers into the nervous system, by means of electrophoresis (Isaacson and Hedwig 2017). Surprisingly such electrodes have not been used to record neuronal activity from the surface of a ganglion or the brain. Here we employ suction electrodes to monitor the activity of auditory neurons in the brain of crickets, to characterise their response properties, and also to identify their structure and the organisation of the neuropil at the recording site by electrophoretic dye injection. We thus provide a proof of principle that the use of surface electrodes is a versatile technique to analyse neural processing in small brains of model systems with little or no neuro-genetic information available.

In crickets, the cell body and the dendrites of the ascending auditory interneurons AN1 and AN2 are located in the prothoracic ganglion while their axons ascend towards the brain, terminating in the ventral anterior protocerebrum (Wohlers and Huber 1982, Schildberger 1984). The structure of these neurons has been identified with intracellular recordings and staining. The spike activity of the AN1 and AN2 neurons has also been recorded with suction electrodes (Stout 1971, Stout and Huber 1972) and hook electrodes from the neck connectives (Hennig 1988; Kostarakos et al. 2008, 2010; Schmidt and Römer 2011). The AN1 auditory activity is tuned to around 5 kHz corresponding to the

frequency range of the cricket calling song, while the AN2 neuron acts within the context of bat detection and responds best to high frequency signals in the range of 15-30 kHz. As AN2 has the larger axon diameter it stands out in such recordings whereas signals from the smaller AN1 axon are more difficult to obtain and sometimes require splitting the connective into axon bundles. A more simple way to record these neurons from the brain with an intact thoracic nervous system would be desirable for long term recordings to study auditory processing and at the same time it would allow to evaluate the recording technique for wider applications.

Methods

Animals: We used adult female bispotted field crickets (*Gryllus bimaculatus*) from a colony at the University of Graz, kept under established housing conditions for crickets. They had continuous access to water, fresh lettuce and fish food.

Electrodes: Tubes for suction electrodes were manually drawn under a dissecting microscope over a hot soldering iron from polycarbonate capillary tubing with 1.0 mm OD and 0.5 mm ID (Paradigm Optics Inc, Vancouver, USA) to an outer diameter of 50-100 µm; tips were cut and heat polished. Electrodes were inserted into a custom made electrode holder using a platinum wire as contact (Isaacson and Hedwig 2017). Its cavity was filled with a solution of 4% of Tylose (Tylose H200 YG4, ShinEtsu, Wiesbaden, Germany) dissolved in cricket saline, composition in g/l: 8.6 NaCL, 0.74 KCL, 0.76 $CaCl_2$, 2.38 HEPES. The electrode shaft was inserted and the lumen of the capillary was filled from the tip with 4% of Lucifer Yellow CH (Sigma-Aldrich, L0259) dissolved in aqueous 4% Tylose by applying a gentle suction to the holder cavity with a syringe, connected to the cavity via a flexible tube.

Recordings: For the recordings, specimens were tethered on a block of Plasticine fitted to a metal holder. The head capsule was opened frontally to expose the brain; it was rinsed with cricket saline to prevent the tissue from drying. A total of 38 female crickets were used to develop and test the method. The electrode tip was gently attached to the ventral surface of the brain where the ascending auditory interneurons AN1 and AN2 terminate. The tip position was altered until a good quality recording of AN1 or AN2 spike activity was obtained, other sensory modalities were recorded at different surface areas of the brain. Good recordings could be obtained even without application of suction, when the electrode was slightly pushed onto the brain surface. The platinum reference electrode was placed into the saline next to the brain. Neuronal activity was amplified 1000X and band pass filtered between 300 Hz and 5 kHz with a differential amplifier (Model 1700, A-M Systems Inc. Carlsborg, WA, USA). It was digitally recorded at a sampling rate of 21 kHz per channel using a CED Micro3-1401 controlled by Spike 2 software (Cambridge Electronics Design, Cambridge, UK). Experiments were performed at 28-32ºC.

Sound stimuli: Sound stimuli were computer generated with Cool Edit Pro 2000 (Syntrillium,Phoenix, AZ, USA, now Adobe Audition) and were delivered at different intensities and frequencies via ultrasound magnetic speakers (MF1-S, Tucker Davis technologies, Alachua, Florida, USA) controlled by a Tucker Davis attenuator system (PA5, Tucker Davis technologies). Four sound pulses of 20 ms duration with 20 ms inter-pulse intervals were grouped in chirps of 140 ms duration and repeated every 460 ms. Other sensory stimuli were provided in a qualitative way by touching the appendages with a paintbrush or moving an object in front of the light source.

Staining: After recording the auditory neurons, Lucifer yellow was injected into the brain at the recording site by hyperpolarizing DC current of -25 µA applied for 10 s to 5 min with a constant current source (Stimulus Isolator A-360, WPI, Sarasota Fl, USA). Thereafter the dye was left to spread in the nervous system while the specimens were kept at 6 deg C° for 24 hours. The CNS was dissected out from the brain to the first thoracic ganglion (TG1), and was fixed and cleared with standard histological techniques. Images of the stained neurons were taken with a Zeiss digital camera (AxioCamERc5s) attached to a Zeiss Axioplan (both Zeiss Wetzlar, Germany) and compared against the structures of AN1, AN2 (Schildberger 1984, Schildberger et al 1989) and local brain neurons (Kostarakos and Hedwig 2012, Schöneich et al. 2015).

Data Analysis: Representative recordings of neurons were selected with Spike 2 and processed in Neurolab (Knepper and Hedwig 1997, Römer et al.2002), with an algorithm that calculated the overall voltage change in a gliding time window of 1.2 ms, corresponding to the duration of a spike, and thereby increased the signal to noise level in the filtered data. For quantitative analysis the timing of the filtered spikes was subsequently detected with a threshold filter and PST histograms were calculated. The mean number of AP/Chirp was calculated over a time window of 10-200 ms after the onset of a chirp.

Results

Placing a suction electrode tip gently on to the ventral surface of the protocerebrum reliably recorded spike activity of underlying neurons. Single units of 50-200 µV amplitude could easily be discerned from a background noise of 20-30 µV. When the tip was positioned at different areas of the brain (Fig. 1A) it selectively picked up neural activity in response to e.g. antennal stimulation, touching the front legs or light stimuli. In this way the method allowed us to scan the surface of the brain for modality specific responses and we obtained visual, antennal and proprioceptive activity. When the tip was positioned on the area of the protocerebrum where the ascending auditory neurons AN1 and AN2 terminate, their spike activity could reliably be recorded in response to repetitive acoustic stimuli (Fig. 1B). The quality of the recording could be improved by very gently pushing the electrode onto the brain or applying gentle suction, which sealed the tip onto the neurolem. The recordings picked up simultaneously the combined activity of AN1 and AN2 or either neuron in a highly selective manner; and could last unchanged for several hours.

Acoustic stimulation (75 dB SPL) allowed us to characterise the spike activity of the recorded neurons based on a quantitative analysis of their frequency tuning and PST histograms (Fig. 2). The activity patterns revealed two different spike amplitudes, with AN2 generally giving a larger response (Fig. 2A); the threshold for AN1 was around 43 dB SPL and for AN2 around 48 dB SPL. Typical responses of AN1 and AN2 were obtained with different stimulus frequencies, with AN1 neurons responding best to 5 kHz sound pulses, whereas AN2 neurons responded best in the high frequency range of 15-40 kHz, as reflected in the frequency tuning curves (Fig. 2B). The PST histograms obtained in response to 5 kHz and 20 kHz pulse patterns (Fig. 2C,D) demonstrate a typical AN1 response coupled to the pulse pattern of the chirp with 20.0±2.3 AP/chirp and a less strong response of 13.1±1.3 AP/Chirp for AN2. On average AN1 and AN2 responded with 24.9±4.0 and 17.1±3.9 AP/Chirp, respectively (N=6 for both neurons). Response latencies to the first sound pulse of a chirp were rather short and were on average 13.7±3.0 ms for AN1 and 14.0±1.2 ms for AN2, as experiments were performed at a high room temperature. These data demonstrated that the activity of identified auditory neurons and of other single units can be selectively recorded through the neuronal sheath from the surface of the intact brain.

We then explored if this extracellular technique could also be used for labelling neurons at the recording site. Based on a recently reported electrophoretic staining method (Isaacson and Hedwig 2017) we used the electrodes to deliver the fluorescent tracer Lucifer yellow into the neural tissue adjacent to the opening of the electrode tip. The staining result depended on the amplitude and duration of the current applied. In experiments where auditory neurons were recorded, subsequent injection of LY reliably labelled the neurons with their cell bodies, neurites and dendrites in the TG1 allowing us to identify the auditory neurons as AN1 and AN2 (Fig. 3A). Labelling the AN1 and AN2 structures in TG1 was successful in 12 out of 14 staining attempts; in two unsuccessful cases two axons could be traced through the suboesophageal ganglion (SEG) towards the TG1. When applying -25 µA for 3 min the dye injection over-stained the brain, here cellular details were not discernible but the auditory neurons could be clearly revealed in TG1. Reducing the electrophoresis time to 15 s still was sufficient to identify the auditory neurons in the prothoracic ganglion, it limited the spread of dye in the brain and labelled neurons just in the vicinity of the recording site (Fig. 3B,C). Stainings in the brain reliably revealed structural details like the ring-like branching pattern of local auditory neurons in the anterior protocerebrum in 11 experiments, and 3 clusters of cell bodies with their primary neurites projecting towards this structure (Fig. 3B). In three more selective stainings, the anterior cluster contained about 16 cell bodies, the lateral one about 13 and the posterior cluster about 25 cell

bodies. These clusters match the position of cell bodies of identified auditory neurons involved in song pattern recognition (Kostarakos and Hedwig 2012, Schöneich et al. 2015). At the recording site of the auditory neuropil, the staining procedure was surprisingly selective for the ascending auditory neurons, only few other axons and neurons from the SEG were picked up as well. The surface electrodes thus not only allowed to record and identify specific neurons, moreover local dye injection revealed structural and organisational details of the surrounding neural tissue. The specific structures labelled, depended on the precise location of the surface electrodes, different details were highlighted in different experiments.

Discussion:

We used surface electrodes to reliably record extracellularly the spike activity of identified ascending auditory neurons terminating ventrally in the brain of crickets and to reveal their thoracic structure by electrophoretic dye injection. Different sensory modalities could also be recorded at different locations, corresponding to the gross organisation of neural processing in the brain. In terms of extracellular approaches to brain activity, single or multiunit metal electrodes have been employed to study sensory and motor processing in the brain of cockroaches (Bender and Ritzmann 2010, Guo and Ritzmann 2013), grasshoppers (Bhavsar et al. 2017), and bees (Brill et al. 2013). As the multielectrodes come with a diameter of at least 20-40 μm some damage is unavoidable when these electrodes are inserted into the tissue, limiting the number of recording sites that can be probed. Although the electrode position can be labelled by depositing metal ions or fluorescent tracers, otherwise no information on the structure of the recorded neurons is obtained. The method described here allows recording of neuronal activity at different brain sites without obvious damage to the tissue, and as a specific advantage neurons adjacent to the recording site can be labelled with fluorescent tracers. This new and surprising possibility of extracellular surface recordings also allows obtaining structural information on the recorded neurons and the local neuron populations. The method seems to be selective to fibres close to the recording site, and in crickets allows easy recordings of ascending auditory neurons, which terminate close to the ventral surface of the brain (Schildberger 1984). We successfully tested the approach to monitor the activity of thoracic motoneurons in locusts and suggest that it can be applied to a wide range of invertebrate nervous systems. Neurons deeper in the brain or a ganglion may also be recorded when more gently force is applied to the electrode tip, this option was not yet systematically explored. Our methodological approach is a new tool to study neural processing in insect central nervous systems. With further technical refinement it may be combined with intracellular recordings, the delivery of calcium sensitive dyes (Isaacson and Hedwig 2017) or the local application of polar neuroactive substances. This offers new possibilities to study the activity and function of invertebrate nervous systems in species, in which genetically engineered calcium indicators are not yet available. Using large scale surface electrodes with multiple contact points should allow simultaneous multi-channel recordings to scan brain activity over a wider range and to simultaneously explore different neuropil regions and functions.

Acknowledgements: The work was supported by the Royal Society in a Newton International Fellowship alumni funding. BH visited the former Newton fellow KK as an external collaborator. The concept of the study was developed by BH. KK and BH jointly performed the experiments and wrote the manuscript. We are grateful to H. Römer for constructive comments on the work.

References:

Bender JA, Pollack AJ, Ritzmann RE (2010) Neural activity in the central complex of the insect brain is linked to locomotor changes. Cur Biol 20(10):921–926.

Bhavsar MB, Heinrich R, Stumpner A (2015) Mini review: Multielectrode recordings in insect brains. Neuroscience Communications.1. e1088. doi: 10.14800/nc.1088.

Bhavsar MB, Stumpner A, Heinrich R (2017) Brain regions for sound processing and song release in a small grasshopper. J Ins Physiol 99:15–24.

Brill MF, Rosenbaum T, Reus I, Kleineidam CJ, Nawrot MP, Rössler W (2013) Parallel processing via a dual olfactory pathway in the honeybee. J Neurosci 33(6):2443–56.

Brown TM, Banks JR, Piggins HD (2006) A novel suction electrode recording technique for monitoring circadian rhythms in single and multiunit discharge from brain slices. J Neurosci Meth 156(1):173–81.

Guo P, Ritzmann RE (2013). Neural activity in the central complex of the cockroach brain is linked to turning behaviors. J Exp Biol. 216(6):992–1002.

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

Hedwig B (1992) On the control of stridulation in the acridid grasshopper *Omocestus viridulus* L. I. Interneurons involved in rhythm generation and bilateral coordination. J Comp Physiol A, 171:117-128

Hennig RM (1988) Ascending auditory interneurons in the cricket *Teleogryllus commodus* (Walker): comparative physiology and direct connections with afferents. J Comp Physiol A, 163(1):135-43.

Isaacson MD, Hedwig B (2017) Electrophoresis of polar fluorescent tracers through the nerve sheath labels neuronal populations for anatomical and functional imaging. Scientific Reports, 7:40433.

Johnson BR, Hauptman SA, Bonow RH (2007) Construction of a simple suction electrode for extracellular recording and stimulation. J Undergrad Neurosci Educ 6(1):A21–26.

Kostarakos K, Hartbauer M, Römer H (2008) Matched filters, mate choice and the evolution of sexually selected traits. PLoS one. 3(8):e3005.

Kostarakos K, Hennig MR, Römer H (2009) Two matched filters and the evolution of mating signals in four species of cricket. Frontiers in Zoology, 6(1):22.

Kostarakos K, Römer H (2010) Sound transmission and directional hearing in field crickets: Neurophysiological studies outdoors. J Comp Physiol A, 196:1–13.

Kostarakos K and Hedwig B (2012) Calling song recognition in female crickets: Temporal tuning of identified brain neurons matches behaviour. J Neuroscience 32:9601-9612.

Knepper M, Hedwig B (1997) NEUROLAB, a PC program for the processing of neurobiological data. Comput Meth Prog Biomed, 52:75-77

Land BR, Wyttenbach RA, Johnson BR (2001) Tools for physiology labs: an inexpensive high-performance amplifier and electrode for extracellular recording. J Neurosci Meth, 106(1):47–55.

Okada R, Ikeda J, Mizunami M (1999) Sensory responses and movement-related activities in extrinsic neurons of the cockroach mushroom bodies. J Comp Physiol A, 185(2):115–129.

Ritzmann RE, Ridgel AL, Pollack AJ (2008) Multi-unit recording of antennal mechano-sensitive units in the central complex of the cockroach, *Blaberus discoidalis*. J Comp Physiol A, 194(4):341–60.

Römer H, Hedwig B, Ott SR (2002) Contralateral inhibition as a sensory bias: the neural basis for a female preference in a synchronously calling bush cricket, *Mecopoda elongata*. Eur J Neurosci, 15:1655-1662

Schildberger K (1984) Temporal selectivity of identified auditory neurons in the cricket brain. J Comp Physiol A, 155(2):171–85.

Schildberger K, Huber F, Wohlers D (1989) Central auditory pathway: Neuronal correlates of phonotactic behavior. In: Huber F, Moore TE and Loher W, eds. Cricket behavior and neurobiology. Cornell University Press; pp: 423–458.

Schöneich S, Kostarakos K and Hedwig B (2015) An auditory feature detection circuit for sound pattern recognition. Sci Adv 1(8) e1500325, doi:10.1126/sciadv.1500325

Schmidt AK, Römer H (2011) Solutions to the cocktail party problem in insects: selective filters, spatial release from masking and gain control in tropical crickets. PLoS One 6:e28593

Stout JF (1971) A technique for recording the activity of single interneurons from free-moving crickets (*Gryllus campestris* L.). J Comp Physiol A, 74(1):26–31.

Stout JF, Huber F (1972) Responses of central auditory neurons of female crickets (*Gryllus campestris* L.) to the calling song of the male. J Comp Physiol A, 76(3):302–313.

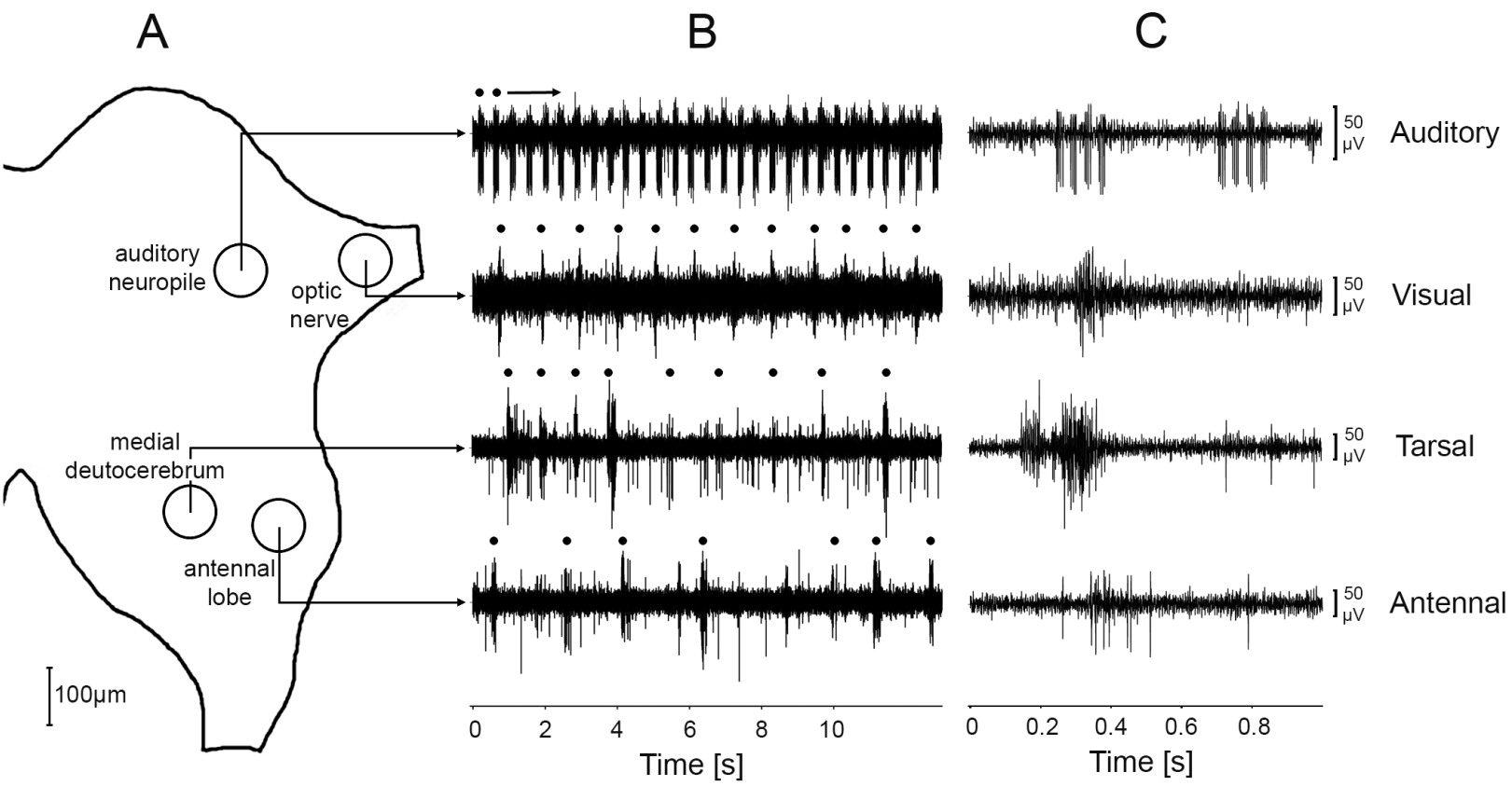
Wohlers DW, Huber F (1982) Processing of sound signals by six types of neurons in the prothoracic ganglion of the cricket, *Gryllus campestris* L. J Comp Physiol A, 146(2):161–73.

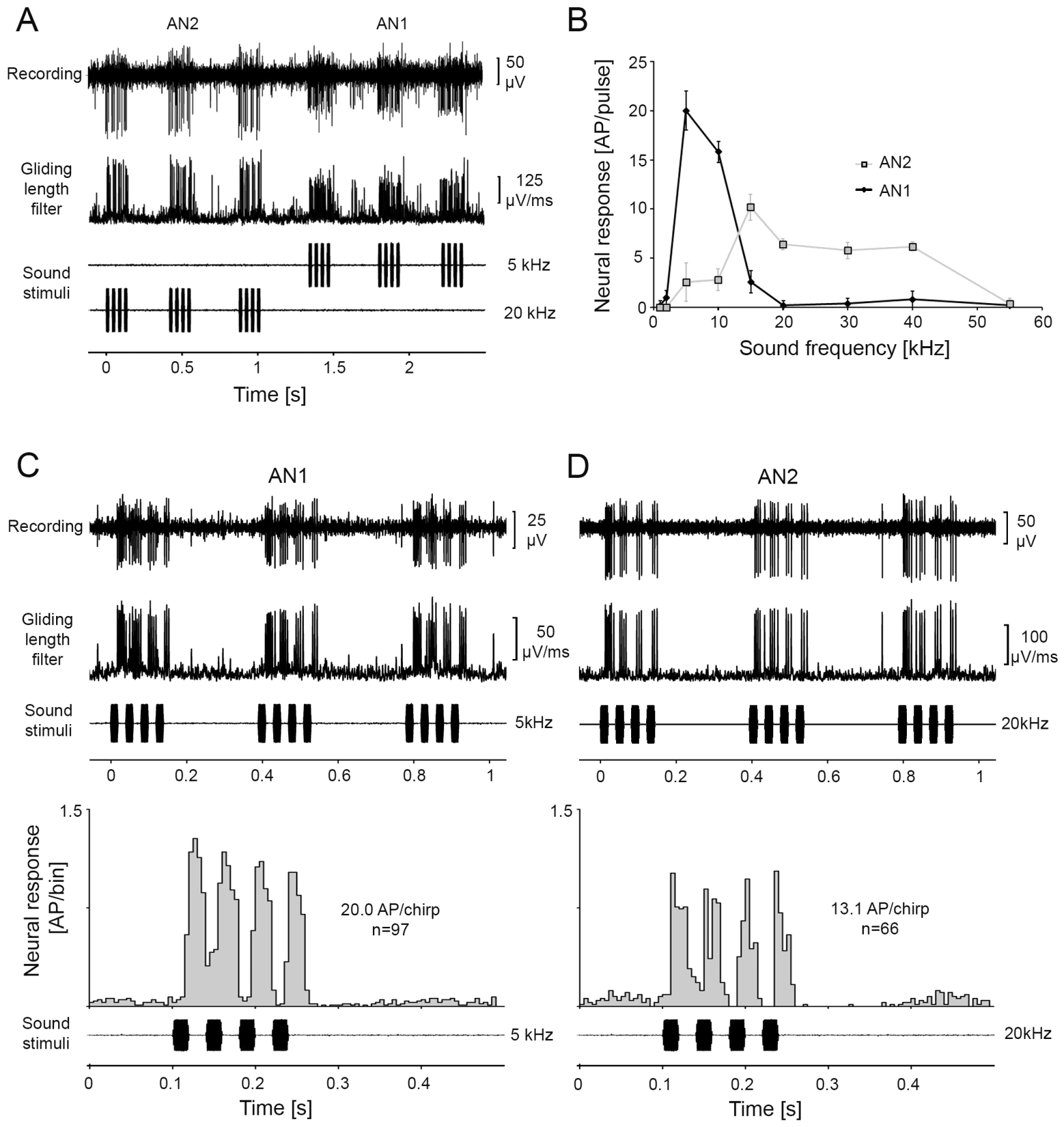
Figures

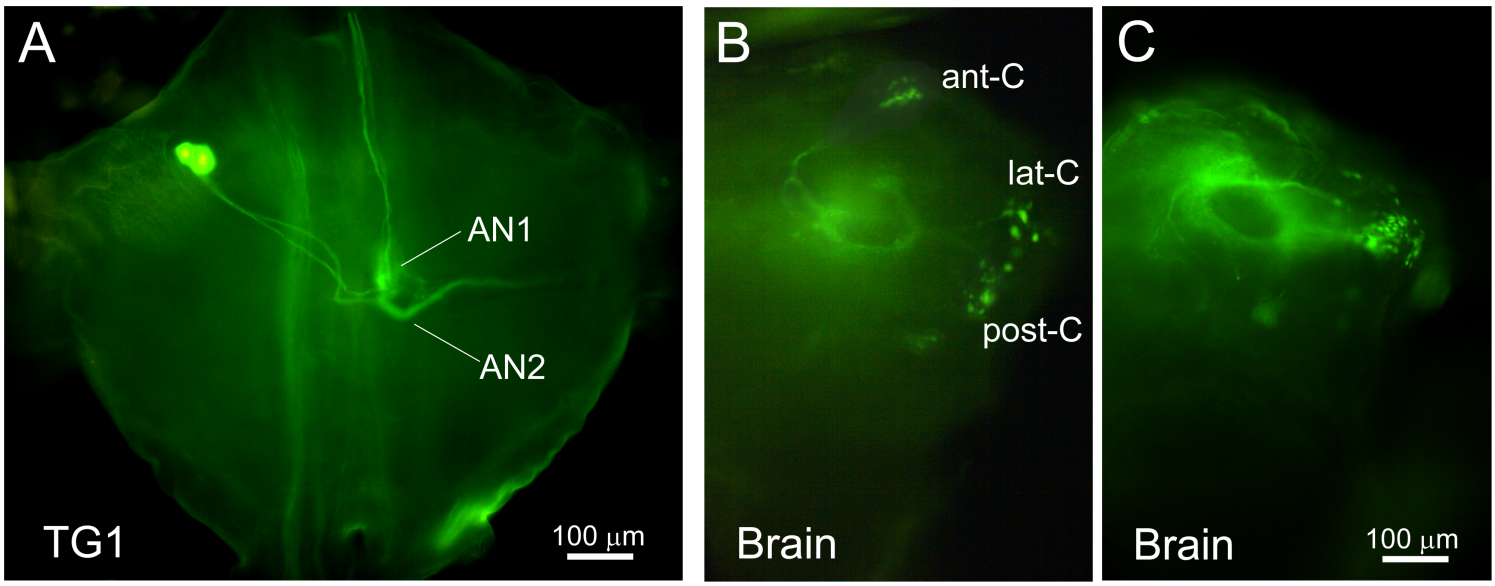
Fig. 1: Neural activity recorded with a surface electrode placed at the ventral side of the brain. (A) Electrode positions at the ventral side of the brain for the different recordings. (B) Responses obtained at different positions in response to acoustic pulse patterns, to changing light intensity; spikes elicited upon touching the ipsilateral antenna or the ipsilateral front leg with a paintbrush. (C) The single unit activity in response to sensory stimulation demonstrates the signal to noise ratio and the selectivity of the recording method.

Fig. 2: Recordings of AN1 and AN2 activity in response to sound pulses presented with different frequencies. (A) The spike patterns of the auditory neurons clearly stand out from the background activity; the signal to noise ratio is increased by applying a gliding length filter, calculating the sum of amplitude changes over a time window of 1.2 ms. A switch in sound frequency from 20 kHz to 5 kHz is accompanied by a shift in neuronal activity from AN2 to AN1. (B) Frequency tuning curves of the auditory activity in response to the acoustic stimuli give the characteristic responses of AN1 and AN2, at each frequency 5 pulses were presented (C,D). Neuronal activity and PST-histograms with a bin width of 5 ms, in response to chirp patterns presented at 5 kHz or 20 kHz reveal the typical temporal activity patterns of AN1 and AN2.

Fig. 3: Electrophoretic labelling of auditory neurons and brain neuropils with surface electrodes. (A) Characteristic structure of AN1 and AN2 with soma positions and dendrites in theTG1, stained after their axon terminals in the brain were labelled with Lucifer yellow for 3 min. (B,C) The ring-like arborisation pattern of the ascending neurons and of local auditory neurons in the anterior protocerebrum revealed by electrophoretic injection of Lucifer yellow for 15 s. Labelled are three separate clusters of ventral cell bodies, with neurites connected to the ring-like arborisation pattern. These clusters are positioned at the anterior protocerebrum (ant-C), the lateral protocerebrum (lat-C) and the lateral posterior protocerebrum (post-C). Due to a different electrode position, the clusters are not as clear in Fig. 3C.


A
B
C
auditory
neuropile
optic
nerve
medial
deutocerebrum
antennal
lobe
100µm
0
2
4
6
8
10
Time [s]
0
0.2
0.4
0.6
0.8
Time [s]
50
µV
Auditory
50
µV
Visual
50
µV
Tarsal
50
µV
Antennal


A
AN2
AN1
Recording
50 µV
Gliding length filter
125 µV/ms
Sound stimuli
5 kHz
20 kHz
0
0.5
1
1.5
2
Time [s]
B
Neural response [AP/pulse]
25
20
15
10
5
0
AN2
AN1
0
10
20
30
40
50
60
Sound frequency [kHz]
C
AN1
Recording
25 µV
Gliding length filter
50 µV/ms
Sound stimuli
5kHz
0
0.2
0.4
0.6
0.8
1
Neural response [AP/bin]
1.5
20.0 AP/chirp
n=97
Sound stimuli
5 kHz
0
0.1
0.2
0.3
0.4
Time [s]
D
AN2
Recording
50 µV
Gliding length filter
100 µV/ms
Sound stimuli
20kHz
0
0.2
0.4
0.6
0.8
1
1.5
13.1 AP/chirp
n=66
Sound stimuli
20kHz
0
0.1
0.2
0.3
0.4
Time [s]


A
AN1
AN2
TG1
100 μm
B
ant-C
lat-C
post-C
Brain
C
Brain
100 μm