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FROM HEARING TO SINGING: SENSORY TO MOTOR INFORMATION PROCESSING IN THE GRASSHOPPER BRAIN

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Declaration of academic honesty

I herewith declare that the Ph.D. thesis entitled “From hearing to singing: sensory to motor information processing in the grasshopper brain” has been written independently and with no other sources and aids than quoted.

Mit Balvantray Bhavsar

Göttingen, February 2016

To my parents……

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Abstract

Grasshoppers, and among them especially the species *Chorthippus biguttulus*, have been used as a model system to study the neuronal basis of 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. Here for the first time multielectrode recordings from the brain of a small grasshopper brain were made. 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. It was possible to separate up to five units by sorting algorithms. Tungsten wires exhibited stable recordings with higher signal-to-noise ratio than copper wires. 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. Physiological identification of units described from intracellular recordings was hard to achieve therefore the focus was on comparing individual units. 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. Decoding the information about the acoustic stimulus was compared between single neurons and set of simultaneously recorded neurons. Information was higher for some data sets with 2 or more simultaneously recorded neurons indicating the existence of a population code inside the brain of grasshopper. Local brain neurons were recorded from lateral protocerebrum, anterior brain and central complex and were separated from ascending neurons based on their longer latencies. One local brain neuron was found discriminating between behaviorally attractive and non-attractive stimuli. Using such multielectrodes, it was also possible to induce singing responses by electrically stimulating different auditory neuropiles in the brain of grasshoppers.

Chapter 1

General introduction

1.1 Communication and its sensory aspects

Communication is a very much fascinating thing. Its study has helped in the general understanding of motor and sensory systems, evolution, and speciation. A major appeal of studying communication is that a researcher can quantify how biologically important information can be coded in particular physical properties of a signal and then experimentally determine if the animals themselves use this information (Gerhardt and Huber 2002). The main use of sounds for vocal communication is widespread among vertebrates and invertebrates which range from mating calls in insects to speech in humans. Sound can transmit broader messages like species identity or narrow messages like the effective state of a caller (Schehka 2009). Communication is a key area of animal behavior because all social interactions among individuals are based on the exchange of information. For communication to occur, a sender has to encode information in a signal, which is then transmitted to a receiver (Shannon and Weaver 1949). Animals have evolved the most astounding ways to pass on messages, by using optical, acoustic, electric or chemical signals. Among them, acoustic signals serve a number of functions and are often part of social behavior. One of the key goals in research on acoustic communication is to explore the wide range of information conveyed in vocal signals.

1.2 Acoustic communication in insects

Acoustic communication is widely spread among vertebrates but, among invertebrates, hearing and acoustic communication are well developed only in insects, in which they serve as detection of predators, the location of mates and of hosts (Pollack 2000). Insects offer several advantages as model systems for neuroethological studies, including robust behavior, easily accessible nervous system and uniquely identifiable neurons that permit one to frame general questions about the neural analysis of signals at the levels of single nerve cells (Nolen and Hoy 1984). Insects are good subjects for studies of the mechanisms underlying signal production and recognition and localization. In insects, interneurons that trigger sound-producing mechanisms have been characterized both anatomically and physiologically (Stumpner and Ronacher 1991). Moreover, the orchestrated activity of motor neurons, muscles or both that pattern acoustic signals has been described in detail (Gans 1973; Hedwig 1994; Heinrich and Elsner 1997a). Insects are also suitable for studying sensory

processes. Many cells have been characterized physiologically, and connections among them are well known. Studying orthopteran insects has an additional advantage that all the biologically relevant information available in the acoustic waveform is conveyed to the brain by a handful of ascending neurons which are individually identifiable (Huber and Thorson 1985; Pollack 1988) and can be recorded in behaving animals. Insects are also suitable for studying the genetic bases of acoustic system and selective responsiveness (Shaw 1996; Ritchie 2000). Some insects have been the subjects of artificial selection experiments that can estimate additive genetic variation and covariation in signal structure and receiver selectivity (Bakker and Pomiankowski 1995).

Acoustic communication in insects presents a diverse and fascinating set of opportunities for biologists interested in sensory mechanisms.

1.3 Grasshoppers *Chorthippus biguttulus* as a model system to study acoustic communication

The complexity and size of sensory systems vary greatly, from the auditory system of a noctuid moth consisting of few neurons (Roeder, 1967), to the primate visual system consisting of 250 million neurons (Hubel, 1988). This size depends on the difficulty of the tasks that a sensory system has to fulfill. Essentially, the auditory system of the noctuid moth only needs to detect bat cries while the visual system of a primate has to analyze and interpret a whole variety of complex visual scenes. The amount of computations needed to perform these tasks differs correspondingly. Somewhere within this range of complexity lies the grasshopper auditory system with a few hundred neurons (Pollack 1998). This system has two advantages: (1) The set of natural stimuli is well known, most importantly the communication signals that are employed by grasshoppers in the mate finding process (Elsner 1974). (2) Despite their simplicity, some grasshoppers show highly evolved behavioral patterns which are accessible to systematic investigations (von Helversen and Elsner 1977).

Work on the species *Ch. biguttulus* has provided valuable insights into the neural basis of song recognition in the early auditory system of acridid grasshoppers. On hot summer days, males produce a calling song by rubbing their hindlegs against a hardened vein on their forewings. A male calling song is a broadband sound having frequency components between 4 and 40 kHz. Species-specific

motor programs produce a movement pattern that modifies this carrier with species- and sex-specific amplitude modulations (Elsner 1974; von Helversen and von Helversen 1997). In the species *Ch.biguttulus*, this envelope consists of 20–30 repetitions of a basic sub-unit called syllable followed by a shorter and much softer pause (von Helversen 1972). If a female grasshopper of the same species hears this song and considers it attractive, it responds with her own song. This female response allows the male to localize and approach the female, resulting eventually in copulation (Schul et al. 1999)

1.4 Neuronal basis of hearing in grasshoppers

The anatomy of the auditory system (Fig.1.1) constrains the processing of auditory inputs. A tympanic membrane is located on each side of the lateral abdomen which is responsible for sound detection. About 70 spiking receptor cells are attached to each membrane (Gray 1960). There are four different kinds of receptor cells and three of these receptor types are most sensitive to low carrier frequencies, the fourth responds most strongly to high carrier frequencies (Römer 1976; Jacobs et al. 1999). As long as a signal contains frequencies in the appropriate range, its amplitude distribution is well encoded by receptor neurons (Machens et al. 2001). The receptor cells project (transfer the information) into the metathoracic ganglion where information is preprocessed before being sent into the head ganglion (brain). As the highest neural processing stage, the brain integrates available information and produces a decision signal. The metathoracic ganglion contains four classes of interneurons. Many have been morphologically and physiologically classified (Stumpner and Ronacher 1991). The ascending neurons (ANs) form a particularly important class. They have probably no direct input from receptor neurons and are the only neurons projecting into the brain. Some neurons (AN1, AN2) encode directional information (Stumpner 1988), whereas others (e.g. AN3, AN4, AN12) are presumably involved in pattern recognition (Stumpner and Ronacher 1991, Krahe et al. 2002). Because of their small number (approximately 20), this group constitutes a bottleneck for the information transmission of the auditory system. In a behaviorally attractive song, one of the ascending neurons, the AN12 marks the beginning of each syllable with a phasic burst provided pauses between syllables are long enough (Stumpner and Ronacher 1991). The AN3 and AN4 respond in a phasic-tonic manner to stimuli and, possibly, they encode onset steepness (Krahe et

al. 2002) and are involved in another behaviorally relevant feature, gap detection (Ronacher and Stumpner 1988). Most described ascending neurons (ANs) originate from metathoracic ganglion, form a bundle, enter each hemisphere of the brain and make branches in the lateral protocerebrum (Eichendorf and Kalmring 1980; Stumpner and Ronacher 1991; Kutzki 2012). The information is then taken up by some local auditory brain neurons (LBNs) for further processing. However, there is limited information available about the locations and the role of local brain neurons in the auditory processing.

1.5 Goal of the project

The project aims at elucidating important steps of neuronal processing involved in the recognition of species-specific acoustic communication signals and in the selection of appropriate acoustic responses. The main goal of the project is to test a newly introduced method in insect science known as multiunit recordings using a tetrode. The whole project is then further divided into three subprojects. The first part is to analyze the combined activity of ascending neurons and detect the potential correlation with specific features of a male song used as auditory stimulus. The second part is to find the locations of the local auditory neurons in the brain and find their potential specialization in the process of song recognition. The third part is electrical stimulation of the auditory neuropiles in the brain to induce a specific motor response (stridulation) to find out locations of these neuropiles involved in stridulation.

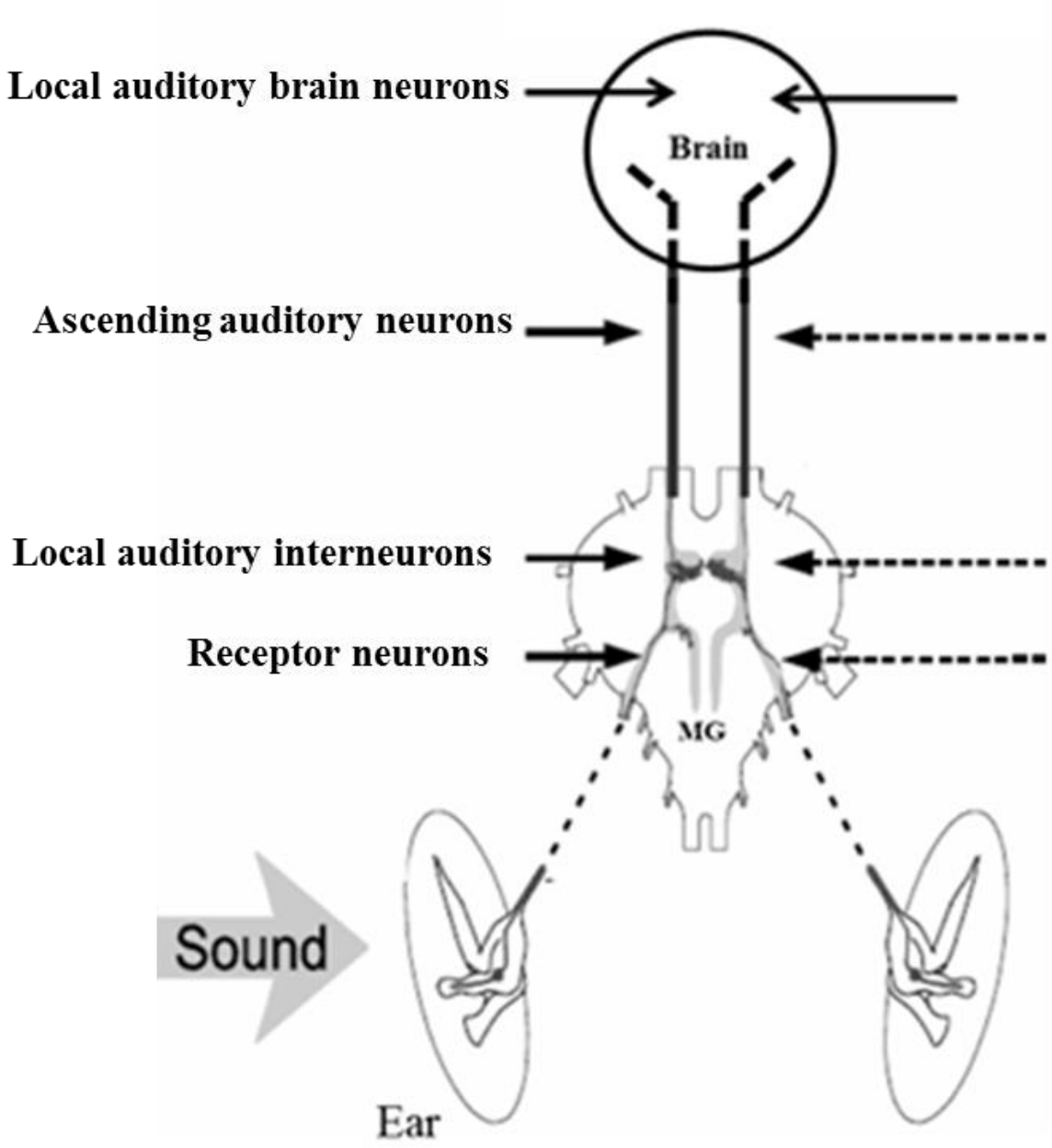

Figure 1.1: **The auditory system of grasshoppers and locusts.** Sound impinges on the two tympana where the receptor neurons translate the sound into neural activity which is forwarded to the metathoracic ganglion (MG). Ascending neurons transmit information upwards to the brain which is then further processed by local brain neurons. Image is from Creutzig (2008).

1.6 Terminology to describe a grasshopper song

Most species of grasshoppers produce the song by stocking a file on the inside of each hindleg femur across a raised vein on the wing (von Helversen and von Helversen 1994). I here include a glossary of terms to describe the song of *Ch.biguttulus*. I follow the nomenclature of (von Helversen and von Helversen 1994) (Figure 1.2).

Pulse: Each partial or uninterrupted upward or downward leg movement produces a pulse.

Syllable: Pulses are grouped into syllables. One syllable consists of one full cycle of upward and downward movements of the legs.

Pause: During the stridulation of *Ch. biguttulus*, the leg stops for about 10-15 ms after end of each syllable. These intervals between two syllables are called a pause.

Gaps: Male *Ch. biguttulus* can lose a hindleg, often due to autotomy during contacts with predators and also occasionally as a result of difficulties in molting (von Helversen and von Helversen 1997). Such males can no longer mask small intervals between the pulses that arise from turning points of leg movements which are called gaps.

Song: A series of syllables separated by pauses are called song. A typical male *Ch. biguttulus* song is 1-3 seconds long.

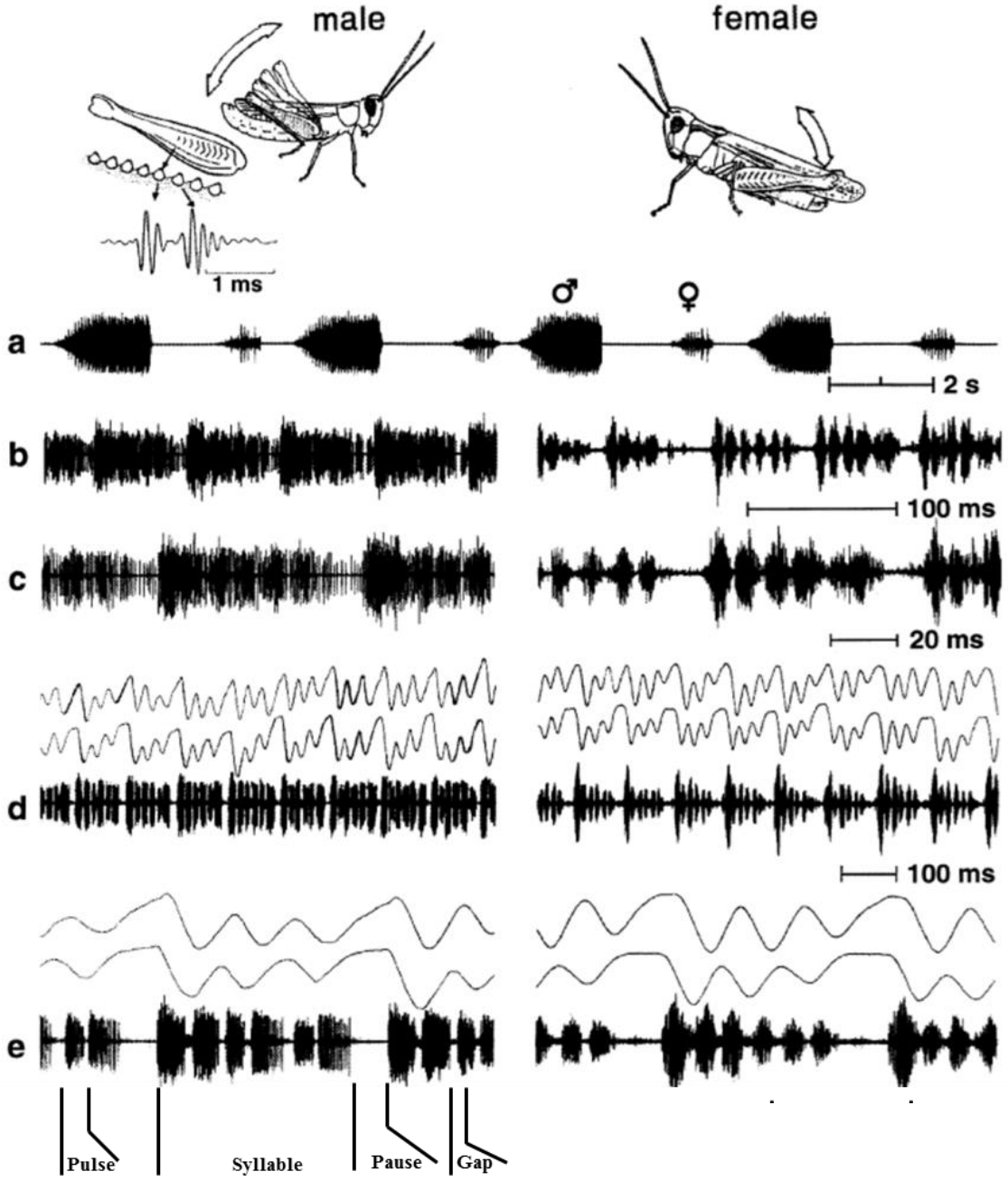

Figure 1.2 **Song patterns of male and female *Ch.biguttulus***. a: duet between male and female. b,c: Parts of song of intact animals stridulating with both hindlegs. d: Movement of hindlegs during stridulation and sound pattern of one leg. e: Same as in d at a larger scale to demonstrate the rectangular modulated pulses of males and the ramp-shaped pulses of females .(Image source: von Helversen and von Helversen 1997)

1.7 Thesis outline

The thesis is divided into chapters where each chapter explains the different part of the project.

Chapter 2 describes the method multielectrode recordings from auditory neurons in the brain of a small grasshopper. This chapter is based on a published manuscript (Bhavsar et al. 2015 a) in the *Journal of Neuroscience methods*.

Chapter 3 is based on the population coding among the ascending auditory neurons in the brain of a small grasshopper. This chapter describes how the information about the stimuli is encoded among the ascending neurons and also the importance of recording from populations of neurons in the same individual.

Chapter 4 describes the locations, role and electrical stimulation of local auditory neurons in the brain of a small grasshopper. This chapter explains where the local auditory neurons are located and the role of one local brain neuron as a feature detector in the brain. This chapter also explains "auditory neuropiles" which have been described by electrical stimulation in the brain of a small grasshopper.

Chapter 5 is the general discussion about the newly introduced method called multiunit recording in the brain of small insects. This chapter mainly describes the pros and cons of using multielectrode recordings in the brain of small insects by comparing the experiences of the people have used this method in different insects to study different sensory processing. This chapter is based on a published review Bhavsar et al. 2015 b in the *Journal of Neuroscience communications*.

Chapter 6 is a summary of the project.

Chapter 2

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

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Author contribution:

Andreas Stumpner and Ralf Heinrich designed the project

Mit Bhavsar performed experiments and analyzed the data

Mit Bhavsar wrote the manuscript draft with help of Andreas Stumpner

Andreas Stumpner and Ralf Heinrich corrected the manuscript

This chapter is from a manuscript published previously in the *Journal of Neuroscience Methods*: (Bhavsar et al. 2015a)

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 minutes or more.

2.1 Introduction

Neuroethology aims at understanding the neuronal basis of animal behaviour. 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 behaviour (Comer and Robertson 2001). In many cases electrophysiological recordings cannot be performed in behaving animals, but neuronal response properties recorded from immobilised animals can be compared to behavioural data (e.g. Roeder 1998). The identified neuron approach often allows extensive comparison across species (e.g.Yager and Svenson 2008). However, understanding elaborated behaviours 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 analyse the activity of simultaneously recorded neurons to understand complex behaviours have been established in vertebrate 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 analyse their potential combined activity (Kostarakos and Hedwig 2012; Meckenhäuser et al. 2014). In order to analyse 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.

2.2 Materials and methods

2.2.1 Animals

Adult female grasshoppers *(Ch. 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).

2.2.2 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).

2.2.3 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). 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 25000 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.

2.2.4 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 towards the speaker, grid on, and a Brüel & Kjær measuring amplifier (type 2602). Sound intensities are given in dB SPL (Sound pressure level) re 2×10^{-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 to 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 to 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.

2.2.5 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 metathoracic ganglion, enter the brain dorsally and project into the lateral dorsal protocerebrum (Stumpner and Ronacher 1991; Kutzki 2012). Local auditory brain neurons are postsynaptic to the ascending auditory neurons. However, so far there is very little information available about these neurons.

For the visualization of the recording site after a successful recording, several methods have been tested. The first method was electrical current-driven deposition of copper which was adopted from a study by Guo and Ritzmann (2013).

The second method intended to mark the recording location with a fluorescent dye. After a successful recording the electrode was retracted from the brain, dipped into the fluorescent dye lucifer yellow (Sigma-Aldrich – St. Louis, USA) and reinserted to the previous location until the auditory activity was detected again. The electrode was kept at this position inside the tissue for 10 seconds to let the dye diffuse into the tissue. Then a drop of Paraformaldehyde (PFA, 4%) was added to fix the

tissue in the vicinity of the electrode and to prevent extensive diffusion of Lucifer yellow. The brain was extracted from the head and fixed in PFA for 2 hours. Then it was dehydrated in an ascending alcohol series (30%, 50%, 70%, 80%, 90%, 96%, and 2 times 100% ethanol, each step 20 min) and finally transferred into methylsalicylate (Sigma-Aldrich – St. Louis, USA). The whole mount preparation was observed with a fluorescence microscope (Axioscope, Zeiss – Jena, Germany). For subsequent analysis, the brain was rehydrated, embedded in albumin-gelatin (Crane and Goldman 1979), fixed in 4% PFA overnight and sectioned transversely or horizontally into 30 µm slices with a vibrating blade microtome (VT1000s Leica – Wetzlar, Germany). The sections were transferred to a slide, enclosed under a cover slip using DABCO as a medium and viewed with a fluorescence microscope (Axioscope, Zeiss – Jena, Germany).

With a third method we tried to coagulate the tissue at the recording site by passing a high current after data collection through one of the tungsten wires. If successful, this coagulates the brain tissue and generates a black spot at the approximate recording location. We tried different currents (up to 0.2 mA, 9V) which were passed between one of the tungsten wires and a reference wire for periods of 5 to 20 minutes. Then the brain was extracted from the head and fixed in PFA for 2 hours. Brains were sectioned and prepared for microscopic analysis as described in the previous paragraph.

2.2.6 Offline spike sorting

Spike sorting is a technique to group spikes based on the similarity of their shape. Given that spikes of each neuron will be recorded with a particular shape depending on the distance between the neuron and the electrode, the resulting clusters represent the activity of different individual neurons (Quiroga 2007). Spike2 7.10 was used for spike sorting. However, spike sorting is sensitive to misclassification (Harris et al. 2000; Joshua et al. 2007; Quiroga 2007) so special care has been taken for this problem. As a first step, a finite impulse response filter was applied on each channel (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. If only three wires were used, one of the three channels was copied and all four channels were analyzed as a

tetrode. The threshold for spike detection was set to ± 3 * standard deviation (SD) of the mean signal amplitude of 10 seconds of recording without acoustic stimulation at the beginning of the experiment (Brill et al. 2013). It was decided not to use subtracted versions of the channels for spike sorting (Brill et al. 2013) since the acoustically stimulated spikes were highly coincident and similar among the three channels and subtraction led to the loss of most of the important information. The time window was set from -0.4 ms before to 1 ms after either positive or negative peak amplitude for the template formation. Acoustic stimuli with high sound pressure levels are known to induce high frequency firing with similar latency in the population of ascending auditory interneurons, especially at the start of the response. Hence, regularly occurring overlapping spikes of different neurons may be interpreted as separate templates (= spike shape of a particular neuron) by the software. To avoid this, template formation was done in responses to stimuli with low sound pressure levels (30 – 60 dB SPL), since all the ascending neurons in *Ch. biguttulus* relevant for song recognition generate action potentials to an acoustic stimulus below 60 dB SPL at lower frequencies and white noise (Stumpner and Ronacher 1991). Generated templates were then applied to the complete range of stimulus intensities used in the experiments after template formation. The sorted units were clustered by applying the clustering dialogues of Spike2. A cluster in the principle component analysis (PCA) 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). After cluster analysis, interval histograms of all sorted units were plotted and the spike shapes were superimposed to make false positive sorting visible. After completion of analysis clusters might represent spikes of individual neurons.

2.2.7 Collision analysis

Spike collision is a common problem in the analysis of multiunit recordings (Pillow et al. 2013). The collisions occur when two or more neurons fire at nearly the same time and the resulting waveform is a summation of the individual spike shapes of these cells (Wehr et al. 1999). It is desirable to extract individual spikes from such collisions in order to assign them to a particular neuron and reduce the inaccuracy of the data analysis. The software Spike2 has an inbuilt “matching

algorithm" which supports the extraction of overlapping spikes. Collisions are identified by an exhaustive search among all possible pairs of templates at all temporal alignments of the identified spike waveform templates. A special program script was written in Spike2 which can automatically go through the entire recording, detect the potential collisions and replace them, wherever possible, with two spikes in single units.

2.2.8 Constancy of recording conditions

Stability of the preparation and quality of the recordings have been analyzed in two different ways. First by quantifying the signal-to-noise ratio and then by analyzing changes in spike shapes. To analyze the stability of the preparation, the intensity recording program (*Methods –Acoustic stimulation*) was repeated at the end of the experiment and the recorded neural activities from both stimulus series were compared. The time difference between the start and the end of the experiment typically was around 15 minutes. To determine the signal-to-noise ratio, the "Signal" value was calculated by taking the root mean squared (RMS) amplitude of the responses to 10 stimuli while the "Noise" was calculated by taking the RMS amplitude from the first 10 seconds of recording without acoustic stimulation.

2.2.9 Unit identification

Ascending auditory neurons have previously been recorded intracellularly in the thorax of *Ch. biguttulus.* These ascending neurons were characterized with respect to their morphology and physiology (Stumpner and Ronacher 1991). After spike sorting and collision analysis of multiunit recordings, identification of the sorted units was attempted by comparing their sound evoked responses with the responses of previously identified neurons.

2.3 Results

2.3.1 Marking the recording locations

Fig.1 shows the locations of the recording with a multielectrode. Fig.1-A displays the sketch of a grasshopper brain with the projection area of most of the auditory ascending neurons (AN-a, AN-b) and local auditory brain neurons (BN). Fig.1-B shows the marking of the two recording locations of local auditory brain neurons using the fluorescent dye lucifer yellow as described in the *Methods*. Shown is the deepest horizontal section with fluorescent dye marking. Localized marking sites were only achieved with PFA fixation shortly after insertion of the dye - coated electrode. Otherwise widespread diffusion of lucifer can mask the location of the recording. The two methods with current injection had different success. Low current with the attempt of precipitating copper did not give reliable results. Weak dark spots appeared only in some of the preparations. Electrocoagulation with higher current, on the other hand, always leads to dark markings and sometimes even holes in the brain tissue. However, passing a current of about 70 µA (5V) for 5 minutes lead to dark markings without damage. The effectivity of the current obviously depends on depositions at the electrode tip. This cannot be controlled during the experiments. In some cases, the brain got damaged even after 5 minutes current injection.

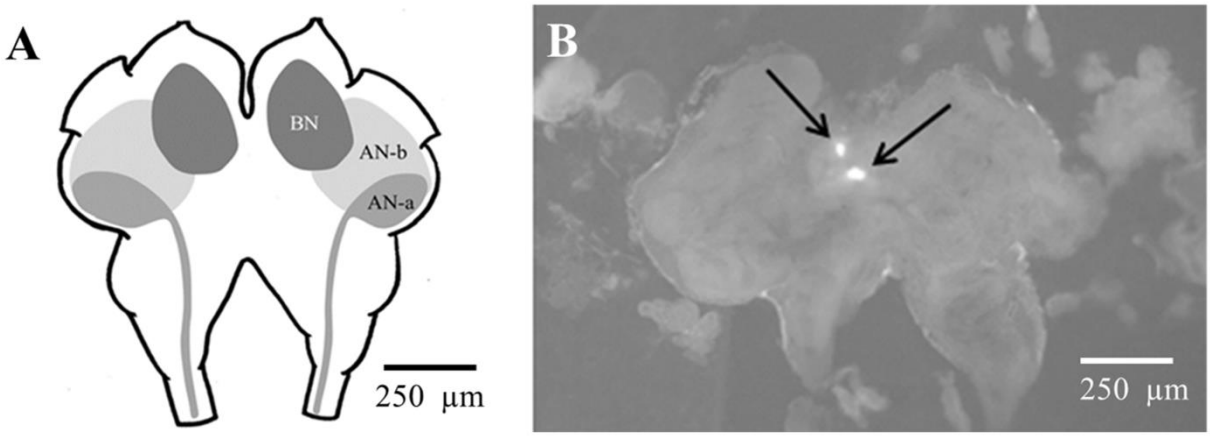

Figure 2.1 **Locations of the recording.** (A) Sketch of a grasshopper brain. AN-a: projection area of the majority of auditory ascending neurons in the lateral protocerebral neuropil; AN-b: alternative projection area of some of the auditory ascending neurons like AN1; BN: the majority of auditory local brain neurons. (B) Marking of the recording location (local brain neurons) using lucifer yellow. Locations are highlighted by black arrows

2.3.2 Comparison between copper and tungsten wire recordings

The first series of experiments was performed with electrodes made from copper wires as described for previous recordings from bee brains (Brill et al. 2013). Penetration of electrodes into the deutocerebrum or the lateral protocerebrum, structures that both contain axonal projections of ascending auditory interneurons (Fig.2.1-A), detected auditory activity with high success rate. However, the signal-to-noise ratio in these recordings was not satisfactory (< 1.5) which lead to problems in template formation during spike sorting. For comparison of wires made from different materials, the signal-to-noise ratio was calculated for the copper wire from 10 different preparations and compared to the signal-to-noise ratio of 10 different recordings with tungsten wires. The signal-to-noise ratio of tungsten wire recordings was clearly higher than that of copper wires which can be seen in the box plot shown in figure 2.1-A (Mann-Whitney U-test: $p = 0.002$). An example recording can be seen in figure 2.2-B. Also the higher tensile strength of tungsten wires compared to copper wires helped to use single multielectrodes repeatedly. Due to these advantages multielectrodes made of tungsten wires were used for subsequent experiments.

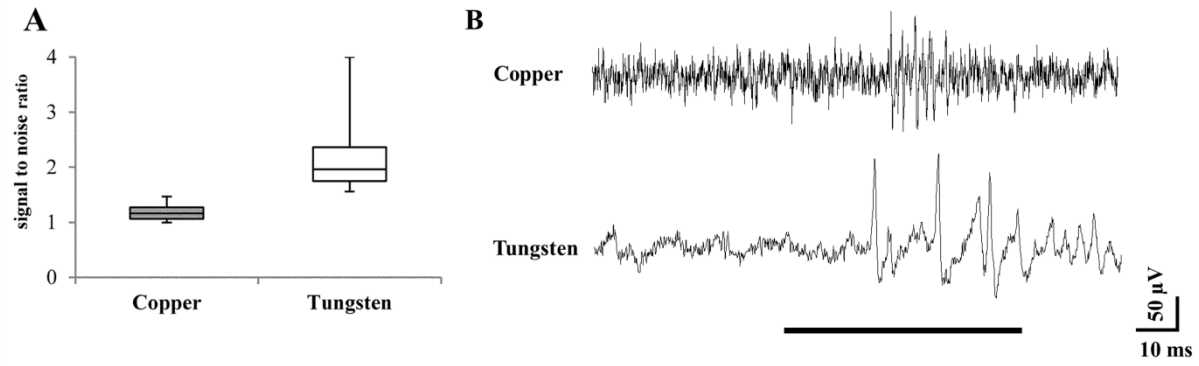

Figure 2.2 **Recordings of auditory activity with multielectrodes made from copper or tungsten wires.**

(A) signal-to-noise ratio was determined from auditory responses to 20 kHz stimuli (Signal) and spontaneous activity without acoustic stimuli (Noise) in 10 preparations for each type of wire. The length of the box represents the interquartile range. The black line in the box represents the median value. The upper and lower whiskers represent the maximum and minimum values respectively. (B) Recording example showing the response to copper and tungsten wires. The black line marks the stimulus.

2.3.3 Spike sorting

Offline spike sorting was done to isolate the activity of single units from the multiunit recording (Fig.2.3). Units were separated based on differences in their spike shape. Spike sorting was done without subtracting between the channels since the auditory spikes were highly similar among the three channels and subtraction would cause loss of most of the important information which can be seen in figure 2.3-B. For clear separation of spikes based on their shapes, the principle component analysis method has been employed as described in the *Materials and method* section. Figure 2.3-D shows three clustered units surrounded by 3.5 times the Mahalanobis distance. The resulting clouds are fairly well separated from each other which may be interpreted as the presence of three different types of spikes being generated by three different neurons. Figure 2.3-E shows the interval histograms for three different units from figure 2.3-D. An overlay of spikes assigned to one cluster (Fig.3-F) serves as a control for the quality of spike sorting. Within the activity of one cluster, one would not expect any spike intervals shorter than 2 ms, relating to the refractory period following an action potential in one neuron. After this procedure, the sorted spikes were plotted on three different channels (Fig.2.3-G).

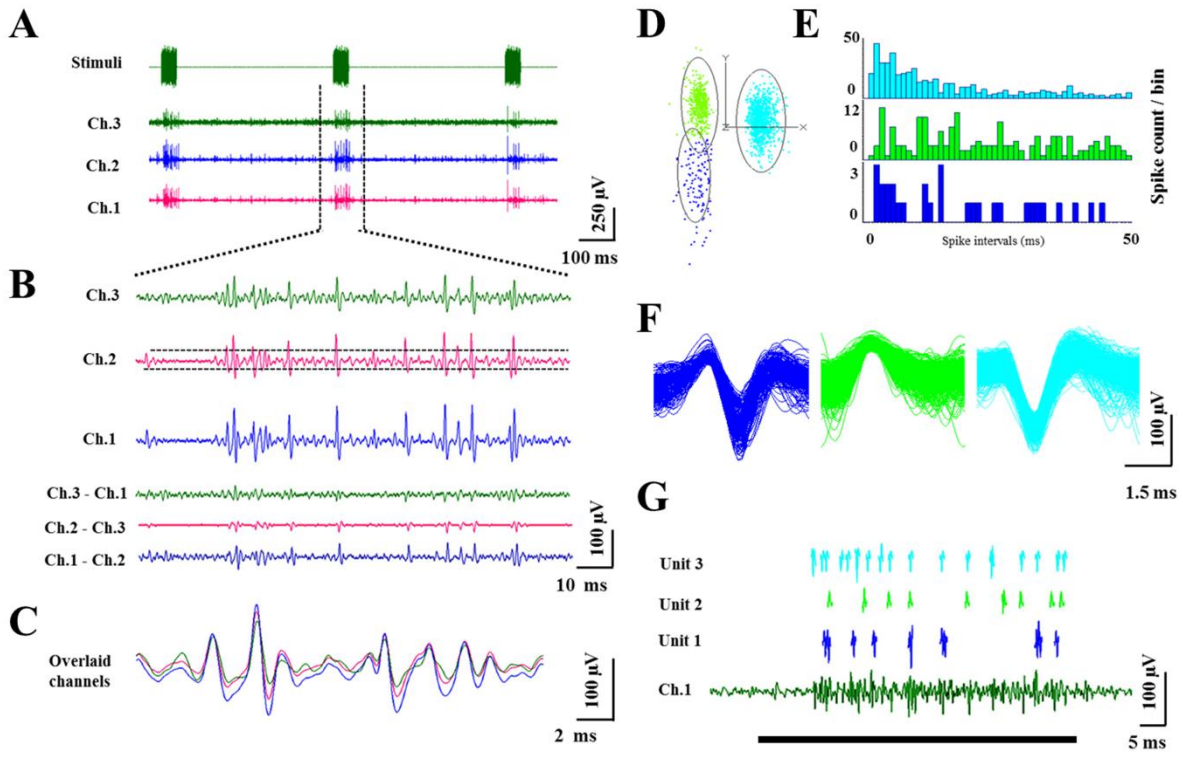

Figure 2.3 **Spike sorting in multiunit recordings of acoustically stimulated activity in ascending auditory interneurons** (A) Response of ascending auditory neurons to acoustic stimuli recorded via three different channels of a multi electrode. (B) Magnified version of channels shown in A. and the result of subtracting the channels with extended scale. The threshold for spike detection (shown as dotted lines in the middle part) was set as: mean (±) 3S.D. during 10s of recording without acoustic stimulation. (C) Superimposed recordings from the three channels to visualize the subtle differences between the signals. (D) The clustered units that emerge from principle component analysis are surrounded by 3.5 times Mahalanobis distance. (E) Interspike interval histograms for all spikes of each sorted unit. F. Superimposed spikes of each sorted unit showing different spike shape and numbers (Unit 1: 517 spikes, Unit 2: 527 spikes, Unit 3: 1174 spikes). (G) Occurrence and waveforms of three sorted units extracted from channel of the multielectrode recording. Black line marks stimulus duration.

2.3.4 Collision analysis

If a channel contains more than one class of spikes and the spikes are independently generated by different neurons, temporal spike collision can prevent spikes from being correctly assigned to a unit. When two cells fire with similar latency to a stimulus, this will produce a complex compound spike shape. Collision analysis was done using the 'Matching algorithm' of the Spike2 software. It detects the collisions by searching for two templates whose sum is most similar within predefined limits to the compound spike and replaces it with spikes aligned to the best matching templates (Fig. 2.4-A). The following strategy has been followed to optimize collision analysis. The spike templates were generated only for low stimulus intensities that initiate rather sparse spiking activity (30 - 60 dB SPL) to avoid superimposed spikes being detected as a new template. Templates derived from periods of low level activity were subsequently used in collision analysis. The result of collision analysis was evaluated by plotting peristimulus time histograms (PSTH) before and after doing collision analysis for intensity recordings (50 - 90 dB SPL) of 20 kHz stimuli. An increase in the total number of spikes is especially seen at shorter latencies when ascending interneurons simultaneously start firing with high frequencies leading to a high degree of overlapping spike activity (Fig.2.4-B). Collision analysis increased the total number of spikes that could be assigned to a particular spike template and hence to a particular neuron by an average of 18% (range 11% – 29%, n=5).

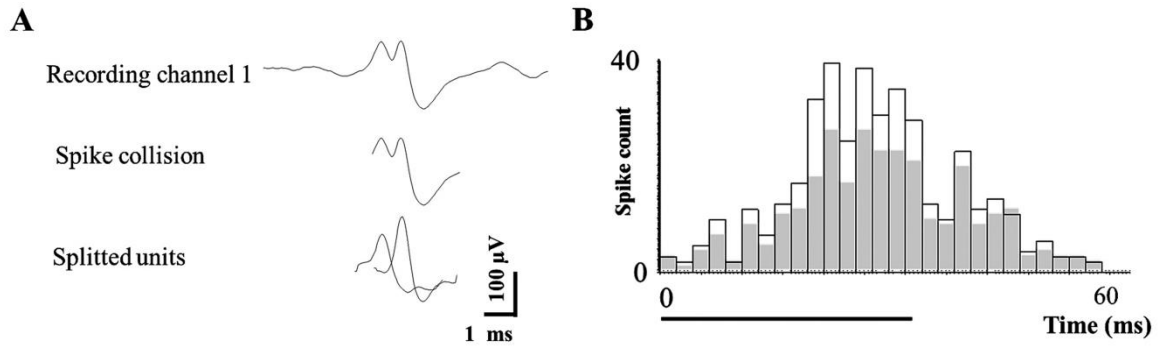

Figure 2.4 **Collision analyses in recordings from ascending auditory interneurons.** (A) Recording from one channel of the multielectrode containing partially overlapping spikes from two different units. The detected compound spike and its separation into spikes of two different single-spike templates extracted from the same recording are shown below. (B) Superimposed PSTHs of total spike activity derived from the same recording during an intensity scan of 50 - 90 dB SPL with 20 kHz stimuli before (gray) and after (white) collision analysis. Collision analysis increases the total number of detected spikes from 87 to 101 especially in the beginning of the acoustically-stimulated response (15 ms latency). The black line marks the stimulus.

2.3.5 Constancy of recording conditions

Figure 2.5-A shows a comparison of signal-to-noise ratios at the beginning and at the end of recording experiments. No significant difference in the signal-to-noise ratio was seen after approximately 15 min. of recording with the multielectrode. To analyze potential changes in spike shapes, spike sorting was done (as described in *Methods*) in the beginning of the experiment and at the end of the experiment and the results were compared. As a typical example, figure 2.5-B shows the result of such an analysis for two sorted units. Alterations of their spike shapes after the 15 minute recording period are minor and do not impact their discrimination.

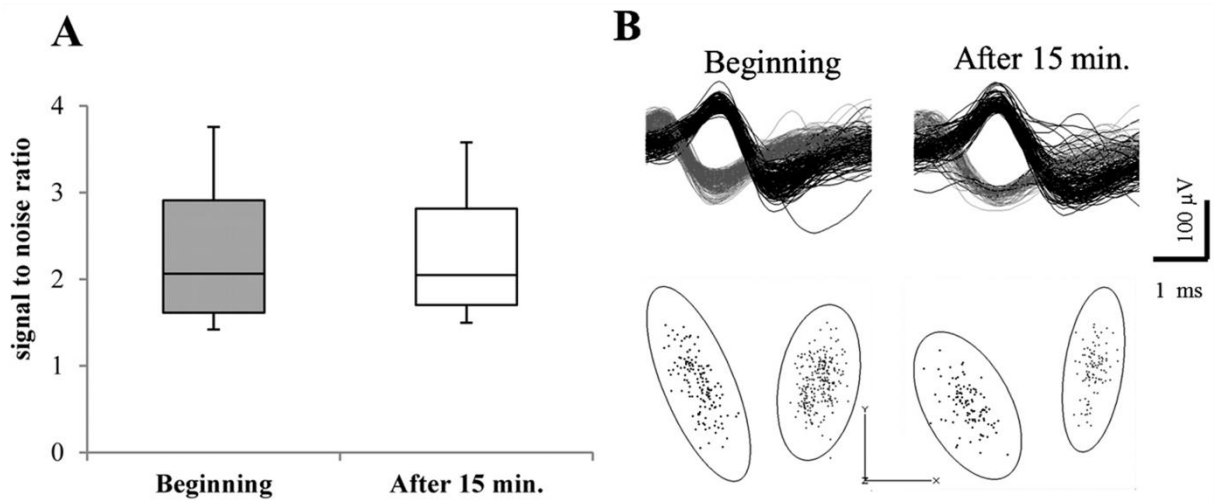

Figure 2.5 **Analysis of the stability of multielectrode recordings from ascending auditory interneurons**. (A) Comparison of signal-to-noise ratios in the beginning and about 15 minutes later of four experiments. The box plot represents the same information as explained in figure 2-A (B) Superimposed sorted spikes of two units along with a cluster analysis from the same channel of a multielectrode in the beginning (left) and at the end (right) of one experiment.

2.3.6 Auditory units

Figure 2.6 shows examples of PSTH of four individual units recorded during a series of 20 kHz acoustic stimuli with varying intensity (50 - 90 dB SPL). Three units increased their firing rates at expected latencies between 13 ms to 15 ms following stimulus onset. Therefore these units were considered as auditory units and used for further analysis. Units which did not show any stimulus dependency like unit 4 were considered as non-auditory and were not included into further analyses (figure 2.6).

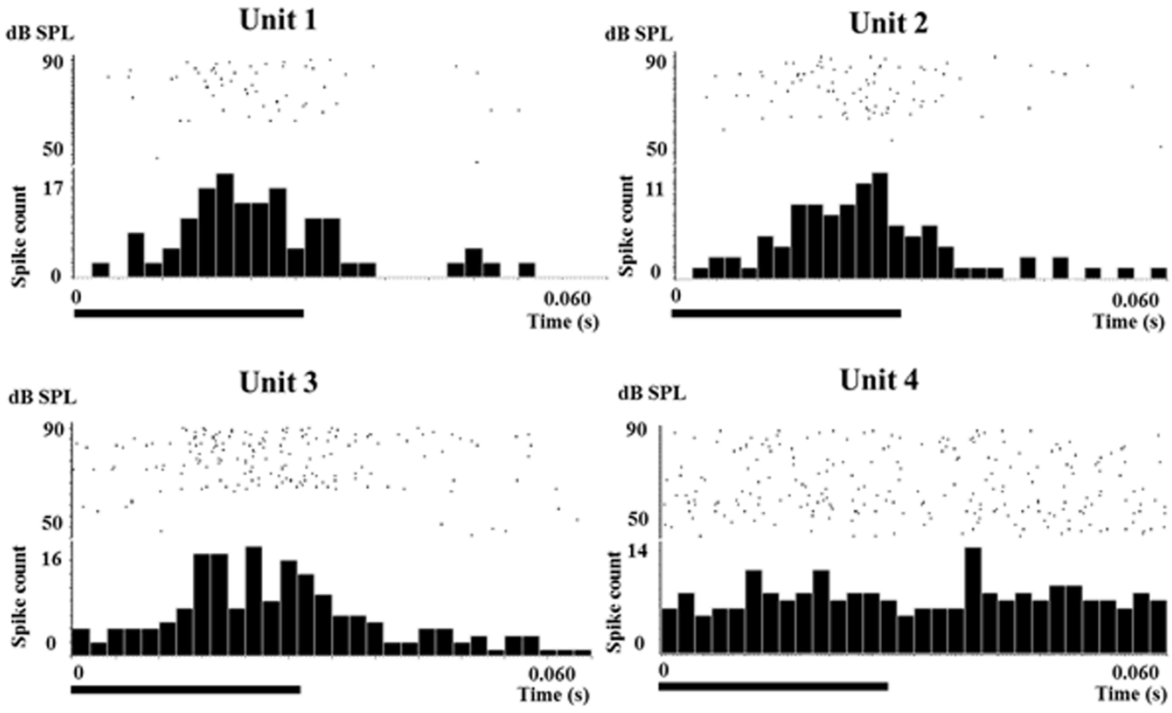

Figure 2.6 **Auditory units.** Responses of single units to acoustic stimuli (20 kHz; 25 ms; 50 - 90 dB SPL) and Peristimulus time histograms of cumulative responses. Auditory units 1-3 increase their firing rate after the expected latency while the firing pattern of the unit 4 is not influenced by the acoustic stimulus. PSTH width: 60 ms, bin size: 2ms, black line marks the stimulus.

2.3.7 Intensity response functions and unit identification

In order to identify the types of ascending auditory interneurons the results of extracellular multielectrode recordings in the brain were compared to previous physiological data acquired by intracellular recordings of ascending interneurons in the metathoracic ganglion of *Ch. biguttulus* (Stumpner 1988; Stumpner and Ronacher 1991). Figure 2.7 compares the responses to ipsi- and contralateral stimulation (defined as the position of the speaker with respect to the side of recording) with white noise and to ipsilateral stimulation with 5 kHz and 20 kHz stimuli between one extracellularly recorded unit and the identified neuron AN2. The extracellularly recorded unit displays a strong difference between ipsi-and contralateral stimulation at intensities ≥ 60 dB SPL (Fig.2.7-A left). Previous studies identified one of the ascending auditory interneurons with a prominently different intensity response to ipsi-and contralateral stimulation as AN2 (Fig.2.7-A right) (Stumpner

and Ronacher 1991). When comparing the responses to 5 kHz and 20 kHz of the extracellularly recorded unit to responses of two different AN2 (Stumpner 1988), the intensity dependence of 20 kHz is somehow similar between the extracellularly recorded unit and one of the intracellularly recorded AN2, though absolute spike numbers differ. At 5 kHz, however, the functions look different especially between 60 and 80 dB SPL (Fig.2.7-B), which is true also for two intracellularly recorded AN2 from two different individuals.

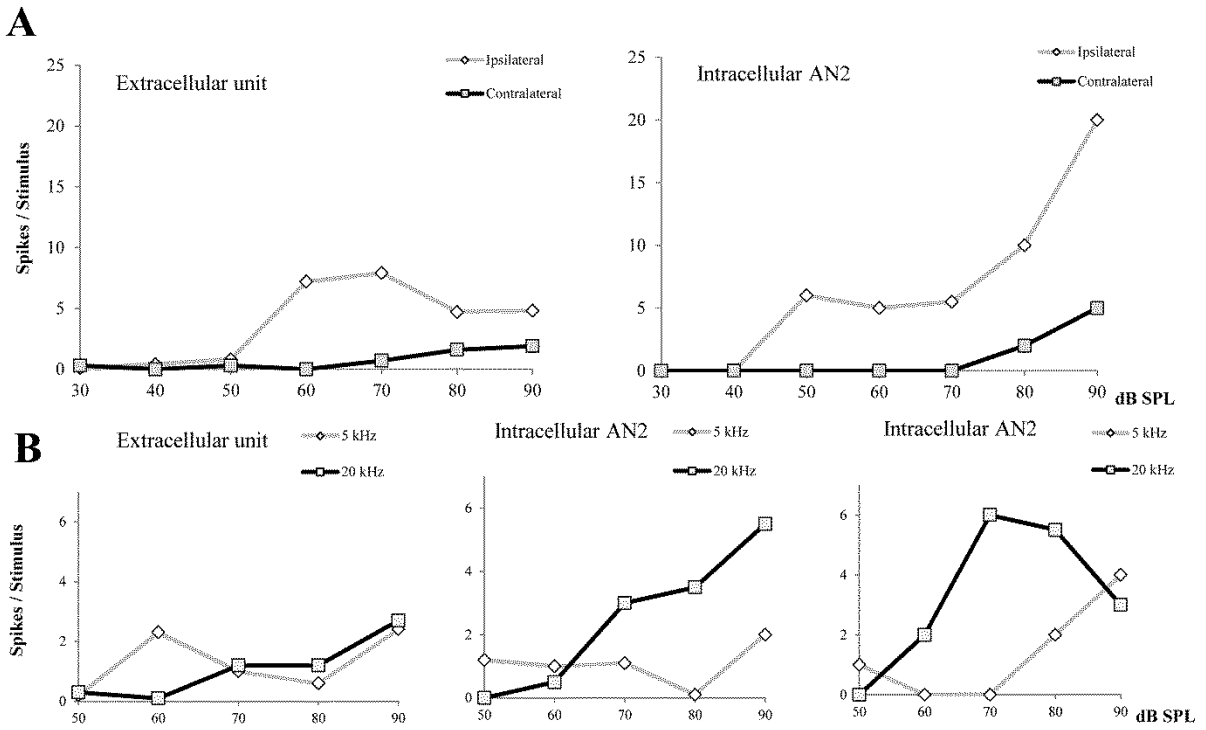

Figure 2.7 **Unit identification** (A) Intensity response functions of a single sorted unit (left) and an intracellularly recorded AN2 (right) for white noise stimuli (100 ms) from ipsilateral and contralateral (B) Intensity response functions of the same unit as in A for 5 kHz and 20 kHz (25 ms) stimulus (left), and the intensity response functions for two intracellularly recorded AN2 (middle and right). Data of AN2 are modified from Stumpner (1988) and Stumpner and Ronacher (1991)

Figure 2.8 shows another comparison between an extracellularly recorded unit and an identified ascending neuron, the AN12. Most obvious is the much lower spike number of the extracellular recording, so that the intensity dependence is hard to compare (Fig. 2.8-A). A special characteristic of AN12 not shared by any other ascending interneuron is its phasic response to the onset of each syllable in *Ch. biguttulus* songs (Stumpner and Ronacher 1991; Creutzig et al. 2009). Artificial songs that retain the typical syllable to pause relation of the natural songs elicit pronounced syllable-onset activity in multiple successive syllables, while artificial songs with too short pauses stimulate strongly reduced responses to syllables of a series (Fig.2.8-B right). This feature is also seen in the extracellularly recorded unit (Fig.2.8-B left). Based on these evidences; the unit was identified as AN12.

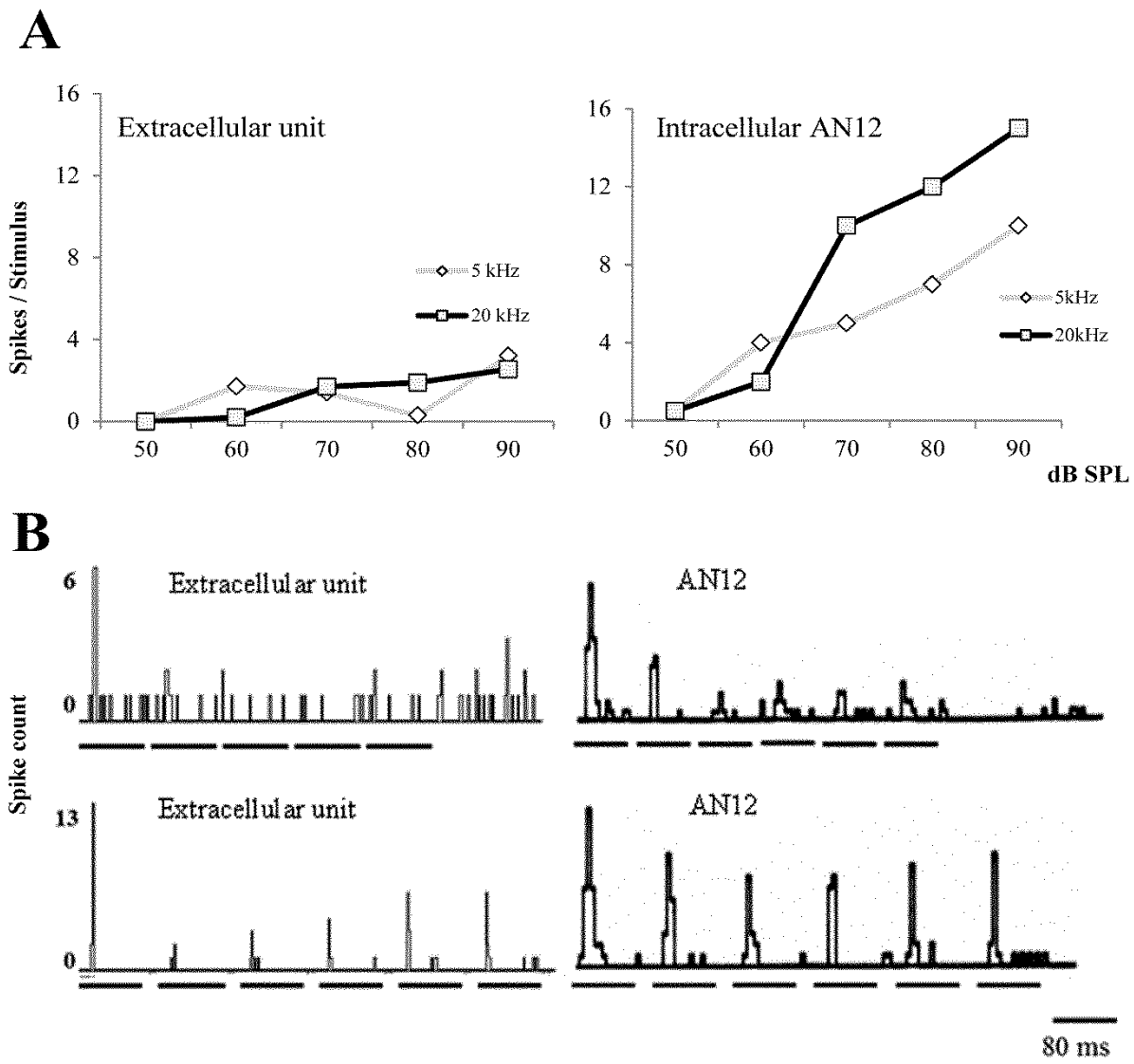

Figure 2.8 **Unit identification**. (A) Intensity response functions of a single sorted unit for 5 kHz and 20 kHz (25 ms) stimuli (left), Intensity response functions of AN12 for 5 kHz and 20 kHz (25 ms) stimulus (right) (B) PSTH showing the response of the single sorted unit to two different artificial grasshopper songs of 80 ms-7.5 ms pattern(left-up) and 80 ms-40 ms pattern (left-down) which is compared to PSTH of two different artificial grasshopper songs of 85 ms-8.8 ms pattern (right-up) and 85 ms-42.6 ms pattern (right-down) for AN12. Data of AN12 are modified from Stumpner (1988)

2.4 Discussion

The activity of several auditory neurons was simultaneously recorded from the brain of the grasshopper *Ch. biguttulus* for the first time using multielectrodes. This type of method has previously been applied in honeybees (Brill et al. 2013), locusts (Saha et al. 2013) and cockroaches (Ritzmann et al. 2008). Our studies demonstrate the stability of multielectrode recordings for sufficiently long periods that allow extensive characterization of neuronal activity for large numbers of stimulus repetitions. In addition we demonstrate reliable isolation of single unit activities from multiunit recordings using spike sorting and spike collision detection. In some cases, single unit activity can be attributed to the identified ascending auditory neurons that have previously been characterized by intracellular recording. Extracellular recordings have the advantage of recording from more than one neuron for a significantly longer period of time than achieved by intracellular recordings from most insect neurons (Vogel et al. 2005; Vogel and Ronacher 2007). Such multiunit recordings also allow examination of population coding by neuronal assemblies. However, multiunit recordings also have some limitations which will be discussed below.

2.4.1 Production of multielectrodes

For recordings in vertebrate central nervous systems, one has the freedom to choose larger diameter electrodes (around 50 to 150 µm) due to the larger sizes of brains and brain regions associated with particular functions (e.g. monkeys: Crist & Lebedev, 2008). In most invertebrates, like in the grasshopper, central nervous structures are much smaller and hence the size of wires and the diameter of the multielectrode is an important factor for the applicability of multielectrode recordings. Insulated copper wires (diameter 15 µm) as have been used in previous studies on other insects (Brill et al. 2013) allowed to record auditory activity in the grasshopper brain. However, signal-to-noise ratio of the recordings was insufficient for reliable spike detection and spike sorting. One reason for a low signal-to-noise ratio could be the high impedance of these wires (around 250 kΩ at 1 kHz). So the copper wires were replaced with tungsten wires (diameter 12 µm) which provided an increased signal-to-noise ratio allowing a more reliable determination of spikes and single unit spike shapes in the recording due to lower impedance of these wires (around 70 kΩ at 1 kHz). In addition to that, the

smaller diameter of tungsten wire electrodes compared to copper wires allows more focal recording from the bundle of ascending auditory neurons and likely causes less damage of brain tissue during penetration. Wires with even smaller diameter are not suited to build electrodes since, even after embedding in dental wax, they will be too fragile and flexible for penetration into brain tissue. Tungsten wires with 12 µm diameter are already requiring careful handling during electrode preparation.

2.4.2 Marking the recording locations

The first method to mark the recording location by electrical current-driven deposition of copper did not give reproducible results. The second method which was marking the locations with the fluorescent dye lucifer yellow gave some reliable results (Fig.2.1-B). Although in all cases auditory activity was immediately found again at the site of insertion, it imposed some uncertainty to match the exact recording position of the electrode. The third method was electrocoagulation of the tissue at the recording site by passing high current through one of the tungsten wires to generate a dark spot of coagulation at the recording sites. With this method there is always a risk of damaging a larger area of surrounding tissue or the whole brain due to high current passing for a longer time. However, carefully controlling the time (around 5 minutes) of current injection can give good and reliable results.

2.4.3 Constancy of recording conditions

One major advantage of multiunit recordings in insects with metal micro wires is the possibility to record for at least 30 minutes from the same set of neurons. Multiunit recordings are extracellular recordings in which electrodes are placed in the vicinity of the neurons. Therefore, the neurons likely remain undamaged which prevents injury-related tampering of neuronal activity, increases the stability of the preparation and thus allows the application of longer stimulus protocols. In intracellular recordings the recording duration is mostly much shorter than one hour – often below 10 minutes because of stability problems. This is especially true, when neural activity can only be recorded from neurites (Chorev et al., 2009). We quantified the stability of multiunit recordings in four different animals by comparing neuronal responses to the same series of acoustic stimuli with varying

intensities at the beginning and end of an experiment. No significant changes in signal-to-noise ratio and shapes of the recorded spikes were observed which demonstrated high stability of the preparation.

2.4.4 Spike sorting and collision analysis

Spike sorting methods should ideally group the spikes based on their shape (Quiroga 2007; Takekawa et al. 2010). We have decided to use Spike2 software for spike sorting instead of some other sorting algorithms like offline sorter (Plexon – Dallas, USA), Waveclus (Quiroga et al. 2004) and a customized MATLAB subroutine for spike sorting (Mathworks – Natick, USA) (Martelli et al. 2013) since Spike2 is a fully developed and easy to handle spike sorting program with inbuilt algorithms for Principle component analysis (PCA) and overlapping spike analysis. There are three main steps involved in Spike sorting: (1) spike detection, (2) feature extraction and (3) spike clustering based on combinations of extracted features (Takekawa et al. 2010). Potential spikes are detected in the first place using an amplitude threshold. Choosing the threshold is critical: If the value of the threshold is too low, noise fluctuations will get detected as spikes and if it is too high, too many low-amplitude spikes will be missed. There is a criterion established to detect the threshold as a multiple of an estimate of the standard deviation of the noise, i.e., threshold = mean ± k * SD, where k is a constant typically between 3 and 5 (Rey et al. 2015). Spikes were detected using a threshold of mean ± 3 SD over 10 seconds of recording without stimulation which in our case gave the best compromise between avoidance of noise fluctuation and detection of auditory spikes. The first three principle components were used to extract features of the spike waveforms and used for the clustering. The clusters were separated by using 3.5 times Mahalanobis distance around the center of gravity (Wölfel and Ekenel 2005). However, it was very difficult to exactly determine the boundaries in the regions where clusters were overlapping just by using this criterion. Therefore, an additional manual approach has been applied to determine the boundaries, especially for the regions where clusters were overlapping. Still, classification errors can arise due to the presence of very similar waveforms in two neurons. This may lead to a cluster which is considered as a ‘single unit’, but in reality contains spikes from two or more neurons (Gray et al. 1995). This type of error can be detected by the presence of interspike intervals shorter than 2 ms indicating the absence of a clear refractory period in the interval

distribution of a spike train. However, even if such short intervals do not occur this does not completely exclude the possibility that two neurons which are mostly active at different times may be classified into one template. So there is no absolute guarantee that spikes assigned to one template are always generated by a single neuron (Gray et al. 1995).

'Collision' is a word used to describe spike waveforms generated by two or more neurons firing nearly at the same time which will lead to spike overlap and produce a distorted waveform in the recording. Normally it is relatively easy to identify such collisions in the recording when a small delay between the spikes is present producing double peaks (Fig.2.4-A). However, if two neurons fire absolutely synchronously with opposite potential deflections, the overlap may lead to "extinction" by resulting in amplitude below threshold value of both spikes. Conventional spike sorting techniques fail to identify such collisions in the recording (Wang et al. 2006) which will lead to loss of information. However the significance of overlapping spikes should not be overlooked, since it can affect the accuracy of spike sorting (Lewicki 1998). Some methods have been proposed to detect such collisions in the recording, for example the "binary pursuit" algorithm (Pillow et al. 2013), the "deconfusion" method (Franke et al. 2010) and a fast Fourier transforms (Wang et al. 2006) all having their advantages and disadvantages. In our study, we used the inbuilt "Matching algorithm" of Spike2 to detect and extract spike collisions present in the recordings. The auditory neurons tend to fire with similar latency especially at the beginning of the stimulus because of the tight coupling to temporally precisely defined stimuli. So the collisions of spikes are expected to occur regularly and especially at the beginning of the stimulus. Therefore the results illustrated in Fig.2.4-B showing higher spike numbers in the classes between about 15 and 25 ms after stimulus onset representing the beginning of the response is in line with these expectations.

2.4.5 Unit identification

The original idea of this study was to compare the physiological responses of extracellularly recorded sorted units to the physiological responses of intracellularly recorded ascending neurons (Stumpner and Ronacher 1991) and to identify the extracellularly recorded neurons based on the similarity of the responses to those recorded intracellularly. However, identification of neurons just by

comparing extracellular data with intracellular data was not as easy as expected which is exemplified for AN2 and AN12. One reason for the identification problem is neuronal variability. Intracellular recordings are more prone to trial-to-trial neuronal variability than extracellular recordings (Ostrowski and Stumpner 2014) (see also similar problems encountered in the leech CNS (Baljon and Wagenaar 2015)). The intracellular recordings have been made from dendritic regions in the metathoracic ganglion where neurons are penetrated with an electrode which causes cell injury and additional depolarization due to intrusion of extracellular fluid which may lead to higher numbers of spikes to a particular acoustic stimulus. Also when recording in the metathoracic ganglion, housing the first center of auditory processing (Römer et al. 1988), opening of the thorax likely affects physical characteristics of the peripheral auditory system which may affect the bioacoustics and may also introduce some variability in the spike response. In contrast, recordings from the brain leave the thoracic auditory system completely intact. The lower spike numbers seen in extracellularly recorded data in the present study and intracellular recordings from the brain in another study (Kutzki 2012) may therefore be more similar to natural auditory information processing. Additionally, there is also interindividual neuronal variability (Stumpner 1989) as shown in figure 2.7-B. Intracellularly recorded responses of AN2 from two different individuals (described in Stumpner 1988) differ considerably. Interindividual neuronal variability has been studied to some detail (Meckenhäuser et al. 2014; Ronacher et al. 2004; Vogel et al. 2005). The sources of such interindividual neuronal variability could be differences in sensitivity of cells, biophysical differences between animals, differences in synaptic strength, number of synapses and stochastic nature of spike generation which can affect the transmission at different stages in the nervous system (Meckenhäuser et al. 2014; Ronacher et al. 2004; Vogel et al. 2005). Adaptation and habituation of neuronal responses may also contribute to spike train variability, since spike count and spike timing change markedly during longer stimuli or with repeated stimulations (Stumpner and Ronacher 1991; Ronacher and Krahe 1998; Givois and Pollack 2000; Krahe et al. 2002a). Due to these reasons the neuronal response is variable within the same animal during repeated trials and among different animals which makes it difficult to compare the responses with extracellularly recorded responses from one single experiment for identification of neurons. However, some ascending neurons encode particular properties of acoustic stimuli in their

physiological responses that may increase the reliability of identification (Stumpner and Ronacher 1994a). For example, AN12 has a phasic characteristic mediating pronounced responses to stimulus onsets (Creutzig et al. 2009), AN4 is involved in gap detection (Ronacher and Stumpner 1988) and neurons like AN1 and AN2 encode directional information (Stumpner and Ronacher 1994). These partially unique characteristics can be useful criteria for the identification of extracellularly recorded auditory units. The unit depicted in figure 2.8 displays a pronounced and phasic response to the onset of each syllable in an artificial song only if the pauses between two syllables are sufficiently long. This equals the typical response of AN12 (Stumpner 1988; Creutzig et al. 2009). Since this response characteristic among ascending auditory interneurons of *Ch. biguttulus* is especially reliably seen in AN12, the extracellularly recorded unit can with high likelihood be identified as AN12. So in addition to comparison of extracellular responses to intracellular physiological responses with standard stimuli, responses specific to stimuli mimicking natural grasshopper songs can be a good criterion for identifying the extracellularly recorded neurons.

2.4.6 Multielectrode recordings and song recognition in grasshoppers

Research in neurophysiology has focused on understanding how populations of neurons encode naturalistic stimuli in vertebrates (Pasupathy & Connor 2002; Petersen et al. 2001). This approach is also gaining attention in invertebrates (Ritzmann et al. 2008; Brill et al. 2013; Campbell et al. 2013; Saha et al. 2013). In the grasshopper auditory system ascending neurons process acoustic stimuli, extract and encode particular features of the signal and convey the preprocessed information to the brain where neural circuits for pattern recognition reside (Bauer and von Helversen 1987; Clemens et al. 2011, 2012). It has been experimentally demonstrated by some studies that there is a better decoding of the stimulus when one analyses the activity of the population of neurons which suggests that the information in the auditory pathway of grasshoppers is represented according to a population code (Clemens et al. 2011; Meckenhäuser et al. 2014). These studies have pooled single cell data from different individuals for modeling the processing in one individual central nervous system (Clemens et al. 2011; Meckenhäuser et al. 2014). This introduces two types of variability – interindividual and trial-to-trial variability. Recording from a population of neurons in the same individual responding to

the same stimuli using a multielectrode approach reduces this variability. There have been few double intracellular recordings performed to study combined responses of different neurons (Vogel et al. 2005; Vogel and Ronacher 2007) but the success rates for simultaneous recordings from two synaptically coupled neurons or two identified neurons in insects is usually quite low. Therefore, recording from a population of neurons simultaneously with a multielectrode may considerably improve the judgement of auditory information processing in grasshoppers, even if identification of described neurons may be hard to achieve.

Chapter 3

Population coding among ascending neurons in the brain of a small grasshopper

Here, I thank Dr. Jan Clemens (Princeton University, USA) for helpful discussions and support in establishing and describing methods related to population coding analysis.

3.1 Introduction

Audition plays an important role especially in the detection of mating partners and natural predators in various animals; how the brain processes this auditory information is one of the fundamental issues in system neuroscience. Evolutionary processes have built auditory systems and behaviors with complexity (Hauser 1996; Bradbury and Lee 1998). As an example, in humans, cortical areas process auditory stimuli, extract language information and generate motor signals which are necessary for proper speech production (Levelt 1993; Ehret and Romand 1997). Auditory systems of insects have a relatively simple architecture with few hundreds of neurons in comparison with mammals. Even with a lower number of neurons, this auditory system is still capable of doing impressive neuronal computations (Machens et al. 2001), making insects ideal models for studying auditory information processing and linking the identified neural circuits to behavior (Campbell et al. 2013).

Acoustic communication of grasshoppers has become a well-known model to investigate principles of neural processing of acoustic stimuli. Grasshoppers produce acoustic signals called "songs" to attract the mating partner. These songs are repetitions of stereotyped subunits (syllable and pause) with species-specific amplitude modulation of a broad band carrier frequency that is produced by rubbing the hind legs against the forewings (von Helversen and von Helversen 1997). The auditory pathway of grasshoppers offers an advantage of identifiable neurons that can be discriminated based on their characteristic morphology (Stumpner and Ronacher 1991). The ears of grasshoppers are located on the side of the first abdominal segment. The receptor neurons transduce the tympanal vibrations into a series of action potentials which travel into the metathoracic ganglion complex, which houses the first auditory processing stage. Metathoracic ganglion contains five classes of neurons: receptor neurons, local neurons (SN), bisegmental neurons (BSN), T-shaped (TN) and ascending neurons (AN). The axons of receptor neurons make contact with local neurons which then contact 20 (so far) identified ascending neurons (Stumpner 1988; Stumpner and Ronacher 1991). The best-described class of ascending neurons ascend up to the brain from the metathoracic ganglion and

establish the likely main auditory input to higher processing circuits and decision centers located in the brain (Eichendorf and Kalmring 1980; Boyan et al. 1993; Kutzki 2012). Since the population of ascending neurons creates a bottleneck for the information available to the brain, they will be in the main focus of this study.

In the brain, the information is divided into the patterns of activity occurring over a population of neurons. Understanding the encoding of information in population activity is important for grasping the fundamental computations underlying brain function (Sanger 2003). The analysis of information processing among a population of neurons is called population coding. The popular way of studying population coding in insects is by recording activity from single neurons in several individuals (or in one individual successively) which then is used to analyze their potential combined activity (Clemens et al. 2011; Meckenhäuser et al. 2014). However, in order to analyze neural information encoded in the activity of the neuronal populations, it would be more appropriate to record the activity of several neurons at the same time in the same individual which reduces effects of neuronal variability (Ronacher et al. 2004).

Here, I recorded from the population of ascending neurons in the brain of *Ch. biguttulus* (which is the best-studied grasshopper species for acoustic behavior) using multielectrodes and investigated if there is any match between the neuronal and the behavior data. Additionally, I also investigated information content carried by a population of ascending neurons (in comparison with the single units) using decoding techniques.

3.2 Materials and methods

3.2.1 Animals

Adult female grasshoppers (*Ch. 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).

3.2.2 Animal preparation

In order to minimize the movements of the animal, front legs were removed and the animal was fixed with its dorsal side up onto a holder using wax resin mixture. 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)

3.2.3 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 loudspeakers (D21/2, Dynaudio – Rosengarten, Germany) situated laterally at a distance of 35cm 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 towards the speaker, grid on, and a Brüel & Kjær measuring amplifier (type 2602). Sound intensities are given in dB SPL (sound pressure level) re 2×10^{-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 to 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. Different stimuli were divided into different stimulus programs which are saved as 'configuration file' in Spike2 7.10 as described below

Search-program. In order to detect auditory neuronal activity from ascending neurons 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. To facilitate finding auditory activity during the search program, an audio monitor (AUDIS-01D/16 NPI Electronic Instruments– Tamm) was used.

Intensity-response program. For obtaining "intensity response characteristics" (responses to changing sound amplitude) 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 from the ipsilateral side (left) and broadband white noise stimuli were delivered between 30 and 90 dB SPL, increasing in 10 dB steps successively from the ipsilateral and contralateral side (right) (Stumpner 1988). The multielectrode was always inserted in the left side of the brain and stimulation is also provided from the left side which is considered as the ipsilateral side (for nearly all ascending neurons this is equivalent to the soma-contralateral side). 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.

Temporal pattern program. In order to test neuronal responses to variation of temporal patterns, male artificial grasshopper songs were presented that were varied in syllable duration (40 ms, 60 ms, 80 ms and 100 ms), pause duration (7.5 ms, 10 ms, 15 ms, 20 ms, 25 ms and 40 ms) and gap duration (2 ms

and 5 ms) (for definitions see introduction). The temporal patterns were generated using broadband white noise (2 ms ramped at the beginning and at the end of a syllable), were presented at 75 dB SPL in a pseudo-randomized order and were separated by 1 s interstimulus intervals. Two temporal programs were made. One program was for different syllable – pause patterns while another program was for Syllable-gap. Each syllable-pause or syllable-gap pattern was repeated 10 times. To standardize and reduce the effect of adaptation, a broadband white noise pulse (1 s, 75 dB SPL) was presented in the beginning.

3.2.4 Offline spike sorting and collision analysis

Spike sorting and collision analysis were done as described in Chapter 2.

3.2.5 Data analysis

Syllable-pause and gap tuning

Syllable-pause and gap tuning plots were made in order to check the correlation between the neuronal and the behavior data. Neuronal data were plotted by calculating the mean spike count against the pause duration. Behavior data regarding syllable-pause tuning were obtained from (von Helversen 1972) and behavior data regarding gap tuning were obtained and averaged from Ronacher and Stumpner (1988). These analyses were done in Excel 2010.

Calculation of feature vectors for PCA-based classification

In order to classify the sorted units, four feature vectors were calculated using the equation shown below.

1. Response to the Onset of stimuli:

$$\frac{No.of\ spikes\ in\ first\ 25\%\ of\ syllable\ (80-40\ ms)\ pattern}{No.of\ spikes\ in\ whole\ syllable\ (80-40)\ ms\ \ pattern}$$

(1 = marking the onset of syllable, 0 = not marking the onset of syllable)

2. Response to the syllable with gaps:

$$\frac{No.of\ spikes\ 0ms\ gaps - No.of\ spikes\ 5ms\ gaps}{No.of\ spikes\ 0ms\ gaps}$$

(1 = Gappy, 0 = non-gappy, Negative = non-gappy)

3. Response to the directional stimuli:

$$\frac{No.of\ spikes\ during\ ipsilateral\ stimulation}{No.of\ spike\ during\ ipsi. + No.of\ spikes\ during\ contra.stimulation}$$

(1 = extremely directional from ipsi. side, 0.5 = non-directional, 0 = extremely directional from contra.side)

4. Overall response to the stimulus (Phasic/Tonic):

$$\frac{No.of\ spikes\ in\ the\ first\ half\ of\ PSTH\ made\ from\ all\ intensities}{No.of\ spikes\ in\ the\ whole\ PSTH\ made\ from\ all\ intensities}$$

(1 = phasic, 0.5 = tonic)

3.2.6 Decoding using confusion matrix

Song identities were decoded from the neuronal responses using a similar decoding approach as used by Clemens et al. 2011. Spike train matrices or confusion matrices were made to decode song identity from the spike trains (Victor and Purpura 1998; van Rossum 2001).

Single unit distance matrices

Single unit distance matrices were constructed as described by Clemens et al. 2011. Spike trains were binned with a resolution of 0.05 ms and filtered with an α-function: $\alpha(t) = \theta(t)\, t \exp(-t/\tau)$, where $\theta(t)$ is Heaviside's function. Classification performance is a function of the metric's temporal resolution τ. Information was optimized with a grid search for τ ranging from 0 to 1000 ms (13 values spaced linearly on a logarithmic scale). The highest probability for correct classification of the stimuli was found at τ = 10 ms. This value of τ was considered in making the single unit matrices. The

distance matrices were made by calculating the Euclidean distances between all pairs of responses (10 repetitions of 6 male song segments of 40 ms syllables) and plotted on a distance matrix.

Combined unit distance matrices

Combined unit distance matrices were constructed by combining the recorded units from a single experimental data file. Application of these matrices amounts to filtering the spike trains with an α-function, embedding the spike trains from multiple cells into a vector space, and then taking the Euclidean distance between different spike trains. The resulting distance matrix for each population is used to quantify stimulus discriminability through the classification algorithm. The only difference to the single unit metric is that the spike trains of the cells comprising a population are embedded into a vector space (Clemens et al. 2011).

Classifier

Responses were classified using a nearest-neighbor clustering algorithm as used in Machens et al. (2001) and Clemens et al. (2011). Nearness was calculated by the single or the combined unit matrices. One template spike train was randomly selected from each of the 10 repetition of a song. The remaining spike trains were classified as being evoked by the song the nearest template belonged to. Out of 10, spike trains of 9 repetitions are used to train the classifier and spike trains of the 10th repetition is used as test data. The classification results were organized in a confusion matrix, which shows the frequency with which a spike train being evoked by a song.

Calculation of mutual information from a confusion matrix

Mutual information of the confusion matrix was calculated as following equation (Quian Quiroga and Panzeri 2009). Mutual Information is given by:

$$I = P(s,r)\ log2\frac{P(s,r)}{P(s) * P(r)}$$

P (s, r) = is the probability of response r given presentation of stimulus s

P(r) = is the probability of responses

P(s) = is the probability of stimuli

Mutual information is 0 bits when the confusion matrix is "uniformly distributed", that is when each entry has the value 1/64. It is maximal (for 8 stimuli $\log_2$ (6) = 2.58 bits) when there is a one-to-one relationship between spike trains and classes, e.g. when all entries are concentrated at the matrix' diagonal. Mutual information was expressed as a rate in bits.

All the analyses regarding stimulus decoding and mutual information calculation were done in MATLAB using the codes written and kindly provided by Dr. Jan Clemens (Princeton University, New Jersey, USA).

3.3 Results

3.3.1 Unit identification

A total of 177 units were sorted from 66 recordings from ascending neurons in the brain of *Ch.biguttulus.* Unit identification has been tried by comparing the extracellular data with previous physiological data acquired by intracellular recordings of ascending interneurons in the metathoracic ganglion of *Ch.biguttulus* (Stumpner 1988; Stumpner et al. 1991). As described by Bhavsar et al. 2015a it was extremely difficult to identify units just by comparing the physiology and so the majority of sorted units remains unidentified.

AN12

AN12 is an ascending neuron with a strong phasic response produced at the onset of a stimulus (Stumpner 1988; Stumpner et al. 1991; Creutzig et al. 2009), which is maintained over a broad intensity range. Among all described ascending neurons AN12 is most reliably influenced by the syllable-pause structure of the songs. The phasic burst in AN12 occurs reliably at the onset of syllables, provided that the pauses between syllables exceed a certain duration (Stumpner 1988; Stumpner et al. 1991). Also, the number of spikes per syllable increases linearly with increasing pause duration. This property was mainly used in identifying the sorted units as AN12. However, there has been quite a lot variation in the intensity response with lower spike numbers for extracellular units in comparison to intracellularly recorded AN12. Among all 177 sorted units, AN12 has been identified 22 times. Figure 1 shows an example of such a unit which is identified as AN12 based on its ability to mark the onsets of the syllables when provided with different model male grasshopper songs. The PSTHs of this unit are compared to the PSTHs of intracellularly recorded AN12 (Stumpner 1988).

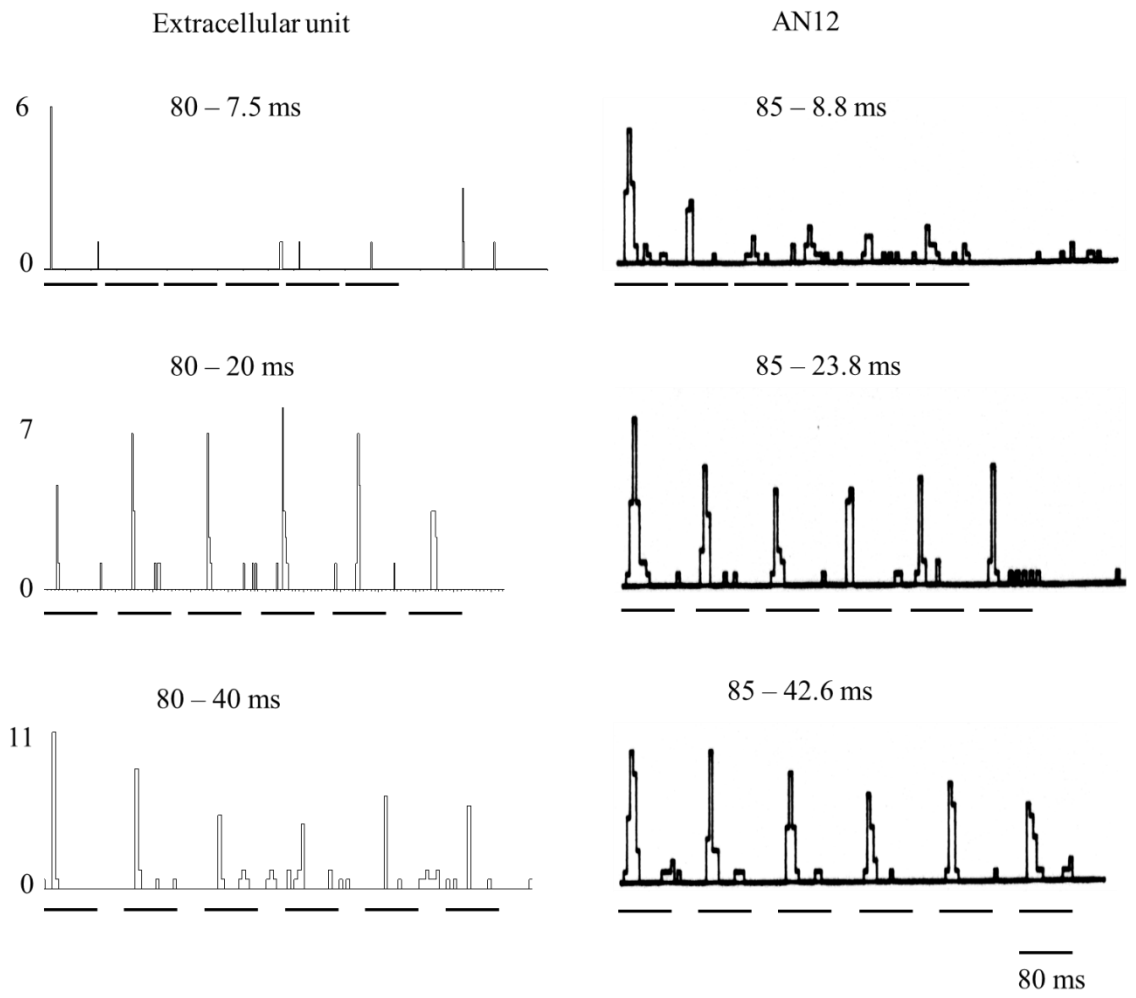

Figure 3.1 **Unit identification**. PSTHs(repetition = 10, bin size = 2 ms) showing the responses of the single sorted unit to three different artificial grasshopper songs of 80 ms syllable with 7.5 ms pause (left-up), 80 ms syllable with 20 ms pause (left-middle) and 80 ms syllable with 40 ms pause pattern (left-down) which are compared to PSTHs of three different artificial grasshopper songs of 85 ms syllable with 8.8 ms pause (right-up), 80 ms syllable with 23.8 ms pause (right-middle) and 85 ms syllable with 42.6 ms pause (right-down) for AN12. Data of AN12 are from (Stumpner 1988) (repetition = between 5 to 8, bin size = 2 spikes/class)

Figure 3.2 shows intensity responses of 5 different units (identified as AN12) to 5 kHz and 20 kHz sine wave stimuli. Most obvious is the much lower spike number of the extracellular units compared to intracellularly recorded AN12 (figure 3.2 A) whereby the intensity responses are hard to compare. Figure 3.2 B shows the mean response of all the 22 units identified as AN12 to 5 kHz and 20 kHz sine wave stimuli.

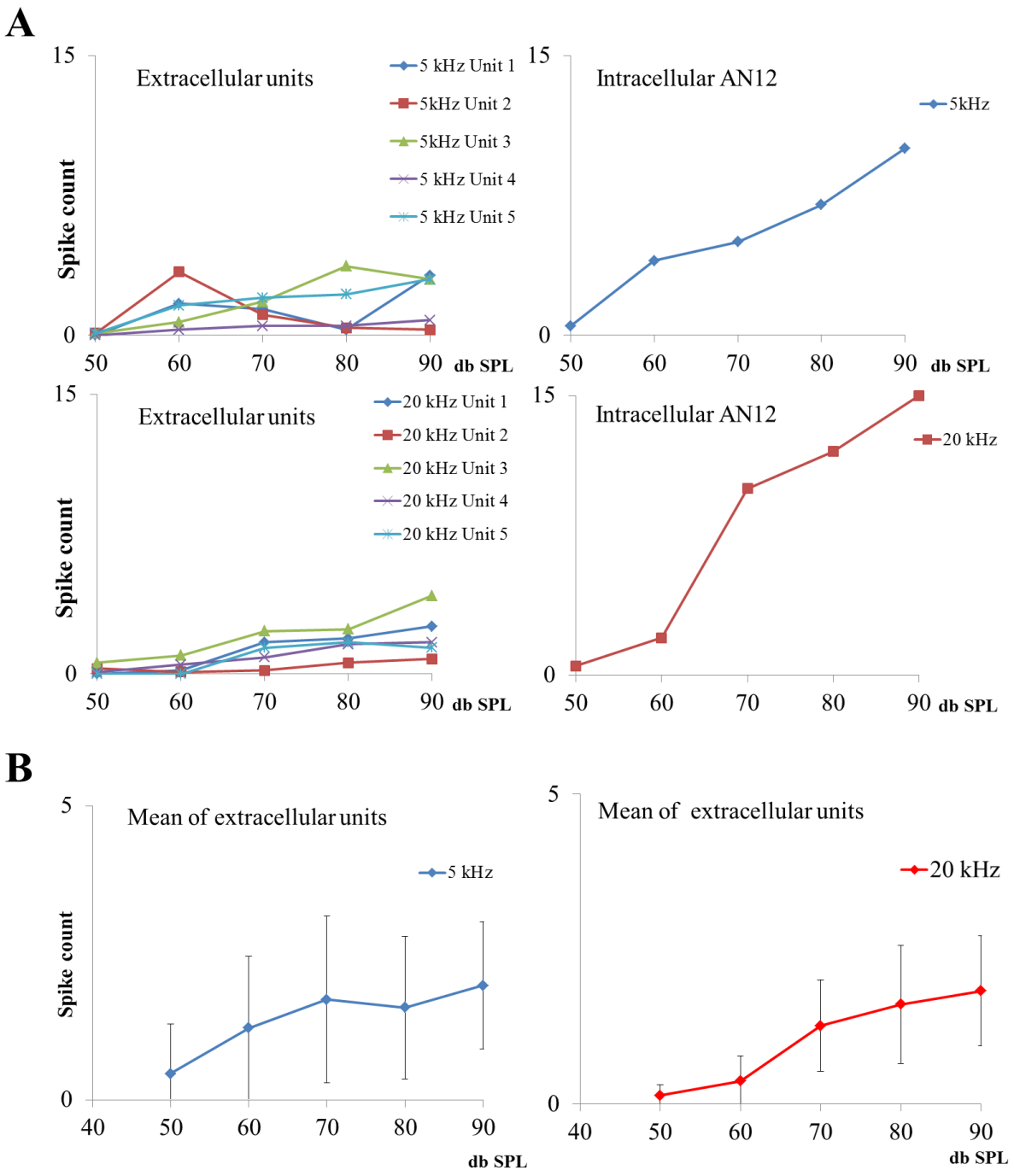

Figure 3.2 **Unit identification AN12.** (A) Intensity response functions of five sorted units for 5 kHz and 20 kHz (25 ms) stimuli (left), Intensity response functions of AN12 for 5 kHz and 20 kHz (25 ms) stimuli (right). Data of AN12 are modified from (Stumpner and Ronacher 1991) (B) Mean response of the sorted units identified as AN12 calculated from 22 preparations along with standard deviation

AN4

AN4 is an ascending neuron which is described as an excellent candidate for detecting gaps within the syllables since its activity is suppressed by syllables with gaps longer than few ms (Ronacher and Stumpner 1988). This characteristic was used to identify sorted units as AN4. AN4 has been identified 9 times among all 177 sorted units. However, there has been quite a lot variation found while comparing the intensity responses of the sorted units with intracellularly recorded AN4. Figure 3.3 shows example PSTHs of such units which are identified as AN4. The unit responds strongly to syllables without gaps while its response is clearly reduced for syllables with gaps of 2ms and 5 ms.

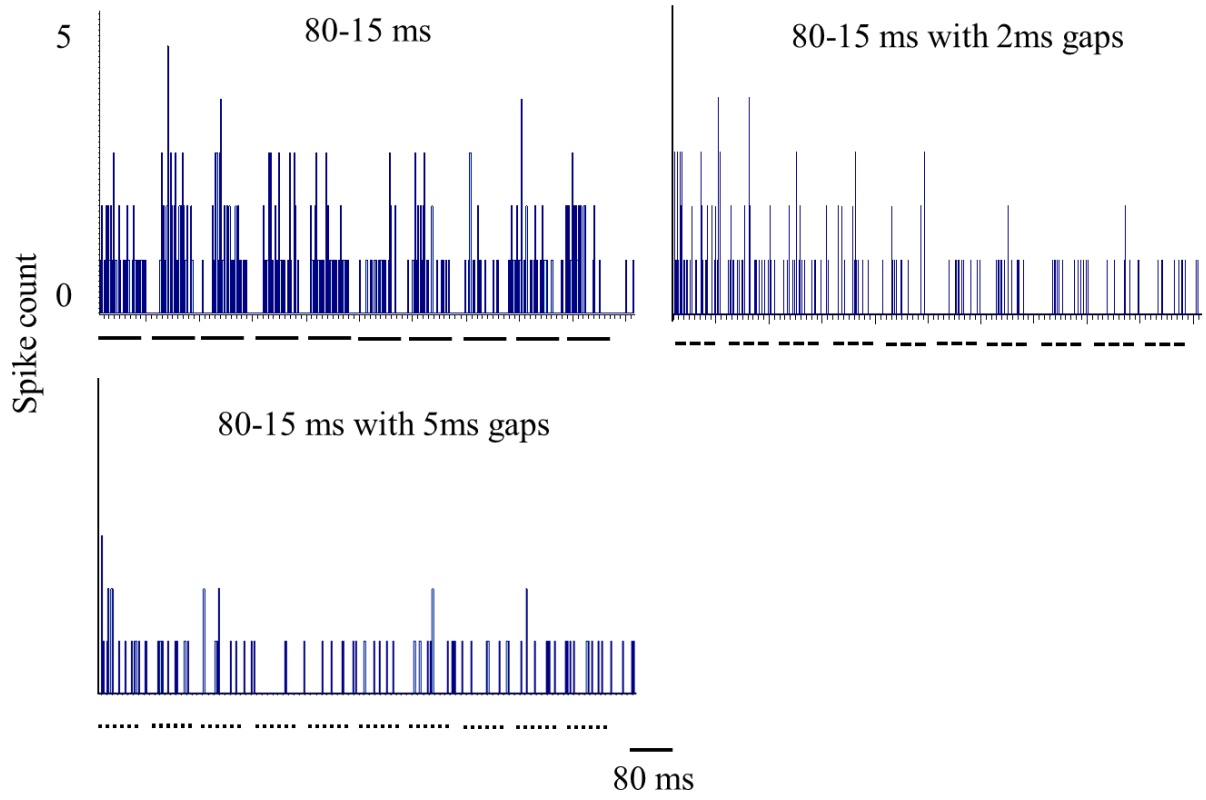

Figure 3.3 **Unit identification AN4.** PSTHs showing the responses of a single sorted unit to three different artificial model grasshopper songs of 80–15 ms with and without gaps (repetition = 10, bin size = 2 ms)

Figure 3.4 shows the intensity response of 5 different units to 5kHz, 20 kHz and white noise stimuli which were identified as AN4. Responses of the extracellular units were variable with lower spike count. Figure 3.4 B shows the mean response of all the 9 units identified as AN4 to 5 kHz and 20 kHz sine wave stimuli.

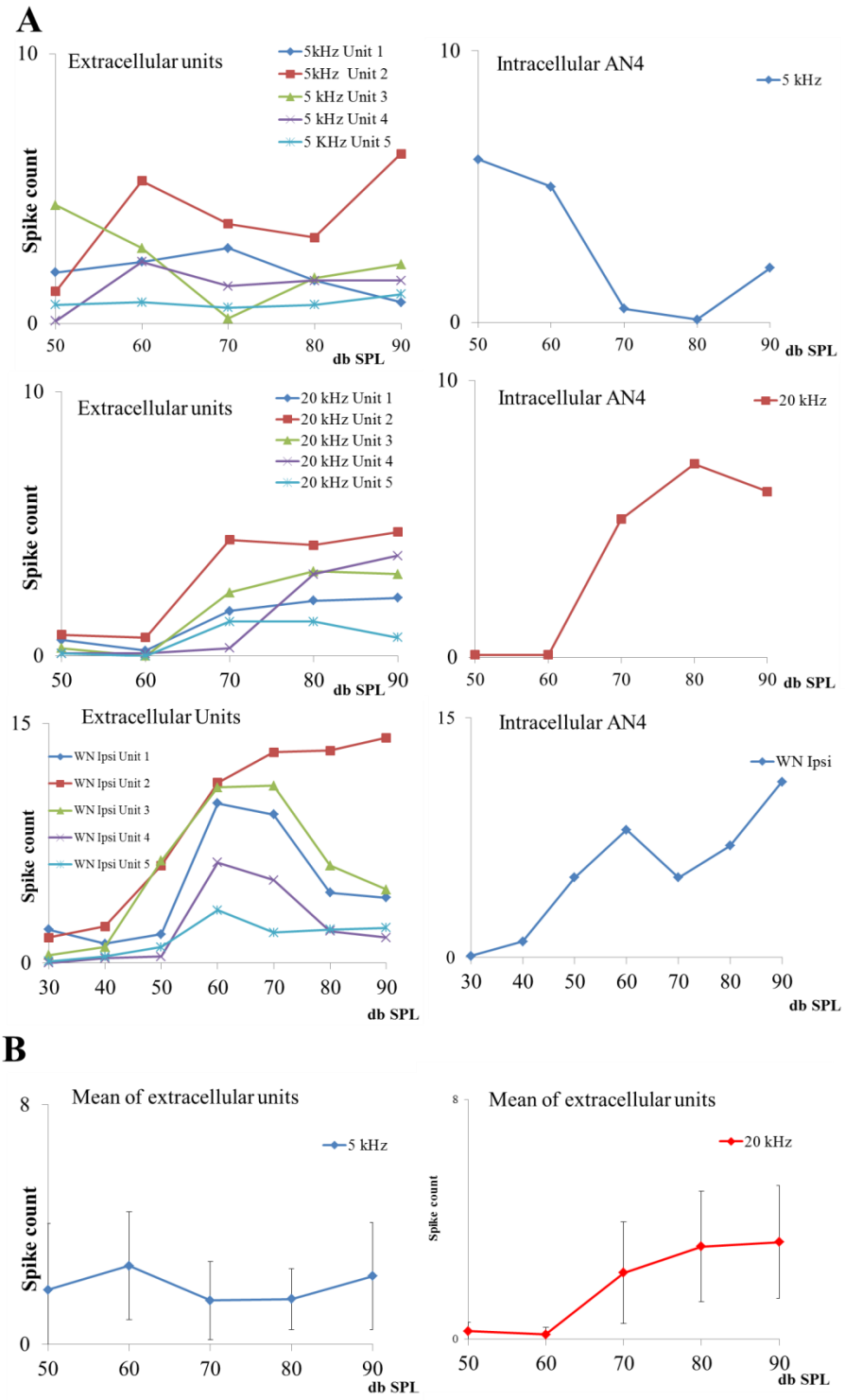

Figure 3.4 **Unit identification AN4.** (A) Intensity response functions of five sorted units for 5 kHz, 20 kHz (25 ms) and white noise (100 ms) stimuli (left), Intensity response functions of AN4 for 5 kHz, 20 kHz (25 ms) and white noise (100 ms) stimulus (right). Data of AN4 are modified from (Stumpner and Ronacher 1991). (B) Mean response of the sorted units identified as AN4 calculated from 9 preparations of along with standard deviation.

AN2

AN2 is an ascending neuron which is drastically influenced by the direction of the stimuli since its activity is mostly suppressed by a soma ipsilateral stimulus up to 80 dB SPL (Stumpner 1988; Stumpner and Ronacher 1991). This property of AN2 was used to identify the sorted unit as AN2. Figure 3.5 shows intensity responses of the units identified as AN2 to 5 kHz and 20 kHz sine wave stimuli and their responses to directional stimuli along with the comparison to intracellularly recorded AN2. Intensity-responses of 5 different units to white noise stimuli provided from the ipsi-lateral side (left) and the contra-lateral side (right) are shown. All the 5 units clearly show suppression in the activity for the contra-lateral stimuli compared to the ipsi-lateral stimuli (figure 3.5). AN2 has been identified 10 times among all 177 sorted units. Figure 3.5 B shows the mean response of all the units identified as AN2 to 5 kHz and 20 kHz sine wave stimuli.

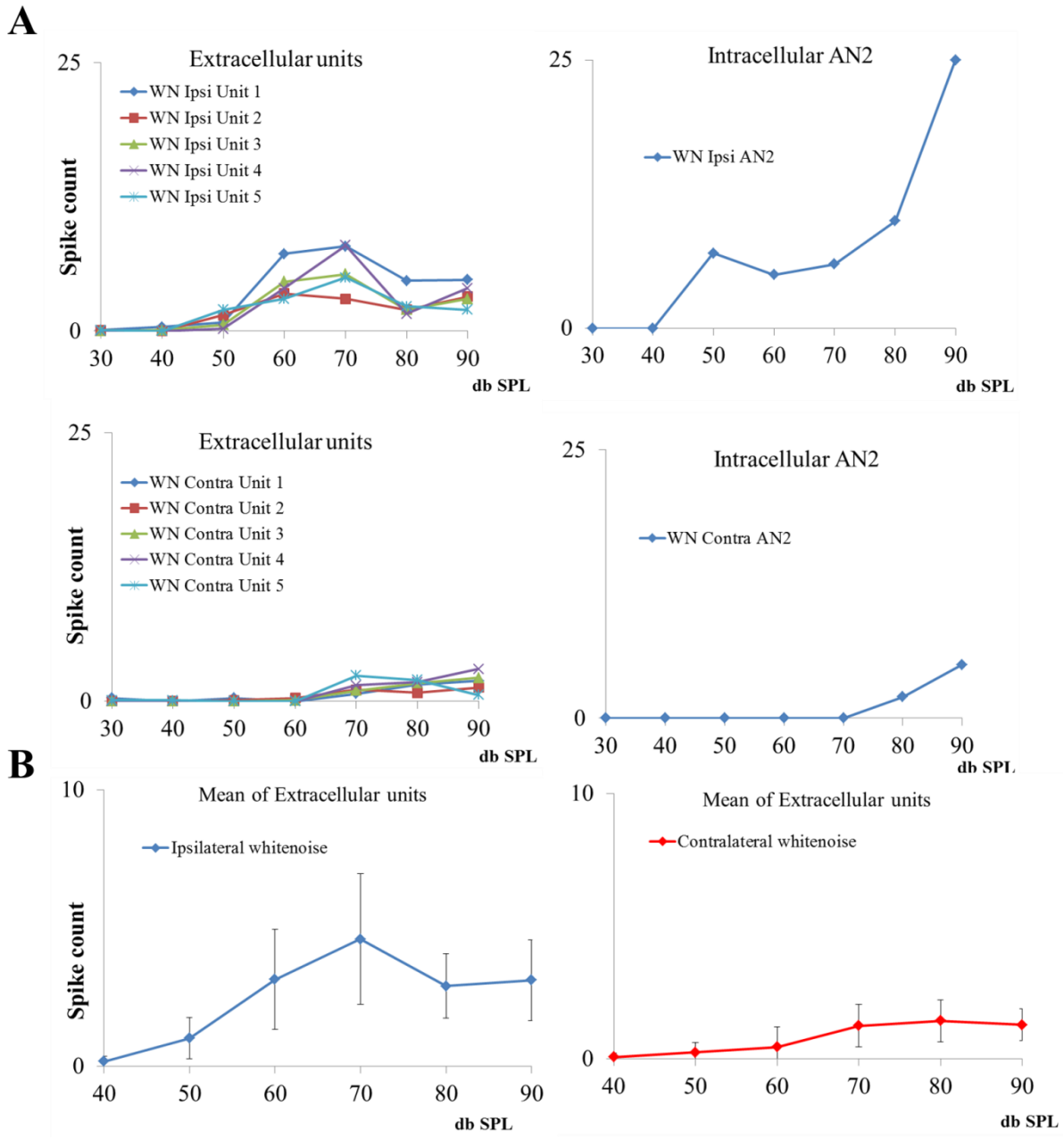

Figure 3.5 **Unit identification AN2.** (A) Intensity response functions of five sorted units for white noise (100 ms) for ipsilateral and contralateral stimuli (left), Intensity response functions of AN2 for white noise (100 ms) for ipsilateral and contralateral stimuli (right). Data of AN2 are modified from (Stumpner and Ronacher 1991). (B) Mean response of the sorted units identified as AN2 calculated from 10 preparations along with standard deviation.

AN6

AN6 is the only described ascending neuron which responds tonically to acoustic stimuli at all intensities and doesn't saturate at highest intensities (Stumpner and Ronacher 1991). This property was used to identify sorted units as AN6. AN6 has been identified 20 times among all 177 sorted units.

Figure 6 shows an example of such units identified as AN6. Figure 6 A shows the response of a unit to 20 kHz sine wave (25 ms) stimuli which can be regarded as a tonic activity. Figure 6 B shows the intensity response of 5 different units to 5kHz and 20 kHz sine wave stimuli. Responses of the extracellular units were linearly increasing with increase in the sound intensity (exception Unit 2 - 5 kHz), however, with a lower spike count in comparison with intracellularly recorded AN6. Figure 6 C shows the mean responses of 20 units identified as AN6 to 5 kHz and 20 kHz sine wave stimuli.

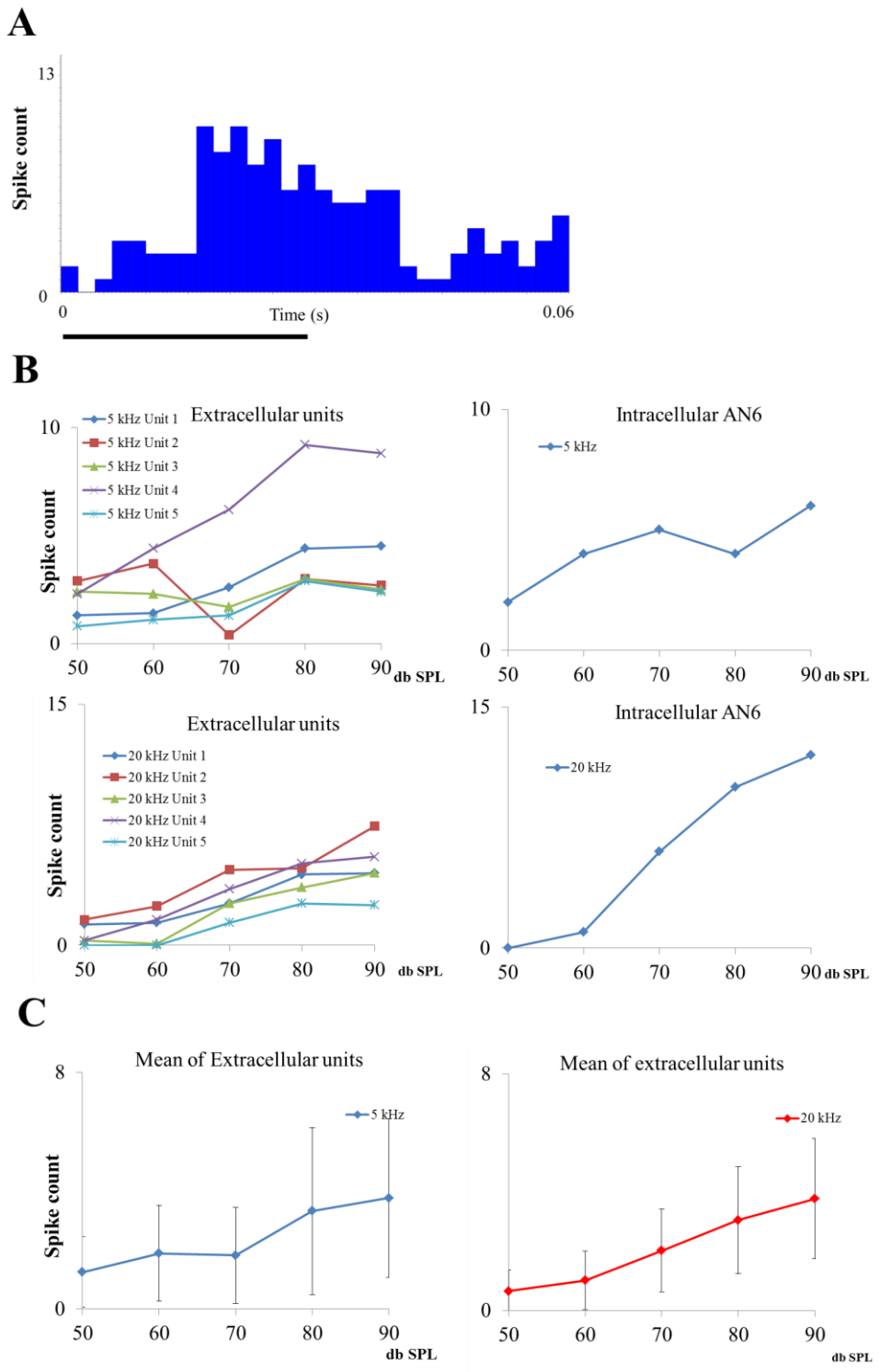

Figure 3.6 **Unit identification AN6.** (A) PSTH showing tonic responses of single unit to acoustic stimuli (20 kHz; 25 ms; 50–90 dB SPL). PSTH width: 60 ms, bin size: 2 ms; the black line marks the stimulus. (B) Intensity response functions of five sorted units for 5 kHz and 20 kHz (25 ms) stimuli (left), Intensity response functions of AN6 for 5 kHz and 20 kHz (25 ms) stimulus (right). Data of AN6 are modified from Stumpner (1988) (C) Mean response of the sorted units identified as AN6 calculated from 20 recordings along with standard deviation.

AN11

AN11 is mainly identified only by comparing the intensity frequency response with intracellular recordings. AN11 mainly respond between 40 to 60 dB SPL with a peak response at 50 dB SPL to white noise stimuli (Stumpner 1988; Stumpner and Ronacher 1991). AN11 has been identified 12 times among all 177 sorted units with much uncertainty. Figure 3.7 A shows an example of 5 different units which are identified as AN11 based on their response to 5 kHz, 20 kHz and white noise stimuli. Figure 3.7 B shows the mean response of the unit identified as AN11 to 5 kHz and 20 kHz sine wave stimuli.

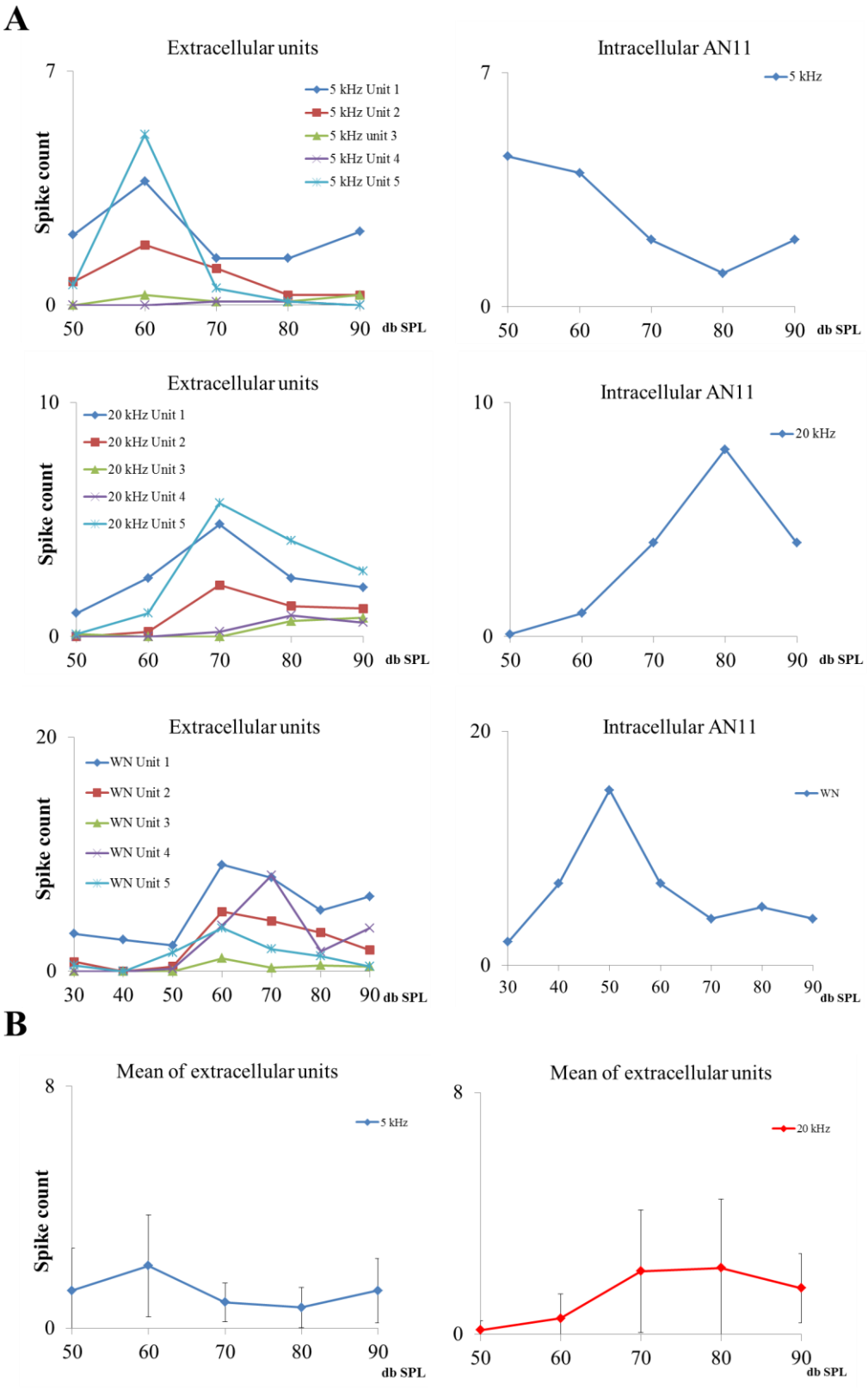

Figure 3.7 **Unit identification AN11.** (A) Intensity response functions of five sorted units for 5 kHz,20 kHz (25 ms) and white noise stimuli (left), Intensity response functions of AN11 for 5 kHz, 20 kHz (25 ms) and white noise stimuli (right). Data of AN11 are modified from (Stumpner and Ronacher 1991). (B) Mean responses of the sorted units identified as AN11 calculated from 12 preparations along with standard deviation.

3.3.2 PCA-based classification of grasshopper ascending neurons

Since the majority of the sorted units remain unidentified, I also tried to classify the sorted units with a PCA-based cluster analysis. For each sorted ascending unit, I calculated (see Chapter 3 - *methods - data analysis*) response to the onset of stimuli, response to directional stimuli, response to syllables with gaps and overall response to the stimuli (phasic/tonic). Figure 3.8 shows first two principle components of all four feature vectors projected, since first two principle components capture majority (63%) of the variance of the data. PCA Clouds representing stimulus onset and directionality were seen however with an area of overlap. Units present in these both clouds were mainly identified as AN12 and AN2 during identification using physiology.

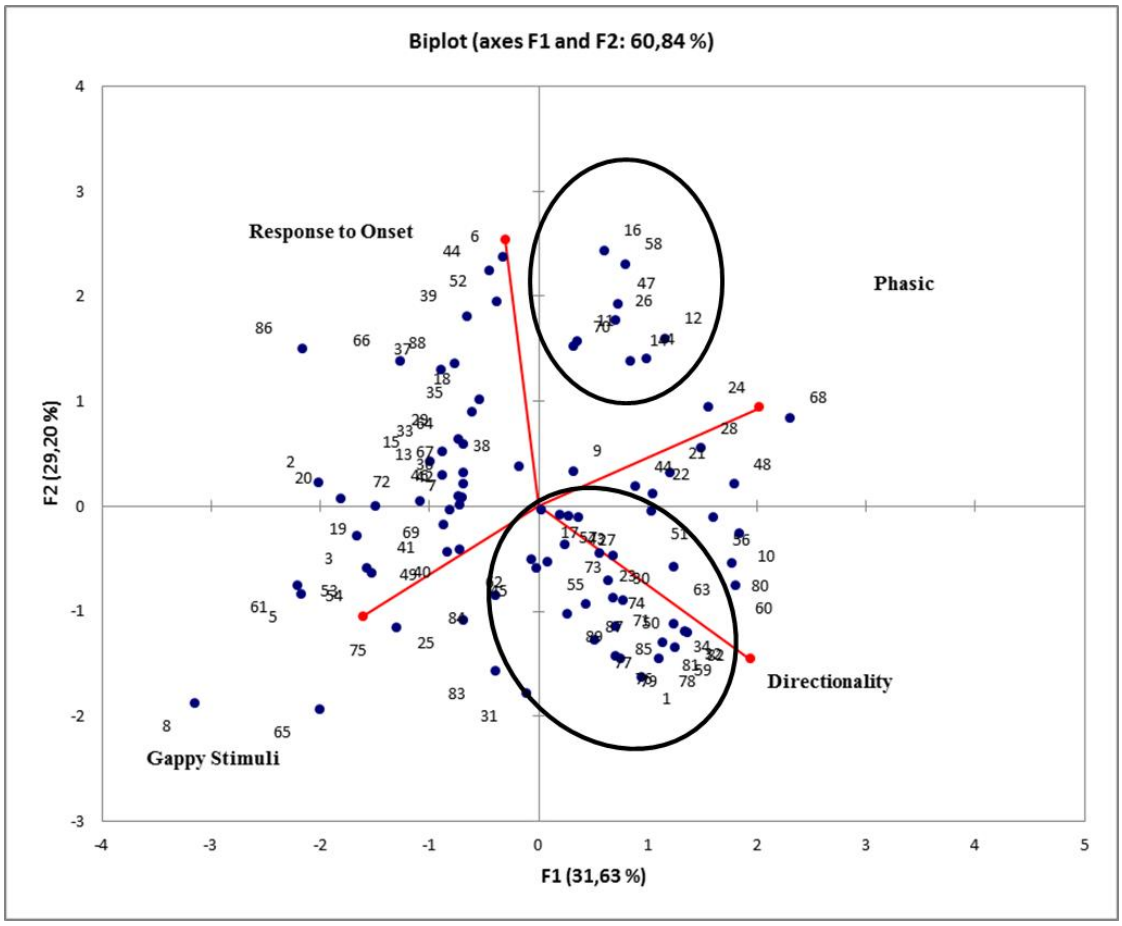

Figure 3.8 **PCA-based cluster analysis of 89 sorted units from spike trains of grasshopper ascending neurons**. Each dot corresponds to a feature vector that describes the response of a single neuron, projected to the first two principal components. Two circles express the group of units representing directionality (lower circle) and stimulus onset responses (upper circle).

3.3.3 Summed activity of the ascending neurons for different syllable – pause patterns

It has been suggested by Clemens et al. 2011 that the population coding take place at the level of ascending neurons in the grasshoppers where each neuron encodes different aspects of the stimulus. This means that the behavior is likely represented by the summed activity of ascending neurons. Ascending neurons were recorded in around 100 preparations and data were analyzed from 66 preparations due to the better quality of the recording. The responses to the artificial male grasshopper songs were analyzed by making peristimulus time histograms (PSTH) for each grasshopper song. In order to quantify the summed response, different techniques have been tested like calculating the mean spikes above a certain level (mean (±) 3*SD) from 10 repetitions, taking the mean maximum spike count from 10 repetitions and calculating the mean number of spikes from 10 repetitions. In the end, the summed response was analyzed by plotting the mean spikes count from 10 repetitions against the pause duration. A clear match between the behavior and the summed response was not seen in any of the recordings.

Figure 3.9 shows a single example of the summed neuronal response of ascending neurons (3 units) along with the behavior data of 40 ms syllables. Figure 3.9 A shows PSTHs of the summed neuronal response for four different types of 40 ms syllable pause pattern. PSTHs were quantified by plotting the mean spikes against different pause durations as shown in figure 3.9 B. It can be inferred from the PSTHs that there is at least one unit present which fires strongly at the onset of the syllables. However, the summed neuronal response was not much affected by the pause durations and remained more or less consistent for the entire pause durations, however, with the high variability in the responses. No clear match between the neuronal and the behavioral data was observed.

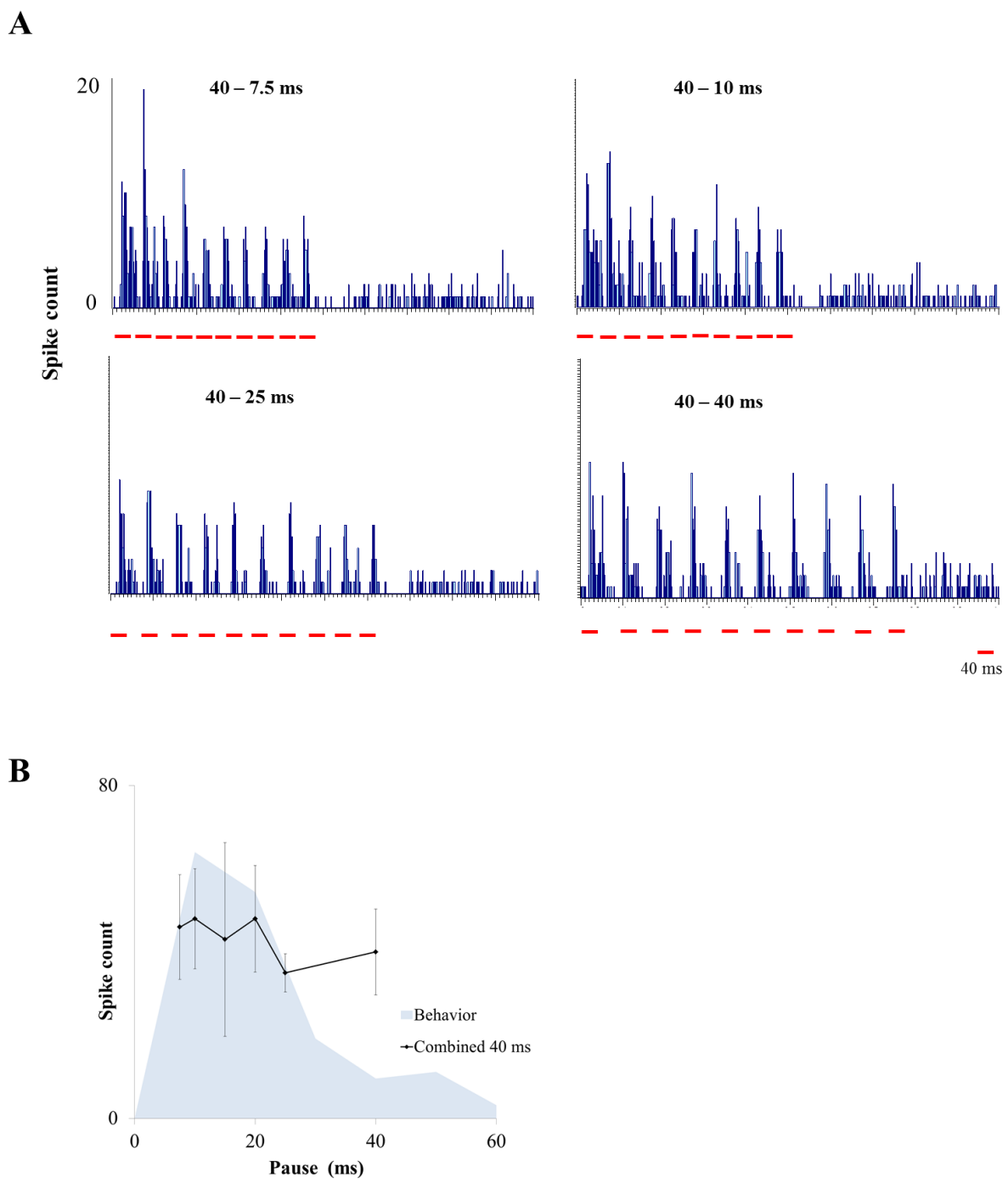

Figure 3.9 **Syllable-pause tuning.** (A) PSTHs of summed response of ascending neurons (3 units) for grasshopper model songs of 40 ms syllables with varying pause durations (repetitions = 10, bin width = 2ms). (B) Plot shows the quantification of the summed neuronal response of ascending neurons along with the behavior data. The average number of spikes from 10 repetitions is plotted against the pause durations. Behavior data are obtained from von Helversen (1972).

Figure 3.10 shows a single example of the summed neuronal response of ascending neurons (3 units) along with the behavior data of 60 ms syllables. Figure 3.10 A shows PSTHs of the summed responses for four different types of 60 ms syllable-pause patterns for 10 repetitions. PSTHs were quantified by plotting the mean spikes against the pause duration as shown in figure 3.10 B. Increase in spike count with a peak at 60-25 ms and then decrease at 60 – 40 ms was seen, however, with the high variability in the responses. Even though, there is a tendency to produce more spikes with pauses above than at 20 ms or below, due to high variability in the responses there is no consistent match between the neuronal and the behavioral data.

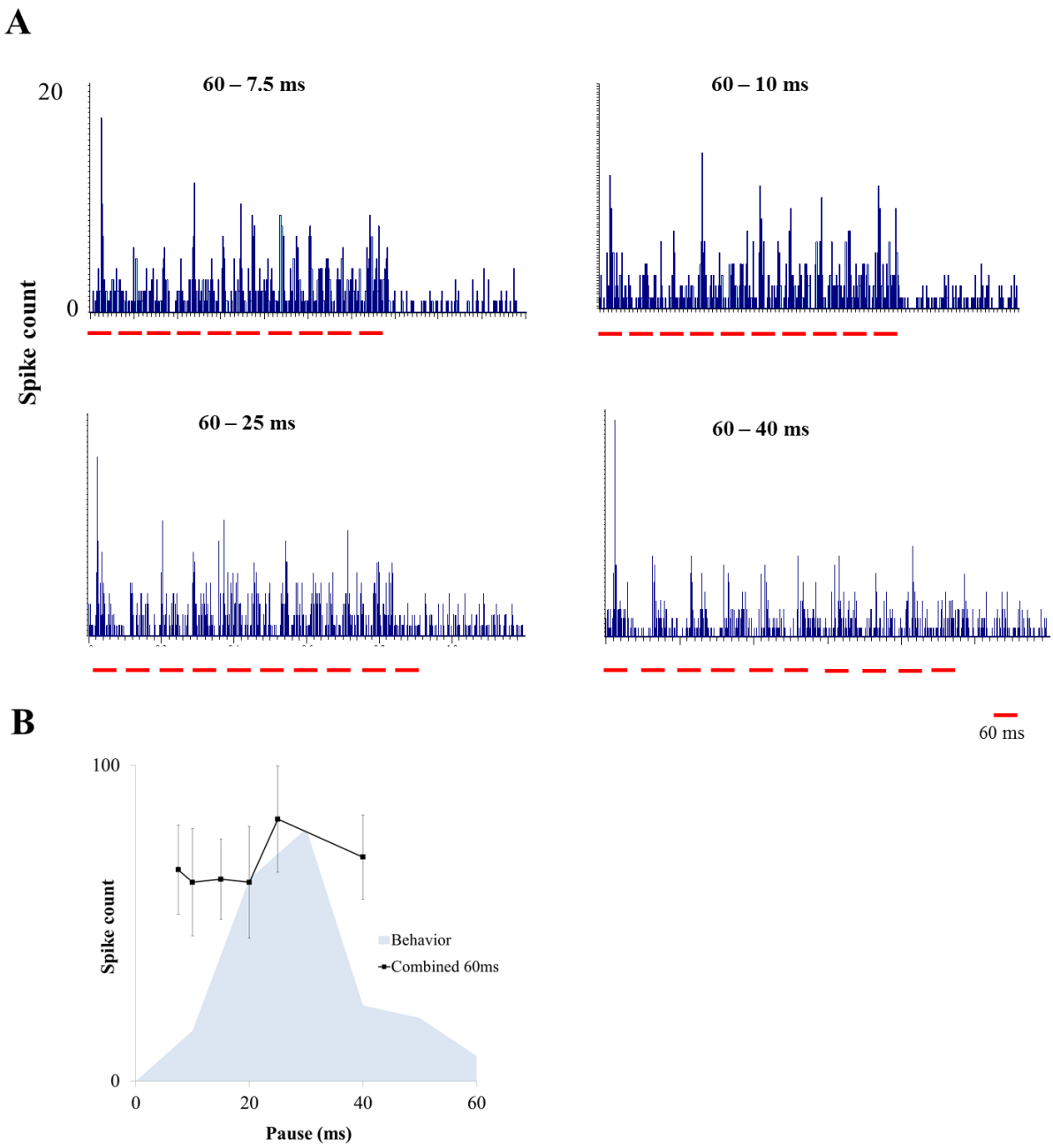

Figure 3.10 **Syllable-pause tuning.** (A) PSTHs of the summed response of ascending neurons (2 units) for grasshopper model songs of syllable 60 ms with varying pause durations (repetitions = 10, bin width = 2ms). (B) Plot shows the quantification of summed neuronal response of ascending neurons along with the behavior data. The average number of spikes from 10 repetitions is plotted against the pause durations. Behavior data were obtained from von Helversen (1972).

Figure 3.11 shows an example of the summed neuronal response of ascending neurons (3 units) along with the behavior data of 80 ms syllable. Figure 3.11 A shows PSTHs of the summed response for four different types of 80 ms syllable pause pattern for 10 repetitions. It can be inferred from the PSTHs that the summed activity increases and the onset of the syllable becomes pronounced with the increase in the pause duration. These PSTHs were quantified by plotting the mean spikes against the pause duration as shown in figure 3.11 B. There is a weak tendency for an overall increase of spike number with increasing pause duration, but with high variability.

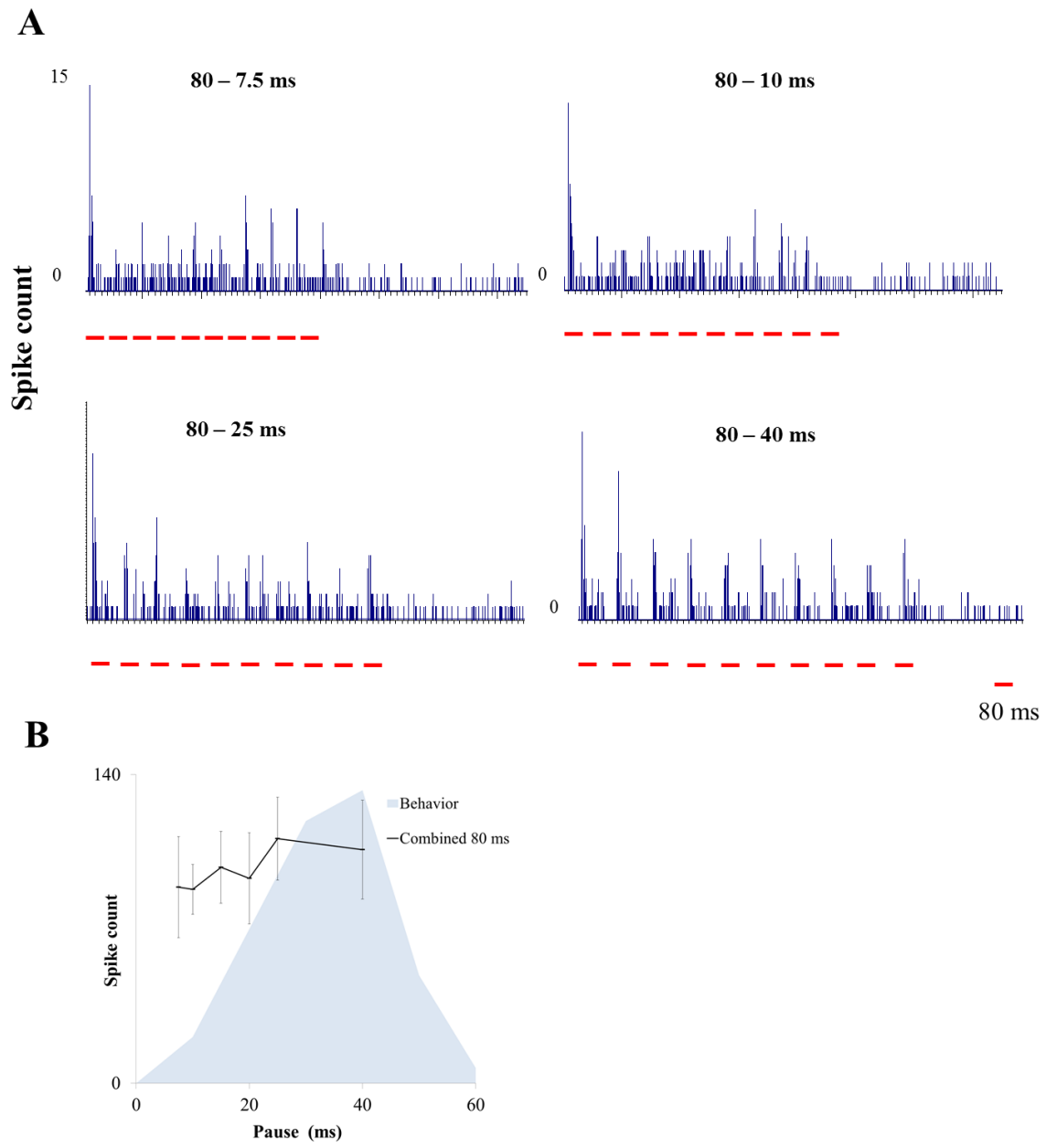

Figure 3.11 **Syllable-pause tuning.** (A) PSTHs of the summed response of ascending neurons (3 units) for grasshopper model songs of 80 ms syllables with varying pause durations (repetitions = 10, bin width = 2ms). (B) Plot shows the quantification of the summed neuronal response of ascending neurons along with the behavior data. The average number of spikes from 10 repetitions is plotted against the pause durations. Behavior data were obtained from von Helversen (1972).

Figure 3.12 shows a single example of the summed response of ascending neurons (3 units) along with the behavior data for 100 ms syllables. Figure 3.12 A shows PSTHs of the summed neuronal responses for four different types of 100 ms syllable pause patterns for 10 repetitions. It can be inferred from the PSTHs that the neuronal responses were dominated by tonically firing units and high neuronal activity was seen at 100 – 20 ms syllable pause duration. PSTHS were quantified by plotting the mean number of spikes against the pause duration as shown in figure 3.12 B. Neuronal responses at shorter and longer pauses were more or less similar except slight increase at 20 ms pause duration. No match between the behavior and the neuronal data was observed.

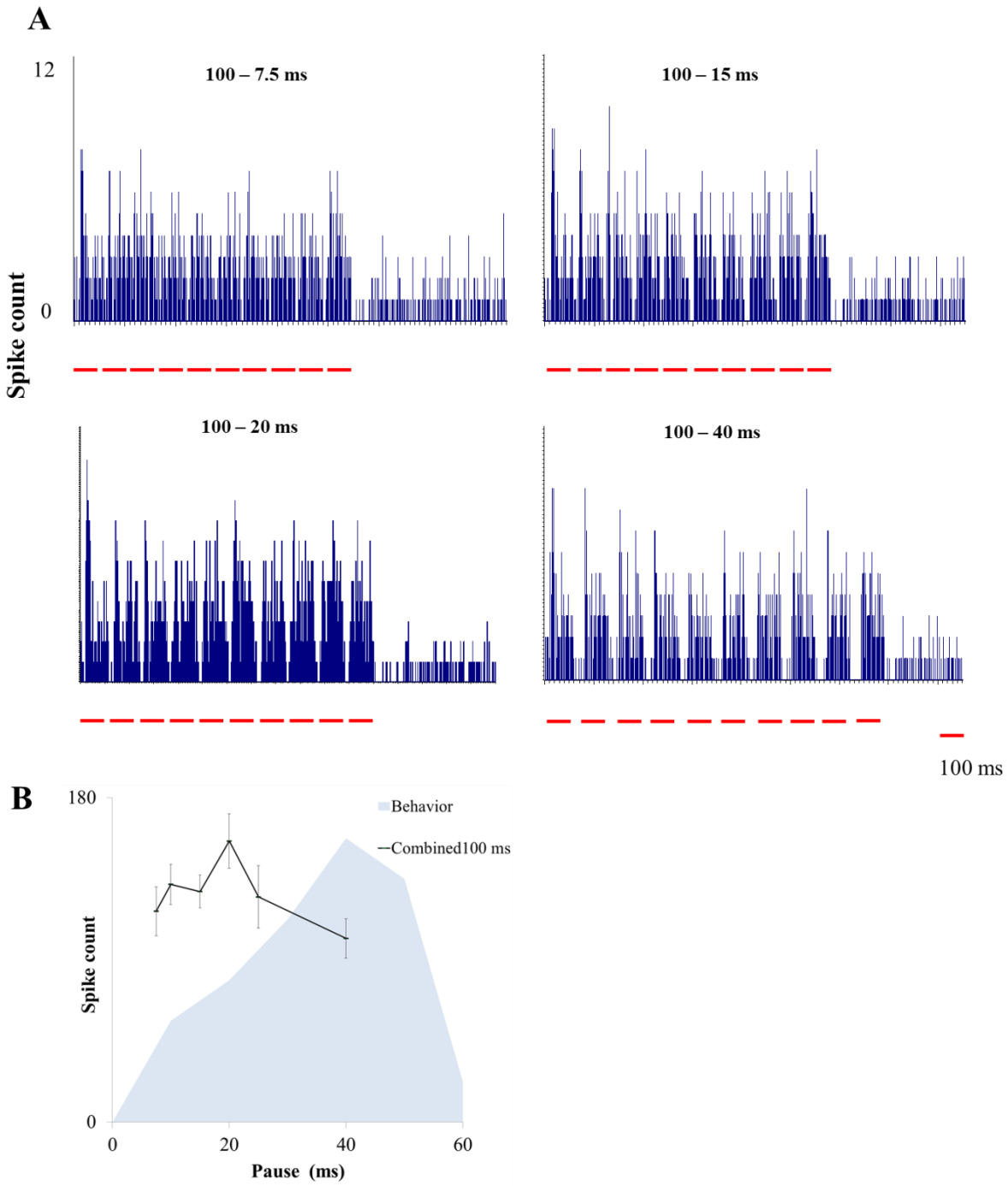

Figure 3.12 **Syllable-pause tuning.** (A) PSTHs of the summed response (3 units) of ascending neurons for grasshopper model songs of 100 ms syllables with varying pause durations (repetitions = 10, bin width = 2ms). (B) Plot shows the quantification of the summed neuronal response of ascending neurons along with the behavior data. The average number of spikes from 10 repetitions is plotted against the pause durations. Behavior data were obtained from von Helversen (1972).

Figure 3.13 shows examples showing the mean summed spike count plotted against the pause durations for four different types of syllables in 10 different recordings. There was a high variability in responses observed among the recordings and a clear match between the behavior and the neuronal response was not seen in any of the recordings.

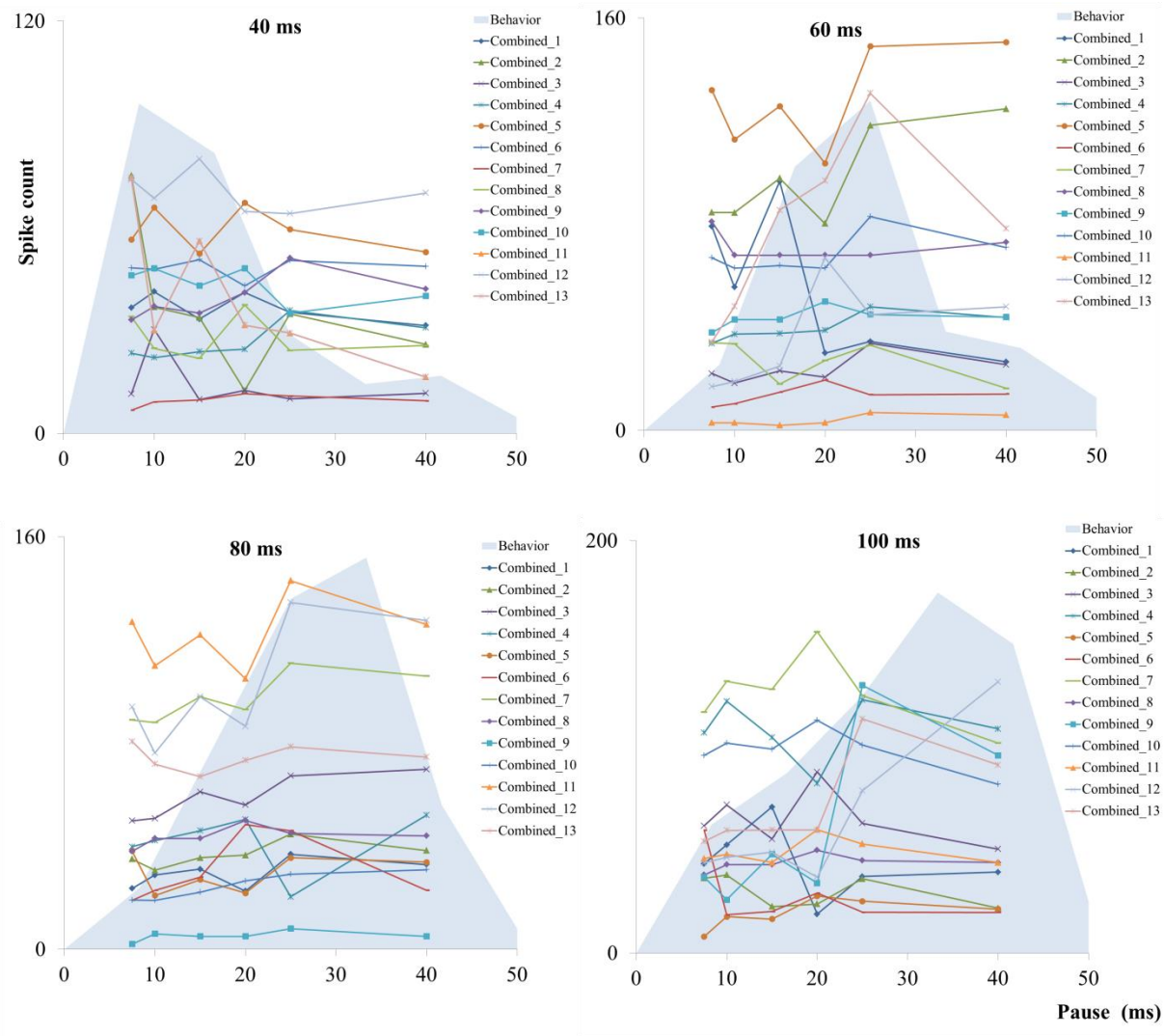

Figure 3.13 **Syllable-pause tuning**. Plots show the behavior and the summed neuronal response of ascending neurons to different male grasshopper model songs with 40 ms, 60 ms, 80 ms and 100 ms syllables. The average number of spikes from 10 repetitions is plotted against the pause durations. Behavior data were obtained from von Helversen (1972).

3.3.4 Summed activity of the ascending neurons for different syllable-gap patterns

Summed responses to different syllable-gap patterns (0 ms, 2 ms, and 5 ms) were analyzed in 48 different preparations. Out of 48, none of the recordings showed a clear match between the behavior and the summed neuronal response. Figure 3.14 shows a single example of the summed response of two sorted ascending units (2 units) to syllable with and without gaps. This example was chosen since it shows the summed neurons response not getting affected by the gap durations (neither strong increase, nor strong decrease). Figure 3.14 A shows PSTHs of the summed neuronal response to syllables with and without gaps. These PSTHs were quantified as shown in figure 3.14 B by plotting the mean spikes against the gap durations. No match between the neuronal and the behavior data was seen.

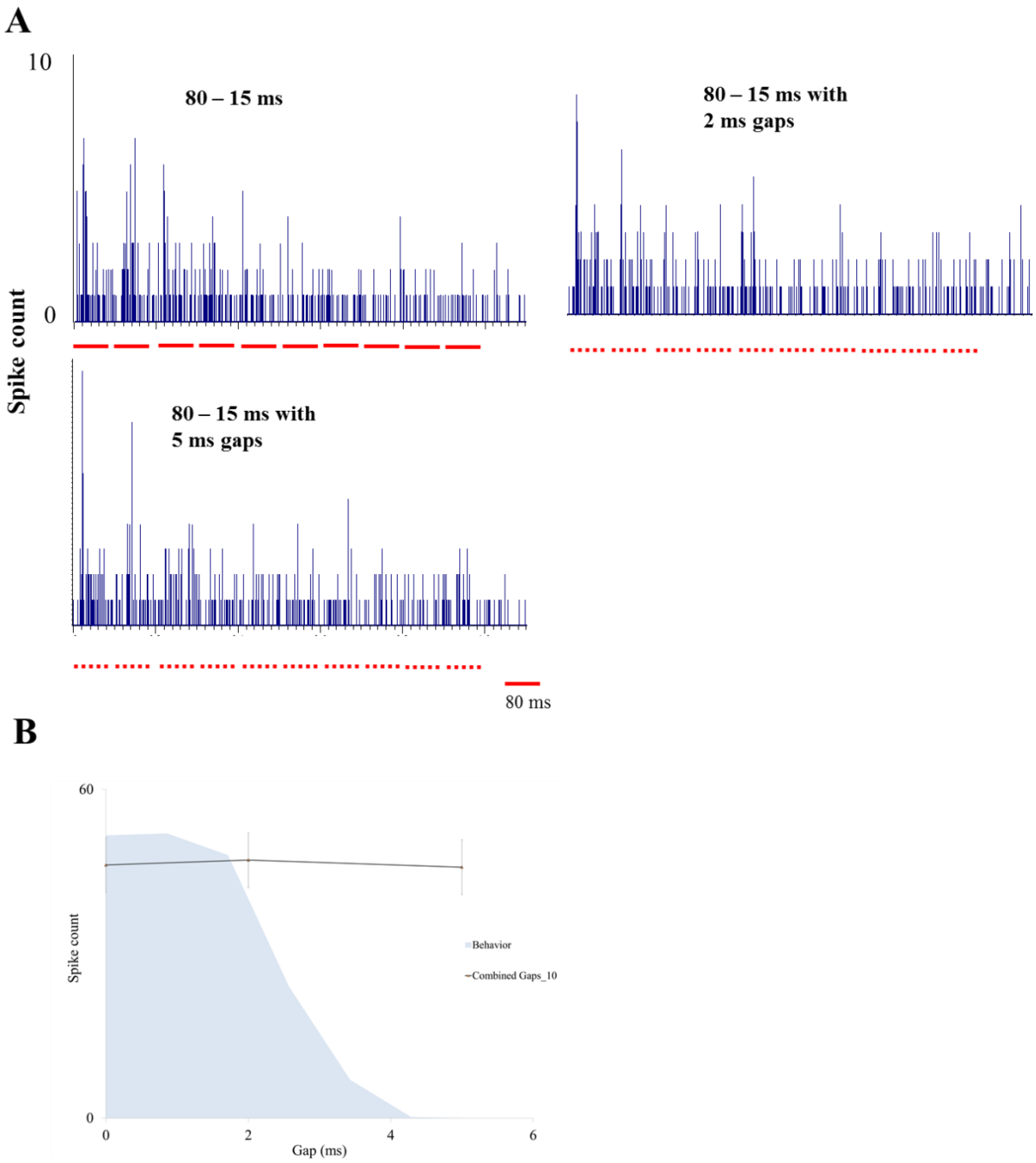

Figure 3.14 **Gap tuning.** (A) PSTHs of the summed response of two ascending neurons to male grasshopper model songs with 80/15 ms syllable/pause duration and variable gaps (repetitions = 10, bin width = 2ms). (B) Plot shows the quantification of the summed neuronal response of ascending neurons (2 units) along with the behavior data. Behavior data were obtained and averaged from Ronacher and Stumpner (1988).

Figure 3.15 shows an example showing the summed spike count of 2 or 3 units recorded simultaneously plotted against the gap duration for 20 different recordings. Most of the recordings did not show any clear match between the behavior and the neuronal data as shown in figure 3.15 A. However, in two recordings, a clear increase in neuronal response with increasing gap duration was as shown in figure 3.15 B. This increase in the neuronal activity was due to the presence of those units which shows stronger response to syllables containing gaps than to those not containing gaps.

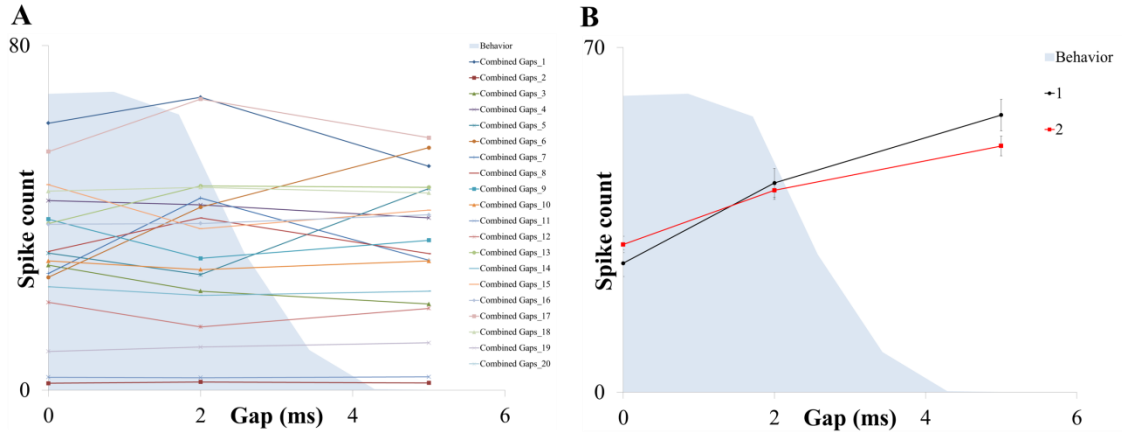

Figure 3.15 **Gap tuning**. (A) Plot shows the summed neuronal response of ascending neurons to male grasshopper model songs with 80/15 ms syllable/pause durations and variable gaps. The average number of spikes from 10 repetitions is plotted against the gap durations. Behavior data were obtained and averaged from Ronacher and Stumpner (1988). (B) Plot shows the increasing summed neuronal response of ascending neurons to different model grasshopper songs with and without gaps. The average number of spikes from 10 repetitions is plotted against the gap durations. Behavior data were obtained and averaged from Ronacher and Stumpner (1988).

Additionally, I also tried to quantify the combined response (2 or 3 units) from those recordings where at-least one unit was identified as AN4 (n=4). One such example of AN4 identification is shown in figure 3.3. However, the response was not decreasing strongly and not a strong match between the behavior and the neuronal data was found when analyzing the summed (combined) response to syllables with and without gaps as shown in figure 3.16. This may be due to presence of those units along with AN4 which show reverse dependence to syllables containing gaps.

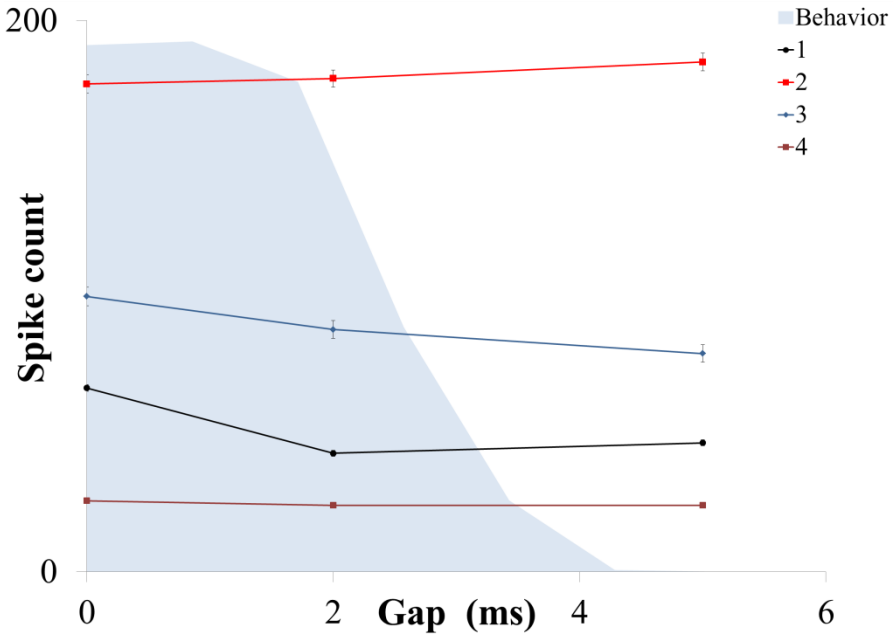

Figure 3.16 **Gap tuning.** Plot shows behavior and the summed neuronal data of ascending neurons where at least one unit was identified as AN4 to different model grasshopper songs with and without gaps. Behavior data were obtained and averaged from Ronacher and Stumpner (1988).

3.3.5 Decoding the stimulus identity from the response

Both decoding and information theory extract quantitative information from the population responses by quantifying the knowledge about the stimulus that is gained from the neuronal population response. Decoding algorithms predict the stimulus that caused the neuronal response. The performance of such decoding algorithms are typically measured by the percentage of correct predictions (Quian Quiroga and Panzeri 2009). Here, I tried to decode the response of a population of ascending neurons to six different artificial syllable pause patterns of 40 ms syllables.

Optimization of matrix parameters

Classification performance is a function of the matrix temporal resolution τ. Information was optimized with a grid search for τ ranging from 0 to 1000 ms (13 values spaced linearly on a logarithmic scale). The performance of τ used for decoding is shown in figure 3.17. Figure 3.17 A

shows the probability of correct classification of the stimulus for the different values of τ. Figure 3.17 B shows the mutual information calculated for the different values of τ. The highest probability for correct classification of the stimuli and the highest mutual information is found at τ = 10 ms, which therefore was used in all decoding analyses.

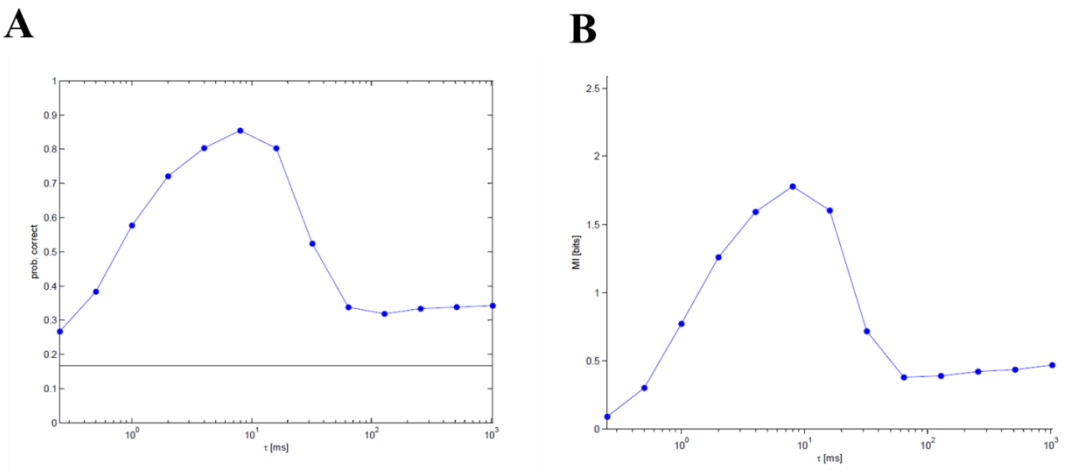

Figure 3.17 **Performance of the decoder**. (A) Plot shows the probability of correct prediction of the stimulus (six grasshopper songs of 40 ms syllables) from the summed response for 13 values of τ spaced linearly on a logarithmic scale. (B) Plot shows the values of the mutual information for 13 values of τ spaced linearly on a logarithmic scale.

Confusion matrices and mutual information

In order to calculate the mutual information, confusion matrices for single sorted units and combined units were constructed and compared. Mutual information was quantified on sorted units of 45 recordings of ascending neurons as shown in figure 3.18. There was an average increase in mutual information seen while comparing the single units with combined units as shown in figure 3.18. After analyzing each recording individually, 18 recordings showed an increase in the mutual information for the population of units in comparison to the single units (Fig.3.19). The other 27 recordings showed no increase in the mutual information when comparing the population of units to the single units.

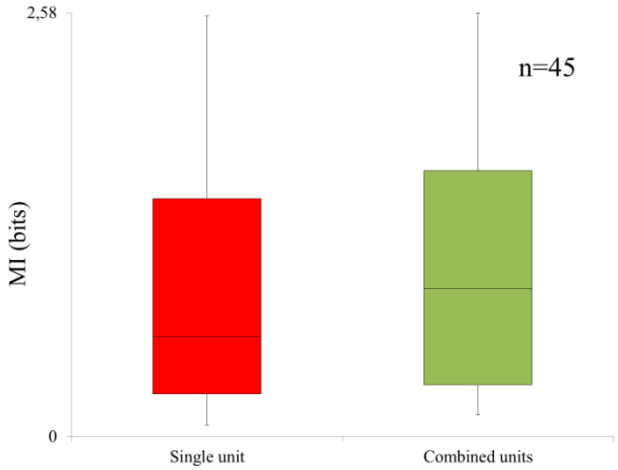

Figure 3.18 **Mutual information.** Box plot showing the comparison of the mutual information for six syllable – pause patterns (40 ms syllables) calculated from the single units and the combined units in 47 data files. The black line in the box represents the median value. The upper and lower whiskers represent the maximum and minimum values respectively. The box is 25% above and below the median.

Figure 3.19 shows an example of a comparison of the mutual information between single sorted units with combined units recorded in the same individual for six different types of male grasshopper songs. Figure 3.19 A and B show that the decoding performance increased for combined units in comparison to three and two single sorted units recorded in the same individual at the same time. Figure 3.19 C shows the comparison of the mutual information of single sorted units with the combined units for 18 recordings. Highest information among the sorted units was considered for single unit MI calculation to avoid any bias. Mutual information for combined units was higher in comparison to the mutual information of single units ($p = 0.0002$, Wilcoxon rank-sum test).

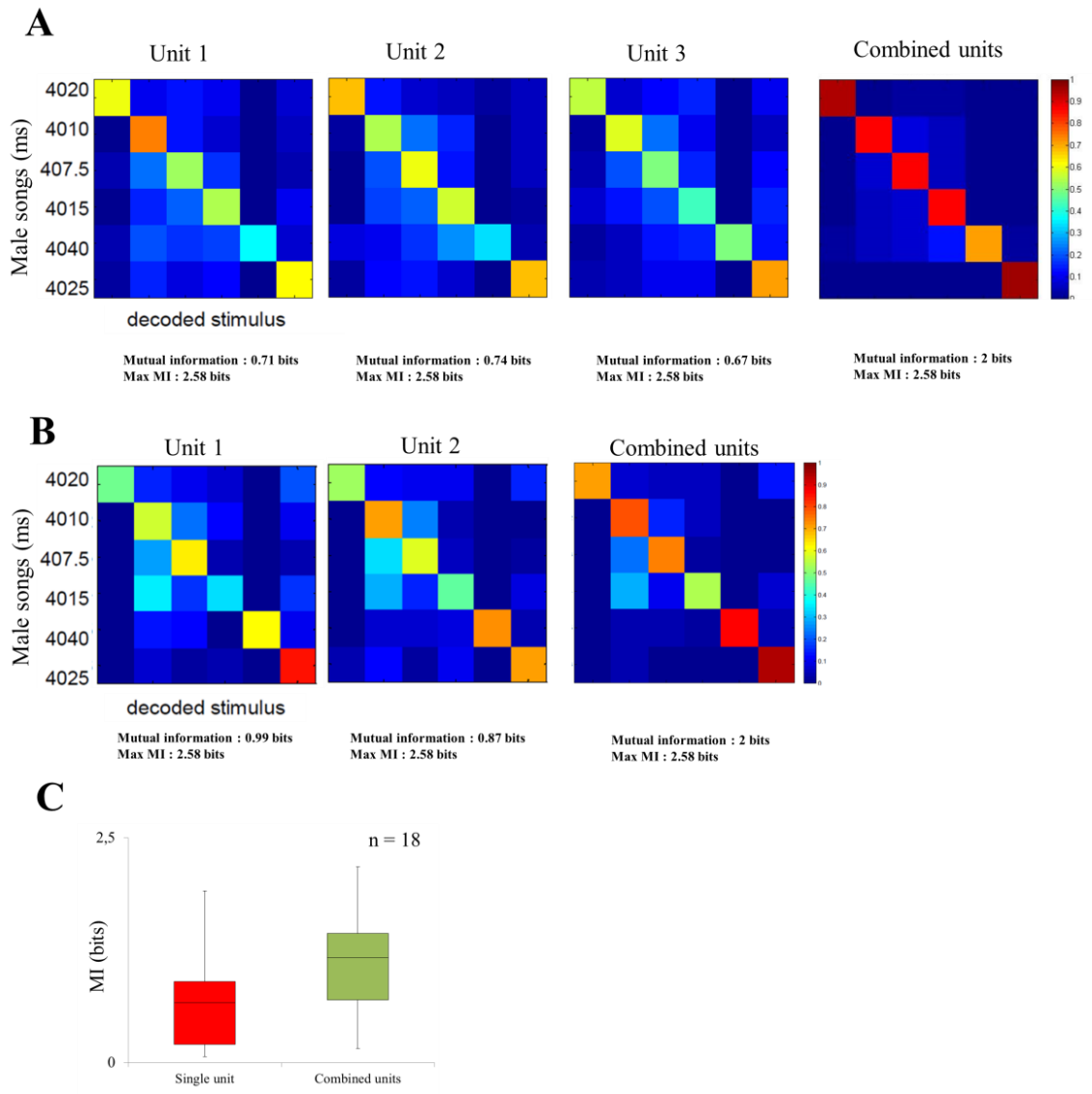

Figure 3.19 **Confusion matrices and the mutual information.** (A) Plots show four confusion matrices for three sorted units and combined units recorded in the same individual for six male grasshopper songs. The order is set as presented during the time of experiments. 4020 means 40 ms syllables, 20 ms pauses etc.. (B) Plots show three confusion matrices for two sorted units and combined units recorded in the same individual for six male grasshopper songs. (C) Box plot showing the comparison of the mutual information calculated from the single units and the combined units in 18 individuals. Highest information among the sorted units was considered for single unit MI calculation. The black line in the box represents the median value. The upper and lower whiskers represent the maximum and minimum values respectively. The box is 25% above and below the median.

To see if the mutual information increases with increase in the number of the recorded units, I compared the mutual information of 2 units with 3 and 4 units recorded in the same individual at the same time as shown in figure 3.20 and figure 3.21. The sorted units have been randomly selected for the mutual information calculation. Figure 3.20 A shows that the decoding performance increased for 3 units in comparison with 2 units in confusion matrices which are recorded in the same individual. Figure 3.20 B shows the comparison of the mutual information of 2 units with 3 units for 9 recordings. Mutual information for three units was higher than mutual information of two units ($p = 0.017$, Wilcoxon rank sum test). Similarly, the decoding performance and the mutual information of 4 units was also compared with 3 and 2 units for three individuals as shown in figure 3.21 A and B. Mutual information and decoding performance increased for 4 units in comparison to 3 and 2 units recorded in the same individual (n=3).

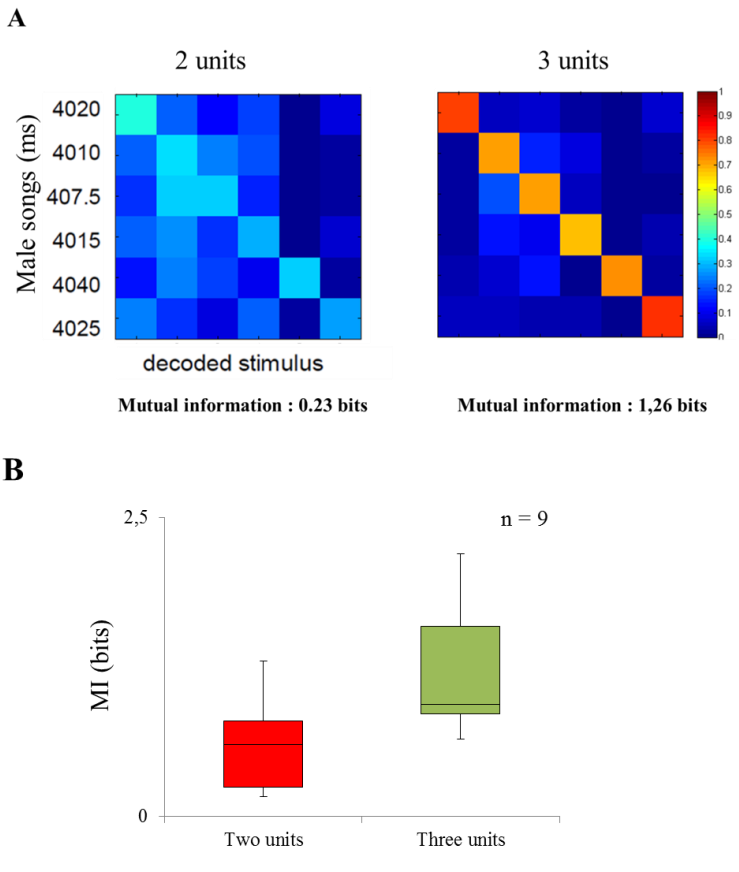

Figure 3.20 **Confusion matrices and the mutual information.** (A) Plots show two confusion matrices for two sorted units (left) and three units (right) recorded in the same individual for six male grasshopper songs. (B) Box plot showing the comparison of the mutual information calculated from two units and three units in 9 individuals. The black line in the box represents the median value. The upper and lower whiskers represent the maximum and minimum values respectively. The box is 25% above and below the median.

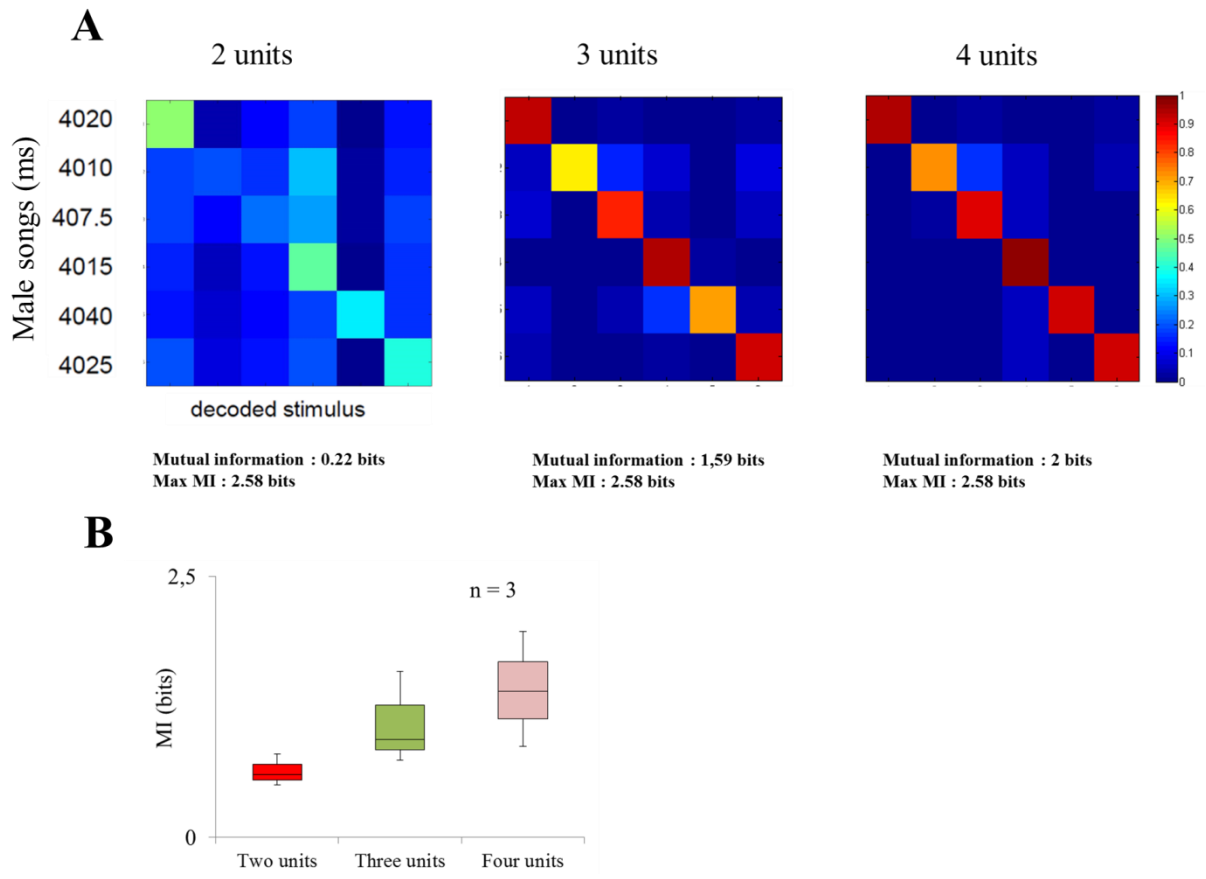

Figure 3.21 **Confusion matrices and the mutual information.** (A) Plots show three confusion matrices for two units (left), three units (middle) and four units (right) recorded in the same individual for six male grasshopper songs. (B) Box plot showing the comparison of the mutual information calculated by combining two, three and four units in 3 individuals. The black line in the box represents the median value. The upper and lower whiskers represent the maximum and minimum values respectively. The box is 25% above and below the median.

Figure 3.22 shows two examples from those 27 recordings which did not show any increase in the mutual information while comparing the single units with the combined units. Figure 3.22 A and B show that the decoding performance remained the same for the combined units in comparison to three and two single sorted units recorded in the same individual at the same time. Figure 3.22 C shows the comparison of the mutual information of single sorted units with combined units for 27 recordings. Mutual information for the combined units was either similar or decreased slightly (n=5) to the mutual information of single units ($p = 0.01$, Wilcoxon rank sum test).

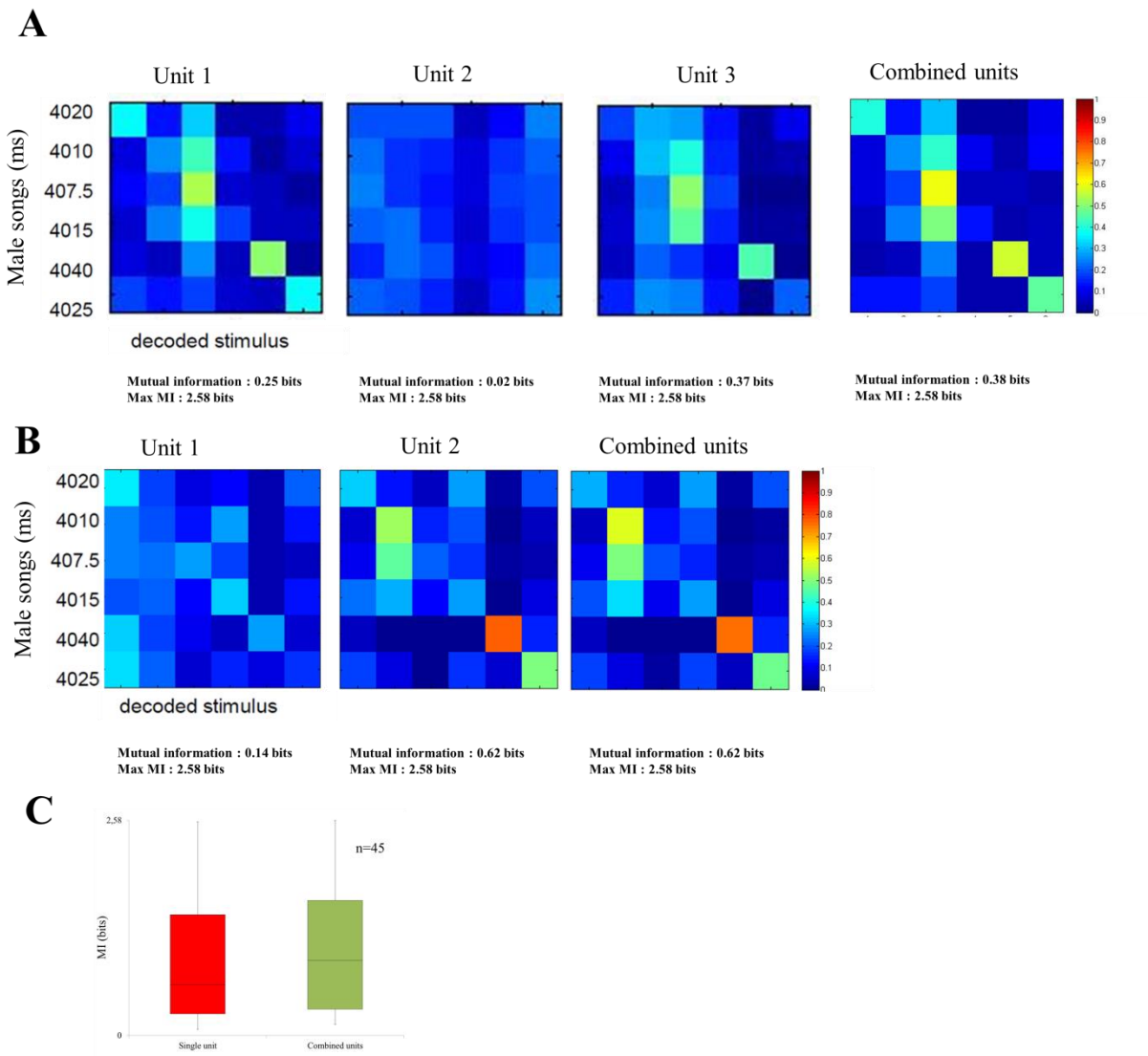

Figure 3.22 **Confusion matrices and the mutual information**. (A) Plots show four confusion matrices for three sorted units and combined units recorded in the same individual for six male grasshopper songs. (B) Plots show three confusion matrices for two sorted units and combined units recorded in the same individual for six male grasshopper songs. (C) Box plot showing the comparison of the mutual information calculated from the single units and the combined units in 29 individuals. The black line in the box represents the median value. The upper and lower whiskers represent the maximum and minimum values respectively. The box is 25% above and below the median.

3.4 Discussion

Information about the relevant aspects of a stimulus must be represented by a population of neurons in the brain. As single neurons are not sufficiently informative, to obtain the complete information about sensory processing some sort of population averaging should be done in the brain. Such information averaging is known as population coding (Averbeck et al. 2006). So far in grasshopper auditory systems, population coding has been studied by combining the responses of single cells recorded intracellularly in several individuals (Clemens et al. 2011; Meckenhäuser et al. 2014). This method suffers from the problem of interindividual and intertrial neuronal variabilities (Ronacher et al. 2004). It would be more appropriate to record the activity of several neurons at the same time in the same individual to reduce such neuronal variabilities. Here, I recorded from the population of ascending auditory neurons in the brain of the grasshopper *Ch. biguttulus* and analyzed population response using summed activity analysis and decoding techniques.

3.4.1 Problems in unit identification

The original idea of this study was to compare the intensity responses of extracellularly recorded units to the intracellularly recorded ascending neurons (Stumpner 1988; Stumpner and Ronacher 1991) and identify the units based on the similarity of the responses to those recorded intracellularly. However, identification of neurons just by comparing the intensity responses of extracellular data with intracellular data turned out to be hard. One reason for the identification problem is neuronal variability as explained in Chapter 2. However, some ascending neurons encode particular properties of acoustic stimuli in their physiological responses that may increase the probability of identification. For example, AN12 has a strictly phasic characteristic mediating pronounced responses to stimulus onsets (Stumpner 1988; Creutzig et al. 2009), activity of AN4 is suppressed by syllables with gaps (Ronacher and Stumpner, 1988), AN2 encodes directional information (Stumpner 1988; Stumpner and Ronacher 1991) and AN6 is the only described neuron showing tonic responses over a large intensity range (Stumpner 1988; Stumpner and Ronacher 1991). These partially unique characteristics can be used as a criteria for the identification of extracellularly recorded auditory units.

Since the identification of neurons just by comparing intensity responses did not give satisfactory results, additionally, a PCA-based classification was done using four feature vectors. It was expected to have completely independent clouds each representing a single class of neurons. Two clouds representing the stimulus onset and the directionality emerged, however, with areas of overlap. Likely reasons for this could be the selection of insufficient number of feature vectors. This leads to lower variance among each class of neurons and clouds with overlaps. It turns out to be difficult to classify units just by using four feature vectors and so the majority of the units remained unidentified.

3.4.2 Population response of ascending neurons

It has been previously suggested by Clemens et al. (2011) that there is a population code taking place at the level of ascending neurons in grasshoppers, where each neuron likely encodes different aspects of the stimulus. To analyze the population response of simultaneously recorded ascending neurons, I have used two different analytical methods.

1. Analyzing the summed response of the recorded neurons.

2. Analysis using decoding techniques to understand the population code.

Since the unit identification turned out to be difficult, these analyses were done without unit identification after sorting the recorded units.

3.4.2.1 Summed response of the recorded neurons

Syllable-pause tuning

Syllable – pause tuning was analyzed in 66 preparations and no clear match was seen between the behavior and the neuronal data. Here, the experiments were performed using simple block stimuli and data have been analyzed only for syllable-pause and syllable-gap patterns. Real grasshopper songs are more complicated having many more features within a song (intensity, frequency, onset etc). It is likely that many more ascending neurons are involved in encoding all features of a natural song. Also, one has to consider that such multielectrodes tend to record from the larger axons of the neurons in the vicinity. Due to this fact, smaller neurons get masked in the recording and one always systematically

miss information from such units in the analysis. Other problems like the sudden spontaneous firing of the recorded units and errors in clearly discriminating the units during the spike sorting will also affect the calculations.

Gap tuning

In the grasshopper *Ch.biguttulus,* some neurons located in the metathoracic ganglion are described as feature detectors. One example is AN4, which is selectively and strongly inhibited by interrupted sound pulses called syllables with gaps (Ronacher and Stumpner 1988). Such gappy songs are generated by male grasshoppers with one hindleg (another hind leg is lost often due to autonomy during contact with predators) who are no longer able to mask small gaps in the song (von Helversen and von Helversen 1994). Female *Ch.biguttulus* uses these gaps in a male song as criteria for rejection in the context of sexual selection (Kriegbaum 1989). I analyzed the gap tuning of summed response of ascending neurons in 48 preparations and none of them showed a clear match with the behavior data even those where at least one unit was identified as AN4. This may be due to the recording from those units which responds better to syllables with gaps and compensate the decrease in the spike counts. Two recordings showed an increase in the neuronal activity with gap durations. This increase in the neuronal activity may be due to the presence of those neurons which shows a stronger response to syllables containing gaps than to those not containing gaps. Such neurons (AN3 and AN12) have been previously described by Stumpner et al. (1991) and are significantly influenced by gaps. It is likely that these neurons (AN3 and AN12) are also involved in transferring the information about gaps to the brain along with AN4.

3.4.2.2 Population coding analysis using decoding techniques

Information about behaviorally important variables such as sensory signals or motor actions is carried by the joint activity of a population of neurons (Panzeri et al. 2015). Population coding is a method to represent stimuli by using the joint activities of a number of neurons. Each neuron encodes different aspect of a stimulus, and the responses of many neurons may be combined to determine some value about the stimulus (Wu et al. 2002).

Clemens et.al (2011) have suggested that there is a population code taking place among the ascending neurons in grasshoppers. Here, I have tried to decode song identity from the neuronal responses to six different grasshopper songs (40 ms syllable-pause patterns) using confusion matrices (Clemens et al. 2011). These matrices were used for mutual information calculation to extract quantitative information from the neuronal responses (Quian Quiroga and Panzeri 2009; Clemens et al. 2011). Such metrices quantify the dissimilarity of spike trains among different stimuli to calculate the information transferred by the recorded neurons (Quian Quiroga and Panzeri 2009). However, this approach underestimates the full information in the statistical sense, one can come closer to what biological system can read from spike trains (Clemens et al. 2011). Out of 45 data sets, 18 showed an increase in the mutual information for the population of units with a better decoding performance in comparison to single units, indicating that the relevant stimulus features of syllable-pause patterns are represented by a population of the recorded ascending neurons. Similar results were also observed by Kobayashi et al. (2013) while studying population coding in the moth antennal lobe. He tried to decode odorant identity from the activity of the recorded neurons using the maximum likelihood method and found that decoding performance rapidly improves with increasing number of neurons. However, in our results, 27 recordings showed no increase in the mutual information for the combined units in comparison to the single units. It is likely that these recorded units do not represent the features of syllable-pause patterns and may be involved in representing other features of a song. Additionally, it was observed that if a non-auditory unit was included during the analysis, decrease in mutual information occurs for combined units.

Here, the analysis has been done using only simple block stimuli of syllable-pause patterns. The real grasshopper songs are much more complicated since they include many features in one song. Using such real songs, one can expect that the decoding from an intact population of at least 20 ANs in grasshoppers would reach considerably higher decoding performance, which supports the hypothesis of a population code in the central nervous system (Clemens et al. 2011; Meckenhäuser et al. 2014) of grasshoppers. In future, it would be interesting to analyze the population response of all 20 ascending neurons to complicated stimuli which include many features.

3.5 Outlook

In summary, the information about the stimulus identity (syllable-pause patterns) is likely represented by the population of specific ascending neurons which supports the previously formulated hypothesis of a population code taking place among the ascending neurons. In future, it would be interesting to use more realistic stimuli consisting of many features in one song. Additionally, it would also be intersting to develop a technique which can simultaneously record from an intact population of 20 ascending neurons (eg. using several multielectrode at different locations in the brain) to get the maximum information about the stimulus.

Chapter 4

Recordings and electrical stimulation of local auditory neurons in the brain of a small grasshopper

4.1 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 is mainly based on the temporal structure of songs arising from combinations and repetitions of stereotypical elements (Gerhardt and Huber 2002). Processing and recognition of these songs are very important for species and gender recognition, localization of reproductive partners, assessment of genetic quality and for mating success (Bradbury and Lee 1998). One of the main challenges for such insects while communicating is recognizing the difference between the call of a conspecific from that of congeneric (Römer and Seikowski 1985). Behavioral experiments with crickets and grasshoppers have shown that they can recognize a song produced by a conspecific and that some temporal features like syllable (for definition see Chapter 1 : General introduction) or intervals are more effective than others in activating the innate releasing mechanism for phonotaxis (Haskell 1958; Dumortier 1963; von Helversen 1972). Many electrophysiological studies have been performed on the auditory pathway of various insects in an attempt to elucidate the neuronal mechanisms underlying song recognition.

Among invertebrates, the acoustic behavior of grasshoppers has attracted the interest of neuroethologists for many years (Elsner 1974). Grasshoppers produce species- and context-specific sound patterns by rhythmically rubbing stridulatory files on the inner sides of their hindlegs 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 1997b), which are connected via local bilaterally arborizing interneurons and thus produce coordinated phase coupled hind leg moments (Hedwig 1992). The decision about when and which song pattern to sing 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). The stridulatory command neurons receive direct or indirect input from local neurons in the brain (Hedwig

2001) and in this way some of such local brain neurons could be directly involved in controlling the stridulation. However, the information on the location and the involvement of such local brain neurons in processing different features of a male grasshopper song and controlling the stridulation is still limited.

Here, I have used multielectrodes and recorded local brain neurons from different areas in the brain of *Ch. biguttulus*. Additionally, I have used the same multielectrodes and extracellularly stimulated these different neuropiles to induce the stridulation.

4.2 Materials and methods

4.2.1 Animals

Adult female grasshoppers (*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).

4.2.2 Animal preparation

In order to minimize the movements of the animal, front legs 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)

4.2.3 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 loudspeakers (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 towards the speaker, grid on, connected to a Brüel & Kjær measuring amplifier (type 2602). Sound intensities are given in dB SPL (Sound pressure level) re 2×10^{-5} N m^{-2} .The microphone has been calibrated using a calibrator (Brüel & Kjær type 4230). The signal was 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. Different stimuli were divided into different stimulus programs which were saved as 'configuration file' in Spike2 7.10 as described below.

Search-program. In order to detect auditory neuronal activity from brain neurons 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. To facilitate finding auditory activity during the search program, an audio monitor (AUDIS-01D/16 NPI Electronic Instruments– Tamm) was used.

Intensity response program. For obtaining "intensity response characteristics" (responses to changing sound amplitude) 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 from the ipsilateral side (left) and broadband white noise stimuli were delivered between 30 and 90 dB SPL, increasing in 10 dB steps from the ipsilateral and contralateral side (right) (Stumpner 1988). The multielectrode was always inserted in the left side of the brain and stimulation is also provided from the left side which is considered as the ipsilateral side. 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 inter-stimulus intervals and repeated 10 times at each sound pressure level.

Temporal pattern program. In order to test neuronal responses to variation of artificial temporal patterns, male grasshopper songs were presented that were varied in syllable duration (40 ms, 60 ms, 80 ms and 100 ms), pause duration (7.5 ms, 10 ms, 15 ms, 20 ms, 25 ms and 40 ms) and gap duration (2 ms and 5 ms) (for definition see introduction). The temporal patterns were generated using broadband white noise, 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. One program was for different syllable – pause patterns while another program was for different syllable - gap patterns. Each syllable - pause pattern or syllable - gap pattern 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.

Attractiveness stimulation program: In order to compare the neuronal data with behavioral data published by Clemens et al. (2014), twenty-five sequences consisting of different combinations of attractive and non-attractive syllables were presented in pseudorandom order. The stimulus design was adapted from Clemens et al. (2014). Attractive and non-attractive syllables were created using the programming script wavemake.s2s. Attractive syllables consisted of 75 ms long noise pulses with broadband white noise (5-40 kHz) of constant amplitude followed by 12ms of pause. Non-attractive syllables were created by introducing gaps (5 ms) in a syllable and removing high frequency (5-12 kHz). The syllables with gaps having low-frequency content are known to reduce the female response probability drastically (Ronacher and Stange 2013).

4.2.4 Electrical stimulation

After recording auditory activity, extracellular electrical stimuli were applied to the same site within the auditory neuropiles in order to elicit the stridulation. Electrical current was injected via one of the tungsten wires and the reference wire to stimulate the neurons in the vicinity of the tip using a stimulus isolation unit (ISO-STIM 01M, NPI Electronic Instruments, Tamm). In the "GATE TTL

Input mode", the output current is generated by the built-in timing unit which was triggered by gate pulses from the interface (Power Mk II, CED – Cambridge, UK). Current pulses (3 ms current followed by 3 ms pause) of 50 µA were applied for at least 5 s. 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). A small reflector (Scotchlight 3 M, type7610) was attached to the hind leg. 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 a 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 Spike 2 7.10. Additionally, a small electrical microphone (Conrad Electronic, sensitive in the audio and near ultrasound range) was placed near the stridulating hindleg to record the sound.

4.2.5 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 minutes. After retraction of the multielectrode, the brain was extracted from the head and fixed in paraformaldehyde (4% in phosphate buffered saline) for 2 hours. For subsequent analysis, the brain was embedded in albumin-gelatin (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 (VT1000s Leica – Wetzlar, Germany). The sections were transferred to a 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).

4.2.6 Offline spike sorting

Spike sorting was done as described in Chapter 2.

4.2.7 Syllable-pause and gap tuning

Syllable-pause and gap tuning plots were made in order to check the correlation between the neuronal and the behavior data. Neuronal data was plotted by calculating the mean spike count against the pause duration. Behavior data regarding syllable - pause tuning were obtained from von Helversen (1972) while average behavior responses to gap tuning were obtained from Ronacher and Stumpner (1988). These analyses were done in Excel 2010.

4.3 Results

4.3.1 Latency criteria

First spike latency (FSL) of a given neuron is defined as the time from the onset of a stimulus to the occurrence of the first spike in response to that stimulus. When neurons are spontaneously active, that is, they spike in the absence of experimenter controlled stimulation, the first spike on a given trial might not be necessarily be evoked by the stimulus. Therefore other definitions of FSL are also in use, for example, the time from stimulus onset to the initial peak in a post-stimulus-time histogram computed from the responses of a neuron to numerous repetitions of that stimulus (Heil 2004).

Local brain neurons were mainly recorded in lateral protocerebrum, anterior protocerebrum and central complex. For clearly discriminating local auditory brain neurons from ascending neurons, a latency criterion was implemented. Since local brain neurons are directly or indirectly postsynaptically connected to the ascending neurons in the brain (Eichendorf and Kalmring 1980; Kutzki 2012), they showed longer first spike latency than ascending neurons in the same individual. The FSL generally shortens monotonically as the overall amplitude of sound increases. Therefore, I compared the FSL of local auditory brain neurons to the ascending neurons recorded in the same individual at the highest sound intensity (90 dB SPL) for the same stimulus as shown in figure 4.1 A and B. The latency of ascending neurons recorded from the deutocerebrum was 15 ms or shorter. The latency of local brain neurons recorded from the lateral protocerebrum, the anterior protocerebrum and the central complex was longer than 15 ms. Figure 4.1 C shows the comparison of the latency in 21

sorted ascending units which are compared with the 21 sorted local brain units (Mann–Whitney U-test: $p \leq 0.05$).

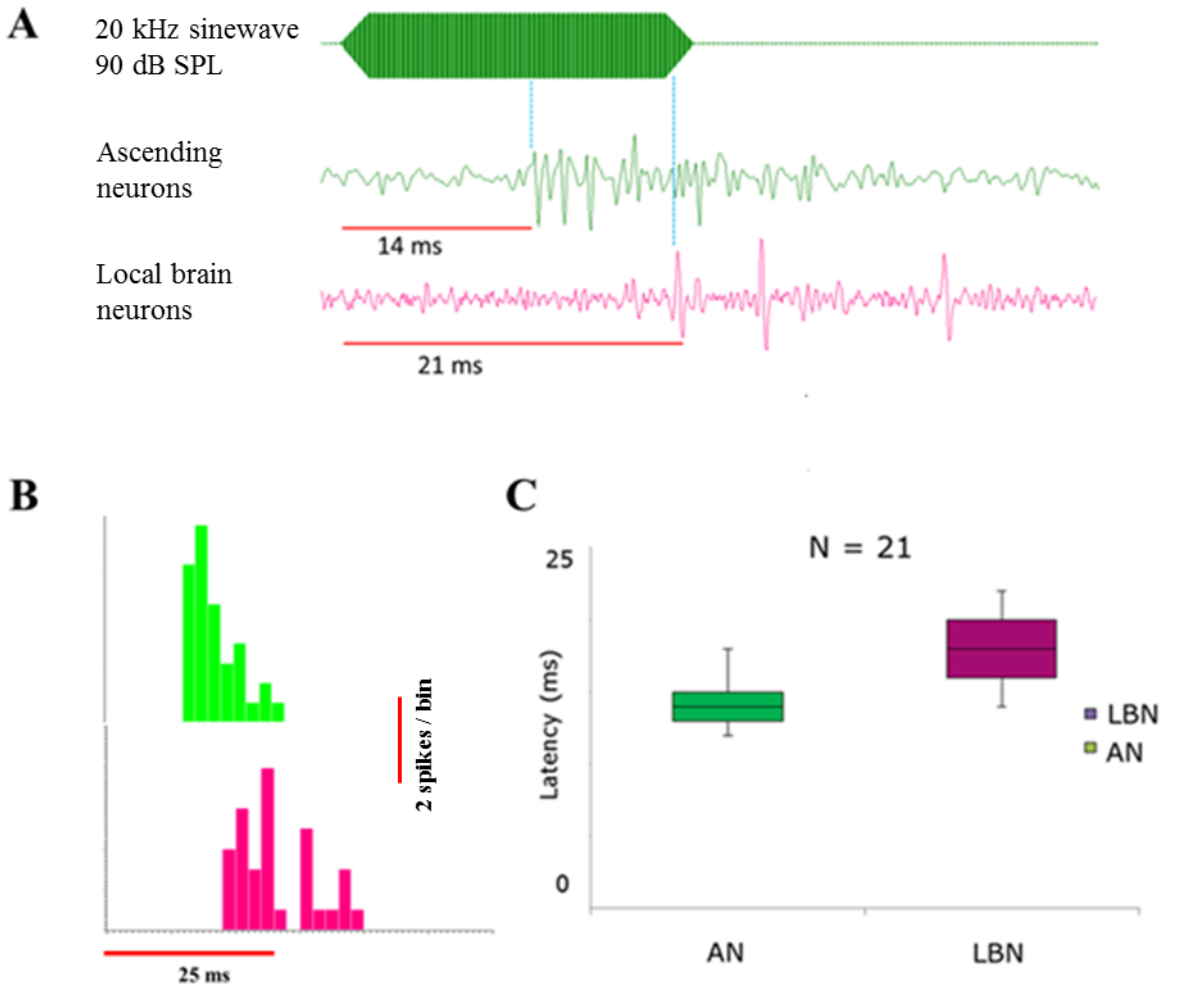

Figure 4.1 **Latency criteria.** (A) Recording traces showing the response of ascending neurons with shorter latency and local brain neurons with longer latency to 20 kHz sinewave stimuli (25 ms) at 90 dB SPL. The recordings have been done in the same individual (B) PSTHs made from 20 kHz sinewave stimuli (25 ms) at 90 dB SPL repeated 10 times (C) Latency difference between local brain neurons and ascending neurons recorded in 21 different individuals (Mann-Whitney U-test: $p \leq 0.05$). Box plot has been plotted from the latency of 21 sorted ascending units and local brain units. The length of the box represents the interquartile range. The black line in the box represents the median value. The upper and lower whiskers represent the maximum and minimum values respectively.

4.3.2 Local brain neurons recorded in the lateral protocerebrum and their exact locations

The best-described class of ascending neurons ascend from the metathoracic ganglion up to the brain and make branches in the lateral protocerebrum (LP) which indicates the presence of local auditory brain neurons which are directly postsynaptically connected to the ascending neurons (Eichendorf and Kalmring 1980; Kutzki 2012). Local brain neurons were recorded from the lateral protocerebrum in 12 preparations and data were evaluated from seven preparations only due to better quality of the recording. The recorded neurons were considered local brain neurons based on their longer first spike latency to the stimulus at the highest intensity (90 dB SPL). A total of nine local auditory brain units were sorted in seven preparations from lateral protocerebrum and their intensity response curves were plotted for 5 kHz, 20 kHz and White noise (Fig.4.2). One out of nine units (Unit 5) showed an increase in response with increase in the sound intensities for 5 kHz and 20 kHz stimuli. Unit 3,4,7,8 and 9 showed increase in response for 5 kHz and decrease in response for 20 kHz stimuli at higher intensities. Unit 1 showed optimum type response for 5 KHz stimuli. Most of the units (except Unit 7) showed similar responses for white noise presented from ipsi- and contralateral side. Unit 7 showed higher response for white noise on ipsilateral side in comparison to contralateral side.

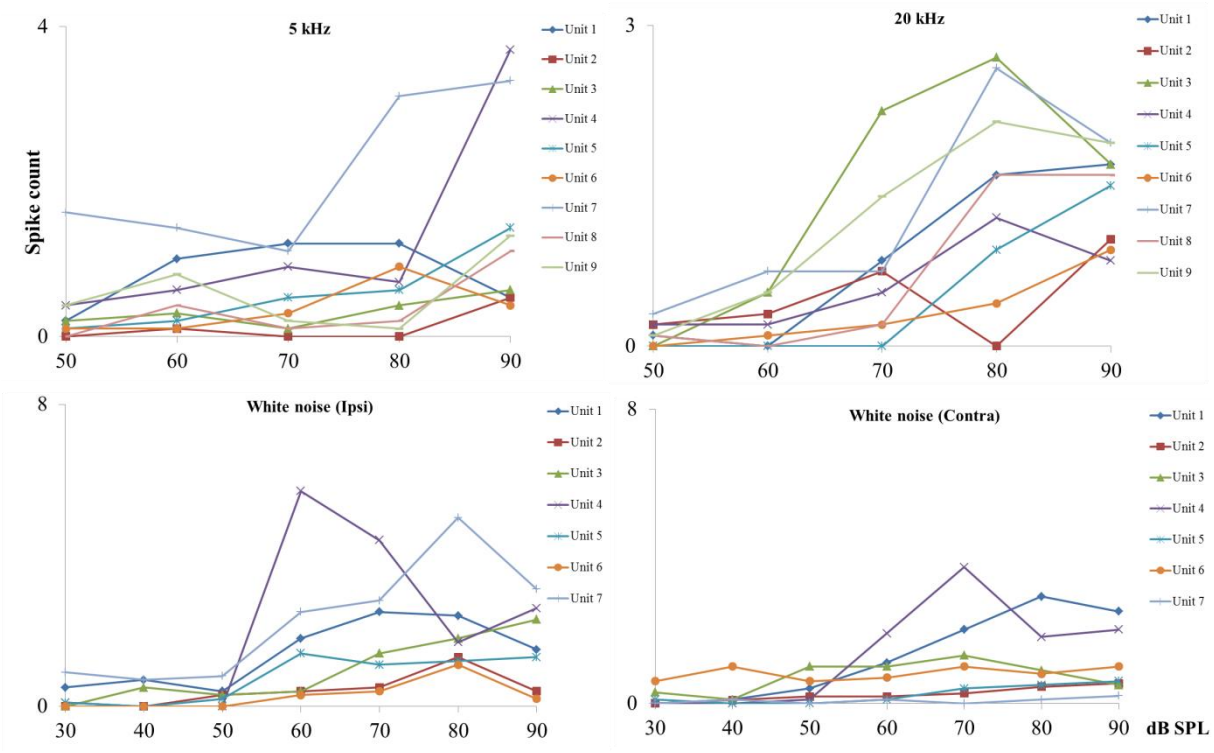

Figure 4.2 **Intensity response curves.** Plots show the intensity dependence of all sorted local brain units recorded from lateral protocerebrum for 5 kHz, 20 kHz and white noise stimuli. 5 kHz sine wave (25 ms) and 20 kHz sine wave (25 ms) stimuli were presented between 50 and 90 dB SPL, increasing in 10 dB steps from the ipsilateral side and broadband white noise (100 ms) stimuli were delivered between 30 and 90 dB SPL, increasing in 10 dB steps from the ipsilateral and contralateral side

Out of nine sorted units, one unit (Fig.4.2 - Unit 7) showed the strongest directional dependence in the response. The response to ipsilateral side was clearly higher than the contralateral side as shown in figure 4.3.

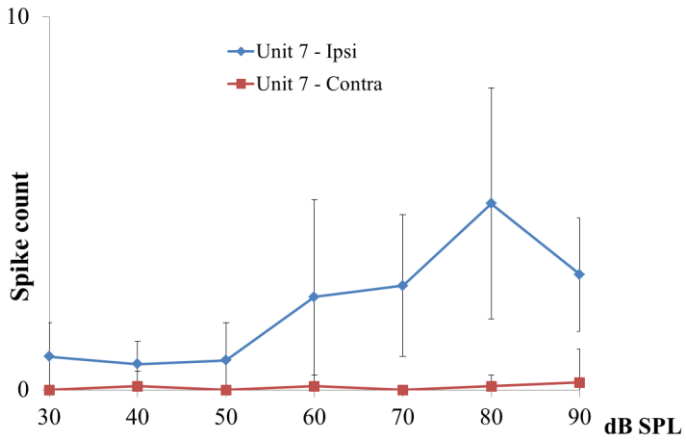

Figure 4.3 **Intensity response curves.** Plot shows the directionality dependence of one sorted local brain unit recorded from the lateral protocerebrum (Fig. 4.2 - Unit 7) for broadband white noise (100 ms).

Syllable-pause tuning was checked for all the sorted units recorded from the lateral protocerebrum and compared with the behavior data of female *Ch. biguttulus* obtained from von Helversen (1972). Some potential dependences of the neuronal activity on different pause durations were seen but a clear match between the neuronal and the behavioral data was not seen in any case (Fig.4.4).

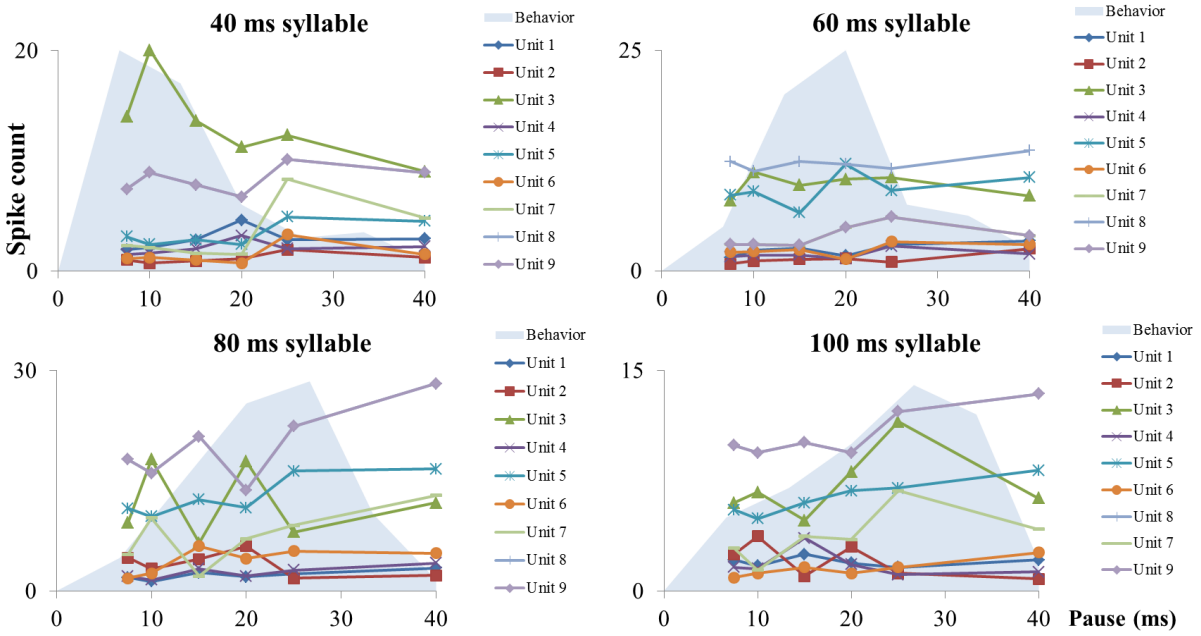

Figure 4.4 **Syllable-pause tuning.** Plots show the neuronal responses of sorted local brain units recorded from lateral protocerebrum to different male grasshopper model songs along with behavior data. The average number of spikes from 10 repetitions is plotted against the pause durations for four different syllables. Behavior data were obtained from von Helversen (1972).

Among all the units shown in figure 4.4, one unit (Unit 3) responded with a phasic burst at the onset of syllables, provided that the pauses between syllables exceed a certain duration as shown in figure 4.5 A. In order to quantify the onset response, the average number of spikes per syllable crossing the horizontal threshold (mean+/- 3*SD) was calculated from the PSTHs and plotted against the pause duration which is shown in figure 4.5 B. The number of spikes per syllable increases linearly with increasing pause duration.

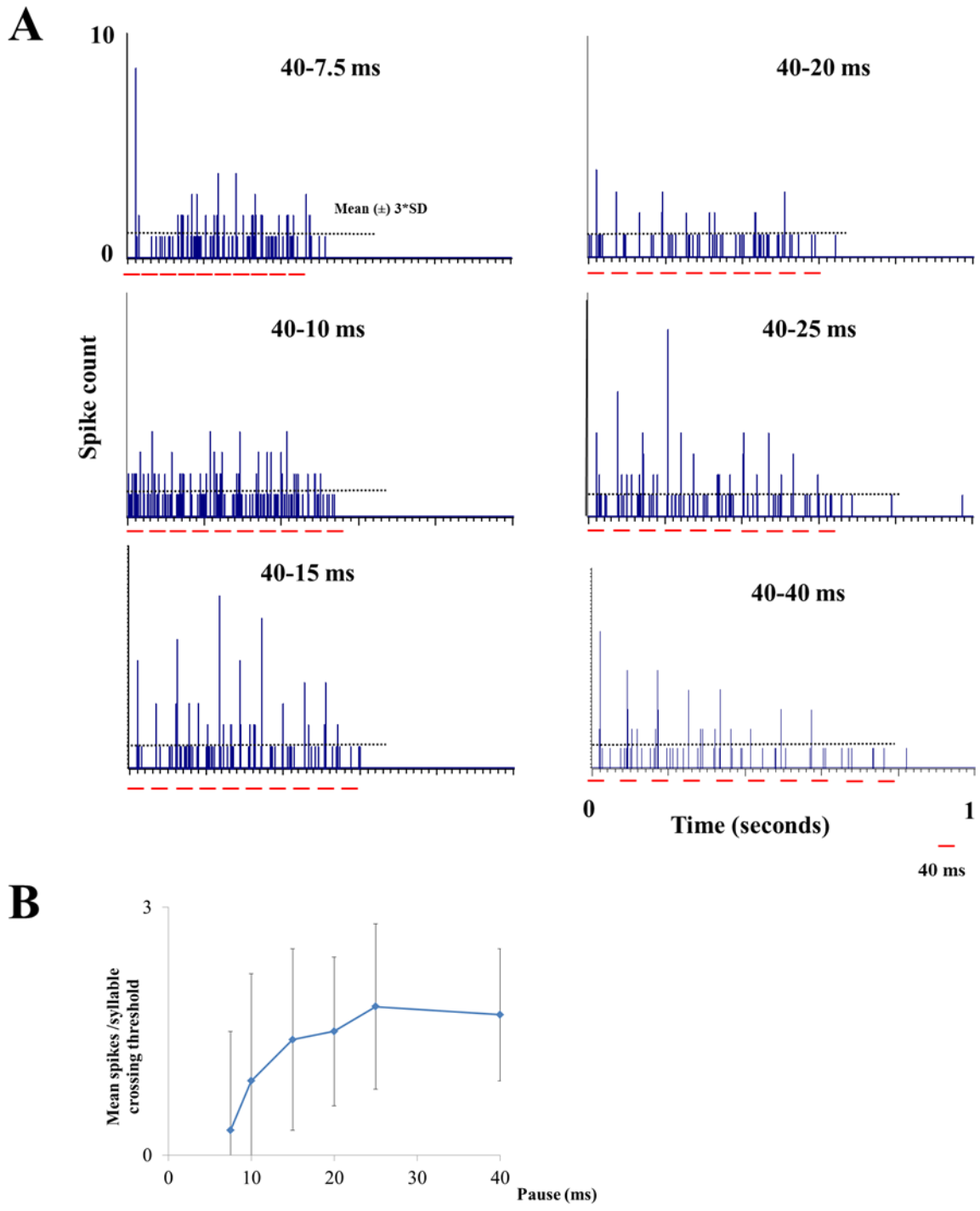

Figure 4.5 **Marking onset of the syllable.** (A) Plots show the PSTHs of one of the sorted local brain unit recorded (Fig. 4.4 - Unit 3) from lateral protocerebrum for different model grasshopper song with syllables of 40 ms and six different pause durations (repetitions = 10, bin width = 2ms) (B) Spike response to the onset of the syllable was quantified by calculating mean spikes per syllable crossing the threshold (mean (±) 3*SD) in PSTHs.

Figure 4.6 shows the responses of one unit (Fig.4.4 - Unit 3) to different syllable-pause patterns along with the behavioral data (von Helversen 1972) of female *Ch. biguttulus*. The mean spike count was plotted against different pause durations. A clear match between the neuronal and the behavioral data was seen for 100 ms syllable patterns as shown in figure 4.6. Lower neuronal activity at shorter and longer pause with peak response at 25 ms matches with the behavioral response. Additionally, a similar good match is seen between the neuronal and the behavioral data at 40 ms syllables. Decrease in the neuronal activity at longer pauses matches the behavior. The neuronal activity was not much affected by 60 ms syllable and no match between the neuronal and the behavioral data was seen for 60 ms and 80 ms syllable-pause patterns.

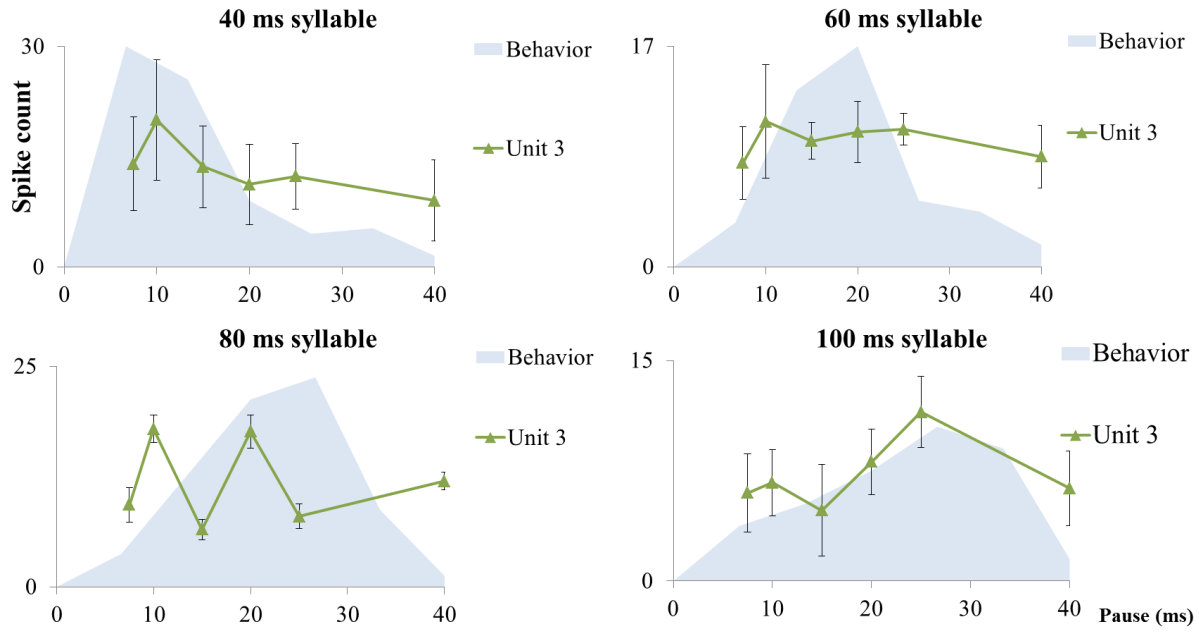

Figure 4.6 **Syllable-pause tuning**. Plots show the neuronal response of one sorted local brain unit (Fig.4.4 - Unit 3) recorded from lateral protocerebrum to different male grasshopper model songs along with the behavior data. The average number of spikes from 10 repetitions is plotted against the pause durations for four different syllables. Behavior data were obtained from von Helversen (1972).

Many of the local brain neurons recorded from lateral protocerebrum were highly adapting to the stimulus. Figure 4.7 shows one example of such a local brain unit showing high adaptation to two different artificial male grasshopper songs.

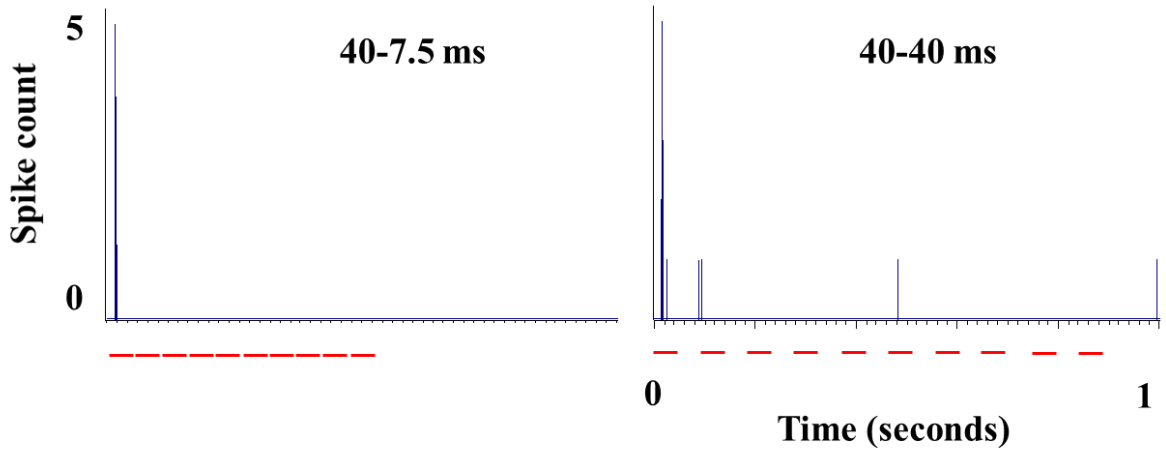

Figure 4.7 **Peristimulus time histograms**. Plots show PSTHs of one sorted local brain unit recorded from lateral protocerebrum for grasshopper model songs with syllable duration of 40 ms and two different pause durations (repetitions = 10, bin width = 2ms).

Syllable-gap patterns were successfully checked in five preparations and the neuronal response of six units was compared with the behavior data of female *Ch. biguttulus* (Ronacher and Stumpner 1988). Unit 4 clearly showed a clear increase in neuronal activity with gap duration while Unit 3 showed a weak tendency in increase in neuronal activity with gap durations as shown in figure 4.8. This dependence on gap durations was not as strong as described for certain ascending neurons (AN12) (Stumpner 1988). The activity of other units (Unit 1, Unit 2, Unit 5 and Unit 6) were not much affected by the gaps of syllables and thus did not show any match with the behavior data as shown in figure 4.8.

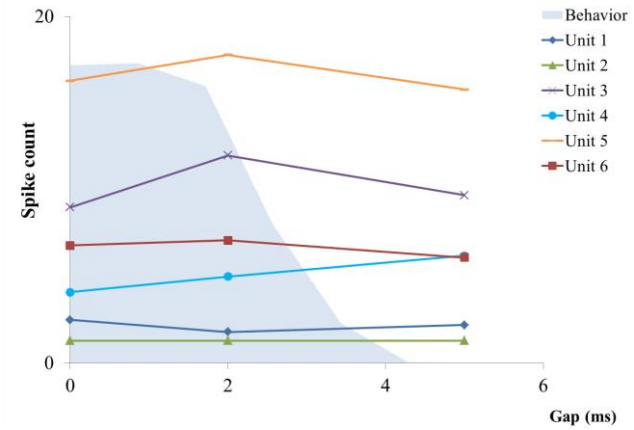

Figure 4.8 **Gap tuning**. Plots show the neuronal responses of sorted local brain units recorded from lateral protocerebrum to male grasshopper model songs with 80/15 ms syllable/pause durations and variable gaps. The average number of spikes from 10 repetitions is plotted against the pause durations. Behavior data were obtained and averaged from Ronacher and Stumpner (1988).

Figure 4.9 A provides a summary of locations in the lateral protocerebrum where auditory activity was recorded from the local brain neurons. After a recording auditory activity successfully, the position of the multielectrode within the brain tissues was marked by passing high continuous current (100 µA) for 5 minutes between one of the tungsten wires and the reference wire or between two tungsten wires. This procedure coagulates the brain tissue and generates a black spot in the immediate vicinity of the electrode as shown in figure 4.9 B highlighted with a red arrow.

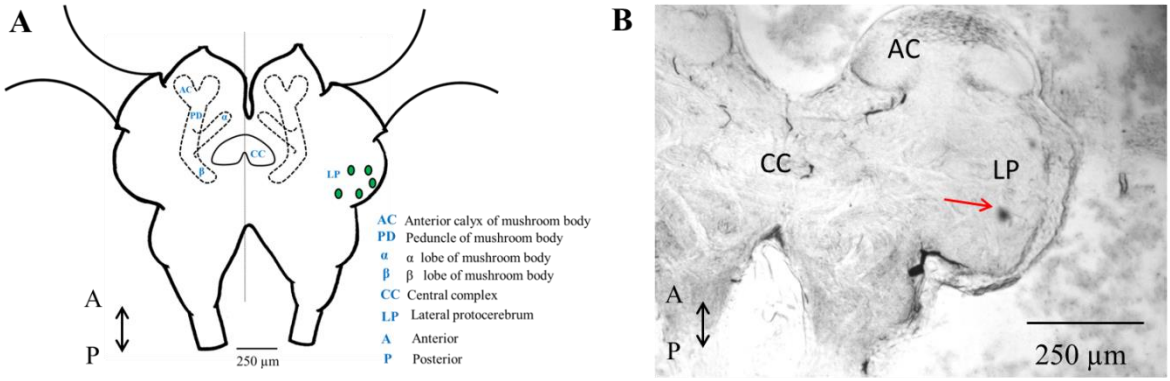

Figure 4.9 M**arking of the recording location.** (A) Sketch of a grasshopper brain with exact recording locations in lateral protocerebrum shown using green dots. (B) Marking of the recording location using electrocoagulation. Location is highlighted by a red arrow.

4.3.3 Local brain neurons recorded in anterior protocerebrum and their exact locations

Local brain neurons in the anterior protocerebrum were recorded in 13 preparations and data were evaluated from 11 preparations due to a better quality of the recording. A total of 12 local auditory brain units were sorted in 11 preparations from anterior protocerebrum and their intensity response curves were plotted as shown in figure 4.10. Eight out of 12 units showed an increase in response with increase in the sound intensities (Unit 1, 2, 3, 4, 5, 9, 10, and 12) for 5 kHz and 20 kHz stimuli. Additionally, three units (Unit 2, 6 and 7) showed optimum type response at 20 kHz stimuli. Most of the units (except Unit 3 and 4) showed similar responses for white noise presented from ipsi- and contralateral side. Unit 3 and 4 showed higher response for white noise at 60 dB SPL on ipsilateral side in comparison to contralateral side, while Unit 11 showed higher response for white noise on contralateral side in comparison to ipsilateral side at higher intensities.

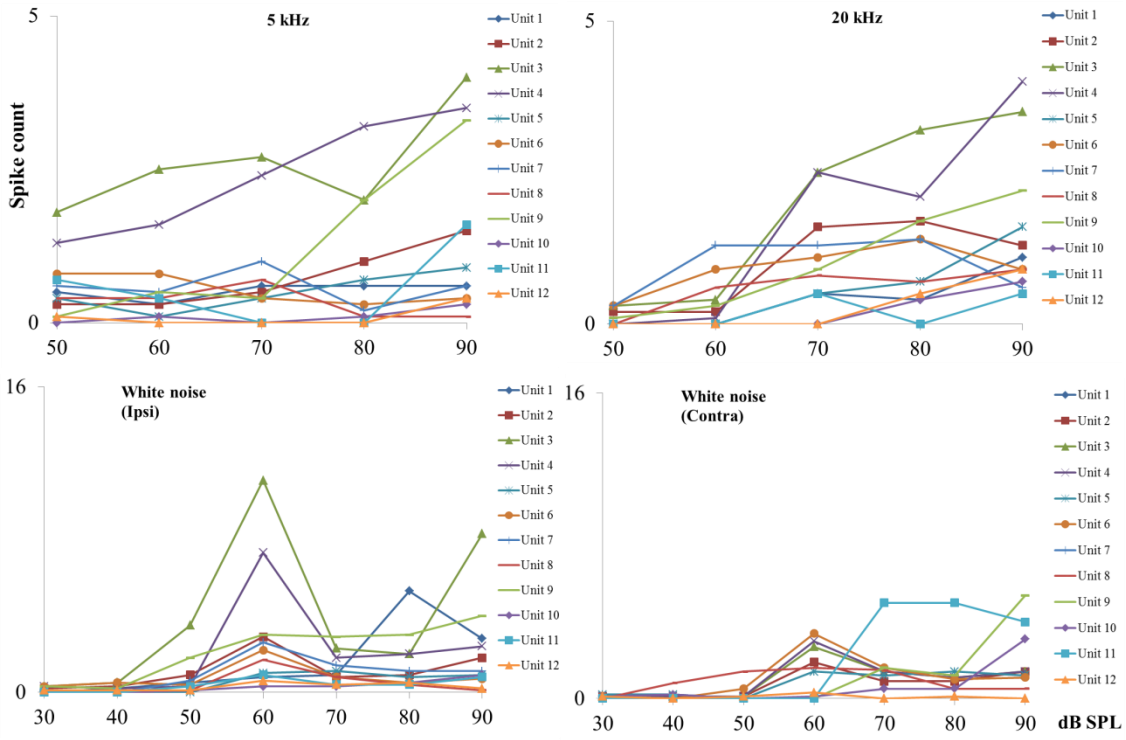

Figure 4.10 **Intensity response curves.** Plots show the intensity dependence of all sorted local brain units recorded from anterior brain for 5 kHz, 20 kHz and white noise stimuli. 5 kHz sine wave (25 ms) and 20 kHz sine wave (25 ms) stimuli were presented between 50 and 90 dB SPL, increasing in 10 dB steps from the ipsilateral side and broadband white noise (100 ms) stimuli were delivered between 30 and 90 dB SPL, increasing in 10 dB steps from the ipsilateral and contralateral side

Syllable-pause tuning was checked for all the sorted units recorded from the anterior protocerebrum and compared with the behavior data of female *Ch. biguttulus* obtained from von Helversen (1972). Some potential dependences of the neuronal activity with different pause durations were seen but a clear match between the neuronal and the behavioral data was not seen in any case as shown in figure 4.11.

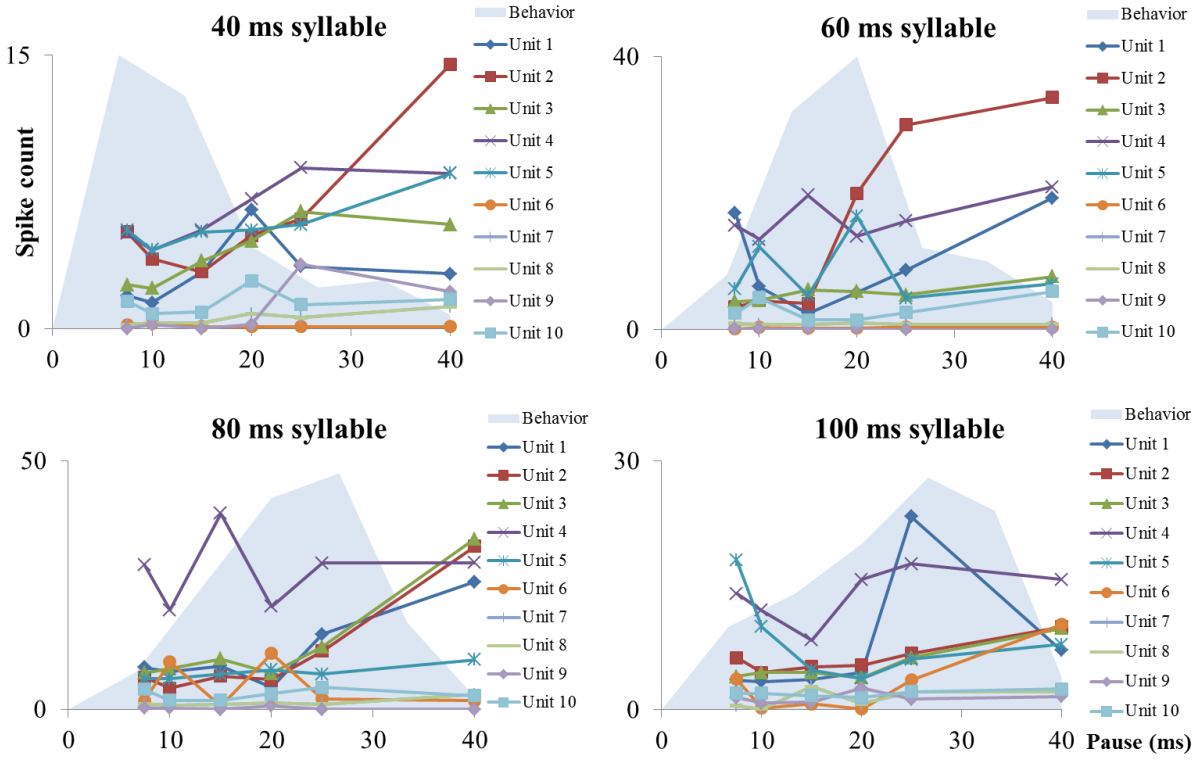

Figure 4.11 **Syllable-pause tuning**. Plots show the neuronal response of sorted local brain units recorded from anterior brain to different male grasshopper model songs along with behavioral data. The average number of spikes from 10 repetitions is plotted against the pause durations for four different syllables. Behavior data were obtained from von Helversen (1972).

Syllable-gap patterns were successfully checked in four preparations and the neuronal response of five units was compared with the behavior data of female *Ch. biguttulus* (Ronacher and Stumpner 1988). Unit 5 showed a strong increase in the neuronal activity while Unit 1 showed a consistent increase in the neuronal activity with the gap durations (Fig.4.12). Unit 2 showed a decrease in neuronal activity with the gap durations (Fig.4.12). The neuronal responses of other units were not much affected by the syllables with 2 ms and 5 ms gaps.

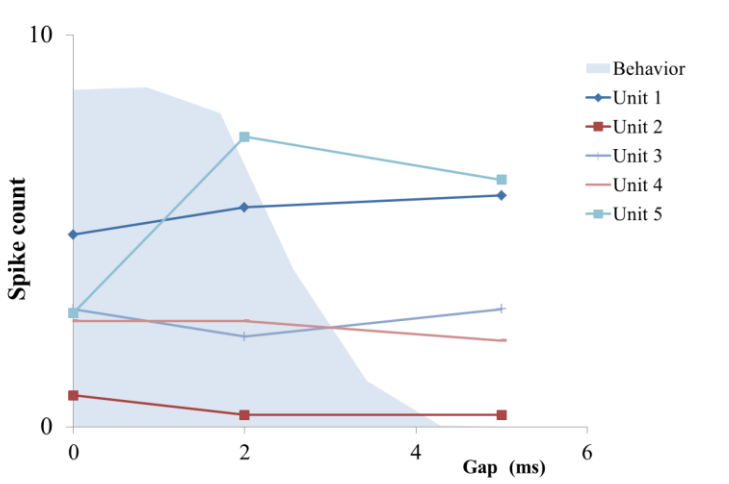

Figure 4.12 **Gap tuning**. Plot shows the neuronal response of sorted local brain units recorded from anterior protocerebrum to male grasshopper model songs with 80/15 ms syllable/pause durations and variable gaps. The average number of spikes from 10 repetitions is plotted against the pause durations. Behavior data were obtained and averaged from Ronacher and Stumpner (1988).

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Figure 4.13 A provides a summary of locations in the anterior protocerebrum where auditory activity was recorded from local brain neurons. After recording auditory activity successfully, the position of the multielectrode within the brain tissues was marked by passing high continuous current (100 µA) for 5 minutes between one of the tungsten wires and the reference wire or between two tungsten wires. This procedure coagulates the brain tissue and generates a black spot in the immediate vicinity of the electrode as shown in figure 4.13 B highlighted with a red arrow.

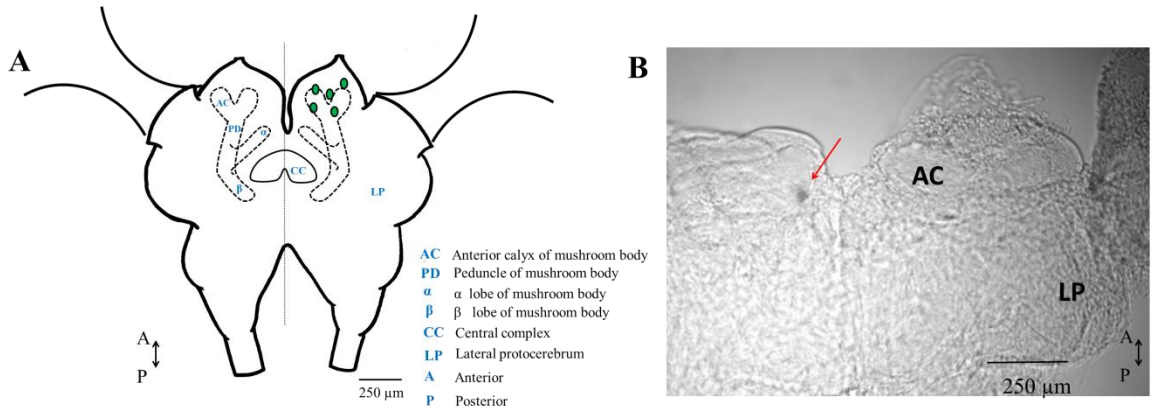

Figure 4.13 **Marking of the recording location. (**A) Sketch of a grasshopper brain with recording locations in the anterior brain shown using green dots. (B) Marking of the recording location using electrocoagulation. Location is highlighted by a red arrow

4.3.4 Local brain neuron showing selectivity between attractive and non-attractive stimuli

Clemens et al. (2014) described how the decisions of female *Ch. biguttulus* grasshoppers to reply to a male grasshopper song change when provided with attractive and non-attractive grasshopper songs *(Chapter 4: Materials and Method)*. In grasshoppers, the auditory information is preprocessed in the metathoracic ganglion before being sent to the brain (Stumpner 1988) where the available information is integrated and decision to generate behavioral response is made. Since the local brain neurons are at higher stage, it is expected that some of the local brain neurons are directly involved in the decision making process. To make the neural implementation of these behavioral experiments (Clemens et al. 2014), I used the same stimuli to see if there is any local brain neuron which shows selectivity between such attractive and non-attractive stimuli and I found one local brain neuron being selective between attractive and non-attractive stimuli as shown in figure 4.14. As a control, these stimuli were also tested on the ascending neurons (recorded in deutocerebrum) and they did not show any discrimination between attractive and non-attractive stimuli (Fig.4.14). Such filtering local brain neuron was recorded a total of four times and data were evaluated from three preparations due to better signal quality. Figure 4.16 shows the responses of this local brain neuron to combinations of different attractive and non-attractive stimuli. It can be seen that this neuron is clearly selective between attractive and non-attractive stimuli irrespective of the number and position of the attractive syllables. Figure 4.17 shows the responses of one sorted ascending neuron to different combinations of attractive and non-attractive stimuli. This ascending neuron was chosen from the several units in the recording to show clear response differences between local brain neuron and ascending neurons for the attractive and non-attractive stimuli. Ascending neuron did not show any discrimination between attractive and non-attractive stimuli. After a successful recording, the location of the recording of this local brain neuron was marked using electrocoagulation and the recording location is identified close to the α lobe of mushroom body as shown in figure 4.15.

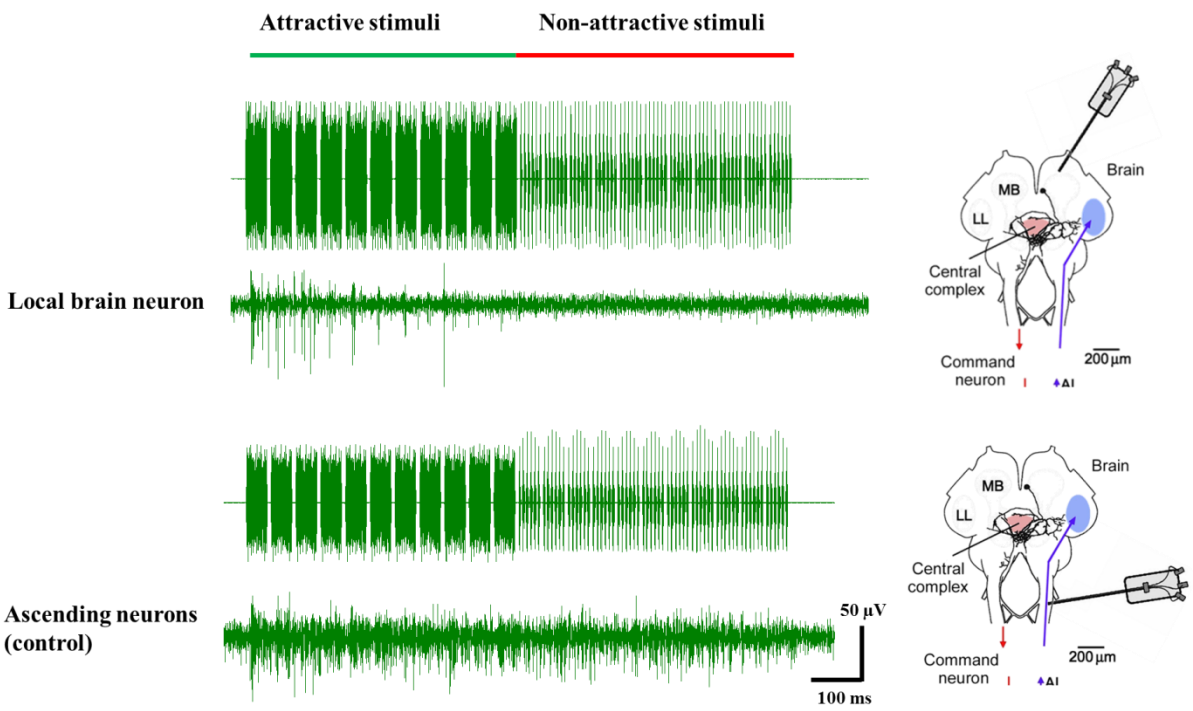

Figure 4.14 **Local brain neuron showing selectivity between attractive and non-attractive stimuli.** Recording trace of a local auditory brain neuron showing selectivity between 10 attractive and non-attractive syllables (above). Recording trace of the response of ascending neurons to 10 attractive and non-attractive stimuli as a control (below). On the right is the recording location of the local auditory brain neuron (above) and ascending neurons (below) in the brain of *Ch. biguttulus*.

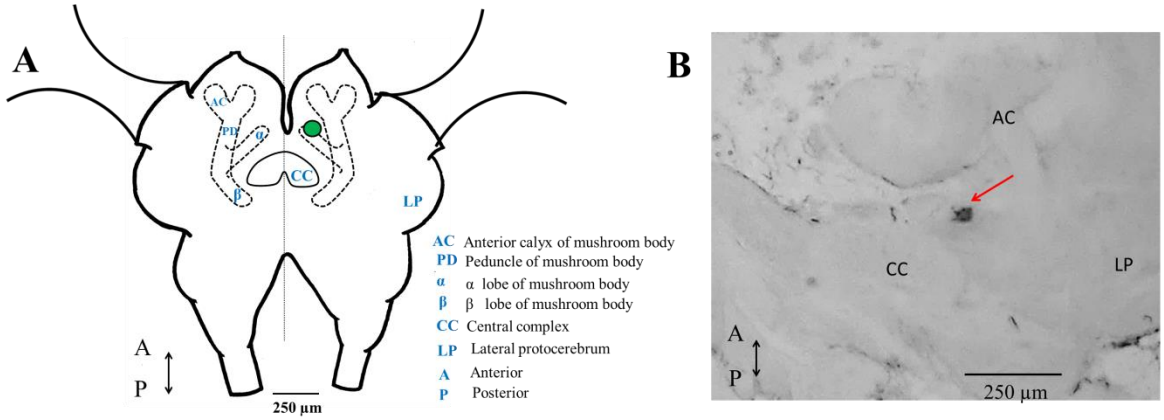

Figure 4.15 **Marking of the recording location. (**A) Sketch of a grasshopper brain with recording location of local brain neuron close to the α lobe of mushroom body shown as green dots (B) Marking of the recording location using electrocoagulation. Location is highlighted by a red arrow.

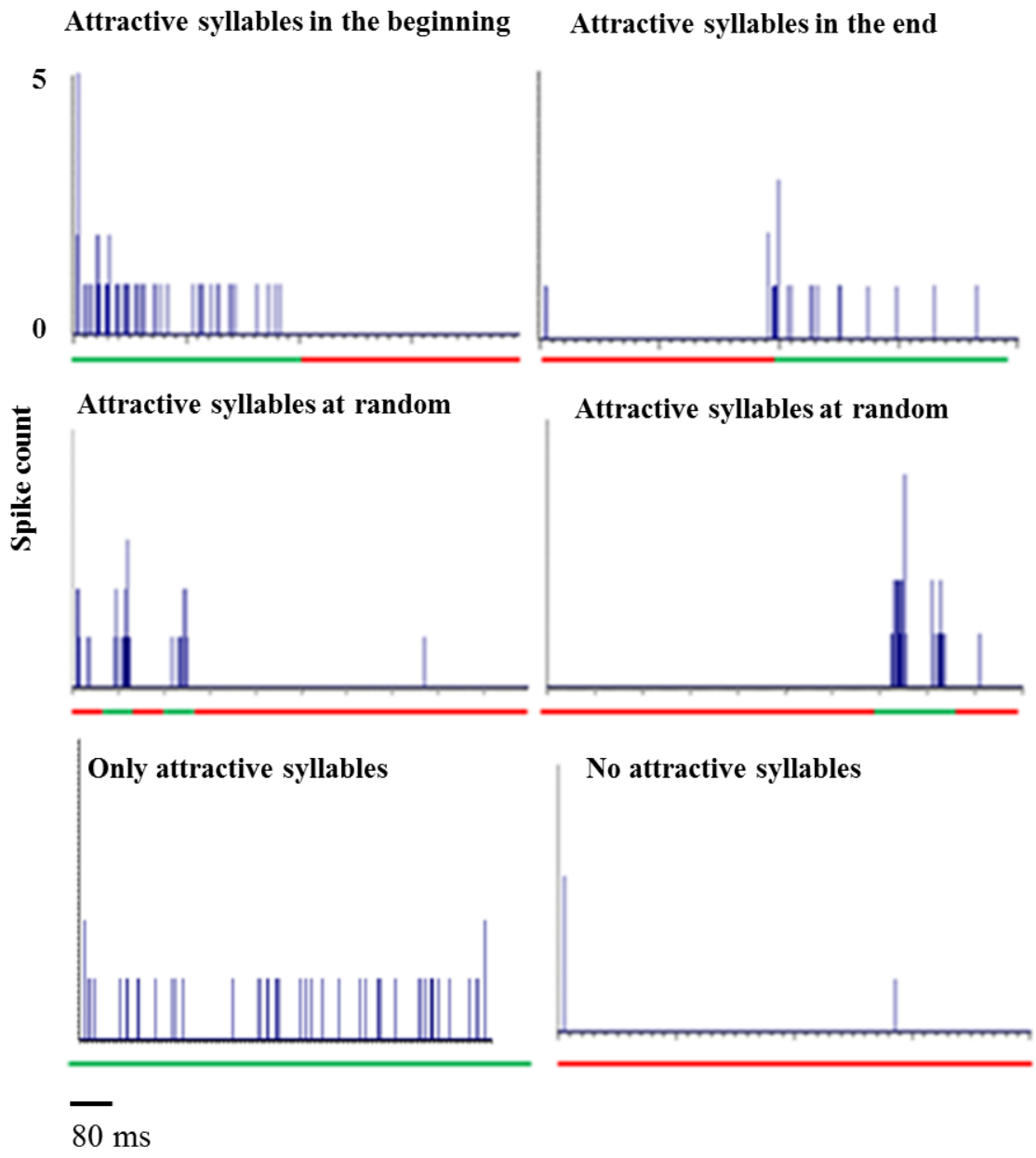

Figure 4.16 **Peristimulus time histograms**. PSTHs showing responses of one of the four times recorded local brain neuron to different combinations of attractive and non-attractive stimuli. Green and red line below the PSTH indicates attractive and non-attractive stimuli repeated 10 times respectively. (bin size: 2ms)

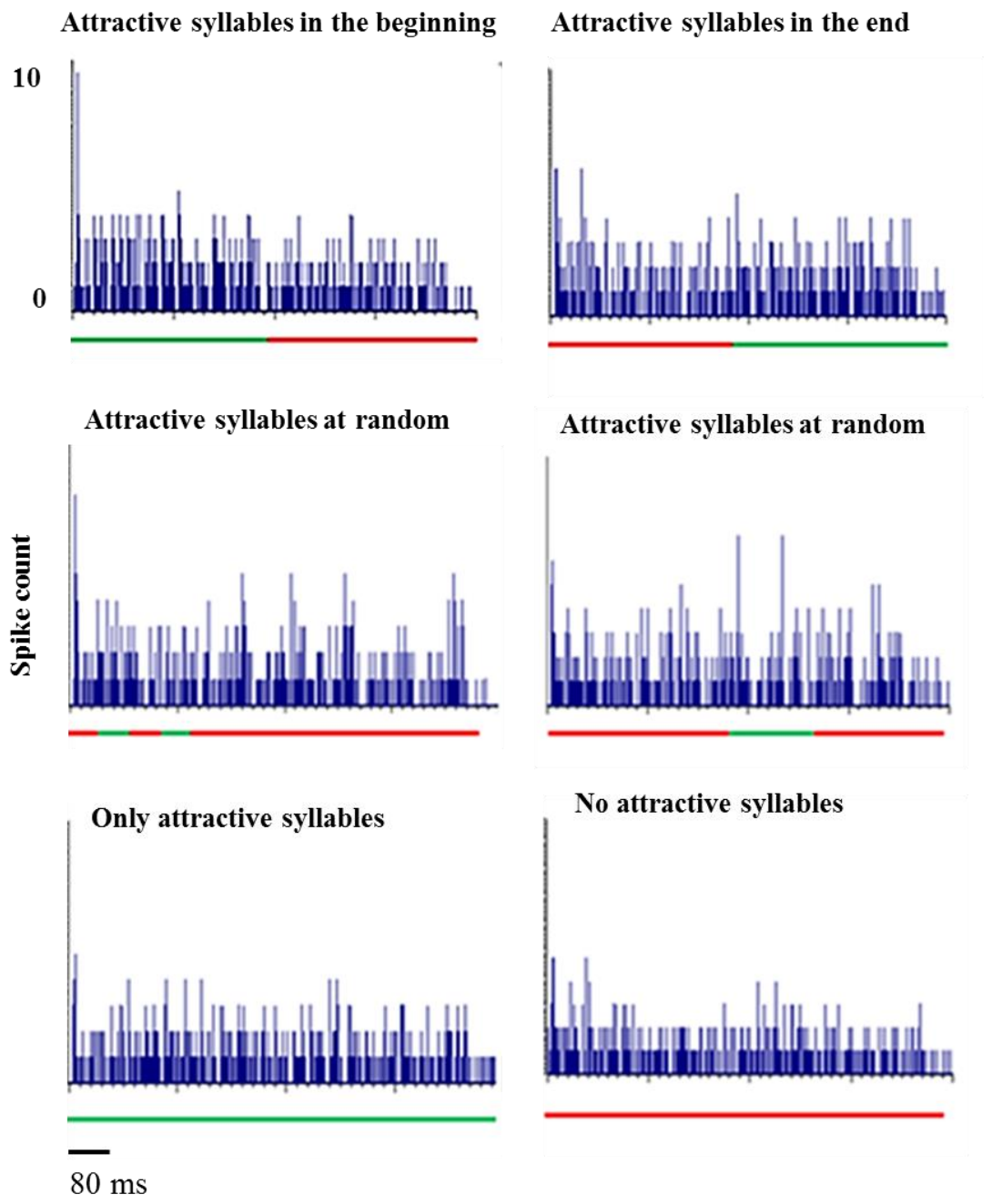

Figure 4.17 **Peristimulus time histograms.** PSTHs showing the responses of one sorted ascending neuron (control) to different combinations of attractive and non-attractive stimuli. Green and red line below the PSTH indicates attractive and non-attractive stimuli repeated 10 times respectively (bin size: 2ms)

The neuronal responses of this local brain neuron were compared with the behavioral data of female *Ch. biguttulus* (Clemens et al. 2014) as shown in figure 4.18. The mean spike count was obtained from three recordings. Local brain neuron was active when the behavioral response was above 50% and vice versa (Fig.4.18 left) while the activity of ascending neuron did not show such dependence on the behavioral response (Fig.4.18 right).

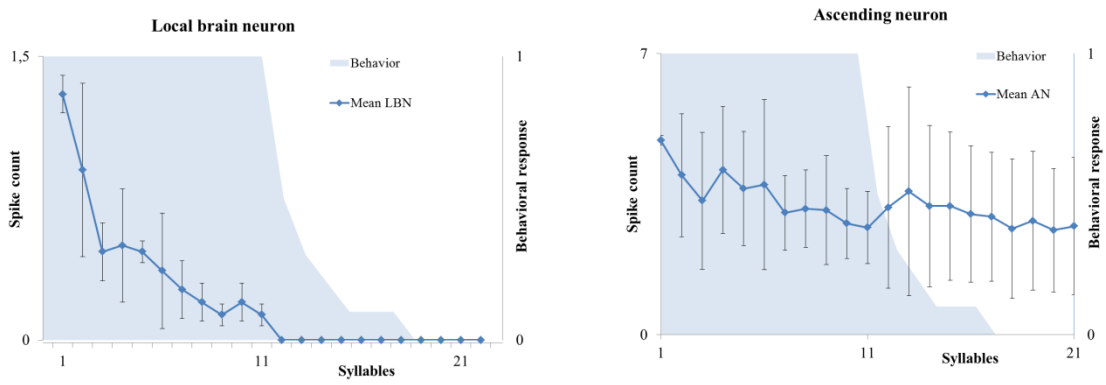

Figure 4.18 **Response tuning of a local brain neuron and ascending neuron.** Left plot shows mean response of local brain (n=3) neuron along with the behavioral data of female *Ch.biguttulus* described in Clemens et. al (2014). Right plot shows the mean response of one sorted ascending neuron (n=2) along with the behavior data described in Clemens et.al (2014). Average numbers of spikes are plotted against the attractive and non-attractive syllables from 10 repetitions.

Additionally, gap tuning was checked for this local brain neuron (n=3) and compared with the behavior data of female *Ch. biguttulus* obtained and averaged from Ronacher and Stumpner (1988). This unit showed decrease in spike count with increasing duration of gaps in syllables (Fig.4.19). However, this decrease in the spike count with the gap duration was not as strong as in the ascending neuron (AN4).

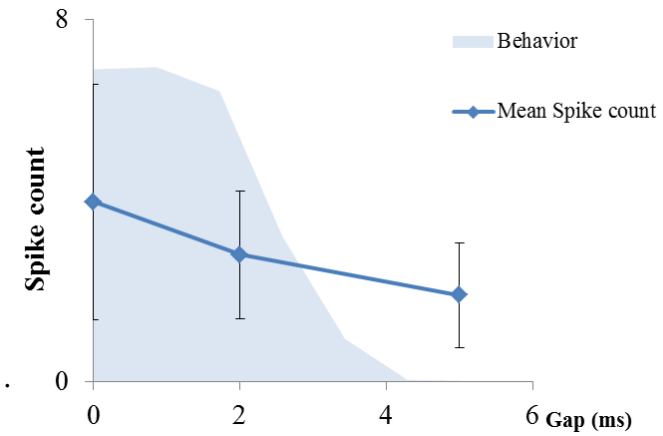

Figure 4.19 **Gap tuning**. Plot shows the neuronal responses of a sorted local brain unit to male grasshopper model songs with 80/15 ms syllable/pause durations and variable gaps. The average number of spikes from 10 repetitions is plotted against the pause durations. Behavior data were obtained and averaged from Ronacher and Stumpner (1988).

This local brain neuron was recorded four times and data were evaluated from three preparations due to better signal quality. Figure 4.20 shows intensity response curves of the local brain neuron. The local brain neuron showed an increase in the neuronal response with increase in the sound intensities to 5 kHz, 20 kHz and white noise stimuli.

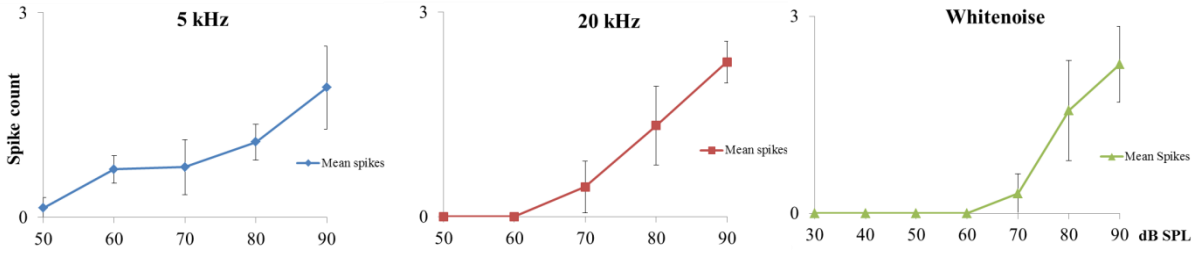

Figure 4.20 **Intensity response curves.** Plots show the intensity dependence of the local brain neuron for 5 kHz, 20 kHz and white noise stimuli. 5 kHz sine wave (25 ms) and 20 kHz sine wave (25 ms) stimuli were presented between 50 and 90 dB SPL, increasing in 10 dB steps from the ipsilateral side and broadband white noise (100 ms) stimuli were delivered between 30 and 90 dB SPL, increasing in 10 dB steps from the ipsilateral side

Additionally, syllable-pause tuning was checked for this local brain neuron and compared with the behavior data of female *Ch. biguttulus* obtained from von Helversen (1972). Dependence of the neuronal activity on different pause durations was seen but a clear match between the neuronal and the behavioral data was not found in any of the syllable-pause patterns as shown in figure 4.21.

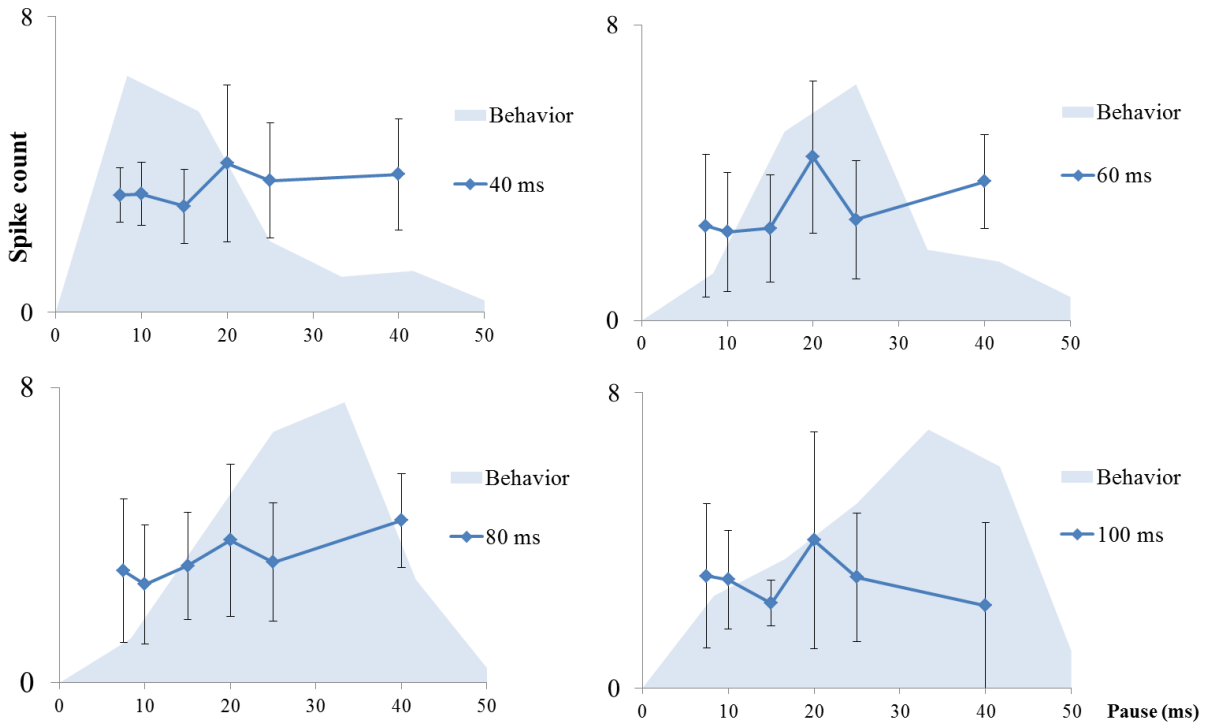

Figure 4.21 **Syllable pause tuning**. Plots show the neuronal responses of one sorted local brain unit to different male grasshopper model songs along with behavior data. The average number of spikes from 10 repetitions is plotted against the pause durations for four different syllables. Behavior data were obtained from von Helversen (1972).

4.3.5 Local brain neurons recorded in the central complex and their exact locations

Local brain neurons were recorded from the central complex in 23 preparations and data were evaluated in 11 preparations due to a better quality of the recording. A total of 13 local auditory brain units were sorted from these preparations and their intensity response curves were plotted as shown in figure 4.22. Seven out of 13 units (Fig.4.22 – Unit 2, 3, 5, 7, 8, 10, and 13) showed an increase in response with increase in intensities for 5 kHz stimuli. Six units (Fig.4.22 – Unit 1, 2, 4, 6, 11 and 12) showed an optimum type of response at 20 kHz stimuli. Unit 9 showed decrease in response at high intensities for 20 kHz stimuli. Ipsilateral and contralateral white noise stimuli were successfully tested in eight preparations out of 12 so only those data are shown in figure 4.22. Unit 6 showed higher response at intermediate intensities from ipsilateral side in comparison to contra lateral side and at higher intensities it reverses. This could be dues to threshold shift between left and right.

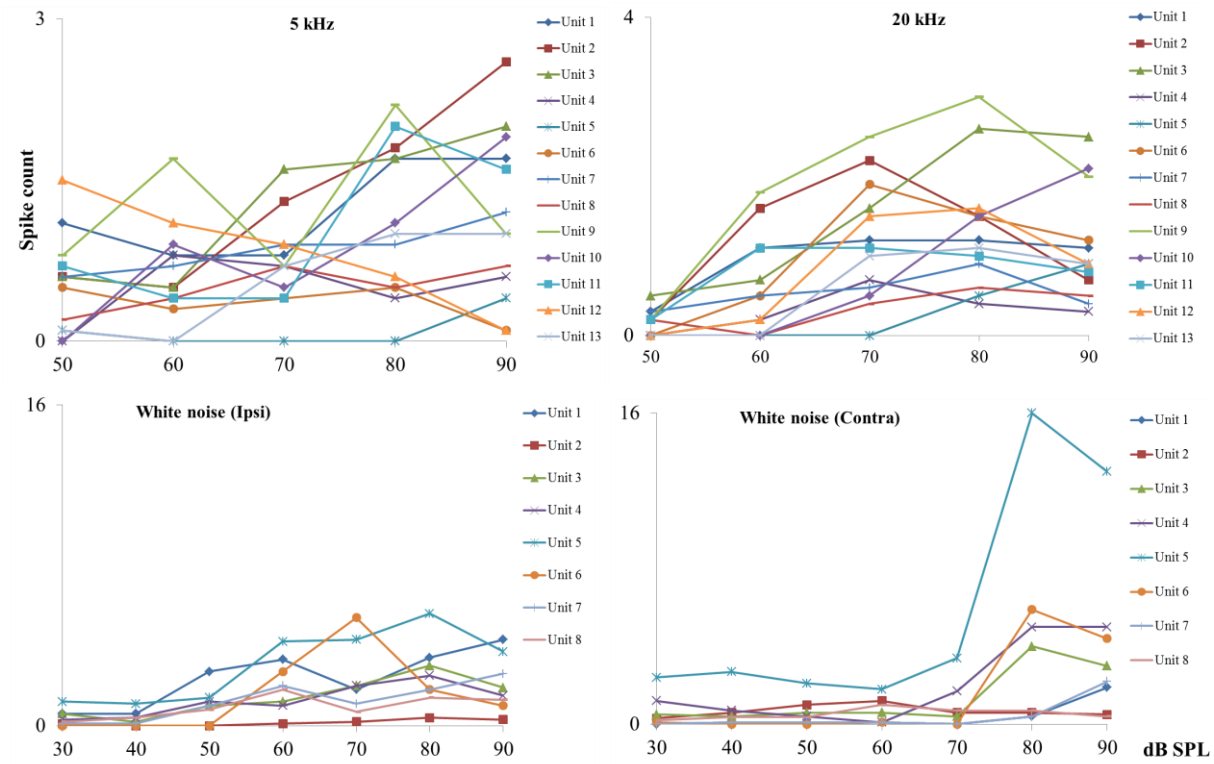

Figure 4.22 **Intensity response curves.** Plots show the intensity dependence of all sorted local brain units recorded from central complex for 5 kHz, 20 kHz and white noise stimuli. 5 kHz sine wave (25 ms) and 20 kHz sine wave (25 ms) stimuli were presented between 50 and 90 dB SPL, increasing in 10 dB steps from the ipsilateral side and broadband white noise (100 ms) stimuli were delivered between 30 and 90 dB SPL, increasing in 10 dB steps from the ipsilateral and contralateral side.

Out of eight sorted units, two units (Fig.4.22 - Unit 1 and Unit 5) showed strong directional dependence in the response. The response of Unit 1 was clearly higher to ipsilateral side than to contralateral side as shown in figure 4.23 (left). The response of Unit 5 was clearly higher to contralateral side than to ipsilateral side at high intensities as shown in figure 4.23 (left).

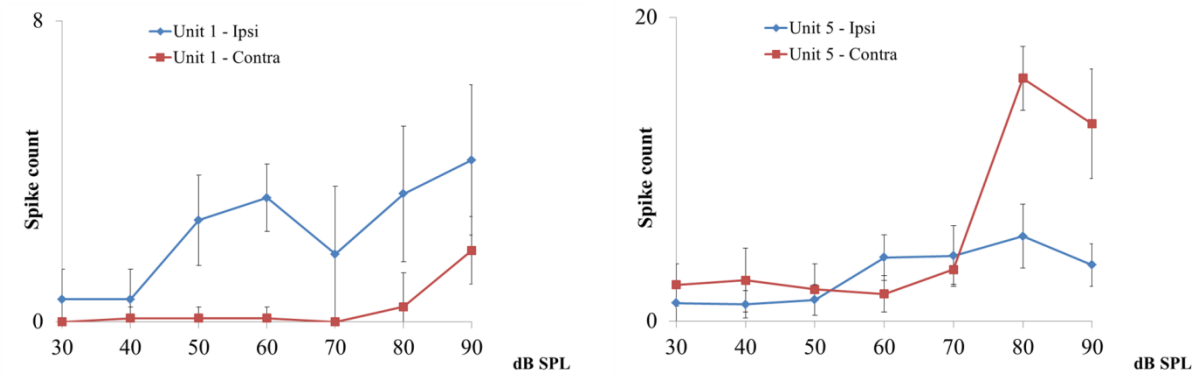

Figure 4.23 **Intensity response curves.** Left plot shows the directionality dependence of one sorted local brain unit recorded from central complex (Fig.4.22 - Unit 1) for broadband white noise (100 ms).Right plot shows the directionality dependence of one sorted local brain unit recorded from central complex (Fig.4.22 - Unit 5) for broadband white noise (100 ms).

Syllable-pause tuning was checked in nine preparations and the neuronal response of 11 units recorded from the central complex was compared with the behavior data of female *Ch. biguttulus* obtained from von Helversen (1972). Dependence of the neuronal activity on different pause durations was seen but a clear match between the neuronal and the behavioral data was not seen in any case as shown in figure 4.24.

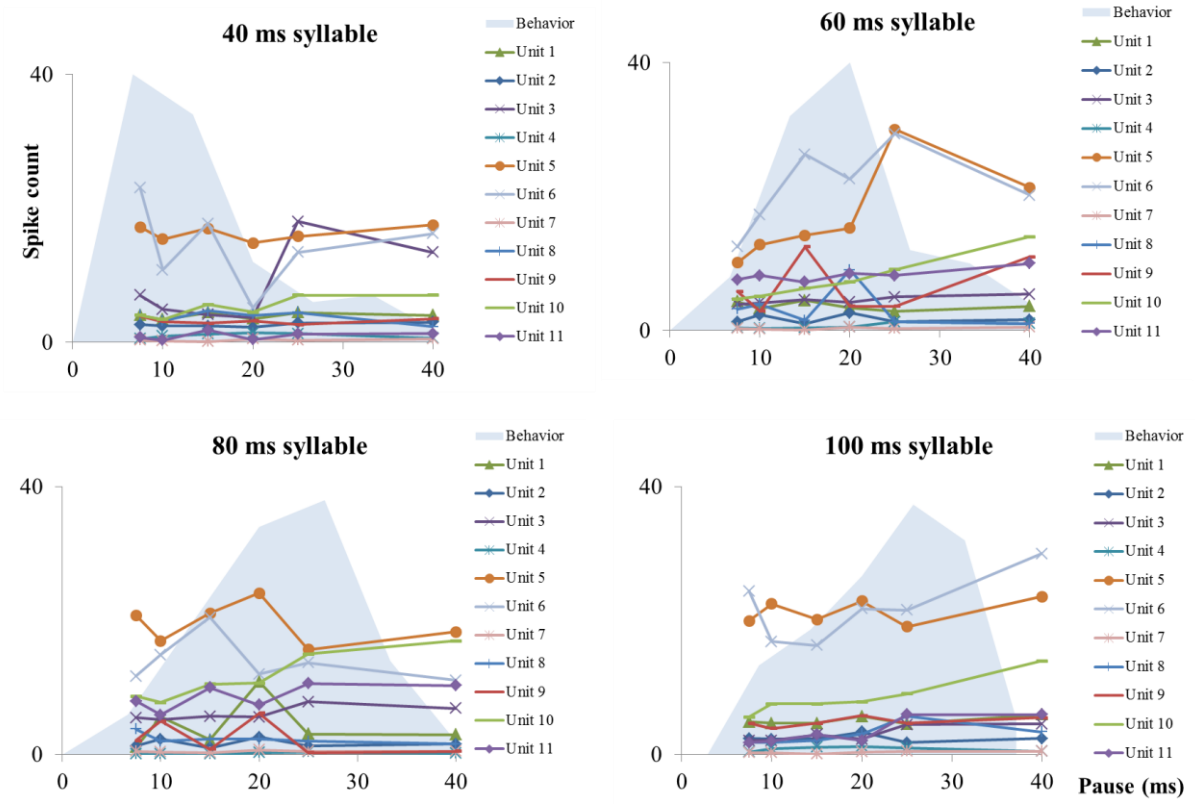

Figure 4.24 **Syllable pause tuning**. Plots show the neuronal responses of sorted local brain units recorded from central complex to different male grasshopper model songs along with the behavioral data. The average number of spikes from 10 repetitions is plotted against the pause durations for four different syllables. Behavior data obtained from von Helversen (1972).

Among all the units shown in figure 4.24, one unit (Unit 10) responded with a phasic burst at the onset of syllables, provided that the pauses between syllables exceed a certain duration as shown in figure 4.25 A. In order to quantify the onset response, average number of spikes per syllable crossing the horizontal threshold (mean+/- 3*SD) was calculated from the PSTHs and plotted against the pause duration which is shown in figure 4.25 B.

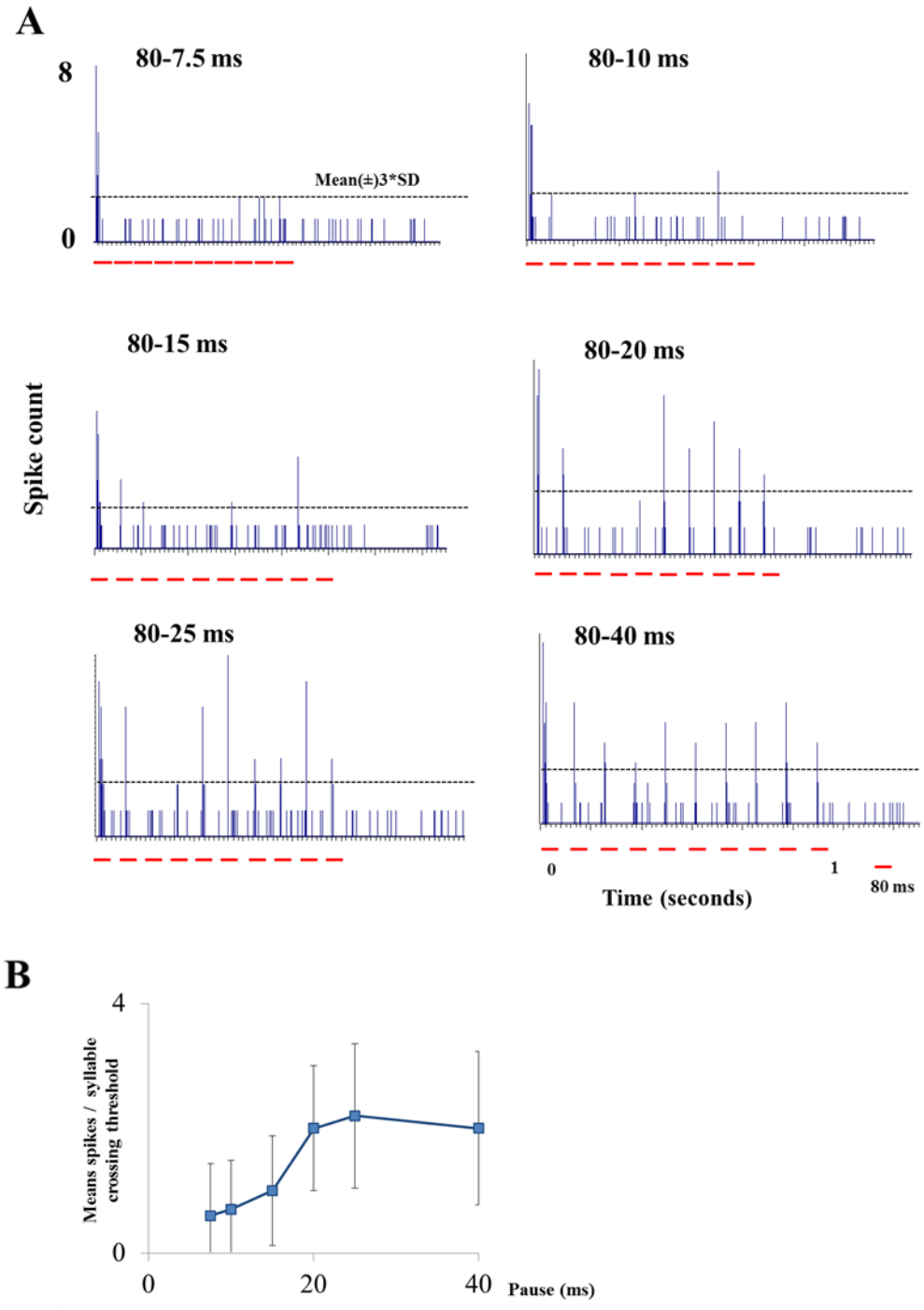

Figure 4.25 **Marking onset of syllable.** (A) Plots show PSTHs of one of the sorted local brain neurons recorded from central complex (Fig.4.24 - Unit 12) for grasshopper model songs with 80 ms syllable and six different pause durations (repetitions = 10, bin size = 2ms) (B) Spike response to the onset of the syllable quantified by calculating the mean spikes per syllable crossing the threshold (mean+/- 3*SD) in the PSTH marked as a horizontal black line.

Figure 4.26 shows syllable-pause tuning of the unit described in figure 4.25 for all the syllable-pause durations. The mean spike count was plotted against different pause durations. Higher number of spikes at longer syllables and an increase in spike count with an increase in pause duration at all syllable durations was seen. However, not a clear match between the behavioral and the neuronal data was found (Fig.4.26).

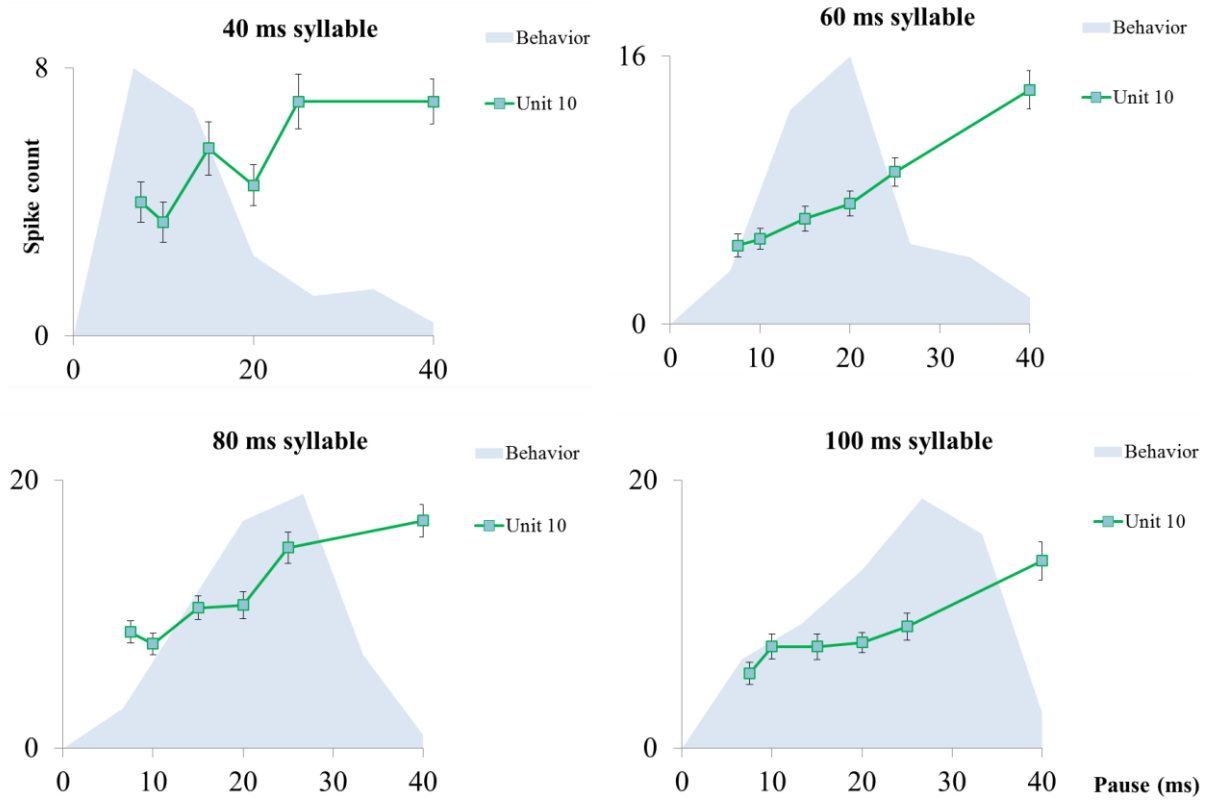

Figure 4.26 **Syllable pause tuning**. Plots show the neuronal response of a sorted local brain unit to different male grasshopper model songs along with the behavioral data. The average number of spikes from 10 repetitions is plotted against the pause durations for four different syllables. Behavioral data were obtained from von Helversen (1972).

Additionally, gap tuning was checked for all the sorted units and compared with the behavior data of female *Ch. biguttulus* obtained and averaged from (Ronacher and Stumpner 1988). Syllables with and without gaps were successfully tested in seven preparations. Figure 4.27 shows gap tuning of nine units sorted in seven preparations. Three out of nine units (Unit 1, 2, 5) showed increase in neuronal response with gap duration and three units (Unit 3, 4, 9) showed decrease in in neuronal

response with gap duration. However, this dependence on gap duration was not so strong as described for certain ascending neurons like AN12 and AN4 (Stumpner 1988).

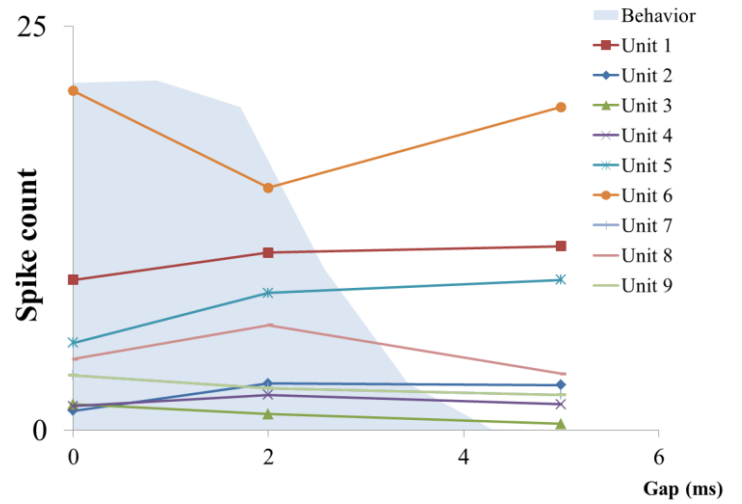

Figure 4.27 **Gap tuning**. Plots show the neuronal responses of sorted local brain units recorded central complex to male grasshopper model songs with 80/15 ms syllable/pause durations and variable gaps. The average number of spikes from 10 repetitions is plotted against the pause durations. Behavior data were obtained and averaged from Ronacher and Stumpner (1988).

Figure 4.28 A provides a summary of locations in the central complex where auditory activity was recorded from the local brain neurons. After recording auditory activity successfully, the positions of the multielectrode within the brain tissues were marked by passing high continuous current (100 µA) for 5 minutes between one of the tungsten wires and the reference wire or between two tungsten wires. This procedure coagulates the brain tissue and generates a black spot in the immediate vicinity of the electrode as shown in figure 4.28 B highlighted with a red arrow.

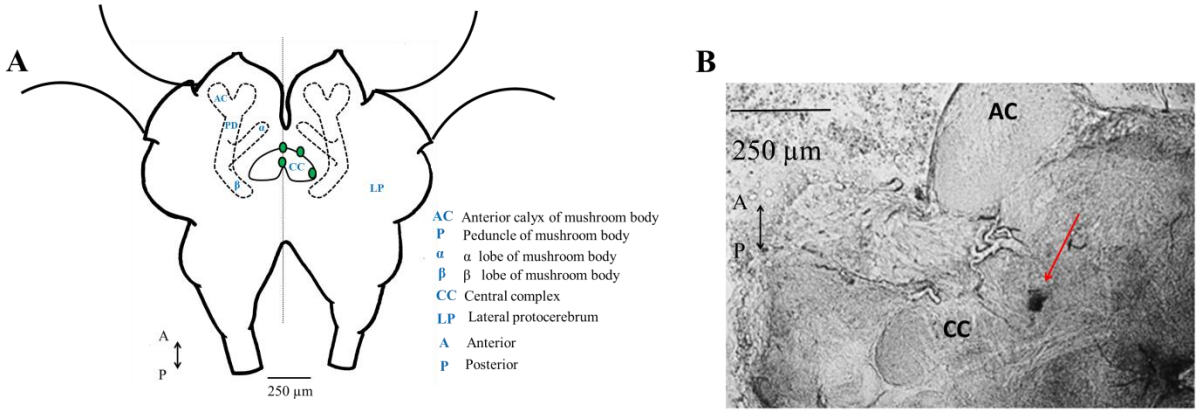

Figure 4.28 **Marking of the recording location. (**A) Sketch of a grasshopper brain with recording locations in the central complex (CC) shown as green dots. (B) Marking of the recording location using electrocoagulation. Location is highlighted by a red arrow.

4.3.6 Electrical stimulation in the auditory neuropile

After a successful recording of auditory activity, extracellular electrical stimulation in the recording site was tried in 25 female *Ch. biguttulus* and it was successful in 21females. At the beginning, different current amplitudes were tested to find out the minimal current required eliciting stridulation and it was around 50 µA as shown in figure 4.29. The electrical stimulation was tried in four different regions of the brain where auditory activity can be recorded, namely posterior brain (deuto/tritocerebrum), lateral protocerebrum, mushroom body (anterior calyx and α lobe) and central complex. Stridulation can be elicited in all regions except for the mushroom body.

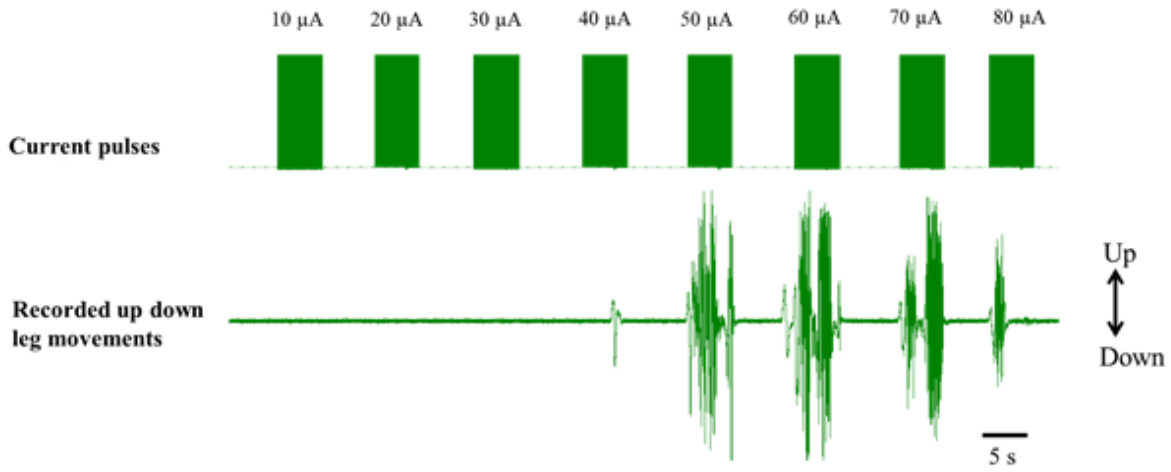

Figure 4.29 **Electrical stimulation in one auditory neuropile**. Upper trace shows the increasing intensity of current pulses and the lower trace shows recording of leg movement using a position detector. The interval between two stimuli is around 5 s. Current was injected for at least 5 s at pulsed at 166 Hz. A position detector was used to detect the leg movements of the animal. The stimulation was done in the posterior brain (deuto/tritocerebrum).

4.3.7 Female *Chorthippus biguttulus* song structure

Figure 4.30 shows a typical example of electrically stimulated stridulation in a *Ch. biguttulus* female. Stimulation of descending pathways in the tritocerebrum instantly induces stridulatory hind leg movements. As it is typical for natural songs of this species, stridulatory activity is structured into song sequences of approximately 2-3 s duration separated by pauses of approximately 1s, that consist of repetitive syllables (70-120 ms duration), each containing two or three up-and-down movements of the hind leg. Continuous electrical stimulation for 10 s elicited four song sequences, with the first sequence being of longer duration than the following ones, which is also typically observed in naturally singing *Ch. biguttulus*.

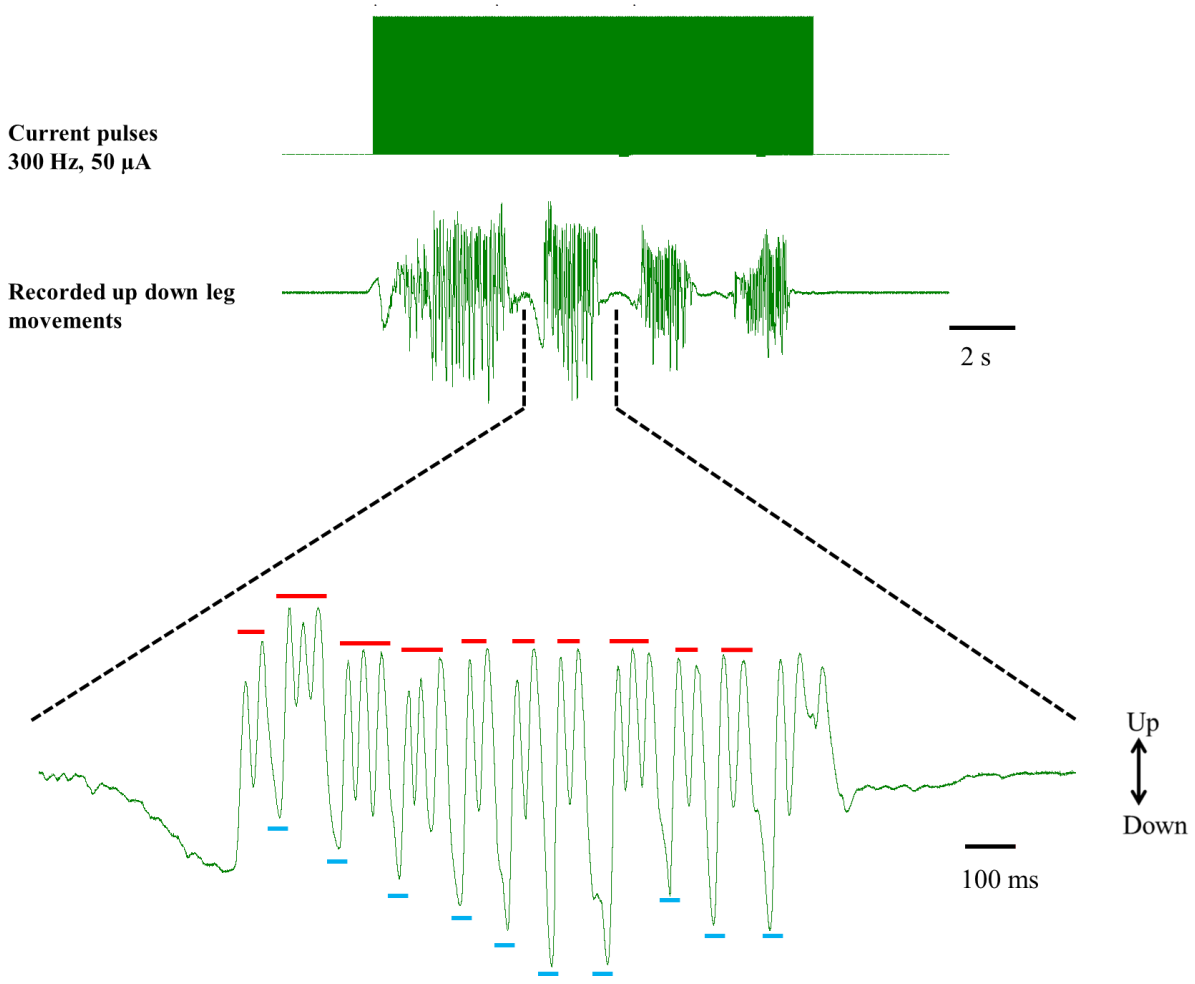

Figure 4.30 **Structure of a female grasshopper song elicited by electrical stimulation**. In the magnified version below typical syllable-pause pattern can be seen. Here syllable is defined as the train of impulses produced by one femoral upward or downward movement (Elsner 1974). The stimulation was done in the posterior brain (deuto/tritocerebrum). Syllables are marked using red lines and pauses are marked with sky blue points. Each syllable is between 70-120 ms long while each pause is around 30-40 ms long.

Additionally, sound produced by the leg movements was recorded using a microphone placed near the left leg of the animal. The sound of the leg movements were recorded as pulsed and irregular syllables by the microphone. The pulses can be grouped into syllables which are defined by the syllable pauses. The frequency spectrum was made from the recorded sound and most of the components were found in low-frequency region between 1 to 5 kHz as shown in figure 4.31.

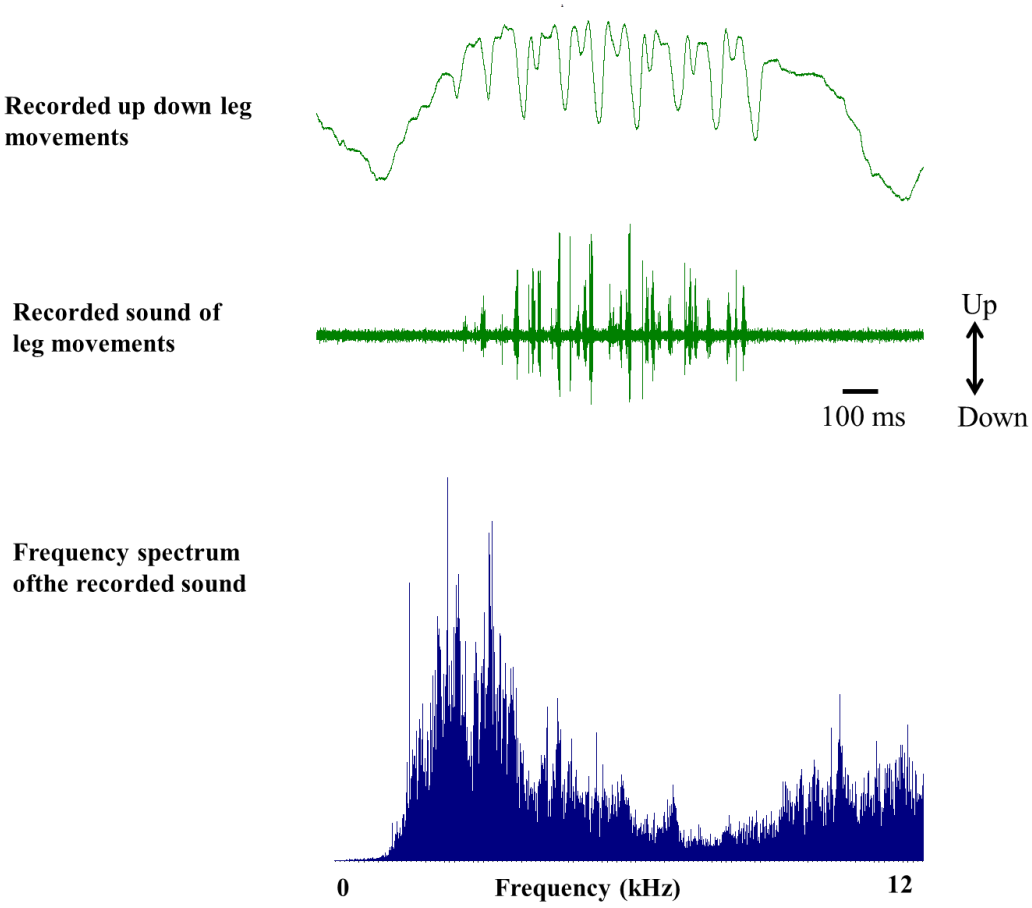

Figure 4.31 **Recording sounds of leg movements.** The sound was recorded from the movements of the left leg using a microphone placed near the leg. Frequency spectrum was made using the recorded sound of the leg movements.

4.3.8 Differences in the singing while stimulating different sites

The electrical stimulation was successfully tried on three different sites in the brain namely the posterior brain (deuto/tritocerebrum), the lateral protocerebrum and the central complex. When stimulated in the deuto/tritocerebrum, grasshoppers continuously generated song sequences throughout the entire stimulation time of 10 s (Fig.4.32). This led to the performance of 4-6 song sequences per stimulus. In contrast, stridulatory activity during electrical stimulation in the lateral protocerebrum and the central complex stopped after 4-6 s of the 10 s stimuli and, in comparison to stimulation in the tritocerebrum, lower numbers of song sequences were generated in all experiments.

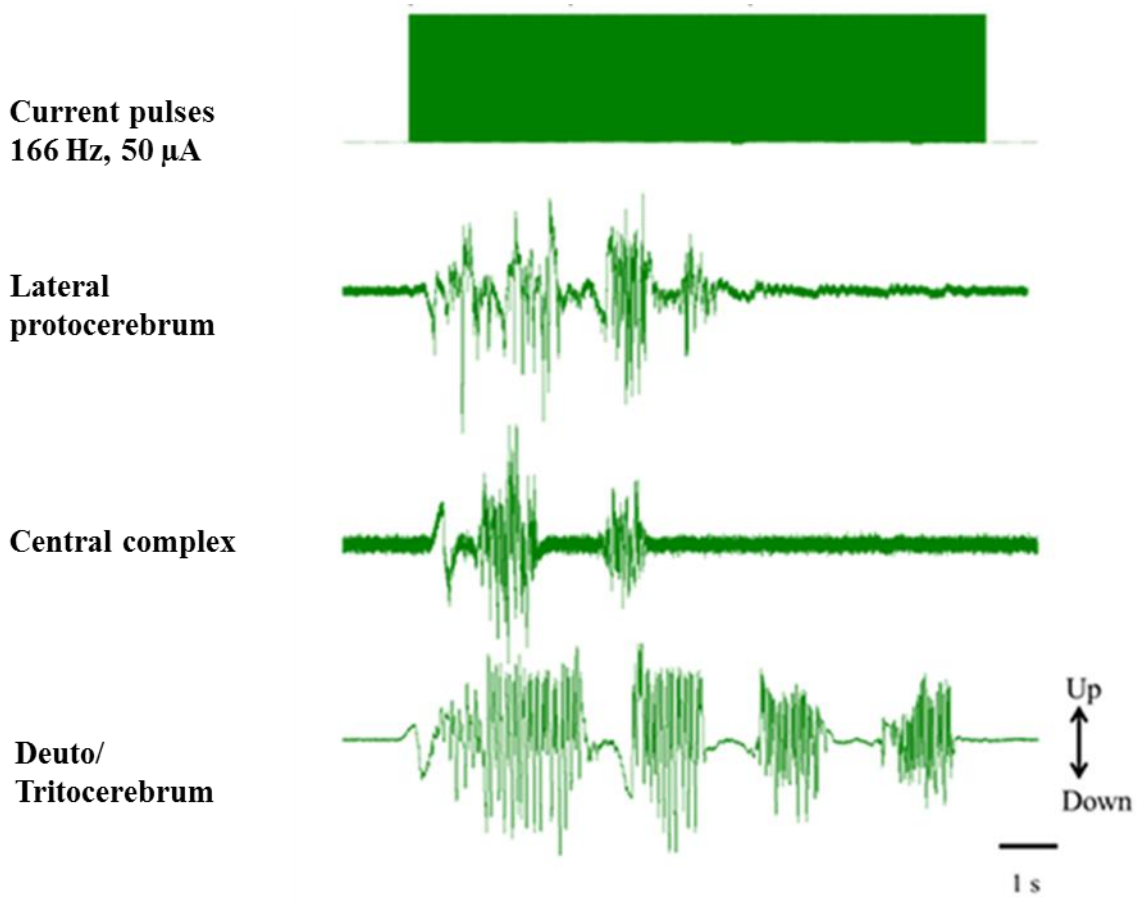

Figure 4.32 **Electrical stimulation of different auditory neuropiles**.50 µA current pulses were injected at 166 Hz in three different brain regions. A position detector was used to detect the leg movements of the animal

4.3.9 Locations of the stimulation sites

Figure 4.33 provides a summary of locations where auditory activity was recorded and stridulation could be induced by current injection. Stridulation could be successfully induced from the auditory neuropiles in the deuto/tritocerebrum, the lateral protocerebrum and the central complex. Stridulation could not be induced from the auditory neuropiles located in the anterior protocerebrum.

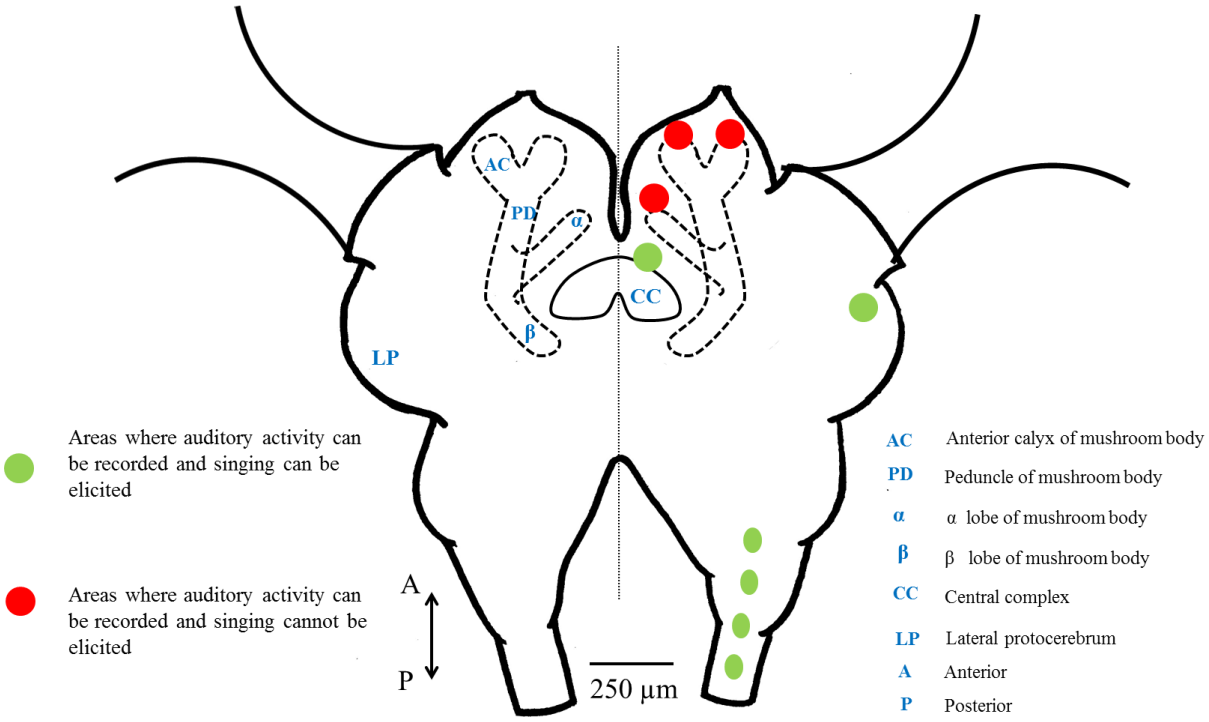

Figure 4.33 **Sketch showing locations of the stimulation sites in a grasshopper brain**. Area of the mushroom body (shown as dotted lines) was not seen in the original electrocoagulation sections and so it is approximated by comparing the sections with immunofluorescence sections. Exact locations may be above or below the α lobe of the mushroom body

4.4 Discussion

The key to understanding the neural basis of recognition is the identification of mechanisms that cause "higher order" neurons to selectively respond to the same stimuli that trigger specific behavior (Schöneich et al. 2015). In *Ch. biguttulus* there is not much information available about the locations and functionality of such "high order" local auditory brain neurons. Here I demonstrated different locations in the brain of the female *Ch. biguttulus* from where the local auditory brain neurons can be recorded and stridulation can be elicited using extracellular electrical stimulation.

4.4.1 Recording auditory activity in grasshoppers

Most described ascending neurons originate from the metathoracic ganglion, enter soma contralateral hemisphere of the brain and terminate in the lateral protocerebrum (Eichendorf and Kalmring 1980; Stumpner and Ronacher 1991; Kutzki 2012). The local brain neurons were mainly recorded from regions like lateral protocerebrum, anterior protocerebrum, and central complex. Additionally, the local brain neurons were separated from ascending neurons based on their longer first spike latencies. One important point of emphasis here is that the signal-to-noise ratio of recording from local brain neurons was lower in comparison to that of ascending neurons. A likely reason for that is that multielectrodes tend to record better signals from larger axons of neurons than from smaller neurites. The local brain neurons were recorded probably from smaller axons (or neurites) in the auditory neuropiles leads to the smaller signal-to-noise ratio.

4.4.2 Local brain neurons recorded in the lateral protocerebrum and in the central complex

Since it is known that most of the ascending neurons terminate in the lateral protocerebrum (Eichendorf and Kalmring 1980; Boyan et al. 1993; Kutzki 2012) , the local brain neurons recorded in this region are likely to be directly postsynaptically connected to the ascending neurons. Such local brain neurons are also previously described by Kutzki 2012. Among all, there was one unit clearly responding with a phasic burst at the onset of syllables, provided that the pauses between syllables exceed a certain length. Such a local brain neuron marking onset of the syllable at longer pauses was also recorded from the central complex. Among all described ascending neurons, AN12 is an

ascending neuron with a strong phasic response produced at the onset of the syllable provided that the pauses between syllables exceed a certain length within a model song (Stumpner 1988; Stumpner and Ronacher 1991) so it is likely that these local brain neurons are directly postsynaptically connected to AN12.

Syllable-pause and gap tuning of the recorded local brain neurons were tested. There was some dependence found on pause and gap durations but no clear match between the behavioral and the neuronal data was seen. One reason is that many of the local brain neurons recorded were highly adapting to the stimulus and not showing any syllable pause tuning. Since in the recordings of such multielectrode spikes from larger axons are more likely to be represented in the recording, one may systematically miss spikes from neurons with smaller branches. Other problems like the sudden spontaneous firing of the recorded units and errors in clearly discriminating the units during the spike sorting can affect the spike count analysis.

4.4.3 A local brain neuron showing selectivity between attractive and non-attractive stimuli

Decision making is mainly a two stage process. First, the task-relevant information is extracted from the input and then the extracted information is integrated to make a decision. Basically in neural systems performing a decision-making task, stimulus cues are extracted by sensory neurons, integrated over some time and combined to inform a motor action (Clemens and Ronacher 2013). This suggests the presence of feature detector and feature integrator neurons in the central nervous system (Clemens et al. 2014). Feature detector neurons are responsible for extracting the information while feature integrator neurons are responsible for combining the extracted information to generate a decision signal. In grasshopper auditory systems some neurons located in the metathoracic ganglion are described as a feature detector like AN4 which is selectively and strongly inhibited by interrupted sound pulses (syllables with shorter or longer gaps). Males with one hindleg produce gaps but these are relatively short and longer gaps may come from males with poor immunocompetence (Ronacher and Stumpner 1988). In grasshoppers auditory systems, the features of a song are detected at the metathoracic ganglion and all these features are integrated in the brain to generate a decision (Stumpner and Ronacher 1994). Since the local auditory neurons are located in the brain, some of

them are expected to be directly involved in the decision-making process. I found one local brain neuron close to the α lobe of mushroom body clearly discriminating between attractive stimuli (high frequency components without any gaps) and non-attractive stimuli (contains low frequency components with gaps in the syllable) and showing a match with the behavior of female *Ch. biguttulus* described in Clemens et al. (2014). Additionally, this neuron showed dependence for syllable-gap patterns and so it is likely that this local brain neuron is getting also input from AN4. However, this neuron did not show clear syllable-pause tuning. This suggests that this local brain neuron relied only on the features like frequency and gap durations of the song and therefore it cannot be the grand "integrator neuron", assuming the presence of an integrator neuron in the brain.

4.4.4 Electrical stimulation of auditory neuropiles

In grasshoppers, the neuronal network that suffices to generate the species-specific excitation pattern is located in the metathoracic ganglion and is controlled by command neurons descending from the brain (Hedwig 1994; Hedwig and Heinrich 1997). Extracellular electrical stimulation of the brain generating specific motoric behavior has been previously described by Wadepuhl (1983) in the grasshopper *Gomphocerus rufus*. He explored the influence of the brain on the acoustic behavior of the grasshopper by means of local electrical stimulation using sharpened metal wires. He successfully induced stridulation by stimulating auditory neuropiles in the central complex and mushroom body regions in the brain of grasshopper *Gomphocerus rufus*. Here I used multielectrodes and successfully induced stridulation by stimulating higher auditory neuropiles (lateral protocerebrum and central complex) and the command systems (deuto/tritocerebrum) in the grasshopper *Ch.biguttulus*. One point of emphasis here is that injecting higher current (100 μA) apparently also leads to neuronal damage at the stimulation site. Additionally, using such extracellular stimulation it is also difficult to assess the effects of the current spread at the recording sites (e.g. number of neurons which are indeed depolarized at the stimulation sites).

The generated stridulatory pattern was always subdivided into song sequences separated by intervals about 1 s. Such songs are typical for *Ch. biguttulus* males and females and are also seen in pharmacological experiments done by Heinrich et al. (1998) and Weinrich et al. (2008). During

electrical stimulation in the deuto/tritocerebrum, grasshoppers persistently generated song sequences (4-6 songs) throughout the entire stimulation. This was in contrasts to stridulatory activity during stimulation in the lateral protocerebrum and the central complex where stridulatory activity stopped after 4-6 s of the 10 s stimulus. Stimulation of lateral protocerebrum or central complex suggests direct activation of local brain neurons which provides input to the descending command neurons. Such local brain neurons involved in song recognition have also been described for *Ch. biguttulus* by Hedwig (2001). He intracellularly stimulated a local brain neuron and induced stridulation with a certain delay and the stridulation continued even after stimulation was stopped. Stimulating the posterior brain (deuto/tritocerebrum) generated stridulation during the complete stimulus suggesting direct activation of descending command neurons independent of any control from local brain networks.

4.5 Outlook

The data presented in this chapter demonstrates some locations at which local auditory brain neurons can be recorded and where stridulation can be elicited. These locations could be used in the future to record and stain neurons using intracellular techniques to understand the morphology and neuronal basis of song recognition.

Chapter 5

Mini review: Multielectrode recordings in insect brains.

Mit Balvantray Bhavsar, Ralf Heinrich, Andreas Stumpner

Author contribution:

Andreas Stumpner and Ralf Heinrich designed the project

Mit Bhavsar wrote the manuscript draft with help of Andreas Stumpner

Andreas Stumpner and Ralf Heinrich corrected the manuscript

This chapter is from a review submitted in the Journal of Neuroscience communication: (Bhavsar et.al 2015 b)

Abstract

Currently, more and more laboratories are acquiring the capability of simultaneously detecting the extracellular activity of neuronal populations in anaesthetized and awake animals by multielectrode recordings. In insects, multielectrode recordings are challenging due to the small size of the nervous system. Nevertheless, multielectrode recordings have been successfully established in brains of cockroaches, honeybees, fruit flies and grasshoppers to study sensory processing related to mechanosensation, olfaction, vision and audition. The number of neurons which can be recorded using such multielectrode did not exceed 5 and likely depends on factors like recorded compartment of the neuron, impedance of the multielectrode, number of wires included in the multielectrode and threshold for spike detection. Signal-to-noise ratio (SNR) of the recordings obviously depends on the material and method used for production of multielectrodes. To mark the location of the recording, different methods like current-driven copper deposition, labelling with fluorescent dye and electrocoagulation of nervous tissue are used. As expected, multielectrode recordings are more difficult in freely moving compared to restricted insects due to movement artifacts and requirement for fixed placement of the multielectrode at a particular recording site in the CNS. Specific differences among different preparations and sensory systems like disentangling spike collisions in auditory stimulation increase in SNR after some time in olfactory systems and photoelectrical effect from compound eye in visual stimulation may require special attention and particular adaptations.

5.1 Introduction

One of the major developments in the field of neurophysiology is the use of multielectrodes (or tetrodes in case of four wires) to simultaneously monitor spiking activity of populations of neurons (Wise and Angell 1975; Recce and O'Keefe 1989) which is used to study fundamental aspects of the functional organization of the nervous system. Long term multielectrode recordings have become routine in mammalian neurophysiology (Nicolelis et al. 1993; Welsh et al. 1995) and at present a large variety of experimental conditions are applied, which include in-vitro preparations using culture or brain slices (Gross et al. 1982; Potter 2001), acute and chronic recordings in anesthetized animals (Ghazanfar and Nicolelis 1997), long term recordings in behaving animals (Laubach et al. 2000) and even short term neurophysiological monitoring in human subjects (Kreiman et al. 2000). However, in insects multielectrode recordings still remain a challenge due to the smaller size of their nervous system. Intracellular recordings with sharp electrodes are very popular in insects since they provide very detailed data on identified neurons. However, this technique is usually limited to one cell at a time (very rarely more), requires a restrained animal and can typically only be stabilized for relatively short periods of time.

Over the last decade considerable attention has been directed on how populations of neurons encode and process different aspects of a sensory stimulus in insects. So far, intracellularly recording single units in several individuals or in one individual successively is a widely used method to analyze their potential combined activity (Meckenhäuser et al. 2014; Schöneich et al. 2015). However, this method suffers from the limitations like trial-to-trial and interindividual neuronal variability (Ronacher et al. 2004). In order to analyze the activity of populations of neurons, however, it would be more appropriate to record the activity of several neurons at the same time in the same individual (Bhavsar et al. 2015a). Multiunit recordings using combined single wires (multielectrodes) can serve this purpose. They allow recording the activity of multiple neurons from a particular recording site via a bundle of 3-4 closely located insulated microwires. Association of spikes with single neurons relies on the fact that both amplitude and shape of spikes change with distance and tissue conductivity between the electrode tip and the cell (Gray et al. 1995; Harris et al. 2000). Since every cell will be at

a different distance from the different contact sites of the electrodes, it will be recorded with a different shape and amplitude at the tips of the wire of a multielectrode. Recordings with this method are typically much more stable than intracellular recordings (Bhavsar et al. 2015a) allowing for long term analysis of the recorded units. In insects, few research groups have successfully used this method for studying mechanosensory processing in cockroaches (Ritzmann et al. 2008), olfactory processing in honeybees (Brill et al. 2013, 2014; de Camp 2013; Duer et al. 2015) and locusts (Saha et al. 2013; Aldworth and Stopfer 2015), visual processing in fruit flies (Zhong et al. 2014) and auditory processing in the grasshoppers (Bhavsar et al. 2015a). Here we discuss the questions they studied in different sensory systems and adaptations they had to make to solve specific problems with using multielectrode recordings in small insects.

5.2 Type of material used for production of multielectrodes

Multielectrodes are typically made from copper, tungsten or nickel-chromium (NiCr) wires. However, signal-to-noise ratio of the recordings differs when using these different metals for multielectrodes. Signal-to-noise ratio (SNR) generally means the dimensionless ratio of signal power to noise power. It allows quantifying the size of signal relative to the fluctuations (called noise) that are outside experimental control (Schultz 2007). High SNR determines the quality of the electrophysiological recordings and the ability to extract and sort single spikes from background noise. The SNR depends on factors like impedance and diameter of the multielectrode, recorded compartment of the neurons and the distance between the neuron and the tip of the electrode. Among these, the impedance of the multielectrode is likely the most influencing factor. It has been shown in Bhavsar et al. (2015a) that for example 12 µm tungsten wires gave better SNR (> 2) than 15 µm copper wires (< 1.5) which is likely due to the lower resistance of tungsten wires (40 - 70 kΩ)

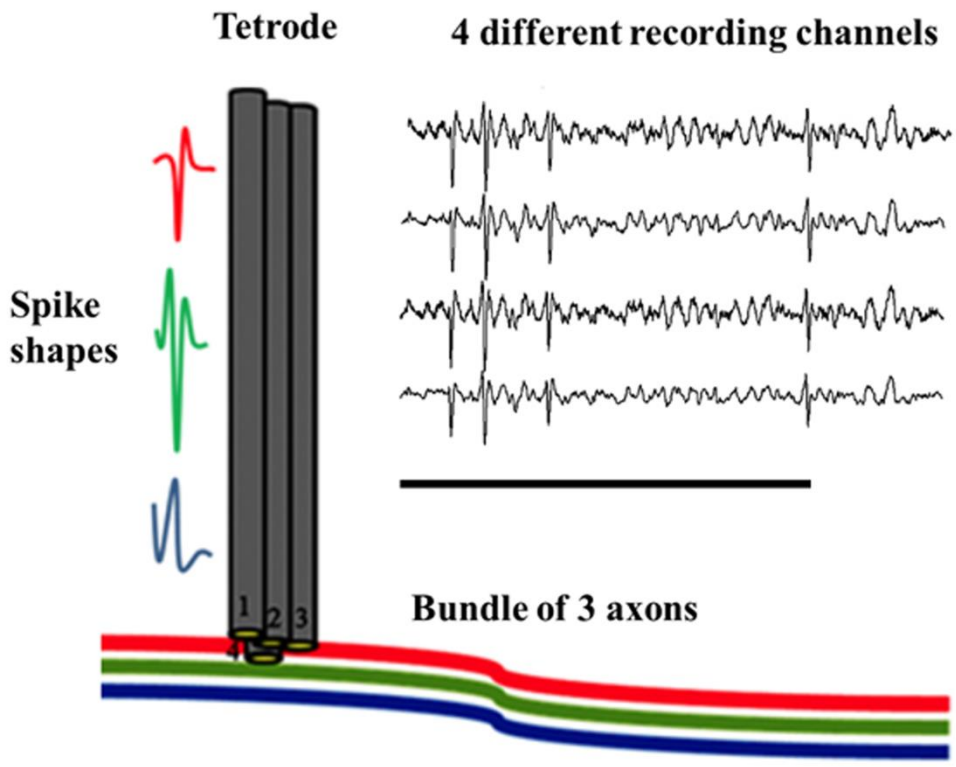

Figure.1 Schematic drawing of a multielectrode (Tetrode with 4 wires) placed near the bundle of 3 axons in invertebrate nervous tissue. The differences between the channels are typically subtle. The black line represents the stimulus. The closer the recorded neurons are positioned to the electrode, the stronger and better signal-to-noise ratios can be achieved. What ultimately leads to principle differences in spike shape is not known.

compared to wires (200-700 kΩ). Additionally, higher tensile strength of the tungsten wires compared to copper wires eases penetration of the tissue and enables the repeated use of one produced multielectrode in multiple experiments. Another method for getting higher SNR in multielectrode recordings is electroplating the recording tip of the multielectrode using noble metals like iridium, gold or platinum. Such an electroplating process basically uses current to reduce dissolved metal cations so that they form a noble metal coating on the electrode. Zhong et al. (2014) have used iridium oxide (IrO2) films which were deposited at the tip of four 17 µm insulated NiCr wires by an anodic deposition process with an electrochemical work station at room temperature. The impedance of the IrO2 modified electrode was almost reduced to 10% of an unmodified NiCr multielectrode. Benefitting from the decreased impedance of the electrodeposited IrO2 films, the SNR was increased to 7.5 which was the highest SNR described in all studies. However, one of the pitfalls of this method is that it is required to plate the multielectrode again after each use because of the low durability of the

plating metal (Desai et al. 2010). Another method to decrease the impedance of multielectrodes is by changing their charge capacity for example with the equipment NanoZ (Neuralynx – Bozeman, USA). It increases the charge capacity of the multielectrode by passing bipolar, constant current square waves to each wire of the multielectrode (User Manual NanoZ). The average decrease in resistance of multielectrodes after using NanoZ was 30% from the original resistance.

One point of emphasis during production of multielectrodes is the fragile nature of these microwires. They are easily bent or damaged if not carefully handled during production and implantation (Guo et al. 2014; Bhavsar et al. 2015a). Wires may be carefully retracted from the preparation after the experiment is completed, allowing for two or three uses. After each use the tip must be cut or cleaned in an ultrasound waterbath to remove any deposit from the tip and plating needs to be refreshed before each use (Guo et al. 2014).

5.3 Number of neurons that can be recorded using multielectrodes

The number of neurons that have been simultaneously recorded with sufficient quality to be isolated as one unit is up to five (Brill et al. 2013; Zhong et al. 2014, Bhavsar unpublished). It depends on different factors like recording compartment of the neuron, impedance of the multielectrode, number of wires of the multielectrode and threshold of spike sorting. For example, in the grasshopper auditory system around 20 auditory neurons ascending from the thorax form a bundle that enters each hemisphere of the brain (Stumpner and Ronacher 1991b; Kutzki 2012). Since the larger axons of ascending auditory neurons generate larger amplitude action potentials, it is possible to record up to five ascending auditory units using multielectrodes with good SNR. However, in a neuropile recording electrical activity recording from smaller dendrites may include more cells but SNR will be low. Another important factor affecting the number of neurons is the impedance of the multielectrode. This has been shown in Zhong et al. (2014) : they were able to record from 5 different neurons using low impedance fabricated IrO2 stereotrodes. The number of neurons that can be distinguished also depends on the number of wires used in the production of the multielectrode. It is obvious that with a larger number of wires, more neurons can be detected. For insect brains it is always advisable to use only 3 to 4 wires per multielectrode, since with increasing number of wires the total diameter of the tip also

increases and therewith also the amount of damage to the nervous tissue during electrode insertion. This is seen especially in very small brains of *Drosophila melanogaster* (Left-right diameter 600 µm) where stereotrodes made of NiCr wires having total diameter of at least 34 µm were described as being still too large for recordings due to insertion damage - even though recordings were with good SNR (Zhong et al. 2014; Lu Yi - personal communication). Threshold of spike detection during spike sorting is a further important factor influencing how many units can be sorted from the recording. A common criterion has been established to choose the threshold as a multiple of an estimate of the standard deviation (SD) of the noise (noise indicates activity that is not related to the stimulus), i.e., threshold = mean (±) k * SD , where k is a constant (Rey et al. 2015). Choosing the value of k is critical: if the value is too low, noise fluctuations will be interpreted as spikes and if it is too high, too many low amplitude spikes will be missed which eventually decreases the number of detected units. Brill et al. (2013) and Bhavsar et al. (2015a) showed that a value of k = 3 provided the best compromise between avoidance of noise fluctuations and detection of spikes in honeybees and grasshoppers. However, higher values like k = 4.5 applied by (Zhong et al. 2014) have also given satisfactory results in fruit flies due to the high SNR of their recordings.

5.4 Methods to mark the location of the recordings

One of the major limitations of multielectrode recordings is that the morphology of the recorded neurons remains unknown unlike in intracellular recorded neurons which often are stained following physiological characterization. However, there are some methods available to at least mark the position of the tip of the multielectrode. Such information about the location of the recording can be used to search for neurons with sharp electrodes and fill them with dye during intracellular recordings. For the visualization of the recording site after a successful extracellular recording, three methods have been used, each with particular merits and demerits.

The first method is electrical current-driven deposition of copper from copper plated NiCr wires as described by Bender et al. (2010) and Mizunami et al. (1998) . At the end of the recording experiment, a 10µA, and 5ms DC current for 5 seconds was passed between one of the tetrode wires and the reference electrode to deposit copper at the recording site. Concentrated brownish deposits

occurring in several adjacent serial sections were identified as the recording location. This method was also tried by Bhavsar et al. (2015a) using unplated copper wires on the grasshopper brain. However, results were not reproducible. The likely explanation is that the maximum current which can be passed via such small diameter unplated copper wires did not dissociate sufficient copper at the recording site. Another explanation could be that the copper from s solid wire can be dissolved with less efficiency than from the plated material at the tip of the multielectrode.

A second method for marking the recording location is the application of fluorescent dyes like Alexa hydrazide (Brill et al. 2014), green fluorescent Nissl stain (Zhong et al. 2014) or lucifer yellow (Bhavsar et al. 2015a). In grasshoppers, after a successful recording, the multielectrode was retracted from the brain, dipped into the fluorescent dye and reinserted to the previous location until the auditory activity was detected again (Bhavsar et al. 2015a). The electrode was kept at this position for 10 s to let the dye diffuse into the tissue. Then with the electrode still in place, a drop of Paraformaldehyde (PFA, 4%) was added to fix the dye in the tissue and prevent extensive diffusion of the dye. This method introduces some uncertainty about the exact recording site since the electrode was removed for dipping in the fluorescent dye and then reinserted. However, in the grasshopper brain recording of auditory activity was re-established immediately in all preparations suggesting that the multielectrode was reintroduced to its previous recording position with reasonable precision (Bhavsar et al. 2015a).

The third method is electric coagulating of brain tissue by passing high current for several minutes, either between one of the tungsten wires and the reference wire (Bhavsar et al. 2015a) or between two tungsten wires (Bhavsar unpublished). The high current (100 µA) coagulates the brain tissue and generates a black spot at the recording location. This method harbors the risk of damaging a larger volume of tissue around the electrode which may go along with shrinkage and deformation. So one needs to control the time of current injection to have reliable results without destroying the brain. Accumulation of gas bubbles at the deposition site may serve as a good indicator for sufficient current

injection. A clear advantage of this method (and the first one mentioned) compared to the previous one is a marking procedure without any changes of electrode position.

5.5 Recording in freely moving insects vs recording in restrained insects

Obviously, multielectrode recordings in freely moving insects are more challenging than in restrained insects, since small movements of the brain relative to the electrode impair the recording. Nevertheless, such recordings have been successfully performed in honeybees (de Camp 2013; Duer et al. 2015) and cockroaches (Guo and Ritzmann 2013; Guo et al. 2014). Most importantly after insertion, the multielectrode must be fixed to stay at the same position during the subsequent recording period. For this, two component silicon glue is applied around the electrodes into the hole of the head capsule (de Camp 2013; Duer et al. 2015). It turned out to be important to remove all the hemolymph surrounding the brain before applying the glue so that the glue can harden quickly (Duer et al. 2015). Another option for fixing the multielectrode at the recording site is to use hot dental wax (Guo et al. 2014). The number of wires used to produce multielectrodes also affects the recording in freely moving insects. Duer et al. (2015) observed that the honey bee was not moving freely with tetrodes since these were too heavy. Therefore, the number of wires in a multielectrode was reduced to two. Also the length of the multielectrode must be adjusted in a way that the animal can move freely in the setup without restrictions and without getting entangled in the wires (Guo et al. 2014). Guo et al. (2014) performed recordings from mechanosensory units from the central complex of 50 freely moving cockroaches and none of the experiments was terminated because the cockroach damaged the wire sets. Nowadays, wireless electrophysiological systems are commercially available which could be more appropriate for small freely moving animals (Harrison et al. 2011; Ghomashchi et al. 2014), but they suffer from limitations like time of data acquisition, battery life and weight (Guo et al. 2014).

In comparison to freely moving insects, the multielectrode recordings in restrained insects are relatively easy and straightforward. The animal can be anesthetized for fixation and appendages may be cut to reduce movements (Brill et al. 2014). Before the insertion of the multielectrode, the brain is typically supported by a metal platform (Saha et al. 2013; Bhavsar et al. 2015a) to reduce its movements as much as possible. In restrained animals one has an additional freedom to record from

more than one area of the brain in the same animal provided that the electrode insertion induces only minor damage. As useful as these restrained preparations have been and will continue to be, they do present some limitations. The sensory processing depends on the behavioral state and often is limited by restraint. In such restrained conditions the behaviors that the insect can perform - if at all - are limited as well. Most restrained preparations are "open loop", that is, they do not allow for normal movement-related feedback to the system (Guo et al. 2014).

5.6 Specific differences between the species and sensory systems

Multielectrode recordings in insects were mainly used in studies of sensory processing (see introduction). Specific differences that affect the analysis of data from multielectrode recordings have been detected in the processing of information from different sensory modalities. For example, while recording from olfactory neurons in a honeybee (Brill et al. 2013), the SNR of the recording improved over 15 -30 minutes after inserting the multielectrode in the brain. This is due to the fact that the hemolymph or ringer surrounding the brain can prevent the electrodes from making a tight contact with glia and neurons. As time passes, the ringer evaporates away and the electrodes get closer to the neurons which will eventually lead to an increased SNR.

In auditory processing it is seen that the auditory neurons tend to fire with similar latency especially at the beginning of the acoustic stimuli because of the tight coupling between the response and the temporally precise stimuli (Bhavsar et al. 2015a). Such coupling will lead to complex spike shapes known as collisions due to spike overlap. Therefore, it is equally important to extract such spike collision from the recording to reduce the loss of important information (Lewicki 1998). Surprisingly spike collision analysis was not performed by most of the studies. The likely reason is, that relevant spike collisions are not as frequent in other systems as in auditory systems due to less precise coupling of activity of several neurons to a stimulus (Roy Ritzmann - personal communication). Nevertheless, the importance of extracting overlapping spikes should not be overlooked since it may provide additional useful information.

Brill et al. (2013) used differential (pairwise subtracted) channels for the spike sorting that contained reduced noise without compromising spike detection too much. However, this is helpful only when the stimulus related activities recorded by the channels are very different. In case of similar activity recorded on different channels (see Fig.1), subtraction also reduces the specific stimulus-related activity and hence is not recommended (Bhavsar et al. 2015a).

Zhong et al. (2014) observed a special problem related to optical stimulation, a photoelectrical effect while recording with multielectrodes from the compound eye of the fruit fly *Drosophila melanogaster*. During stimulating with blue light, the metals (NiCr) used for the production of multielectrode emit electrons when light shines upon them which affect the potential of the photoreceptors.

5.7 Conclusion

Here, the pros and cons of multielectrode recordings in different sensory systems were reviewed. Lower resistance wires like tungsten or gold plated NiCr are probably best suited for multielectrode recordings. Compared to other physiological recording techniques in insects, multielectrode recordings ensure long-time access to population neuronal activity in behaving insects. Multielectrode recordings can also be successfully applied to freely moving insects. A major pitfall is missing neuronal identification as compared to intracellular studies. In the ideal case, both studies are combined in the same species and may allow identification of neurons based on their physiology also in multielectrode recordings. Interindividual neuronal variability, however, may be the main obstacle in this approach.

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Chapter 6

Summary

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

Three 12µm tungsten wires (combined in a multielectrode) were used 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. 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 of ascending neurons was hard to achieve. 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.

6.2 Population coding among ascending neurons in the brain of a small grasshopper

In the brain, information is represented by activity occurring over populations of neurons (Gerstein et al. 1985; Sanger 2003; Houghton and Kreuz 2013; Kobayashi et al. 2013). Understanding the encoding of information in neural population activity is important for understanding fundamental computations underlying brain functions (Quian Quiroga and Panzeri 2009). I tried to understand population coding among ascending neurons in the brain of female *Ch.biguttulus* using multiunit recordings. The identification of ascending neurons just by comparing the physiology to intracellularly recorded neurons turned out to be difficult due to inter-trial and interindividual variability. Also, PCA classification of the sorted units using just four feature vectors did not give satisfactory results so the summed response of sorted units has been analyzed without identification. Data has been analyzed for syllable pause and gap tuning. Clear syllable-pause or gap tuning was not seen except few data showing partly correlations. Likely reasons could be the complexity of a grasshopper song and

limitation of a multielectrode to record from smaller axons. Song identity was decoded using single and multi-neuronal confusion matrices. 40% of the all data sets showed an increase in mutual information. The decoding performance improved when comparing single units to population of up to 4 units, which indicates that a population code takes place among ascending neurons. However, rest of the data did not show any improvement in decoding performance and this is likely due to these data representing neurons not encoding features like syllable and pause. In future, it would be interesting to analyze the population response to more realistic stimuli.

6.3 Recordings and electrical stimulation of local auditory neurons in the brain of a small grasshopper

The key to understanding the neural basis of recognition processes is the identification of "high order" brain neurons which selectively respond to same stimuli that trigger specific behavior (Schoneich et al. 2015). Here, I recorded from local auditory brain neurons in the lateral protocerebrum, anterior brain and central complex. The local brain neurons were separated from the ascending neurons based on their longer spike latency. The signal-to-noise ratio was low while recording from the local brain neurons in comparison to ascending neurons. Local brain neurons recorded from lateral protocerebrum and central complex were found marking onset of the syllables and encoding directionality of the stimulus. Additionally, one local brain neuron was found showing a selective response to artificial grasshopper songs made from attractive and non-attractive stimuli. This local brain neuron also showed reduced response to the syllables with gaps. Extracellular electrical stimulation was tried mainly in lateral protocerebrum, central complex and posterior regions of deuto-tritocerebrum. Stimulating the posterior regions (likely stimulating command neurons) generated continuous song sequences throughout the stimulation while stimulating central complex or lateral protocerebrum (likely stimulating local neurons) generated singing only for first 2-3 s of stimulation. Differences in stridulation while activating local brain neurons and command neurons suggests that the stridulation is likely controlled by the higher centers in the brain located in the central complex and lateral protocerebrum.

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Codes

Splitting Spike collisions (Spike2)

```
var ss%;                ' handle of spike shape dialog
var num%;
var data%;
var chan%;
var lastCh%;
var list%[33];
var ok%;
var text$;
var ct%;
var MeanWdt%:=1;
var minErr := 1;
var maxErr := 1.5;
var SplitId%:=1;

Data%:=FrontView();

If viewkind(data%)<> 0 then
   Message("Not a data view. Halting");
   Halt;
Endif
Chanlist(list%[], 16);
If list%[0] = 0 then
   Message("No WaveMark channels available. Halting");
   Halt;
endif

Chan%:=List%[1];
Ct%:=Count(list%[1], 0, MaxTime());
text$:=Print$("Total spikes %d", ct%);
      num%:=ct%;

DlgCreate("Setup");  'Start new dialog
DlgAllow(0, 0, Change%);
DlgChan(1,"Channel to process", 16+4096+16384+2097152);
DlgInteger(2,"Number of spikes to process",1, ct%);
DlgLabel(3, Text$);
DlgCheck(4, "Mean width");
DlgReal(5, "Choose Mean error limit", 0, 100);
DlgCheck(6, "Split as ideal?");

DlgButton(1,"OK");
DlgButton(0,"Cancel");



ok% := DlgShow(Chan%, Num%, Text$, MeanWdt%, minErr, SplitId%);


ss% := SSOpen(1,1);         ' Open an Edit WaveMark dialog
SSChan(chan%);
if ss% < 0 then halt endif;
SSButton(13,1);             ' Set collision analysis mode
```

```
if MeanWdt% = 1 then
   SSButton(14,1);
else
   SSButton(14,0);
endif

if SplitId% = 1 then
   SSButton(15,1);
else
   SSButton(15,0);
endif


var n%,n1%,n2%,p1,p2,err;
var i%;
var j%;



for i% := 1 to Num% do  'go through all the wave marks

   n% := SSColInfo(err,n1%,n2%,p1,p2);

   if (n% = 2) and (err > minErr) then ' if err is in between 0.2 and 2.5

      SSColApply(2); 'apply the best match criteria to split the spike

      PrintLog("%10.5f %5.2f %2d %2d %5.2f %5.2f\n",
      View(ViewLink()).Cursor(0), err, n1%, n2%, p1, p2); ' write the locations of the found collisions in the log
window, min error, and the classes in which it is splitted

   endif;

   SSRun(1);                  ' step to the next item
next;

Func Change%(item%);
Var val%;
Var New$;

If item% = 1 then
   Chan%:=Dlgvalue(1);
   if lastCh% <> Chan% then
      ct%:=Count(Chan%, 0, maxtime());
      New$:=Print$("Total spikes %d", ct%);
      DlgValue$(3, New$);
      LastCh%:=Chan%;
   endif
endif
return 1
end


View(App(3)).WindowVisible(0);
```

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