

Fachgebiet für Zoologie der Technischen Universität München

**Neural responses to synthetic and natural stimuli in auditory forebrain centres
of juvenile and adult zebra finches**

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1. Introduction

1.1. Important facts about the field L complex of songbirds

The field L complex of songbirds is the highest processing centre of the ascending auditory pathway (Boord, 1969). It receives auditory input from the thalamic nucleus ovoidalis (Karten, 1968) and projects into the song system via the shelf region of the high vocal centre (HVC) and into adjacent auditory nuclei, like the caudomedial neostriatum (NCM) or the caudal hyperstriatum ventrale (Kelly and Nottebohm, 1979; Vates et al., 1996). It is analogous to the auditory cortex of mammals (Heil and Scheich, 1991; Heil et al., 1992).

This brain area in the caudal neostriatum was first described in 1914 by Rose as a region of small, densely packed and darkly stained neurones in Nissl preparation (Rose, 1914). Anatomically and functionally it can be divided into several areas. Former anatomical classifications describe five subdivisions due to cells of different size and density (Fortune and Margoliash, 1992; Wild et al., 1993; Vates et al., 1996). These anatomical subdivisions are called L, L1, L2a, L2b, L3.

In male, adult zebra finches it is possible to distinguish six different areas in the neostriatum on the basis of their tonotopic gradients and response behaviours to pure tones (Gehr et al., 1999; Gehr and Leppelsack, 2000). These areas are called NA-L, NA2a, NA2b, NA2c, NA3 and NA4 (Figure 1), according to their anatomical counterparts. The areas NA-L, NA2a, NA3 and NA4 respond to pure tones with phasic on- plus sustained excitations. NA-L shows additionally clear sidebands of lateral inhibition dorsal and ventral to excited regions. NA2b and NA2c show only phasic responses to pure tones. A similar differentiation of the field L can be seen in starlings. In European starlings and zebra finches, all functional areas represent the frequency distribution of the basilar papilla of the inner ear in a tonotopic way from low to high frequencies (Leppelsack, 1992; Capsius and Leppelsack, 1996; Capsius and Leppelsack, 1999; Gehr et al., 1999; Gehr and Leppelsack, 2000). The different response properties in the areas indicate different tasks in the processing of auditory stimuli (Capsius and Leppelsack, 1999). Natural auditory stimuli are especially the songs of conspecific males for individual recognition in the colony or the birds' own songs, which are important for sexual selection and therefore mating success.

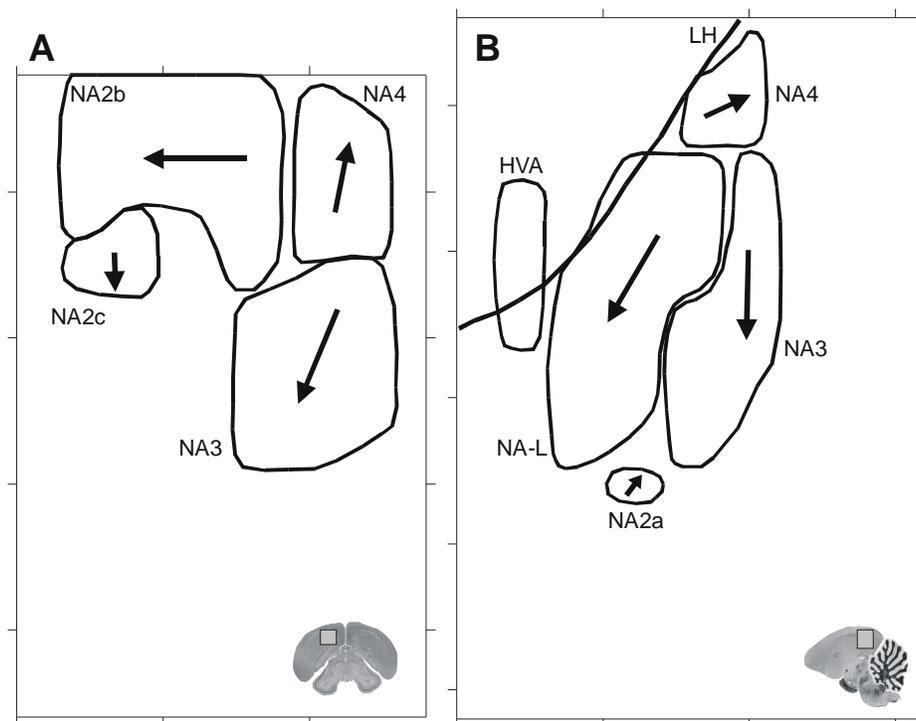


Figure 1. Functional organisation of the field L complex in adult male zebra finches in (A) frontal and (B) sagittal section. Arrows indicate tonotopic gradients of the single areas. The field L complex is located in the caudal neostriatum between the lamina hyperstriatica (LH) and the lamina medullaris dorsalis. Positions of the two maps are in the grey rectangles shown on the insets. (A) A frontal section of the caudal part of field L complex contains the areas

NA2b, NA2c, NA3 and NA4. (B) The medial sagittal section includes the areas NA-L, NA2a, NA3 and NA4 in the neostriatum. The area HVA (auditory part of the hyperstriatum ventrale), situated rostral to the NA-L, is pervaded by the lamina hyperstriatica (LH) in its lower third. A tonotopy is not obvious. Marks on the frames are separated by 0.5 mm.

1.2. The song of zebra finches

Zebra finches (*Taeniopygia guttata*) are easy to maintain and breed in the laboratory. With their stereotyped song, they are a famous species among neuroscientists to study the perception and generation of bird song.

Male zebra finches learn their song from a tutor during a certain time in ontogeny. The sensory phase of song learning starts after post-hatch day (PHD) 25-30. Whilst this period, the song of the tutor is acquired and stored as a template in the brain. The sensory-motor phase starts after PHD 60 (Mooney, 1999). The young bird compares its own singing with the template until the mature song crystallises after PHD 90. The crystallised song is similar to the tutor's song, but it is not an identical copy (Immelmann, 1969; Marler and Peters, 1982).

The zebra finch song is highly stereotyped and was described by several authors in the past (e.g. Immelmann, 1969; Zann, 1993; Sturdy et al., 1999 a,b). Several notes can be classified in the song. These notes are distinguishable by various lengths, harmonic contents and modulations. The characteristics of zebra finch song are harmonics with a highly variable fundamental frequency and quick modulations in frequency and amplitude.

1.3. Auditory feedback and neural selectivity to song in zebra finches

Auditory feedback via the field L complex is crucial for song development during ontogeny (Leonardo and Konishi, 1999). The response properties of auditory neurones of nuclei of the song system, like e.g. the lateral magnocellular nucleus of the anterior neostriatum (IMAN) or the area X, to various song stimuli change during ontogeny in male zebra finches, as evaluated in anaesthetised birds (Doupe, 1997; Solis and Doupe, 1999). No differences in the neural selectivity can be found in the IMAN of PHD 30 birds between the tutor's and conspecific song. 60 days old birds have developed a neural responsiveness that distinguishes between the bird's own song (BOS) or the tutor's song and conspecific song. The majority of IMAN neurones responds significantly higher to the BOS and the tutor's than to the conspecific song. In these reports, it can clearly be seen that a selectivity for BOS arises during ontogeny in nuclei of the song system.

Auditory neurones of the HVC, which receives auditory input from field L, show also a high selectivity for BOS in anaesthetised, adult songbirds (Margoliash, 1986; Margoliash and Fortune, 1992; Lewicki and Arthur, 1996; Theunissen and Doupe, 1998; Janata and Margoliash, 1999). Selectivity in the auditory pathway increases from non-telencephalic centres to the field L complex of European starlings (Leppelsack et al., 1986), but single units in field L do not show a preference for the BOS over other songtypes (Lewicki and Arthur, 1996). However, the responses of neurones in field L are correlated with the spike patterns of HVC neurones (Janata and Margoliash, 1999), which proves the neuronal connection of these two centres. The question arises, where in the brain or when in ontogeny, song selectivity develops.

Auditory input into the song system is important to maintain a stable song in adult birds (Nordeen and Nordeen, 1992; Leonardo and Konishi, 1999; White and Mooney, 1999). This input is processed within the field L.

In this study the functional organisation of the field L complex was investigated with a multi unit mapping technique in awake 30 and 60 days old juveniles and adult birds to draw conclusions about the ability of auditory processing in the field L complex.

2. Material and methods

2.1. Animals

Ten juvenile, and nine adult, male zebra finches (*Taeniopygia guttata*) were investigated neurophysiologically. In all juvenile and adult subjects, the field L complex of the left hemisphere was visualised (data is called JT plus the number of the juvenile bird). Whereas in six adults, recordings were additionally performed in the field L complex of the right hemisphere (data of the the left hemisphere is called AT or WT, data of the the right hemisphere is called BT or VT plus the number of the bird) (Table 1). AT and BT data originate from white, albino type-birds, VT and WT data originate from grey, wild-type birds. In one additional grey, wild-type adult bird (bird: GP2), the entire field L complex of the left hemisphere was mapped.

The birds were bred and raised in the laboratory by four couples in different cages. Six of the young birds were investigated at post-hatch day (PHD) 30, four at PHD 60. Experimental subjects were kept in small social colonies of usually five to ten birds within their cages until surgery and before and after the experiments. Birds were prepared one to three days before the electrophysiological experiments by stereotaxic implantation of a stainless steel well onto the skull with the bill in an angle of about 55° - 60° to horizontal. The well was implanted with dental cement (Resin Cement and Fluid, Ivoclar). The bifurcation of the sinus sagittalis rostral to the cerebellum was used as reference point. Surgery was done under halothane (Hoechst) anaesthesia using pure oxygen as fuel gas. Moistened oxygen flow was kept at 800 cm³/min during surgery. The halothane proportion was usually kept at 1.3 Vol.% (Halothane Vapor 19.3, Dräger). Lidocaine (Xylocain, Astra Chemicals) was used for local anaesthesia of skin and skull. The well served as an indifferent electrode and for fixing the bird within the recording set-up. Before each penetration, the dura mater was opened at the desired position with a fresh and sharp hypodermic needle. During recording sessions the fixation of the steel well and the position of the electrode in the brain was reproducible with an accuracy of 10 µm.

<i>Adult birds</i>						
<i>Bird</i>	<i>Tutor</i>	<i>Penetrations</i>	<i>Recording plane [mm]</i>	<i>Mean body mass (\pm SD) [g]</i>		
AT3 /	Clyde	11	1.3 frontal left	14.20 \pm 0.60 (N = 20)		
BT3	“	13	1.3 frontal right	“		
AT4 /	Heino	8	0.8 sagittal left	14.21 \pm 0.50 (N = 13)		
BT4	“	12	0.8 sagittal right	“		
AT5 /	Clyde	13	1.2 sagittal left	13.77 \pm 0.26 (N = 15)		
BT5	“	13	1.2 sagittal right	“		
AT6 /	Heino	12	1.0 frontal left	15.18 \pm 0.54 (N = 23)		
BT6	“	12	1.0 frontal right	“		
WT1 /	Willi	13	0.6 sagittal left	16.78 \pm 0.31 (N = 23)		
VT1	“	13	0.6 sagittal right	“		
WT3	Fred	10	1.0 sagittal left	14.99 \pm 0.47 (N = 8)		
WT4 /	Willi	12	1.5 frontal left	14.29 \pm 0.47 (N = 11)		
VT4	“	12	1.5 frontal right	“		
WT5	Willi	10	2.0 frontal left	15.58 \pm 0.29 (N = 9)		
GP2	Willi	70	1.2 – 2.8 f, 0.2 – 1-6 s	15.44 \pm 0.47 (N = 8)		
<i>Juvenile birds</i>						
<i>Bird</i>	<i>Tutor</i>	<i>Penetrations</i>	<i>Recording plane [mm]</i>	<i>Body mass [g]</i>	<i>Age (PHD)</i>	<i>CON</i>
JT1	Heino	10	1.5 frontal	12.8	30	Willi
JT2	Fred	10	1.2 frontal	13.9	60	WT5
JT3	Fred	9	0.8 sagittal	13.1	60	Clyde
JT4	Heino	7	0.6 sagittal	14.4	30	AT3
JT5	Heino	8	1.0 sagittal	13.0	30	Clyde
JT6	Willi	9	0.8 sagittal	17.0	30	L72
JT7	Willi	6	1.2 frontal	14.3	30	Heino
JT8	Fred	10	1.5 frontal	15.5	60	L62
JT9	Willi	8	0.4 sagittal	12.7	60	WT5
JT10	Heino	9	0.4 sagittal	13.9	30	AT5

Table 1. Individual data of all experimental subjects. The upper part of the table shows tutor, number of penetrations, position and orientation of the recording plane and the mean body mass (N = number of recording sessions) of each adult bird. The position of the recording planes is shown

according to the reference point and the respective hemisphere (Figure 6). Data of one bird are visualised by one box in the first column (AT and BT resp. WT and VT). The lower part of the table shows tutor, number of penetrations, position and orientation of the recording plane in the left hemisphere (Figure 6), body mass, age and the conspecific song, which was used for acoustic stimulation, of each juvenile bird.

Body mass was measured before each recording session to control the state of health of the birds. PHD 30 birds had a mean (\pm standard deviation (SD)) body mass of $14.23 \text{ g} \pm 1.51$ (one value per bird, $N = 6$). The mean (\pm SD) body mass of PHD 60 birds was $13.80 \text{ g} \pm 1.24$ (one value per bird, $N = 4$). Individual mean (\pm SD) body masses ranged between $13.77 \text{ g} \pm 0.26$ and $16.78 \text{ g} \pm 0.31$ in adult birds.

2.2. Acoustic stimuli

2.2.1. Juvenile birds

The stimulus ensemble consisted of white noise, a pure tone sequence and two natural songs. The pure tone sequence was made up of seven stimuli of 60 ms duration including 5 ms rise and 5 ms fall time. The frequencies used were 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 kHz. The song of the birds' tutor (TUT) and of one conspecific (CON) animal out of eight, which was completely unknown to the experimental subject, were used as natural stimuli. The sonograms of the natural stimuli are shown in Figure 2, the spectra are shown in Figure 3. An own TUT-CON combination was used for each bird (Table 1). The whole stimulus ensemble was generated to be short (usually less than a minute per recording site, ten repetitions) to be able to record one complete plane per recording session. This was done to prevent the recording of hypothetical, functional inter-day changes of the field L complex. The maximum sound pressure of the stimuli at the ear of the bird was about 55 dB SPL (re 20 μ P), measured using a 0.5" microphone (GenRad).

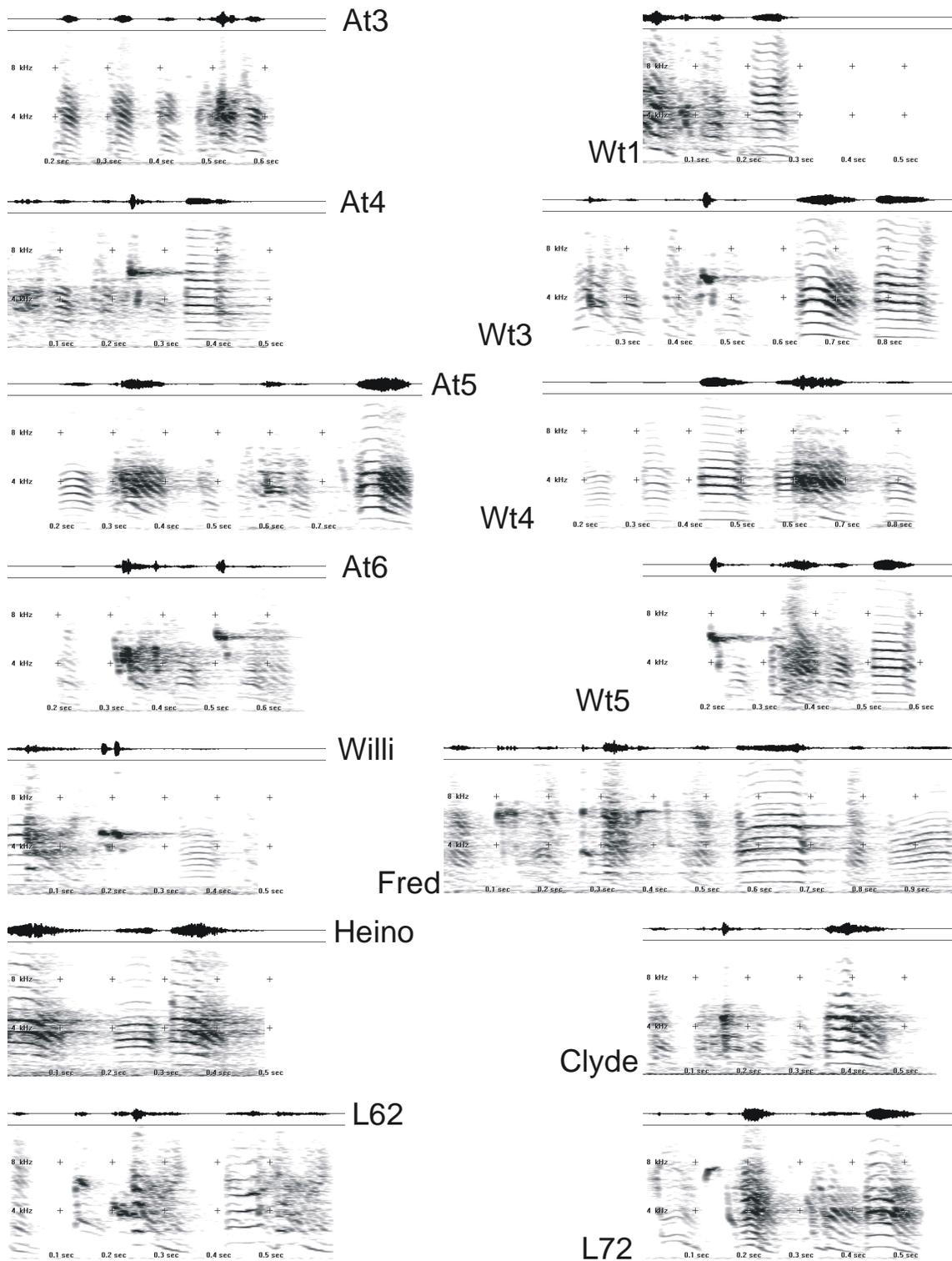


Figure 2. Sonograms of all natural stimuli. The first four lines show the BOS of the adult birds. The next two lines show all four tutor (TUT) songs. The last line shows two additional conspecific (CON) stimuli of the birds JT8 and JT6. In sonograms frequency is plotted against time. The darkness is a scale for stimulus intensity. Amplitudes are shown above each sonogram. Scale marks are separated by 0.1 s in the time domain and 4.0 kHz in the frequency domain.

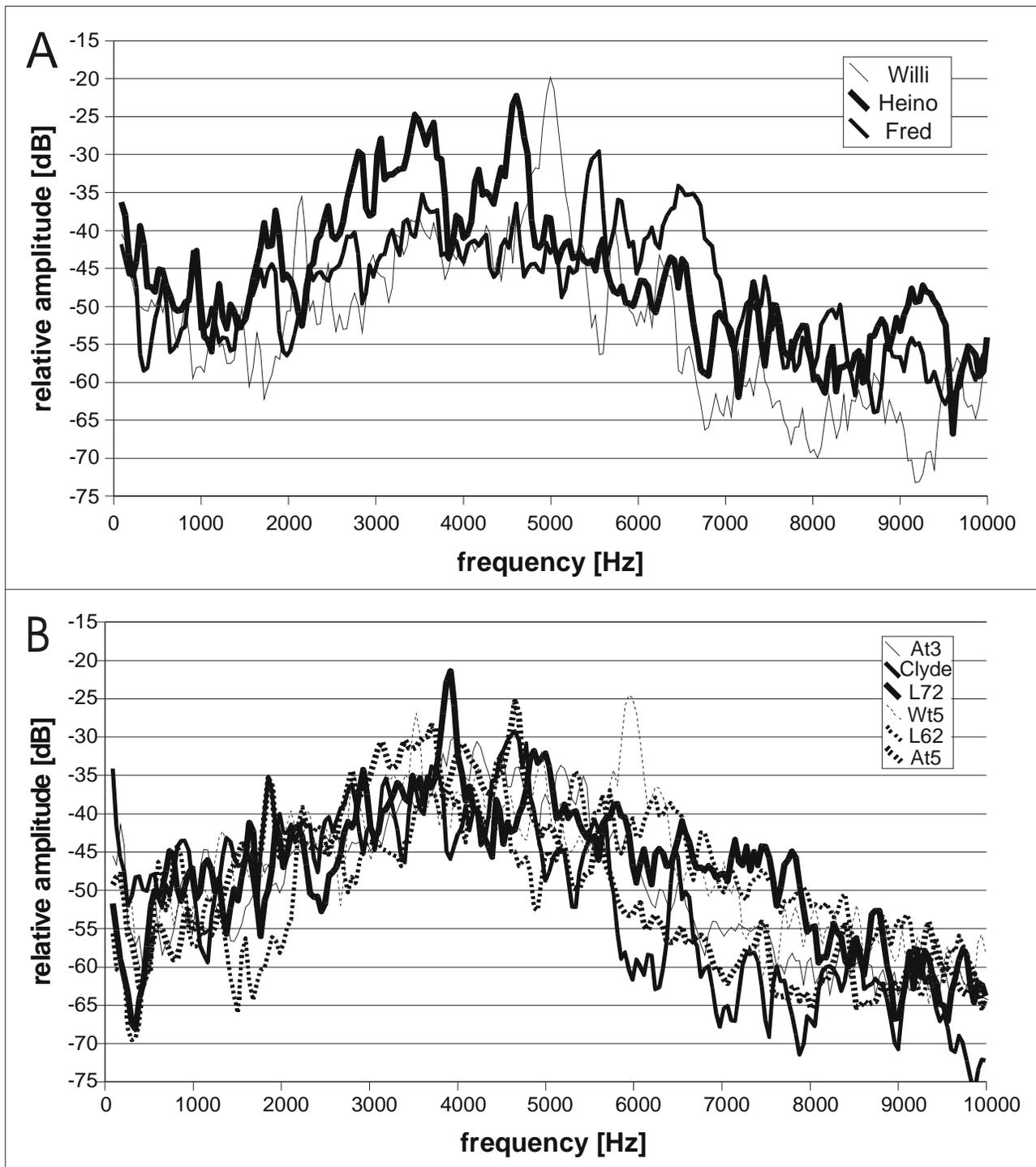


Figure 3. (A) Frequency spectra of the three tutors of the juvenile birds. Please pay attention to the various positions of maxima above -35 dB relative amplitude: Heino: 2.5 kHz – 4.5 kHz, Willi: 4.5 kHz – 5.0 kHz, Fred: 5.5 kHz – 6.0 kHz. (B) Frequency spectra of six conspecific birds. Relative amplitude [dB] is drawn against frequency [Hz]. The tutors of the adult birds are Willi, Fred, Heino, Clyde.

2.2.2. *Adult birds*

The stimulus ensemble for the birds AT, BT, VT and WT consisted of a pure tone sequence, four harmonic complexes and five natural songs. The synthetic stimuli had a duration of 80 ms including 5 ms rise and 5 ms fall time each. The eight pure tone frequencies used were 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 kHz. The harmonic complexes consisted of a base frequency, i.e. the fundamental (1.0 and 2.0 kHz) and the first to fourth harmonics. The harmonic stimuli were presented with the fundamentals and without them resulting in a total of four harmonic stimuli. The harmonic complexes lacking the fundamentals will be called “high-pass filtered harmonic complexes” in the further text. The BOS of each individual experimental bird and the songs of all four possible tutor birds were used as natural stimuli. Therefore, each subject listened to its own song, the song of its tutor and the songs of three conspecifics. The natural stimuli were all presented in normal and time-reversed form. The sonograms of the natural stimuli are shown in Figure 2, the spectra are shown in Figure 3. The playback lasted around 2 minutes and 15 seconds per recording site (ten repetitions). The stimulus ensemble for the bird GP2 consisted only of synthetic stimuli. They were the same as the synthetic stimuli for juvenile birds. The maximum sound pressure of the stimuli at the ear of the bird was about 55 dB SPL (re 20 μ P), measured using a 0.5” microphone (GenRad).

Synthetic stimuli were generated on a PC with a Pentium CPU and a Soundblaster sound card. The Software used was Soundforge (Sonic Foundry), Goldwave v4.18 (<http://www.goldwave.com/>) and Cooledit96 (Syntrillium). Natural stimuli were reversed with Soundforge software.

2.3. *Multi unit recordings*

Electrolytically sharpened and glass insulated platinum-iridium wire (70/30) with an impedance of 0.5 – 1.0 M Ω was used as recording electrode. Multi unit recordings were performed in awake, restrained birds in an anechoic chamber (Grünzweig and Hartmann) with a loudspeaker (Heco KC52) placed 80 cm in front of the bird's head. The frequency response of the chamber was measured and described by Leppelsack (1992). The sound pressure differs from the desired value only by ± 1.5 dB in the frequency band between 650 and 7800 Hz. Acoustic stimuli were played by a

DAT recorder (Sony DAT 59 ES). Action potentials of usually two to four neurones were amplified, filtered and recorded simultaneously via a window discriminator (Frederick Haer & Co.) and stored onto a computer (LSI 11/73). The threshold of the window discriminator was set manually at each recording site. The neuronal recordings were controlled via an audio monitor (Heco KC52), an oscilloscope (Tektronix 5113) and raster plots on paper (Integrex Colourjet 132) (Figure 4).

Neural impulses were recorded by the electrode at different depths between 400 and 4500 μm below the brain surface within one penetration. These recording sites were separated from each other by 100 μm in juveniles and 80 μm in adults. Frontal or sagittal maps were obtained by performing the recordings in adjacent penetrations, separated by 200 μm in juveniles and 150 μm in adults. One recording plane was mapped in each juvenile bird within one day. The distances of the recording grid of the bird GP2 were the same as for juvenile birds, namely 100 μm in vertical direction and 200 μm in horizontal direction. Due to the short stimulus ensemble, which was used for that bird, it was possible to perform up to 18 penetrations per recording session. A recording session in adult birds lasted 3 to 4 hours per day and about 2 penetrations could be performed during one session. Eight to 23 recording sessions were required to acquire the data of the adult birds. The recording sessions in juvenile birds lasted up to 5 hours. At each recording site ten repetitions of the acoustic set of stimuli were presented.

2.4. Response maps

Within one recording plane peri-stimulus-time histograms (PSTHs) were calculated for each recording site using 20 ms time bins. Latency times were determined, using white noise as broadband stimulus or an adequate pure tone, with an accuracy of 1 ms under consideration of the 80 cm distance between sound source and ear (delay = 2.3 ms, speed of sound = 343.42 m/s at 20 °C). Neuronal response strength was defined as the ratio between actual discharge rate and spontaneous activity. A binomial test with an error probability of less than 5% was performed for

each time bin at each recording site. The spatial distribution of significantly active response sites was plotted as a response map (Surfer 7.0, Golden Software). Response maps, showing the recording planes, represented the neural activity within 20 ms time bins of responses to pure tones (Figure 5). The positions of excited regions to various frequencies were compared and the tonotopic gradients defined. In the following text, the functional areas of different ages are marked with indices quoting the PHD like e.g. NA-L₃₀ in the bird JT1. Areas of adult birds have no indices.

Response maps, which made it possible to compare the neuronal activity to several stimuli in one map were computed by a software provided by Goldsche (1997). In these comparison maps recording sites are shown responding to various stimuli, if there is at least a significant excitation in one 20 ms time bin during the whole stimulus duration. Responses to the different stimuli are marked by distinct numbers. Compare Figure 11 in chapter 3.2.2.

Response area extension histograms (RAEHs) were calculated to characterise the response behaviour of the different auditory centres using 20 ms time bins. Here the number of significantly excited recording sites of an area to the single time bins of a certain stimulus was compared with the complete number of significantly responding recording sites of this area for the whole duration of all presented stimuli. An example to make this clear will be explained on the basis of the response map in Figure 5: The map shows the on-response to the 3.0 kHz pure tone stimulus. Out of 72 recording sites in the area NA-L₆₀, 24 recording sites are significantly excited, four sites are inhibited and the other 44 sites do not respond. The ratios of number of responding sites versus number of all sites are computed. In the example 33.3% of the whole area size is excited and 5.5% of the area are inhibited in the first time bin of the given stimulus. These data for all frequencies and all planes are finally pooled so that one histogram results per functional area (compare Figure 8).

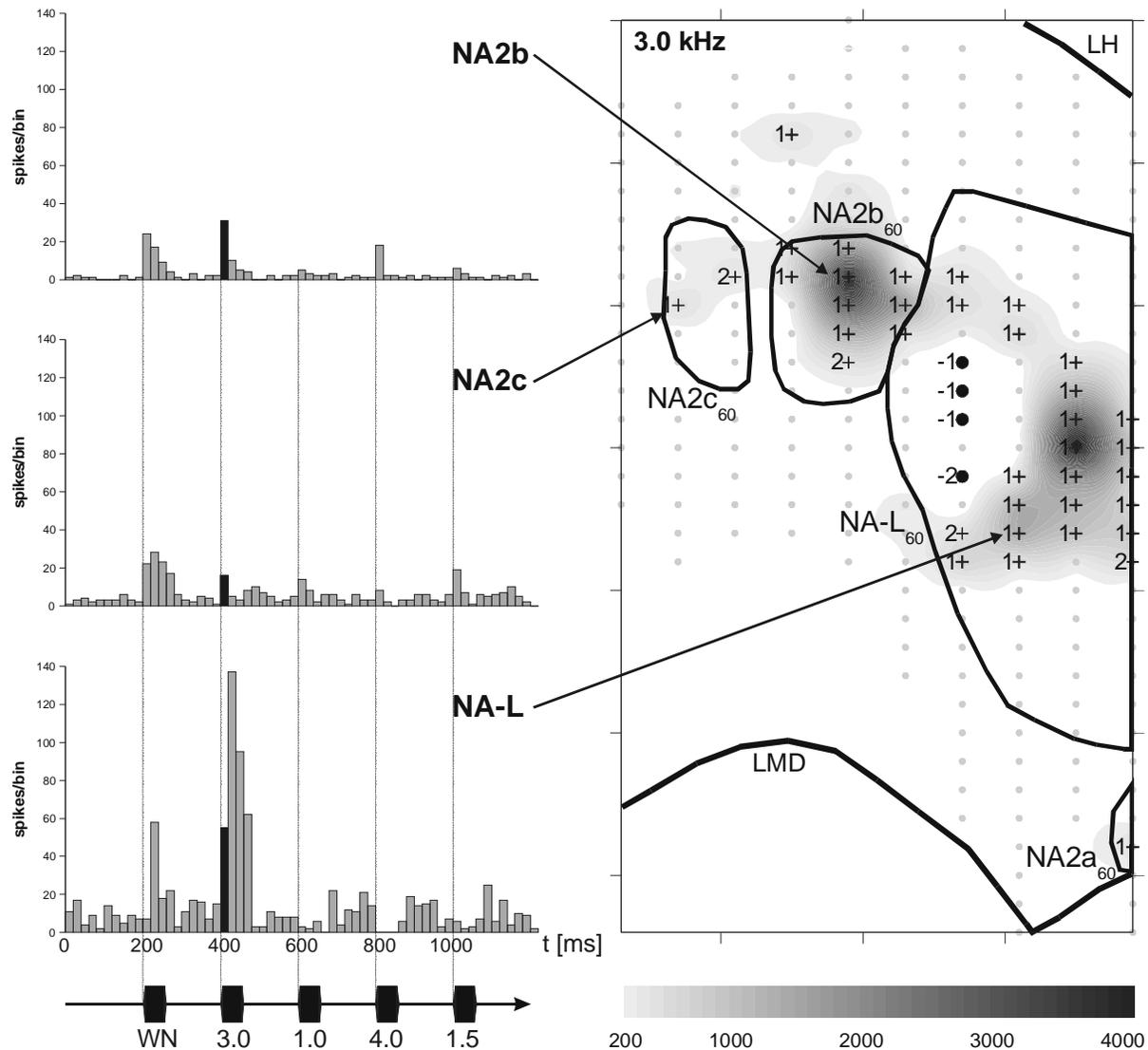


Figure 5. Neuronal responses and response maps. *Left:* Responses to a part of the stimulus ensemble are presented as PST histograms at the left with a bin width of 20 ms. Stimuli are white noise (WN) and pure tones with the frequencies 3.0, 1.0, 4.0 and 1.5 kHz. Stimulus length is 60 ms each. Neuronal responses to ten repetitions of the stimuli are shown for three selected recording sites. Typical responses are: NA-L: a sustained excitation to 3.0 kHz and a sustained inhibition at 4.0 kHz. NA2b: phasic on-responses to pure tones (3.0 and 4.0 kHz) and a sustained excitation to white noise, which is characterised by its amplitude modulations. The response behaviour of recording sites of NA2c is similar to NA2b. *Right:* Arrows originating from the PSTHs indicate the positions of the relating recording sites in the recording grid of the map on the right side of the figure. Black bars in the histograms relate to the time bin of the example map, which represents the onset response to the 3.0 kHz pure tone. The complete recording grid is visualised by grey dots in the map. Recording sites with significant responses are indicated by numbered symbols (Excitation (+): 1: $p < 2.5\%$, 2: $p < 5.0\%$. Inhibition (black dot): -1: $p < 2.5\%$, -2: $p < 5.0\%$; binomial test). The scalebar below the map indicates the neuronal excitation in % (spontaneous activity is 100%). The distance between two marks on the frame is 500 μm . Functional auditory areas of the field L complex of a PHD 60 bird are drawn into the histology of the forebrain with the lamina hyperstriatica (LH) and the lamina medullaris dorsalis (LMD).

2.5. Histology

After recordings were finished, two recording sites were marked by injections of alcian blue (6% in phosphate buffer, pH 5.2) to provide landmarks for transferring the response map onto the outlines of the anatomy of the brain. Injections were made by air pressure (custom-made pump; 2 bar, 100 ms) from a glass micropipette with a tip diameter of about 25 μm . One to two days later the animal was killed by an overdose of 6% pentobarbital (Narcofen, Rhone Merieux) injected into the pectoral muscle. The bird was perfused transcardially by 50 ml of an isotonic, heparinised sodium chloride solution followed by 100 ml 10% formaldehyde in 1 M phosphate buffer (pH 7.2). Blood and perfusion solutions could flow off through a cut in the right jugular vein. A perfusion lasted normally 2 h. After the perfusion, the brain was carefully removed from the skull and kept in the formaldehyde solution for at least one more day. For cryo-sectioning, the brain was transferred into a 10% sucrose solution for at least 12 h to prevent freezing artefacts, and embedded into egg yolk, treated with sucrose and glutaraldehyde. Frozen sections of 50 μm were cut either in frontal or in sagittal orientation depending on the orientation of the recording plane (Leitz 1720). Alternating sections were dyed using cresyl violet and silver staining techniques, respectively. Dyed sections were projected (Leitz Prado Universal) onto a screen and drawn on paper to reconstruct the histology of the recording planes. Finally, histology was digitised into the functional maps using Surfer software (V 7.0, Golden Software).

The present experiments were carried out under animal experimentation licence number 211-2531-59/96 from the Government of Upper Bavaria.

3. Results

3.1. Recording details

3.1.1. Recording details of juvenile birds

Data were collected in the left hemisphere. In PHD 30 birds neuronal recordings were performed in six planes: four sagittal planes 0.4, 0.6, 0.8 and 1.0 mm lateral of the sinus sagittalis and two frontal planes 1.2 and 1.5 mm rostral of the bifurcation of the sinus. In PHD 60 birds, data were collected in four recording planes: two frontal (1.2 and 1.5 mm) and two sagittal planes (0.4 and 0.8 mm) (Figure 6A).

The recording planes consist of 6 to 10 electrode penetrations (Table 1) and 177 to 288 recording sites per plane. There is a total number of 2230 recording sites (mean \pm SD = 223 \pm 37) in all juvenile birds.

3.1.2. Recording details of adult birds

Data in the left hemisphere were collected in eight birds. There are four sagittal planes 0.6, 0.8, 1.0 and 1.2 mm lateral of the sinus sagittalis and four frontal planes 1.0, 1.3, 1.5 and 2.0 mm rostral of the bifurcation of the sinus. In six of these birds, complete planes were additionally recorded in the right hemisphere with the same reference coordinates as in the left hemisphere of those birds. There are three sagittal planes 0.6, 0.8 and 1.2 mm lateral of the sinus sagittalis and three frontal planes 1.0, 1.3 and 1.5 mm rostral of the bifurcation of the sinus (Figure 6B).

The recording planes in adults consist of 8 to 13 electrode penetrations (Table 1) and 203 to 440 recording sites per plane. There is a total number of 4557 recording sites (mean \pm SD = 326 \pm 61) in all adult birds, excluding subject GP2. The higher mean in comparison to juveniles results from the recording grid, which is more dense.

In the bird GP2, eight frontal planes 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6 and 2.8 mm rostral of the bifurcation of the sinus and nine sagittal planes 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 mm lateral of the sinus were recorded. A total of 70 penetrations and 1263 recording sites was required to obtain the data in that bird.

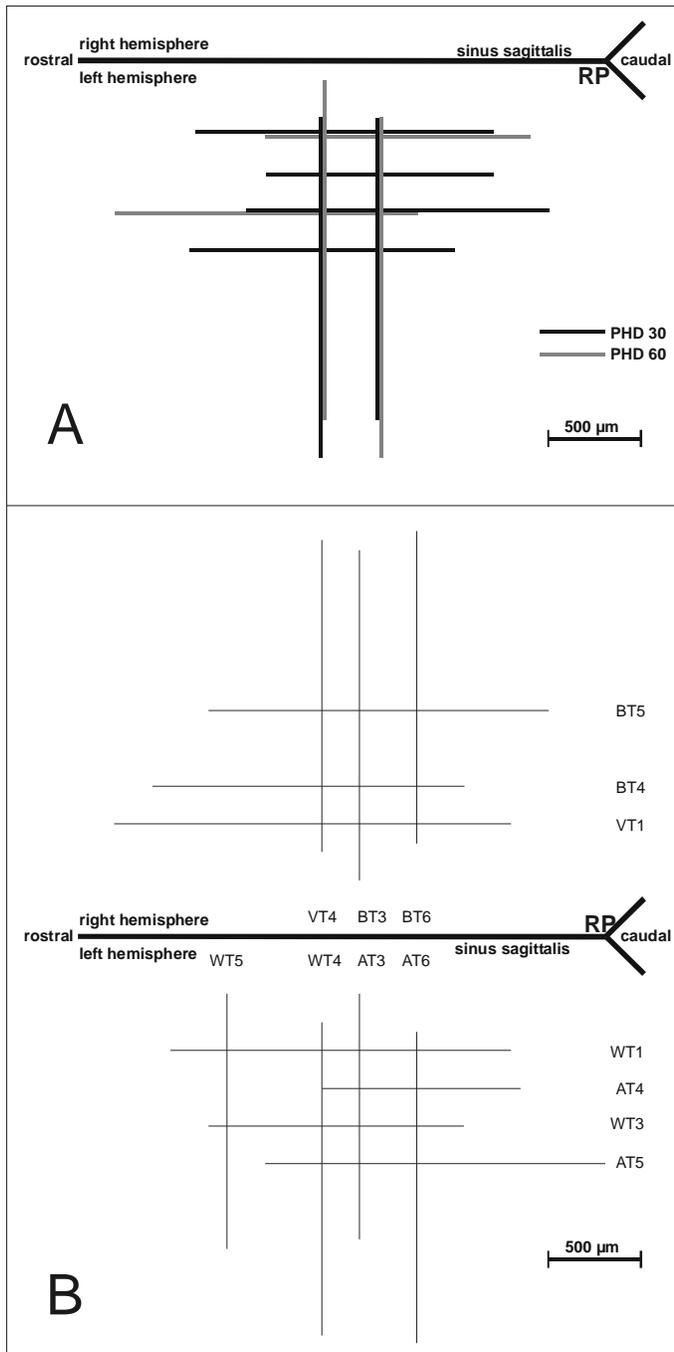


Figure 6. Positions of recording planes on the surface of the zebra finch brain. The sagittal sinus can be seen with its bifurcation rostral to the cerebellum, which serves as reference point (RP). (A) Analysed planes of PHD 30 birds are represented by black lines, those of PHD 60 birds by grey lines. (B) Recording planes of adult birds in both hemispheres are drawn next to the birds' names.

3.2. *Adult birds*

3.2.1. *Functional organisation*

3.2.1.1. Left hemisphere

In the recordings of the left hemisphere of adult birds, six functional areas could be separated within the neostriatum (Figure 7). These six areas are already described in the literature (Gehr et al., 1999; Gehr and Leppelsack, 2000). They are NA-L, NA2a, NA2b, NA2c, NA3 and NA4. The response properties of the areas can be seen in the RAE histograms (Figure 8). Data of the bird GP2 are not included in the histograms, due to the various lengths of the pure tone stimuli (80 ms vs. 60 ms).

NA-L is the largest area and covers Rose's (1914) field L well. The tonotopic gradient from low to high frequencies runs from caudodorsal to rostroventral. The neurones of NA-L typically respond to pure tones with a phasic on-response, followed by sustained excitation and spatially accompanied by sustained inhibition. Inhibitory sidebands, which lie dorsal and ventral to the excited area, are a characteristic feature of NA-L. Excitations to the whole spectrum of presented pure tones, which ranges from 0.5 to 5.0 kHz, can be seen.

NA2a is positioned rostroventral to NA-L. A weak tonotopic gradient runs from rostral to caudal. The neuronal response behaviour is a weak on-response followed by an irregularly spread, sustained excitation to frequencies between 1.5 and 5.0 kHz. Due to the lack of a sufficient number of recording sites, a further analysis of responses to song was not performed.

NA2b is lying lateral to the caudodorsal part of the area NA-L. The main tonotopic gradient runs from medial to lateral with an additional rostro-dorsal portion. The pure tone response of NA2b is characterised by strong phasic on-excitation, followed by a rare sustained excitation and accompanied by a rare sustained inhibition. The neurones also show a rare off-response after the pure tone stimulation. Excitations can be seen to frequencies between 0.5 and 5.0 kHz.

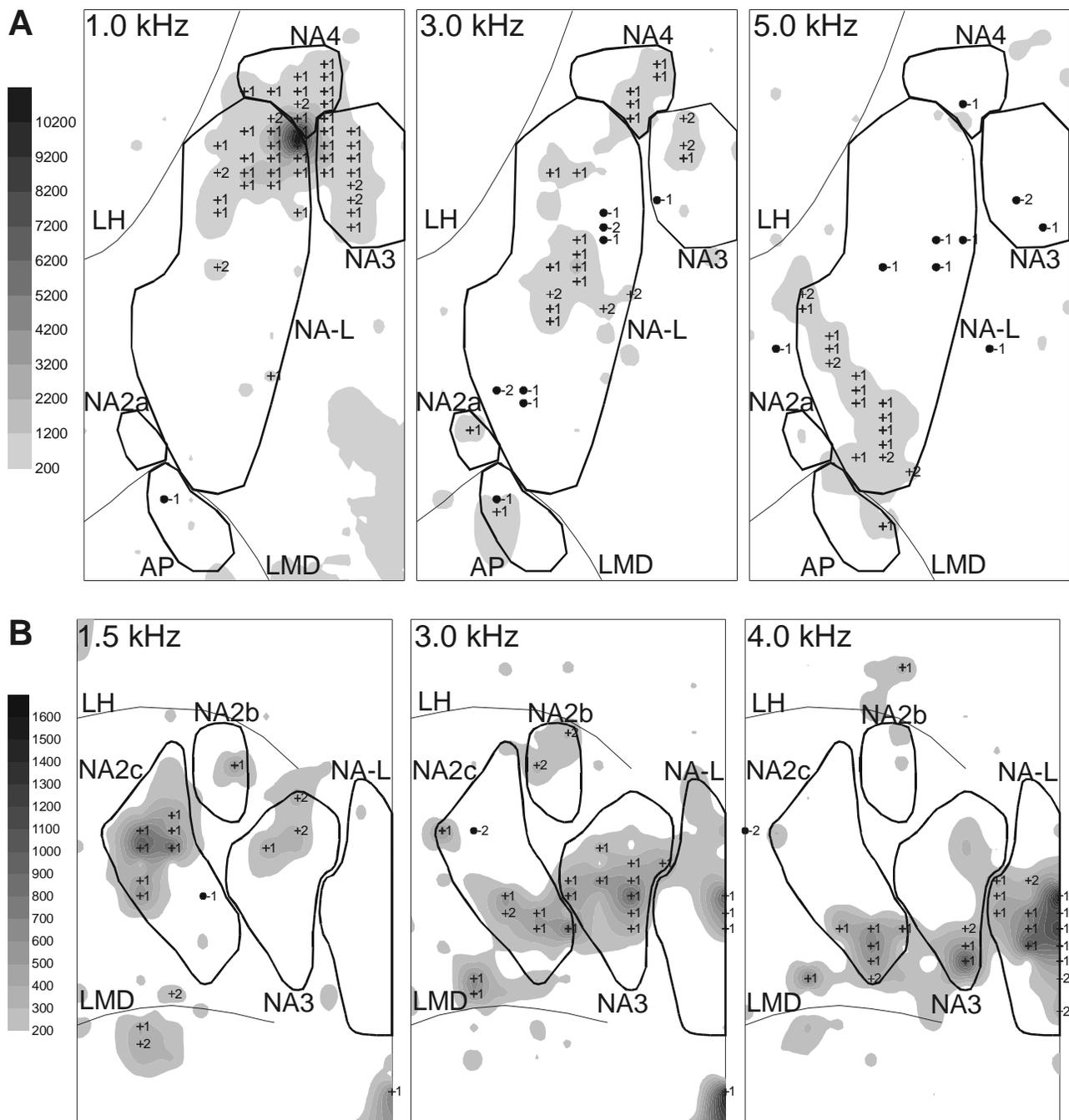


Figure 7. Response areas in the field L complex of the left hemisphere. The distance between two adjacent recording sites is 80 μm in horizontal and 150 μm in vertical direction. For further details see Figure 5. (A) This sagittal plane is located 0.6 mm lateral to the RP in the bird WT1. It contains the areas NA-L, NA2a, NA3 and NA4 with their onset responses to 1.0, 3.0 and 5.0 kHz. Responses in the auditory palaeostriatum augmentatum (AP) can be seen ventral to the LMD. (B) This frontal plane lies 1.25 mm frontal to the RP containing the auditory centres, NA-L, NA2b, NA2c and NA3 and their on-responses to 1.5, 3.0 and 4.0 kHz. It was recorded in the bird AT3. Responses in the palaeostriatum augmentatum can be seen ventral to the LMD.

NA2c is situated lateral to NA2b. The tonotopic gradient is equally orientated like that of NA-L, namely from caudodorsal to rostroventral. The response behaviour and the spectrum of responded pure tones of NA2c are similar to that of NA2b, apart from the missing off-response at the end of the stimuli.

NA3 is positioned caudal to NA-L. It extends rostral by surrounding NA-L laterally. The tonotopic gradient shows a similar orientation to that of NA-L. NA3 neurones respond to pure tones with phasic on- plus sustained excitation with rare sustained inhibition. Neurones in NA3 respond to frequencies between 0.5 and 5.0 kHz.

NA4 is lying caudodorsal to NA-L and could only be found in two medially situated, sagittal recording planes. The tonotopic gradient runs in the opposite direction to that of NA-L. The response behaviour is mainly a phasic on-excitation. Frequencies that are responded by excitations range between 0.5 and 5.0 kHz.

Additionally to the neuronal responses in the neostriatum it was possible to record significant responses at six recording sites in the auditory part of the palaeostriatum augmentatum ventral to the lamina medullaris dorsalis. A tonotopic gradient could not be defined as well as a regular response behaviour.

There were also significant auditory responses in parts (8 recording sites, GP2) of the hyperstriatum ventrale (HVA), dorsal of the lamina hyperstriatica. The response behaviour to pure tones in that brain region is an on-excitation, followed by rare excitations in the second time bin of the histogram and accompanied by sustained inhibition. A tonotopic gradient is not obvious, because there are only excitations to high frequencies of the stimulus ensemble (4.0, 5.0 kHz). These areas, which do not lie in the neostriatum will not be considered for further analysis of song data.

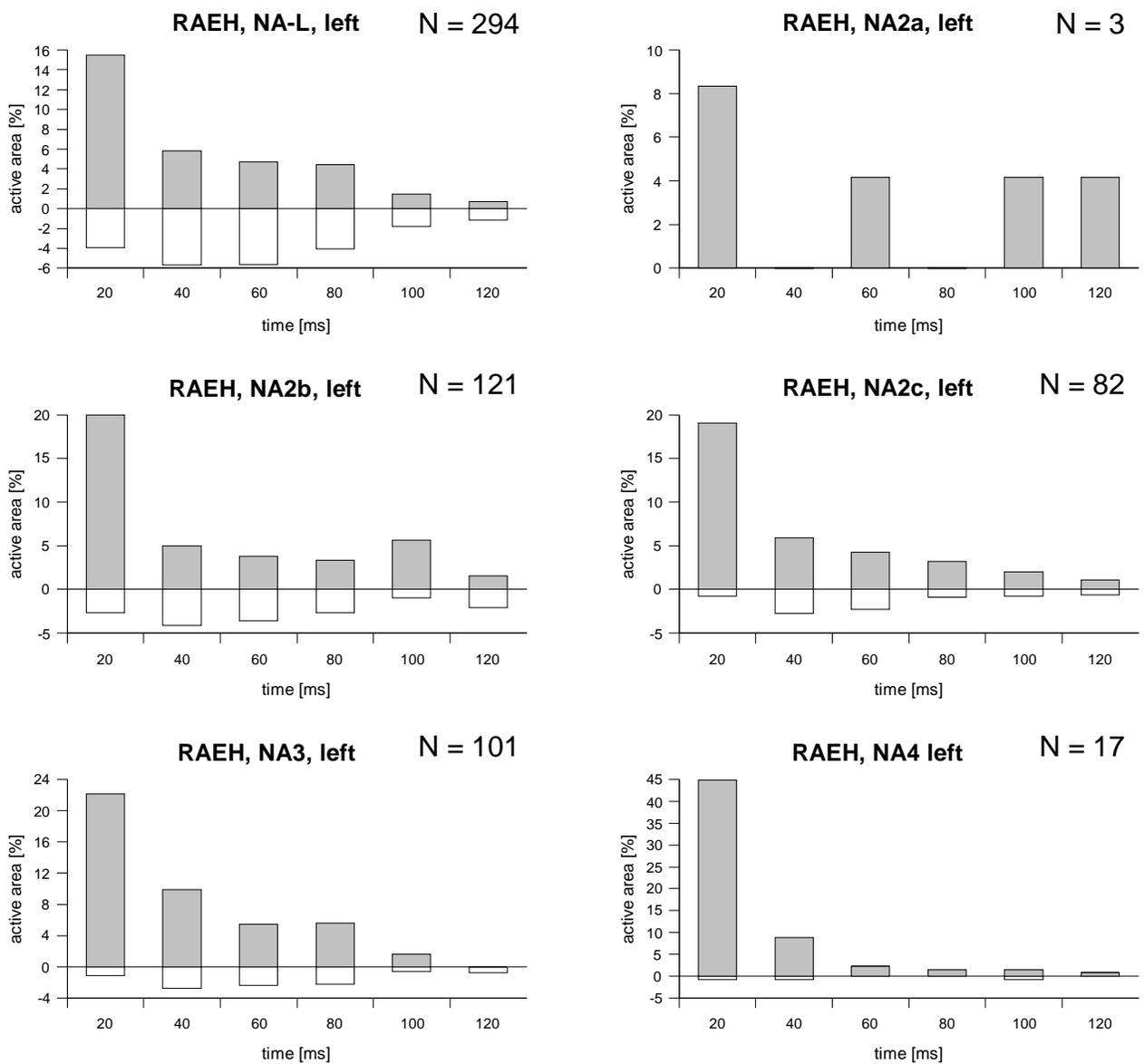


Figure 8. Response Area Extension Histograms of the left hemisphere. Neuronal activity to pure tones is shown for each area in 20 ms time bins. Excitation: grey bars, inhibition: white bars. The pure tone stimuli had a duration of 80 ms. See text for further details.

3.2.1.2. Right hemisphere

All functional areas that are present in the left hemisphere can be found in the right hemisphere (Figure 9). An exception may be NA4, which could not be seen in any of the recording planes. Since this area is only found in the most medially situated sagittal planes in the left hemisphere, it most probably exists in the right hemisphere, too. It was just not covered by the recording grid.

The response behaviours (Figure 10) and tonotopic gradients of the areas in the right hemisphere are the same as in the left hemisphere (compare chapter 3.2.1.1.). Each area responds with excitations to the complete spectrum of presented pure tone stimuli (0.5 – 5.0 kHz).

NA2a is only present in one recording plane, like in the left hemisphere, with 29 active recording sites.

Auditory responses can be seen at 39 recording sites in two planes in the area HVA. The response behaviour to pure tones is an on-excitation, followed by rare sustained excitation and accompanied by sustained inhibition. Neuronal excitations occur to all pure tone frequencies of the stimulus ensemble. The tonotopic gradient has the same orientation as that of NA-L, namely from caudodorsal to rostroventral.

Due to the analogousness of the two hemispheres concerning response behaviour and relative positions of the areas, the data of both will be pooled for further analysis. Consequently there is a total of 14 recording planes in adult birds.

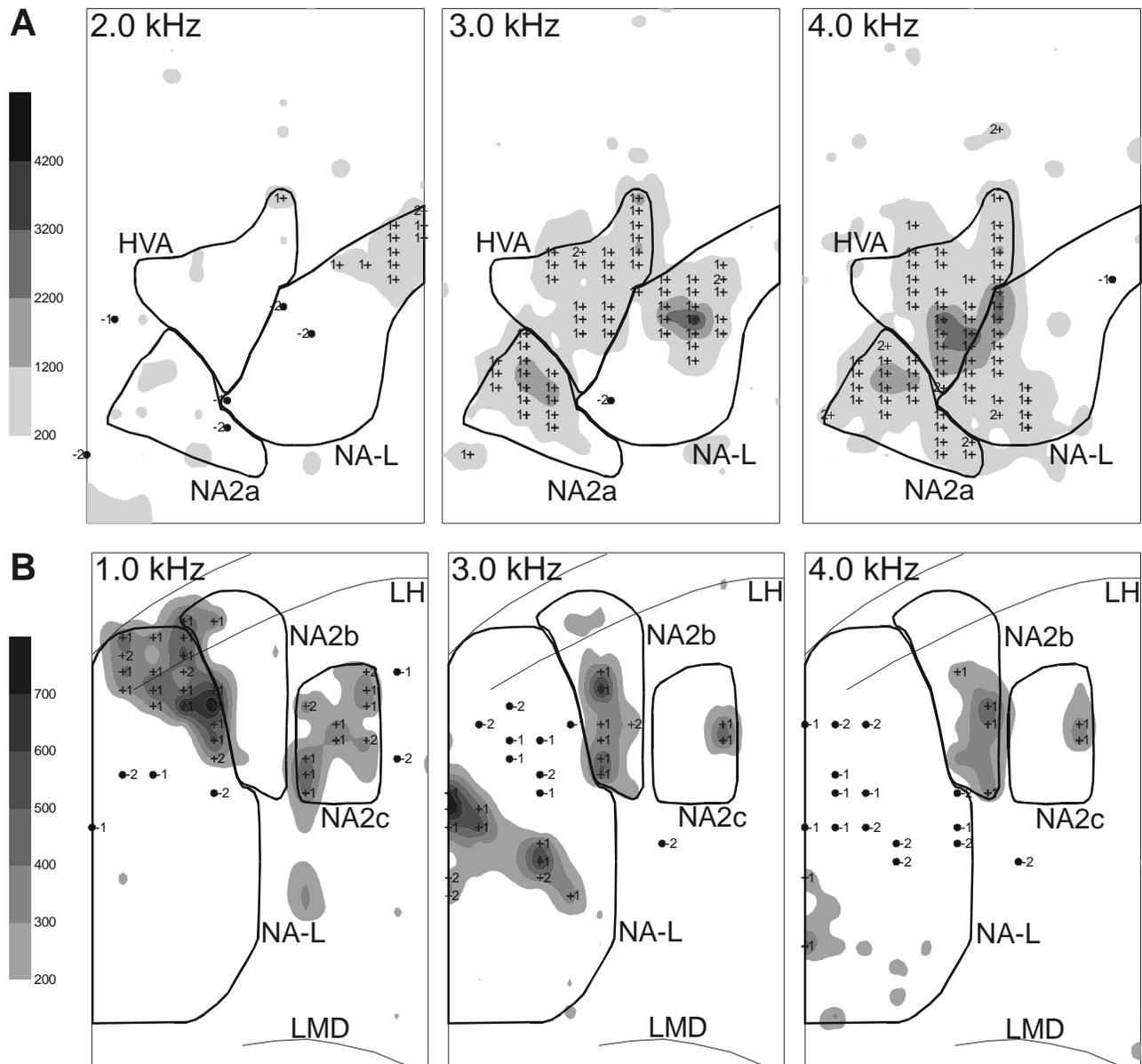


Figure 9. Response areas in the field L complex of the right hemisphere. For details see Figure 5. The distance between two adjacent recording sites is 80 μm in horizontal and 150 μm in vertical direction. (A) This sagittal plane in the bird VT1 is located 0.6 mm lateral of the RP. It contains the areas NA-L, NA2a and HVA (which is situated in the hyperstriatum ventral to the LH) with their onset responses to 2.0, 3.0 and 4.0 kHz. Histology could not be reproduced for that recording plane. (B) Frontal plane 1.5 mm (bird VT4) rostral of the RP containing the auditory centres, NA-L, NA2b and NA2c and their on-responses to 1.0, 3.0 and 4.0 kHz.

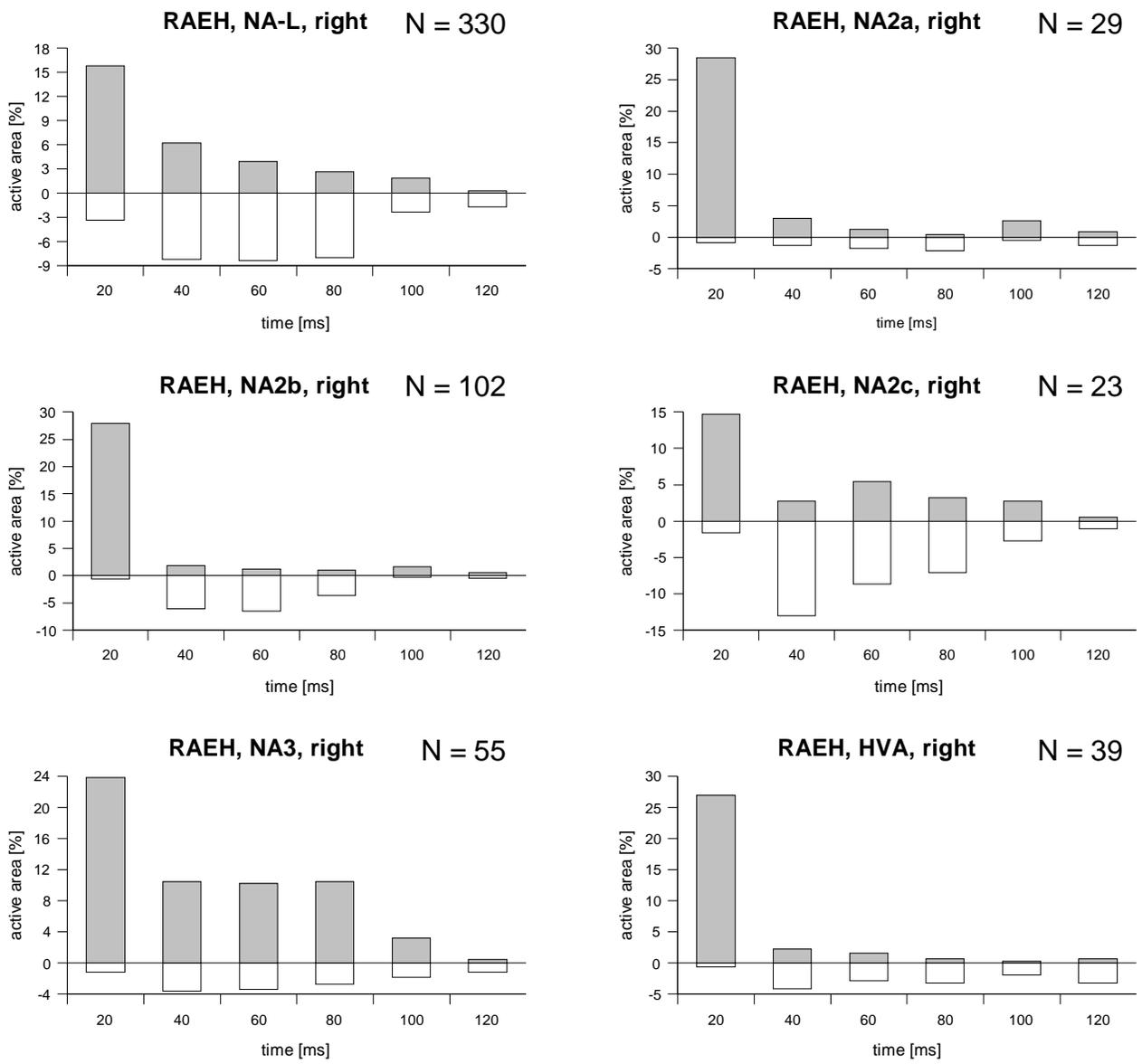


Figure 10. Response Area Extension Histograms of the right hemisphere. Neuronal activity to pure tones is shown in 20 ms time bins. Excitation: grey bars, inhibition: white bars. The pure tone stimuli had a duration of 80 ms. Please note that there is no area NA4. The RAE histogram of the area HVA is implemented into the figure instead. See text for further details concerning response behaviours.

3.2.1.3. Functional organisation of symmetrically recorded planes

Data were recorded in six pairs of symmetrical recording planes (Table 1, Figure 6B). A single pair consisted of one plane in the left hemisphere and one plane in the right hemisphere of one experimental bird. For the entire functional organisation of the mentioned recording planes see Table 2. The areas appear in the following ratio: NA-L:NA2a:NA2b:NA2c:NA3:NA4 = 4:1:4:3:4:2 (left) and 5:1:4:2:2:0 (right hemisphere). No recording plane pairs with a completely identical composition of functional areas could be found. The closest correspondence identified within one pair was the missing of one functional area in hemisphere A, which was in contrast present in the area make-up of hemisphere B. Examples for that are the three frontal planes. It was also possible to find one pair with absolutely different area compositions, which are the sagittal planes of the bird AT5/BT5. An intermediate state between those two possibilities showed up in the two other sagittal plane pairs, which contain identical and different functional areas in both hemispheres (compare Figures 7A and 9A).

NA-L is the most prominent functional area. It can be seen in nine of the twelve recording planes. This is consistent with the data about the area sizes (see below). The areas NA2a, NA4 and HVA are only present in the most medially located planes

<i>Position of the plane pair</i>	<i>Areas in left hemisphere</i>	<i>Areas in right hemisphere</i>
1.0 mm frontal (AT6)	NA-L, NA2b, NA2c	NA2b, NA2c
1.3 mm frontal (AT3)	NA-L, NA2b, NA2c, NA3	NA-L, NA2b, NA3
1.5 mm frontal (WT4)	NA-L, NA2b	NA-L, NA2b, NA2c
0.6 mm sagittal (WT1)	NA-L, NA2a, NA3, NA4	NA-L, NA2a, HVA
0.8 mm sagittal (AT4)	NA2b, NA3, NA4	NA-L, NA3, HVA
1.2 mm sagittal (AT5)	NA2c, NA3	NA-L, NA2b

Table 2. Functional areas in six pairs of symmetrical recording planes in the two hemispheres. In the column indicating the position, the nomenclature of the left hemisphere is additionally mentioned. Data of one recording plane pair were recorded in a single subject.

- Functional organisation in wild-type and albino-type birds

Data were recorded in six planes of four grey, wild-type individuals and eight planes of four white, albino-type birds (Table 1, Figure 6B). All functional areas, except the area NA2a, were found in the wild-type (WT), as well as in the albino-type (AT) birds (Table 2).

NA2a is located medially and frontally in the field L complex. It can only be seen in the 0.6 mm sagittal planes of WT1 and VT1, or the 0.2 mm and 0.4 mm sagittal, resp. 2.2 mm and 2.4 mm frontal planes of the bird GP2. There are no planes of a similar position in the albino-type birds (compare Figure 6B).

3.2.1.4. Size of the field L complex

The number of active recording sites per area is defined by responses to pure tones of AT, BT, VT and WT birds. Normalised sizes of the areas are calculated by pooling the mean number of recording sites per plane and multiplying them with the size of one two-dimensional grid unit ($A_{GU} = 0.080 * 0.150 \text{ mm}^2 = 0.012 \text{ mm}^2$) (Table 3).

NA-L consists of 294 recording sites in the left hemisphere. This area could be found in six out of eight recording planes (49.0 recording sites per plane). In the right hemisphere, there are 330 recording sites in five planes (66.0 sites/ plane). This results in a pooled normalised size of 56.7 sites per plane or 0.680 mm^2 per plane.

As already mentioned above, NA2a is a rather small area, which was only found in one plane per hemisphere. There are 3 recording sites in the left and 29 recording sites in the right hemisphere. Pooled data are 16.0 sites per plane and a normalised size of 0.192 mm^2 .

NA2b was found in five recording planes with 121 recording sites responding to pure tones (24.2 recording sites per plane) in the left hemisphere. In the right hemisphere, there are 102 recording sites in four planes (25.2 sites/ plane). Pooled data are 24.8 sites per plane and a normalised size of 0.298 mm^2 .

NA2c was analysed in three planes with 82 recording sites, responding to pure tones (27.3 recording sites per plane) in the left hemisphere. There are 23 recording sites in two planes in the

right hemisphere (11.5 sites/ plane). Pooled data are 21.0 sites per plane and a normalised size of 0.252 mm².

NA3 could be found in four planes with 101 recording sites (25.3 recording sites per plane). The right hemisphere shows up with 55 recording sites in two planes (27.5 sites/ plane). Pooled data are 26.0 sites per plane and a normalised size of 0.312 mm².

Neurons of NA4 are active at 17 recording sites in two planes (8.5 recording sites per plane). No responses were found in the left hemisphere. The normalised size is 0.102 mm².

Area sizes between the two hemispheres are called similar, if the mean hemispherical values do not differ by more than 20% from the overall pooled number of recording sites. This is true for the areas NA-L, NA2b and NA3. These areas have in common that they were found in at least six recording planes with more than 150 recording sites (in both hemispheres).

A total of 1158 recording sites show significant responses to pure tones and can be assigned to a functional area. This is almost a fourth of all recording sites (4557). The numbers of responding recording sites to pure tones were compared with the responses to harmonic complexes and song (Table 4).

Since the complete field L complex of one bird was mapped in the subject GP2 in a three-dimensional grid, it is possible to estimate the volumes of single functional areas. Volumes of the areas in the bird GP2 were estimated by multiplying the number of grid points (i.e. the total number of recording sites) per area with the volume of one grid unit ($V_{GU} = 0.100 * 0.200 * 0.200 \text{ mm}^3$) (Table 3). The complete field L complex of the left hemisphere in the bird GP2 has therefore an estimated volume of 0.888 mm³ including the area HVA in the hyperstriatum. NA-L and NA3 are the largest areas with a volume bigger than 0.200 mm³. The field L complex extends up to 4500 μm below the brain surface.

<i>Area</i>	<i>Left hemisph.</i>	<i>Right hemisph.</i>	<i>Mean number of grid points</i>	<i>Normalised size [mm²]</i>	<i>Number of grid points</i>	<i>Volume [mm³]</i>
NA-L	49.0 (6)	66.0 (5)	56.7	0.680	64	0.256
NA2a	3 (1)	29 (1)	16	0.192	10	0.040
NA2b	24.2 (5)	25.2 (4)	24.8	0.298	28	0.112
NA2c	27.3 (3)	11.5 (2)	21	0.252	31	0.124
NA3	25.3 (4)	27.5 (2)	26	0.312	56	0.224
NA4	--	8.5 (2)	8.5	0.102	25	0.100
HVA	--	--	--	--	8	0.032

Table 3. Normalised sizes and volume estimates of the different functional areas. Relative area sizes of the different hemispheres are shown in the first 2 data columns (number of planes is shown in brackets). Normalised sizes result from pure tone responses in AT, BT, VT and WT birds. Extension of a grid unit: $A_{GU} = 0.080 * 0.150 \text{ mm}^2$. Volume estimates result after mapping the complete field L complex of the bird GP2. NA-L and NA3 are the largest areas with individual volumes $> 0.2 \text{ mm}^3$. Volume of a grid unit: $V_{GU} = 0.100 * 0.200 * 0.200 \text{ mm}^3$.

3.2.2. Responses to harmonic complexes

Harmonic complexes consist of a fundamental (1.0 or 2.0 kHz) and four harmonics. The fundamental could be included and removed from the complex. They were used for stimulation, to look for neuronal correlates of pitch detection ability by the aid of harmonics above the (missing) fundamental. Additionally, harmonics are an important feature of zebra finch song.

There is a total of 1052 recording sites showing significant responses to harmonic complexes. The exact distribution among the different functional areas can be seen in Table 4. This is a significantly lower number than the number of responding sites to pure tones (1158) (Wilcoxon, $p = 0.043$). The numbers of responding recording sites to harmonic complexes were additionally compared with the responses to song (Table 4).

The responses of recording sites, which only respond to 1.0 or 2.0 kHz and no higher frequencies of the pure tone stimulus ensemble, to the high-pass filtered harmonic complexes were analysed in further detail (Figure 11). This was done to search for neuronal clusters in the auditory cortex analogue of zebra finches, which make it possible for the individual bird to perceive the pitch of the fundamental only by harmonics. This phenomenon is well known in psychophysics for

songbirds (Cynx and Shapiro, 1986) and humans (Seebeck, 1841; Schouten, 1938; both after Cynx and Shapiro, 1986). In this study recording sites were searched for, which respond to pure tones of 1.0 and 2.0 kHz. These sites are marked in the figure by a diamond, which is numbered above with a 1 (1.0 kHz) or 2 (2.0 kHz). They were tested with comparison maps for significant responses to the high-pass filtered harmonic complexes, which do not contain the fundamental of 1.0 or 2.0 kHz respectively. These sites are marked by a cross, numbered below with 1 (1.0 kHz missing) or 2 (2.0 kHz missing). Numbered combinations of interest are consequently 1 (above) – 1 (below), 2 – 2, 1 – 2, 1 – 12.

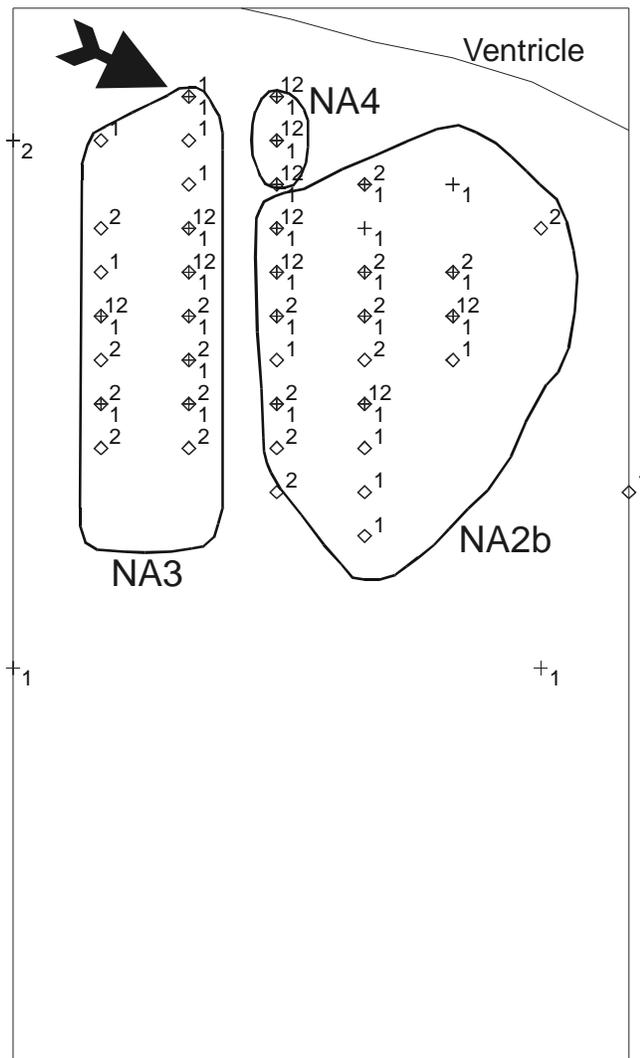


Figure 11. Comparison of responses to harmonic complexes. An example map of the comparison of 1.0 and 2.0 kHz pure tone stimuli with high-pass filtered harmonic complexes is presented. It is a sagittal plane lying 0.8 mm lateral to the RP in the bird AT4. Recording sites that respond to a pure tone with an excitation are marked with a diamond and a number above that diamond indicating the frequency eliciting the response. Recording sites that respond to a high-pass filtered harmonic complex with an excitation are marked with a plus and a number below that plus indicating the fundamental frequency of the harmonic complex eliciting the response. Only recording sites with the number combinations: 1 (above) - 1 (below), 2 - 2, 1 - 2 or 1 - 12 are of interest. One recording site in that map shows that feature. It is highlighted by the arrow. This sites does not respond to higher pure tone frequencies and lies directly at the border of the area NA3. The areas NA2b and NA4 were also found in that plane. The distance between two adjacent recording sites is 80 μm in horizontal and 150 μm in vertical direction.

There are twelve recording sites with these properties that enable them to perceive the pitch of a missing fundamental by the help of harmonics. This number of sites is 1.14% of all active 1052 recording sites. The recording sites are distributed among the functional areas as follows: seven

recording sites in NA-L; NA2b and NA2c each with two recording sites; and one recording site in NA3.

It is noticeable that eight of those twelve recording sites (75%) are located exactly at the boundaries of the areas. The remaining four sites are situated one recording site away from the borders. The positioning of the sites at the periphery of the functional auditory areas is obvious. These twelve recording sites are located between 0 to 150 μm away from the areas' borders.

3.2.3. Responses to song

It is reasonable to assume that the auditory cortex analogue of songbirds is optimised by evolution to perceive stimuli of the birds' natural, auditory environment, which are behaviourally relevant. Birdsong is an important component of that environment. Therefore, it is of some interest of how the field L complex in the caudal neostriatum of the zebra finch is able to code song in general, and bird's own song (BOS), tutor song (TUT) and conspecific song (CON) in particular. Differences and similarities in coding between the various song types, which originate from different individuals, and their time-reversed forms give an insight into the ability to discriminate between individuals on this level of the auditory pathway.

There is a total of 1539 recording sites showing significant responses to the natural song stimuli of the stimulus ensemble. The exact distribution among the different functional areas can be seen in Table 4. There is an overall significant difference between the data sets of all three stimulus conditions (Friedman, $p = 0.003$). A significantly higher number of recording sites responds to song than to pure tones (1158) (Wilcoxon, $p = 0.028$) and harmonic complexes (1052) (Wilcoxon, $p = 0.028$). Every functional area shows the largest extension under song stimulation and the smallest extension if harmonic complexes are used for stimulation. The numbers of recording sites responding to pure tones range between these minimum and maximum values for each area except NA2b. NA2b shows the same extension of 223 sites, when responding to pure tones and harmonic complexes.

<i>Area</i>	<i>Sites responding to pure tones</i>	<i>Sites responding to harmonic complexes</i>	<i>Sites responding to song</i>
NA-L	624	555	849
NA2a	33	25	47
NA2b	223	223	260
NA2c	105	85	174
NA3	156	150	242
NA4	17	14	21

Table 4. Number of significantly responding recording sites per functional area under the stimulus conditions: pure tones, harmonic complexes and song. There are significant differences between all data sets (Friedman, $p = 0.003$), as well as between all possible pairs of data sets (Wilcoxon, pure tones vs. harmonic complexes: $p = 0.043$, pure tones vs. song: $p = 0.028$, harmonic complexes vs. song: 0.028).

3.2.3.1. Data based on comparison maps

The analysis based on comparison maps was done to test song selectivities. A more detailed analysis, which is based on RAE histograms is presented in the chapter 3.2.3.2.

Responses to the different song types were analysed using comparison maps as already shown in Figure 11. A recording site was counted as responding to a stimulus, if at least one 20 ms time bin showed a significant excitation during that stimulus. The ratios of recording sites significantly responding to one song type and its time-reversed form, and to the song or its time-reversed form, exclusively, were calculated for all functional areas (Table 5). The time-reversed forms are marked by an “r” in front of the song type.

The average proportion of recording sites responding to the original songs and their time-reversed counterparts is 71% over all areas and song types. Whereas 16% and 12% of the recording sites respond exclusively to the original songs and their time-reversed counterparts, respectively. The exact relative distributions per area and song type can be seen in Table 5. The three columns BOS+rBOS, TUT+rTUT and CON+rCON were compared, as well as the three columns of the original songs and the three columns of the time-reversed songs. There is neither a difference between the first columns of the three song types (Friedman, $p = 0.115$), representing the sites that

do not respond selectively to the original or time-reversed stimuli, nor between the second columns, representing the recording sites, which respond exclusively to the original song (Friedman, $p = 0.438$). However, there is a significant difference between the third columns, representing the recording sites, which respond exclusively to the time-reversed forms (Friedman, $p = 0.032$). Areas NA2a and NA4 differ in their rate of responding sites from the the rest of the areas. NA2a shows a high proportion under rBOS (0.28) and rCON (0.31) stimulation and a low proportion under rTUT (0.05) stimulation. The average value of all areas is 0.12. NA4 shows no specialised recording sites responding exclusively to rBOS stimulation and high proportions of non-selective sites under BOS+rBOS (0.90) and TUT+rTUT (0.82) stimulus conditions.

Furthermore, the amount of recording sites responding exclusively to original song were compared with the number of those responding exclusively to the time-reversed form. No differences were found for any of the three song types (Wilcoxon, BOS vs. rBOS: $p = 0.249$, TUT vs. rTUT: $p = 0.104$, CON vs. rCON: $p = 0.345$).

The ratio of the three response possibilities (original song + reversed song, original song, reversed song) was compared between the different functional areas for the three song types. There is a significant difference between the areas under BOS (chi-square, $p < 0.001$) and CON (chi-square, $p = 0.004$) stimulation. Under BOS stimulation, the areas NA2a and NA4 differ significantly from the all the other areas (chi-square, $p < 0.05$). Under CON stimulation, the area NA2a differs significantly from the rest of the areas (chi-square, $p < 0.05$). NA2a and NA4 are both the smallest areas under song stimulation with 47 and 21 active recording sites (in two planes each) responding to song, respectively.

<i>Area</i>	<i>BOS+rBOS</i>	<i>BOS</i>	<i>rBOS</i>	<i>TUT+rTUT</i>	<i>TUT</i>	<i>rTUT</i>	<i>CON+rCON</i>	<i>CON</i>	<i>rCON</i>
NA-L	0.77	0.14	0.09	0.73	0.13	0.14	0.75	0.13	0.12
NA2a	0.55	0.17	0.28	0.73	0.23	0.05	0.51	0.19	0.31
NA2b	0.70	0.21	0.09	0.75	0.13	0.12	0.71	0.19	0.10
NA2c	0.73	0.16	0.10	0.71	0.18	0.11	0.66	0.21	0.13
NA3	0.75	0.14	0.11	0.68	0.17	0.15	0.67	0.18	0.15
NA4	0.90	0.10	0	0.82	0.09	0.09	0.74	0.16	0.11

Table 5. Ratios of recording sites responding to the three different song types and their time-reversed forms. The first column of each song type indicates the ratio of recording sites responding to both the song and its time-reversed form. The second and third column of each song type indicate the ratio of recording sites responding exclusively to the song or its time-reversed form, respectively.

Similar comparisons were made for the ratios between the responses to BOS+TUT, BOS and TUT (Table 6). The average proportion of recording sites responding to the BOS and TUT counterparts is 68% over all areas. Whereas 16% and 15% of the recording sites respond exclusively to the BOS and the TUT, respectively. This is about the same ratio between song-selective and non-selective recording sites as before (71%, 16%, 12%), when comparing original with time-reversed forms. There is no difference between the amount of recording sites responding selectively to BOS and TUT (Wilcoxon, $p = 0.498$).

Finally, the ratio of the three response possibilities (BOS+TUT, BOS and TUT) was compared between the different functional areas. Only the smallest area NA4 with 21 active recording sites in two planes showed significant differences to NA2a (chi-square, $p = 0.004$), NA2b (chi-square, $p = 0.005$), NA2c (chi-square, $p = 0.050$) and NA3 (chi-square, $p = 0.022$). Again, NA4 shows a high proportion of non-selective recording sites (0.82 vs. 0.68 average). All other area-area combinations do not differ (chi-square, $p > 0.05$). An overall significant difference could be determined (chi-square, $p = 0.028$).

<i>Area</i>	<i>BOS+TUT</i>	<i>BOS</i>	<i>TUT</i>
NA-L	0.71	0.15	0.14
NA2a	0.61	0.21	0.18
NA2b	0.62	0.23	0.14
NA2c	0.67	0.18	0.15
NA3	0.66	0.12	0.22
NA4	0.82	0.09	0.09

Table 6. Ratio of recording sites significantly responding in at least one 20 ms time bin to BOS and TUT, or BOS and TUT, exclusively.

Due to the similarities between various song types, their time-reversed forms, among the song types and their average ratios, this analysis was not performed for CON stimuli. A detailed analysis, including responses to CON stimuli, which is based on average response strength is shown in the following chapter.

3.2.3.2. Song analysis based on RAE histograms

Response Area Extension histograms with 20 ms time bins were calculated for the neuronal responses to original song stimuli. The time domain of histograms covers the complete natural stimulus duration plus five more time bins at the end to include possible responses after the stimulation. The percentage values of all time bins in every histogram were averaged. These averaged values were pooled for each functional area. Individual area sizes were considered during the pooling procedure. Finally, one value for excitation and one value for inhibition resulted for each area. CON data were acquired from three songs per experimental subject. For each song type and area, one value for excitation and one value for inhibition were achieved (Table 7). In contrast to the data based on simple comparison maps, the response distribution, which is coded here by the number of active recording sites over time, is determined.

<i>Area</i>	<i>BOS +</i>	<i>BOS -</i>	<i>TUT +</i>	<i>TUT -</i>	<i>CON +</i>	<i>CON -</i>	<i>Planes</i>
NA-L	10.78	-1.43	8.33	-1.21	9.76	-1.19	12
NA2a	11.45	-1.01	8.85	-1.92	7.36	-0.83	2
NA2b	9.22	-1.28	7.97	-1.18	8.67	-1.04	9
NA2c	8.00	-0.91	7.36	-0.93	6.47	-0.84	5
NA3	10.76	-0.60	10.57	-0.66	8.36	-0.75	6
NA4	11.11	-3.40	7.03	0	11.90	-0.05	2

Table 7. Average values of RAE histograms to the three song types BOS, TUT and CON. The average values of excitations (+) and inhibitions (-) are plotted in the column of the respective song type. The number of recording planes in which the functional areas were recorded are mentioned in the last column.

The excitations were compared between the three song types. Significant differences can be seen between the responses to all song types (Friedman, $p < 0.001$). This is a result of different excitations to BOS and TUT (Wilcoxon, $p < 0.001$), as well as BOS and CON (Wilcoxon, $p < 0.001$). The average multi unit excitations to BOS are greater than those to TUT or CON. Whereas there is no significant difference between the responses to TUT and CON (Wilcoxon, $p = 0.646$).

3.3. Juvenile birds

3.3.1. Field L complex of PHD 30 birds

3.3.1.1. Functional organisation

In the field L complex of PHD 30 birds, three different functional areas could be separated (Figure 12). At this age NA-L₃₀, NA2b₃₀ and NA2c₃₀ can be distinguished.

plane is located 0.8 mm lateral of the RP in the bird JT6. It contains the area NA-L₃₀ and its onset responses to 1.5, 3.0 and 5.0 kHz.

In response maps based on PST-histograms of neural responses, NA-L₃₀ shows the largest extension and covers Rose's anatomically defined field L (Rose, 1914). The tonotopic gradient from low to high frequencies runs from caudodorsolateral to rostroventromedial. Neurons in NA-L₃₀ respond to pure tones with a phasic on-response, followed by sustained excitation and spatially accompanied by sustained inhibition (Figures 12B, 13A). Inhibitory sidebands, dorsal and ventral to the excited area, are a characteristic feature of NA-L, as it is seen in adult birds.

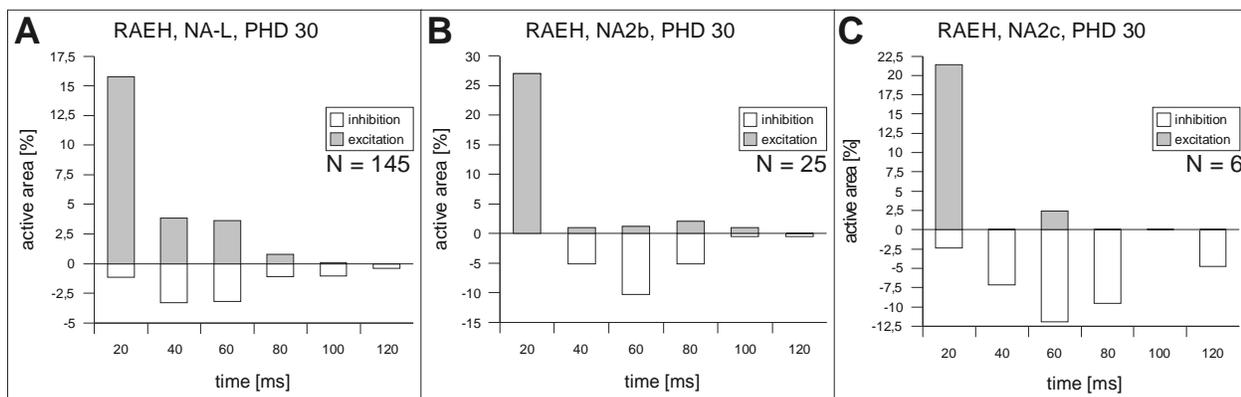


Figure 13. Response Area Extension Histograms of PHD 30 birds. Neuronal activity to pure tones is shown in 20 ms time bins for each area. Excitation: grey bars, inhibition: white bars. (A) NA-L₃₀ shows a wide distribution of on-response plus sustained excitation and is also clearly inhibited. (B) NA2b₃₀ is characterised by phasic on-response followed by sustained inhibition. (C) NA2c₃₀ is characterised by phasic on-response followed by sustained inhibition, like NA2b₃₀. The pure tone stimuli had a duration of 60 ms.

NA2b₃₀ is positioned caudodorsolateral to the area NA-L₃₀ and shows a tonotopic gradient, which runs from medial to lateral. The responses within NA2b₃₀ are characterised by an on-excitation with subsequent inhibition (Figure 13B). These two areas respond to all pure tones presented, i.e. 1.0 to 5.0 kHz.

NA2c₃₀ was only found in one frontal plane, consisting of only six recording sites, and is situated lateral to NA2b₃₀ with no definite tonotopic gradient, which is a result of the orientation of the recording plane. The response behaviour is similar to that of NA2b₃₀ (Figure 13C). NA2c₃₀ in this plane responds only to pure tone frequencies between 1.0 and 2.5 kHz. However, this is a clear

sign for the existence of a tonotopy, which extends perpendicular to the recording plane. Due to the small number of recording sites in that area, a further analysis of responses to song was not performed.

Resulting normalised area sizes based on pure tone responses can be seen in Table 10. The size of a grid unit is $A_{GU} = 0.100 * 0.200 \text{ mm}^2$. As in adults, NA-L is the largest area. The normalised sizes are also compared in the Table 10 with those of the adult and PHD 60 birds.

Latency times of NA-L₃₀ and NA2b₃₀ ranged from 10 to 14 ms and 11 to 13 ms, respectively. NA2c₃₀ responds with an onset latency of 13 ms.

Not all pure tone frequencies could be expected to be represented in each functional area and recording plane, because the orientation of tonotopies and the position of the plane did not match in each case.

The diamond in the 5.0 kHz maps shows the probable position of NA3, which appears at this position in PHD 60 and adult birds. The asterisk in the bottom 5.0 kHz map indicates the location of HVA in the hyperstriatum of adult birds.

3.3.1.2. Responses to song

All recording sites were investigated for neuronal activation by the song of the individual bird's tutor (TUT) and the song of another conspecific (CON), using comparison maps (Figure 14A) and averaged RAE histograms (Table 8). A recording site was defined to be excited by one stimulus in the comparison maps, if at least one 20 ms time bin showed significant excitation.

Neurones at 65% of the recording sites in NA-L₃₀ responded to both stimuli. 4% responded only to stimulation by TUT and 31% to stimulation by CON, exclusively. In NA2b₃₀, neurones at 74% of the active recording sites responded to both song stimuli. Neurones at 10% of the sites responded only to stimulation by TUT and 16% only to stimulation by CON.

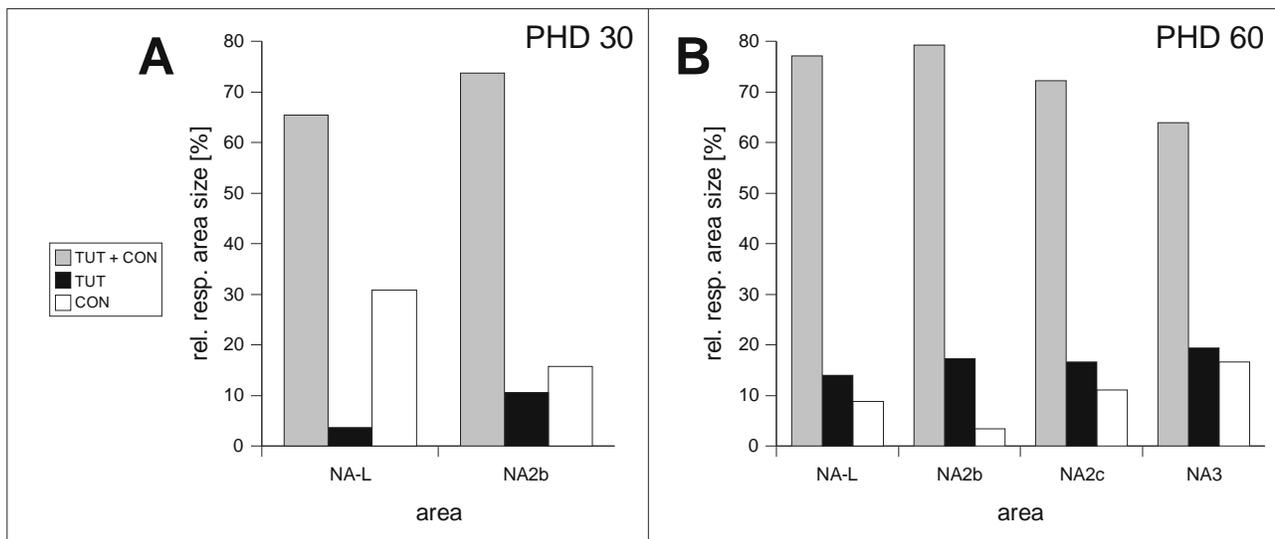


Figure 14. (A) Responses to song at different recording sites of PHD 30 birds, N (NA-L₃₀) = 126, N (NA2b₃₀) = 21. (B) Responses to song of PHD 60 birds, N (NA-L₆₀) = 211, N (NA2b₆₀) = 30, N (NA2c₆₀) = 21, N (NA3₆₀) = 41. The amount of recording sites responding to TUT and CON stimuli is indicated by grey bars. The amount of recording sites responding only to TUT or only to CON is specified by black and white bars, respectively.

In both areas, neurones at about two thirds of the recording sites, responding to song stimuli in comparison maps, do not distinguish between the song of the tutor and the song of a conspecific male. Similar values can be seen in adult comparison map data, when comparing the responses to BOS and TUT (Table 6).

Response Area Extension histograms with 20 ms time bins were calculated for the neuronal responses to natural song stimuli. The percentage values were averaged as in adult birds (Table 8). The excitations were compared between the two song types. Significant differences can be seen between the responses to the song types (Wilcoxon, $p = 0.015$). The average responses to CON are higher than the average responses to TUT. The major cause for these findings may be the combination of the stimulus ensembles and the responded frequency ranges of the different recording planes. These facts will be discussed below.

<i>Area</i>	<i>TUT +</i>	<i>TUT -</i>	<i>CON +</i>	<i>CON -</i>	<i>Planes</i>
NA-L ₃₀	5.48	-1.21	10.31	-1.43	5
NA2b ₃₀	10.18	-3.57	14.93	-1.59	2
NA2c ₃₀	2.92	-0.42	0.83	-2.50	1

Table 8. Average values of RAE histograms to the two song types TUT and CON. The average values of excitations (+) and inhibitions (-) are plotted in the column of the respective song type. The number of recording planes in which the functional areas were recorded are mentioned in the last column.

3.3.1.3. Size of the PHD 30 field L complex

In NA-L₃₀, there are 145 active recording sites in five recording planes (29 sites/ plane) responding to pure tones. 126 recording sites show responses to song stimuli (25.2 sites/ plane). The area NA2b₃₀ consists of 25 active recording sites in two recording planes (12.5 sites/ plane), which respond to pure tones. 21 sites in that area are significantly excited by natural stimuli (11.5 sites/ plane). NA2c₃₀ is only found in one recording plane with six recording sites responding to tones and eight sites responding to song. No significant differences in area size were found between the two stimulus situations (Wilcoxon, $p = 0.324$). In chapter 3.4 and Table 10, the average area sizes, based on pure tones are compared with the two other age groups.

3.3.2. Field L complex of PHD 60 birds

3.3.2.1. Functional organisation

In this age group, there are five different areas shaping the functional field L complex in the neostriatum (Figure 15). The areas NA-L₆₀ (recorded in 4 planes), NA2b₆₀ (3 planes) and NA2c₆₀ (2 planes) were present as in PHD 30 birds. NA2a₆₀ (2 planes) and NA3₆₀ (2 planes) appeared as new areas and had not been found in the younger age group.

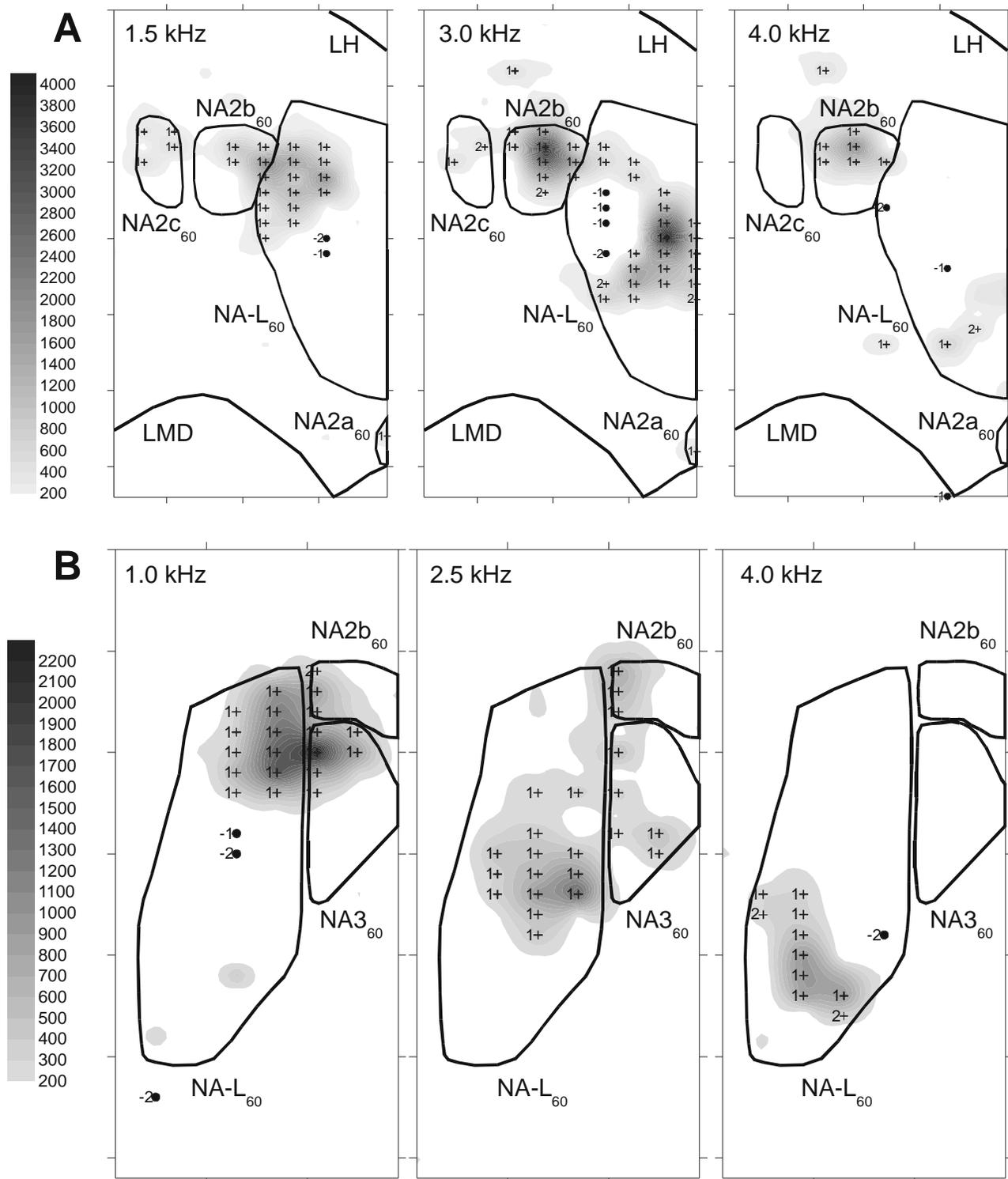


Figure 15. Response areas in the field L complex of PHD 60 birds. For details see Figure 5. (A) This frontal plane is located 1.5 mm rostral of the RP in the bird JT8. It contains the areas NA-L₆₀, NA2a₆₀, NA2b₆₀ and NA2c₆₀ with their onset responses to 1.5, 3.0 and 4.0 kHz. The tonotopic gradients of NA2b₆₀ and NA2c₆₀ are orientated perpendicular to the recording plane and are not visible. (B) This sagittal plane lies 0.4 mm lateral of the RP in the bird JT9. It contains the auditory centres, NA-L₆₀, NA2b₆₀ and NA3₆₀ and their on-responses to 1.0, 2.5 and 4.0 kHz.

NA-L₆₀ is also the largest area and covers Rose's field L (Rose, 1914). The tonotopic gradient runs from caudodorsolateral to rostroventromedial. NA-L₆₀ neurones respond to pure tones with a phasic on-response, followed by sustained excitation. Spatially they are accompanied by neurones that respond with sustained inhibition to the pure tone stimuli (Figures 15, 16A). So inhibitory areas are surrounding an excitatory region. Latency times of NA-L₆₀ range from 9 to 15 ms.

NA2a₆₀ is positioned rostroventral to NA-L₆₀. This area was found in only two planes with five active recording sites. On this data basis, a definition of the tonotopic gradient was not possible. The neuronal response behaviour is a weak on-response followed by sustained excitation. A further analysis of responses to song was not performed.

NA2b₆₀ is situated caudodorsolateral to the area NA-L₆₀. Its tonotopic gradient runs from medial to lateral. The response behaviour within NA2b₆₀ is characterised by a phasic on-response with a weak subsequent inhibition (Figure 16B). Responses occur after a latency time of 11 – 18 ms. Neurones of these three areas respond to the complete frequency range of the pure tone stimuli (1.0 – 5.0 kHz).

NA2c₆₀ is situated lateral to NA2b₆₀. A tonotopy is not obvious due to the frontal orientation of the recording planes, in which this area was discovered. The pure tones eliciting a phasic on- plus off-response together with sustained inhibition (Figure 16C) are in the frequency range between 1.0 and 4.0 kHz (latency times: 11 ms, 12 ms). Again, this is a sign for an existing tonotopy, which extends perpendicular to the frontally orientated recording planes.

NA3₆₀ is positioned caudal to NA-L₆₀. It extends rostral by surrounding NA-L₆₀ laterally. The tonotopic gradient shows a similar orientation to that of NA-L₆₀. NA3₆₀ neurones respond to pure tones with phasic on- plus sustained excitation with rare sustained inhibition (Figure 16D). Neurones in NA3₆₀ respond to frequencies between 1.0 and 5.0 kHz after a latency time of 14 – 16 ms.

Resulting normalised area sizes based on pure tone responses can be seen in Table 10. The size of a grid unit is $A_{GU} = 0.100 * 0.200 \text{ mm}^2$. As in adults, NA-L and NA3 are the largest areas of the field L complex. The normalised sizes are also compared in the Table 10 with those of the adult and PHD 30 birds.

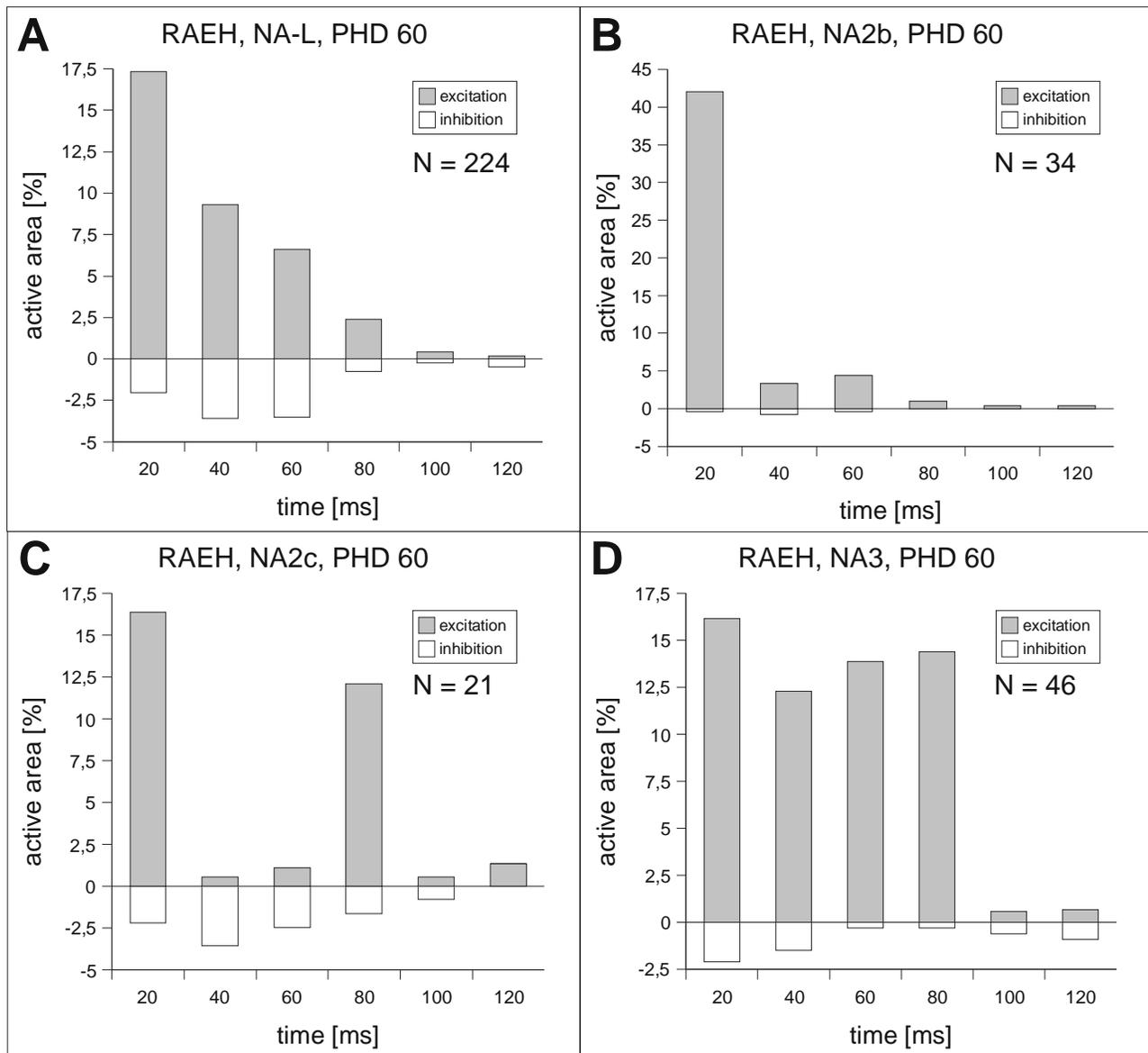


Figure 16. Response Area Extension Histograms of PHD 60 birds. Histograms were calculated according to the neuronal responses to pure tones using 20 ms time bins. Excitation: grey bars, inhibition: white bars. (A) NA-L₆₀ shows on-responses plus sustained excitation and is also clearly inhibited. N = 224 recording sites responded to pure tones. (B) NA2b₆₀ is characterised by phasic on-responses. N = 34. (C) NA2c₆₀ exhibits phasic on- and off-responses, accompanied by sustained inhibition. N = 21. (D) NA3₆₀ responds similar to NA-L₆₀ with less inhibitory and more excitatory recording sites after the on-response. N = 46. As the pure tone stimuli had a length of 60 ms, responses appearing in the time class 80 ms are defined as off-responses. NA2a₆₀ is not included due to a lack of sufficient recording sites.

3.3.2.2. Responses to song

All recording sites were investigated for responses to TUT and CON stimuli (Figure 14B) as it was done in PHD 30 birds. In 77% of the recording sites in NA-L₆₀ neurones respond to both stimuli. 14% respond only to stimulation by TUT and 9% only to stimulation by CON. In NA2b₆₀, neurones at 79% of the recording sites respond to both song stimuli. Neurones at 17% of the sites respond to stimulation by TUT and 4% to stimulation by CON, exclusively. In the area NA2c₆₀ 72% of the recording sites respond to both stimuli. 17% and 11% respond to TUT and CON, respectively. Of the recording sites in NA3₆₀, 64% respond to both, TUT and CON, 19% respond to TUT, 17% respond to CON.

In PHD 60 birds, all areas respond to both song stimuli with approximately 70% of their recording sites, making no discrimination between tutor song and conspecific song.

Response Area Extension histograms with 20 ms time bins were calculated for the neuronal responses to natural song stimuli as in adult and PHD 30 birds (Table 9). The values for excitations were compared between the two song types. No significant differences can be seen between the average responses to the song types (Wilcoxon, $p = 0.219$).

<i>Area</i>	<i>TUT +</i>	<i>TUT -</i>	<i>CON +</i>	<i>CON -</i>	<i>Planes</i>
NA-L ₆₀	12.11	-0.79	15.76	-0.89	4
NA2a ₆₀	6.85	-0.93	5.39	0	2
NA2b ₆₀	14.84	-0.75	12.80	-0.33	3
NA2c ₆₀	8.23	-1.55	6.96	-2.38	2
NA3 ₆₀	5.08	-0.25	10.51	-0.41	2

Table 9. Average values of RAE histograms to the two song types TUT and CON. The average values of excitations (+) and inhibitions (-) are plotted in the column of the respective song type. The number of recording planes in which the functional areas were recorded are mentioned in the last column.

3.3.2.3. Size of the PHD 60 field L complex

In NA-L₆₀, there are 224 active recording sites in four recording planes (56 sites/ plane) responding to pure tones. 211 recording sites responded to the natural stimuli. NA2a₆₀ was found in only two planes with five active recording sites (2.5 sites/ plane) under pure tone stimulation and 6 recording sites under song stimulation. In the area NA2b₆₀, there are 34 active recording sites in three recording planes (11.3 sites/ plane), which respond to pure tones and 30 responding sites to birdsong. In NA2c₆₀ are 21 active recording sites in two recording planes (10.5 sites/ plane) under pure tone stimulation. The same number of recording sites responds to song. The area NA3₆₀ consists of 46 active recording sites in two recording planes (23 sites/ plane) responding to pure tones and 41 sites, which respond significantly to song. No significant differences in area size were found between the two stimulus situations (Wilcoxon, $p = 0.123$). In chapter 3.4 and Table 10, the average area sizes, based on pure tones are compared for the three age groups.

3.4. Comparisons between different developmental stages

In all juvenile age groups, the neuronal response properties show mature features (Figures 8, 10, 13, 16). This is true for the temporal resolution of on-responses in RAE histograms, the bandwidth of responded pure tone frequencies as well as for the relative appearance and strength of sustained responses. NA2c₆₀ shows strong and frequent phasic off-responses to pure tones. This can not be observed in the younger and the older age group.

The major difference between PHD 30, PHD 60 and adult birds is the number of functional areas of the field L complex. There are only three areas in the younger age group, namely NA-L₃₀, NA2b₃₀ and NA2c₃₀. In comparison to that, five areas (NA-L₆₀, NA2a₆₀, NA2b₆₀, NA2c₆₀ and NA3₆₀) are functioning in PHD 60 birds. Finally, the field L complex of adult birds consists of six areas in the neostriatum plus HVA in the hyperstriatum. Neither HVA nor NA4 were found in juvenile birds of 30 and 60 days of age.

The area NA-L covers Rose's field L (Rose, 1914) in all age groups from PHD 30 to adult. The relative positions of all the other areas found in comparison to that of NA-L do not change either during ontogeny. Although there are no significant differences, the area sizes, based on pure

tone stimulation seem to grow with increasing age. NA-L of PHD 30 birds exhibits 145 different recording sites in five planes, responding to any stimulus, with an average of 29 active sites per bird. In PHD 60 birds 224 active recording sites exist in four planes. This results in 56 active recording sites in each PHD 60 bird. The area NA-L of adult birds has a mean size of 56.7 active sites per bird, whereby the smaller grid unit size in adults has to be considered ($A_{GUjuvenile} = 0.020 \text{ mm}^2$, $A_{GUadult} = 0.012 \text{ mm}^2$). To receive contrastable values, the normalised sizes of the areas were calculated and compared (Table 10). No differences could be found concerning normalised area sizes between the age groups (Kruskal-Wallis, $p = 0.991$).

<i>Area</i>	<i>PHD 30</i>		<i>PHD 60</i>		<i>Adult</i>	
	<i>Grid points</i>	<i>Norm. size</i>	<i>Grid points</i>	<i>Norm. size</i>	<i>Grid points</i>	<i>Norm. size</i>
NA-L	29.0	0.580	56.0	1.120	56.7	0.680
NA2a	--	--	2.0	0.040	16.0	0.192
NA2b	12.5	0.250	11.3	0.226	24.8	0.298
NA2c	6.0	0.120	10.5	0.210	21.0	0.252
NA3	--	--	23.0	0.460	26.0	0.312
NA4	--	--	--	--	8.5	0.102

Table 10. Comparison of normalised sizes [mm^2] of all age groups. The number of grid points is the mean number of recording sites per area responding significantly to pure tones. The size of a grid unit of the PHD birds is $A_{GUjuvenile} = 0.100 * 0.200 \text{ mm}^2$. The size of an adult grid unit is $A_{GUadult} = 0.080 * 0.150 \text{ mm}^2$. There are no significant differences in normalised area size between the age groups (Kruskal-Wallis, $p = 0.991$).

The tonotopic gradients in NA-L and NA2b are similar in 30 and 60 days old birds. Their main axis is orientated from caudodorsolateral to rostroventromedial. In adult zebra finches the orientation lacks the lateromedial component in the area NA-L. But the main orientation of tonotopies show similar tendencies between comparable areas of various age groups.

No significant latency differences were observed between the age groups of this study for single functional areas. Neuronal latency times in adults range between 9 and 21 ms. In the functional areas of PHD 30 and 60 birds an identical time span is covered (9 – 18 ms).

The neuronal responses of juvenile birds to song stimuli based on comparison maps show an obvious difference (Figure 14). In PHD 30 birds, the ratio of recording sites responding only to CON and only to TUT is larger than one, whereas in PHD 60 birds the ratio of recording sites (CON/TUT) is smaller than one. The difference between the two age groups is significant (Mann-Whitney-U test, $p < 0.05$). A similar trend can be observed in the data based on averaged RAE histograms (Tables 8, 9). The major cause for these findings may be the combination of the stimulus ensembles and the responded frequency ranges of the different recording planes. These facts will be discussed below. In adults, there are no differences between responses to TUT and CON. However, the responses to BOS are stronger than those to TUT and CON.

4. Discussion

4.1. Ontogeny of the functional organisation and neuronal properties

4.1.1. Ontogeny of the functional organisation

The functional organisation of the field L complex changes from PHD 30 with only three functionally active areas to six areas in adulthood. Volumes and dimensions of brains do not vary between the different age groups (Gehr, unpublished). Neuronal space for the upcoming functional areas exists already within PHD 30 birds (compare Figure 12). Functional areas do not grow, once they appear. An example is the nicely represented tonotopic gradient of NA-L, which does exist already in its complete extension by PHD 30 (compare Figure 12B). These facts lead to the conclusion that the growing number of areas during ontogeny can be explained by an extending amount of recruited neurones for auditory processing in the field L complex, which exist already in younger birds, but are not actively working. Capsius and Leppelsack (1999) argue that NA-L in the starling with its phasic plus sustained excitations is predestined for spectral analysis. NA2b or NA2c with their phasic on-responses are important for temporal coding. Areas like NA3 and NA4, which appear later, deal with more complex tasks. Response behaviours are similar in starlings and zebra finches. Therefore, sound features like pitch and temporal aspects can already be processed at PHD 30 within the areas NA-L, NA2b and NA2c. A more complex task of NA3 or NA4 is e.g. the coding of frequency modulations (FM). Their function is important for the recognition of individual songs. Zebra finch song is characterised among others by harmonic complexes and FMs. The ability to distinguish between different songs obviously appears later in ontogeny. The fact of only three areas coding natural sounds in PHD 30 birds may explain the nonselectivity of song system neurones in that age.

In mammalian forebrains, sensory cortices maintain their functional plasticity throughout development until maturity. An example for plasticity in adult animals is presented in a study on owl monkeys, where after training certain frequencies use more neuronal space in the auditory cortex than others (Recanzone et al., 1993). A plasticity is also existing in several nuclei of the bird brain.

This plasticity can either be shown histochemically by using a labelling technique for brain-derived neurotrophic factor (e.g. Akutagawa and Konishi, 1998; Iyengar et al., 1999; Johnson et al., 2000) or functionally in the field L (Heil and Scheich, 1992). In contrast to nuclei of the song system, the field L of zebra finches does not express significant amounts of this neurotrophic factor during critical periods of song learning (Akutagawa and Konishi, 1998). It remains an open question, if the neural plasticity during ontogeny differs in principle between birds and mammals. Tonotopy changes in chick field L complex during early development (between PHD 0 and PHD 27) propose a spectral shift in the neuronal tuning properties as a function of time (Heil and Scheich, 1992). The conclusion is apparent that the neural substrate of the zebra finch field L complex exists completely and develops quite early in ontogeny and does not undergo such shifts as found in chicks. The two species are different with regard to the developmental stage. The chick is a precocial bird and the zebra finch is an altricial bird. Around PHD 20 the songbird fledges.

The only change observed in this study is a slight shift in the tonotopy in the zebra finch field L complex. An example for the tonotopy shift is the area NA-L with the tonotopic gradient running from caudodorsolateral to rostroventromedial at PHD 30, in contrast to the tonotopy in the adult NA-L from caudodorsal to rostroventral (Gehr et al., 1999). The adult situation in zebra finches of the organisation and response properties is similar in other songbird species, like the European starling (Capsius and Leppelsack, 1996 & 1999) or the white-browed robin chat (Leppelsack, unpublished data).

4.1.2. Developmental aspects of the neuronal response behaviour

The development of functional properties in auditory neurones are described by several authors. Latency times in cat auditory cortex are decreasing from kittens (40 to 70 ms) to adult cats, they reach adult values (17 ms) by the age of three weeks (Brugge et al., 1988). The latency discrepancies between young and adult seem to be a result of the degree of myelinisation and synaptic delays (Eggermont, 1991). Latency times in the field L complex do not vary between the observed age groups in this present study. It has to be mentioned that a zebra finch at PHD 30 is closer to sexual maturity than a three weeks old kitten. Such latency shift effects could occur in much younger zebra finches. An incomplete representation of frequencies in the tonotopic map of the primary auditory cortex of kittens is reported (Brugge et al., 1988). All used frequencies are

represented in active functional areas of the field L complex. Therefore, myelination and neuronal connections with the periphery of the auditory system might be complete in areas that respond to auditory stimulation.

Single unit data in rat auditory cortex show decreasing EPSP durations, increasing peak amplitudes and decreasing latencies during development (Aramakis et al., 2000). Similar observations can be made in vocal-control nuclei in zebra finches of similar ages as those used in this present study (Livingston and Mooney, 1997; Bottjer et al., 1998). EPSP durations and latencies decrease in IMAN of the developing songbird forebrain, whereas the peak amplitudes stay more or less the same for all ages. Response patterns to rectangular current injections do not change significantly during ontogeny, but juvenile IMAN projection cells tend to show bursts of action potentials around the age of PHD 30 (Livingston and Mooney, 1997). Such plasticity effects appear in brain areas with strong reorganisations, e.g. in song control nuclei during the period of song learning. There are two imaginable possibilities how these effects can be explained. Plasticity in a higher order centre (e.g. IMAN) may be elicited by an even higher plasticity in afferent centres (e.g. areas of the field L). The other possibility is that plasticity may not occur in sensory areas that provide the plastic areas with input, which has to be reliable. The field L is an accumulation of those sensory centres. The data presented here of static neuronal properties (response behaviour, latency times) throughout ontogeny support the second idea.

The maturation of electrophysiological properties correlates with the development of the dendritic connectivity within a neuronal ensemble in rat supraoptic neurones (Chevalyere et al., 2001). This result describes a common neuronal property, because the morphology of a neurone has got high influences on its firing patterns and response behaviour, as described in a neuronal modelling study by Mainen and Sejnowski (1996). The multi unit response properties to pure tones are similar between the different age groups in the present study. This leads to the conclusion that dendritic ramification (Chevalyere et al., 2001) of single neurones has reached a mature state as soon as the functional area is activated. The missing of significant concentrations of brain-derived neurotrophic factor in field L at these developmental stages is explained by that fact (Akutagawa and Konishi, 1998), because no axons, synapses and dendrites have to be rearranged anymore. The functional maturity is in accordance with the response patterns of IMAN neurones described by Bottjer et al. (1998), who report a similar neuronal morphology in juvenile (PHD 38 – 42) and adult birds (> 90 days) and also mature neuronal properties like e.g. resting potentials and time constants. The latency times of field L complex functional areas do not show an age-dependent shift like the

examples in cat auditory cortex (Brugge et al., 1988; Eggermont, 1991) and song-control nuclei of zebra finches (Livingston and Mooney, 1997; Bottjer et al., 1998). Myelination and neuronal connectivity seem to be accomplished in the functioning areas of the field L complex at the age of 30 and 60 days.

Actually, such effects cannot be denied in the developing field L complex. The two temporal sections of 30 and 60 days might simply be not adequate to observe such phenomena, since neurones within a neuronal cluster form their connections only during a critical time window (e.g. Zhang et al., 1998). However, the fact that in PHD 30 birds the areas NA3, NA2a and HVA are not existing, but well functioning in PHD 60 birds, indicates that the time span of 30 days brings these areas to complete maturation. As one has to assume that all maturation processes observed in auditory neurones also take place in the field L complex, the fact that none of them can be observed in PHD 30 and PHD 60 birds has to be taken as an indication that they last only for a very short time. This seems to include myelination, which is thought to be an important prerequisite for mature functioning.

4.2. New aspects of functional and organisational properties in adult field L complex

4.2.1. Lateralisation

Various sensory or motor systems of vertebrate brains show different distinct occurrences of lateralisation. Examples are the human auditory cortex with differences in coding frequency and amplitude modulations between hemispheres (Pardo et al., 1999), the visual tectofugal system of pigeons (Keysers et al., 2000) or song control nuclei of songbirds (e.g. Greenspon and Stein, 1983). Often there are dominances of one hemisphere over the other. In the zebra finch song control system the HVC nuclei have different tasks in the two hemispheres (Floody and Arnold, 1997). Apart from slight organisational asymmetries, which are not unusual in biological systems, the field L complex, which provides the auditory input into the HVC (Vates et al., 1996) does not show any interhemispherical differences in response behaviour and number of areas in adult male zebra finches. The song system is able to receive similarly coded input from the auditory cortex analogue in both hemispheres. This fact is of interest as there are no interhemispherical connections in the

telencephalic parts of the ascending auditory pathway in the avian brain (Ehret, 1996), which would enable the hemispheres to interact or communicate.

4.2.2. Harmonic complexes in the field L

Harmonic complexes are an important attribute of zebra finch song (Sturdy et al., 1999 a, b; compare Figure 2). Four different synthetic harmonic stimuli were used to investigate the abilities of the six functional areas to respond to these “song model stimuli”. Lim and Kim (1997) studied single unit responses of field L to harmonic complexes. They describe simple and stereotyped responses in field L2 and widely varying responses in the fields L1 and L3. They compare the responses in L1 and L3 with those of song selective neurones of HVC and are able to finally conclude that some of the L1 and L3 responses might be a pre-stage to song selectivity in HVC. To discuss their data with the results of this present study, it is important to know the functional equivalents of the anatomical fields first. As published before (Gehr et al., 1999), L1 is completely covered by NA-L. The anatomical input area of the field L complex L2 contains mainly parts of NA-L. It is not clear whether the functional areas NA2a, NA2b and NA2c can be assigned to L2, too. The only study that divided the field L into L1, L2 and L3 (Bonke et al., 1979) explains the anatomical subdivisions according to sagittal sections, there are no frontal sections, which explain the mediolateral extension of L2. L3 may consist of parts of NA3 and parts of NA-L. It is also imaginable that NA3 is equivalent to the caudal part of the neostriatum (NCM). Therefore, the simple and stereotyped responses may occur in the functional areas NA-L, and probably NA2a, NA2b and NA2c. The complex and variable responses should show up mainly in NA-L. Since NA-L is present in all three anatomical areas, it is clear that NA-L is a highly variable functional area with complex and simple responses. The other functional areas seem to be more specialised. But, in this present study, all functional areas showed widely spread significant multi unit responses to harmonic complexes without discrepancies.

4.2.3. Perception of missing fundamentals of a harmonic complex

It is a well known fact in psychacoustics, that songbirds are able to perceive the pitch of missing fundamentals (Cynx and Shapiro, 1986). A very small portion (1.14%) of the responding recording sites are able to detect the pitch of a missing fundamental. The neuronal correlate to code this phenomenon exists and consists only of a few neurones in the field L complex. These neurones can be found in at least four functional areas, namely NA-L, NA2b, NA2c and NA3 and therefore in all three major anatomical areas. It is not surprising that such specialised neuronal clusters occur in the frequency coding areas NA-L and NA3 with their sustained response properties to pure tones. However, the existence of such recording sites in the functional areas NA2b and NA2c is a very interesting fact. These two areas show strong phasic on-responses to pure tones with only weak and rare sustained excitations. According to their multi unit response behaviour, they are thought to be predestined for temporal coding (Gehr, 1998; Capsius and Leppelsack, 1999). This new result shows that NA2b and NA2c are capable of coding harmonics. The neuronal sensation of harmonics and the the pitch of a missing fundamental is based on strong spectral coding abilities. Combined with the knowledge of existing tonotopic gradients in these two areas, it is obvious that temporal and spectral informations are computed parallel to each other in NA2b and NA2c.

The positioning of the pitch perceiving recording sites at the border of the functional areas is a logical outcome of the nature of the harmonic complexes combined with the hearing range of zebra finches. Assuming that at least two harmonics are required to code the pitch of the missing fundamental with the frequency $f_0 = n$, the auditory system has to be able to recognise the second harmonic with the frequency $f_2 = 3 * n$. The hearing range of zebra finches lies between 0.3 and up to 8.0 kHz (Okanoya and Dooling, 1987). Consequently, the birds' brains are only able to detect the pitch of a missing fundamental with a frequency of $f_0 = f_2 / 3 = 8.0 \text{ kHz} / 3 = 2.67 \text{ kHz}$ with the help of harmonics. Theses low frequencies are represented in the low-frequency third, which is close to the border at the low frequency end of an area. At higher fundamental frequencies, no neurone would be able to respond to the second harmonic, which is a prerequisite for this effect.

4.2.4. Area sizes

The different sizes and volumes between functional areas under pure tone stimulation is striking (Tables 3, 10). The two areas with phasic plus sustained excited responses to pure tones, NA-L and NA3, are the largest areas with volumes $> 0.2 \text{ mm}^3$ each. The knowledge that neurones with such response properties, which are found in NA-L and NA3 are predestined for spectral coding (Capsius and Leppelsack, 1999) leads to the conclusion that spectral coding needs more neuronal space than temporal coding of acoustic stimuli. Temporal coding is mainly done in the areas NA2b and NA2c that have half the size of NA-L and NA3.

There are not only differences in size between the areas but also within them under various stimulations. Various synthetic and natural stimulus conditions lead to significantly different numbers of active recording sites per area (Table 4). Song elicits responses at the highest number of recording sites in comparison to pure tones and harmonic complexes. Song is characterised by a large variety of spectro-temporal aspects (e.g. Sturdy et al., 1999 a,b). Neurones with four different types of spectro-temporal receptive fields are distributed in each functional area of the field L complex in starlings (Gehr and Leppelsack, 1999). Considering all the functional similarities between starlings and zebra finches (e.g. Capsius and Leppelsack, 1999; Gehr et al., 1999), one should assume that such a distribution of receptive field types exists also in auditory forebrain neurones of the zebra finch. Neurones with a rather complex receptive field do not respond very well to simple synthetic stimuli, but are tuned for typical elements of conspecific song. This explains the huge amount of multi unit recording sites responding to song.

The low number of recording sites responding to harmonic complexes in comparison to pure tones can easily be explained by lateral inhibitory effects. There are inhibited regions above and below the best-frequency in the tuning curves of auditory neurones. Several frequencies are presented at the same time with the harmonic complexes. Excited and inhibited regions of the neuronal receptive fields are covered simultaneously by that type of stimulus. The responses (excitation + inhibition) can cancel each other. These effects do not show up under pure tone stimulation, which elicits responses at a higher number of recording sites as harmonic stimuli.

4.2.5. *Coding abilities of NA-L*

NA-L with its strong sustained responses to pure tones is thought to be predestined for spectral coding so far (Capsius and Leppelsack, 1999). The inhibitory sidebands in the spectral domain of NA-L are another argument for the frequency coding abilities of the area. The sidebands occur at recording sites that are tuned to higher and lower frequencies than the actual responded frequency. They show up simultaneously with the excitations. Strong inhibitory receptive fields are discussed to have a high impact on the abilities of primary auditory cortex in mammals to code complex natural sounds (Sutter et al., 1999; Schreiner et al., 2000). This should also hold for NA-L due to several functional analogies between the auditory cortex and the field L complex, like e.g. the existence of maps or tonotopies (for a review on auditory cortex see: Ehret, 1997). Furthermore, the data of the comparison maps show that there is a reasonable amount of recording sites in NA-L, which respond exclusively to original or time-reversed song. This indicates that NA-L is also well able to code temporal aspects of sound, since this is the only difference between those two stimuli, as the spectral content was not altered. Combined with the data about specialised neurones in the anatomical counterpart of that area (Lim and Kim, 1997), it is possible to say that NA-L contains highly specialised as well as quite unspecific neurones and processes spectral and temporal information parallel to each other.

4.3. *Song selectivity*

Song selectivity occurs when a neurone or a neuronal ensemble responds significantly stronger to one song type than to another. Different song types in songbirds can be the bird's own song (BOS), the tutor song (TUT) or conspecific song (CON).

4.3.1. *Neuronal selectivities in different age groups*

One obvious difference in the functional analysis of auditory forebrain neurones at the age of 30 and 60 days is the response to songs. The results presented imply changing selectivities for TUT

and CON. There is a preference in PHD 30 birds of CON over TUT, in contrast to PHD 60 birds, which has to be explained. The combinations of tutor songs with the conspecific songs lead to a limited number of stimulus ensembles, since there is a finite number of tutors and conspecific birds. Individual variances in the song stimuli, which do occur due to the reason of finite combinations, have a significant impact on the responses of the single recording planes. Additionally, the whole frequency spectrum cannot be covered in the different planes equally well. Similar responses can never be expected.

The young birds originate from different nests and the small number of tutors ($N = 3$) does not cover both age groups equally. The different song selectivity might be explained as an artefact caused by the tutor bird distribution in the two age groups (Table 1). The tutor combination in the younger group has its maxima in the spectrum between 2.5 and 5.2 kHz (majority of the peaks are above -35 dB (rel. Ampl.)). The tutor combination of the older group has its maxima in the spectrum between 4.7 and 6.9 kHz (Figure 3). Furthermore there are also different temporal properties between individual songs. This diversity, combined with the incompletely represented frequency spectrum in each recording plane, may be the reason for the inverse song selectivity (CON > TUT in PHD 30; TUT > CON in PHD 60) in both age groups. Consequently, in juvenile field L complex mainly the spectral and temporal content of a song stimulus – not its meaning or type – is coded by the neuronal ensembles.

Apart from sensitivities to spectral patterns (Leppelsack and Vogt, 1976; Leppelsack, 1978) and modulated sounds (Leppelsack, 1983; Müller and Leppelsack, 1985), there are no preferences reported for certain song types, like BOS or TUT, in single neurones or individual recording sites of the field L of adult male songbirds (Margoliash, 1986; Lewicki and Arthur, 1996). Nuclei of the anaesthetised adult (Margoliash and Fortune, 1992; Lewicki and Arthur, 1996; Theunissen and Doupe, 1998; Janata and Margoliash, 1999) or anaesthetised juvenile (Doupe, 1997; Solis and Doupe, 1999) song system show clear selectivities for BOS or TUT, respectively. Another interesting fact is that the caudal part of the neostriatum (NCM), adjacent to the field L, shows an experience-dependent neuronal activation, measured by increased expression of immediate early genes in awake birds. This could be important for the recognition of TUT and its comparison with the BOS (Bolhuis et al., 2000) in a brain region very close to the field L complex. A pre-processing of the auditory song-type information, which elicits this effect is done in the field L (Ball and Gentner, 1998). A song recognition mechanism in the field L complex is conceivable.

The simple spectral coding mechanism of juvenile birds is also imaginable in the field L complex of adult birds. But the neuronal responses to the various song types are much more stable, except the higher responses of the neuronal ensembles to BOS (BOS > CON = TUT). Tutors are better distributed among the adult birds, than they are among the juveniles (Table 1). There is no difference between CON and TUT, as it can be seen in the irregularly responding juvenile field L. It is more likely that another mechanism takes over in adult birds. The neurones in juvenile birds are tuned to simple stimuli due to the lack of sufficient auditory experiences. During ontogeny neurones become better tuned to familiar or more complex stimuli (Eggermont, 1990). With increasing age of the bird, first the TUT and later the BOS are more important than the CON. The receptive fields of auditory neurones will adapt to those important stimuli.

For the first time it has now been shown that a song selectivity for BOS exists in the functional areas of the field L complex in awake adult birds. The earlier studies concentrated on single recording sites or neurones, whereas in this study whole neuronal populations were investigated within complete functional areas. There are definite interactions between single cells and the neuronal ensemble, in which they are embedded (Bernander et al., 1991; Abeles et al., 1994; Tsodyks et al., 1999), but the whole neuronal ensemble that forms a functional area anatomically is responsible for its complex integration abilities. The simultaneous coding of complex, behaviourally relevant sounds throughout the whole primary auditory cortex has been shown before (Creutzfeldt et al., 1980; Ehret, 1997; Wang et al., 1995; Kilgard and Merzenich, 1999; Gehr et al., 2000). According to the presented data, this is also valid for the auditory cortex analogue of songbirds. Song is represented and coded throughout all functional areas of the field L complex. Each area has certain tasks in sound and song processing, according to its response behaviour. Song selectivities are expressed by the complete neuronal ensembles of the six different areas, whereas each area is more or less specialised with its coding abilities (Capsius and Leppelsack, 1999; Gehr and Leppelsack, 1999), and are forwarded into the song system and filtered via the shelf region of the HVC (Vates et al., 1996; Lim and Kim, 1997).

Song selectivity in nuclei of the song system seems to be based upon the neuronal processing of stimuli in afferent auditory centres like the field L complex. A lack of complexity in the field L complex at the age PHD 30, with only three functionally working areas explains the missing selectivity in the song system nuclei.

4.3.2. *Song selectivity and anaesthesia*

The influence of anaesthesia should not be underestimated when discussing neuronal selectivities in the song system. Anaesthesia influences neural activities. There are e.g. obvious differences in auditory processing between awake and anaesthetised animals. The areas and neurones of the field L complex, which provide the song system with auditory input (Vates et al., 1996), as well as neurones of the HVC respond differently under awake and anaesthetised conditions (field L: Capsius and Leppelsack, 1996; HVC: Schmidt and Konishi, 1998). The arising of the described song preferences (Margoliash and Fortune, 1992; Lewicki and Arthur, 1996; Doupe, 1997; Theunissen and Doupe, 1998; Solis and Doupe, 1999; Janata and Margoliash, 1999) during ontogeny in song system single neurones of songbirds remains still unclear and should be tested under awake experimental conditions, too.

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5. Abstract

5.1. English abstract

Multi unit recordings were performed in awake 30 and 60 days old and adult male zebra finches investigating the responses of the field L complex to synthetic and natural song stimuli during essential periods of song learning and adulthood.

- ◆ In adult birds there are six different functional areas in the neostriatum; they are NA-L, NA2a, NA2b, NA2c, NA3 and NA4 and can be defined by their tonotopic gradients, i.e. how pure tones are represented from low to high frequencies and their response behaviours to pure tones. These areas are present in both hemispheres.
- ◆ Sizes and volumes of the areas are determined with the result that the largest areas (NA-L, NA3) seem to be specialised for spectral coding, whereas smaller areas (NA2b, NA2c) seem to be specialised for temporal coding.
- ◆ All areas respond to harmonic complexes, but only 1.14% of the recording sites are able to detect the pitch of a missing fundamental with the help of harmonics.
- ◆ It was possible for the first time to show, that there is a neuronal selectivity for the bird's own song in the field L complex. This selectivity can only be found in the complete neuronal responses of whole functional areas and not in the responses of single neurones or recording sites.
- ◆ In juveniles, three different functional areas, NA-L₃₀, NA2b₃₀ and NA2c₃₀, can be confirmed in 30 days old birds. In 60 days old birds, five different functional areas are defined, namely NA-L₆₀, NA2a₆₀, NA2b₆₀, NA2c₆₀ and NA3₆₀. Area sizes do not change during ontogeny.
- ◆ The different areas of the juvenile age groups show already mature response behaviours, which means that myelination is complete and synaptic properties reveal adulthood. No neuronal preference for a certain song type can be found in the juvenile age groups.
- ◆ Areas with basic sound coding abilities, like spectral and temporal analysis exist already in PHD 30 birds, whereas areas with more complex tasks, like the coding of frequency modulations appear later in ontogeny. But spectral and temporal properties of sound can be coded parallelly

within one area. Dendritic connections of single neurones seem to be at a mature state, as soon as their functional area is activated.

A pre-processing of various song types is performed in the adult field L, which lies afferent to the song system. The incomplete functional organisation of the field L complex in young birds (30 days) may be a reason for the neuronal nonselectivity for various song types in the song system at this age.

5.2. Deutsche Zusammenfassung

Neuronale Mehrzelleitungen wurden im Vorderhirn von wachen 30 tagigen, 60 tagigen und adulten, mannlichen Zebrafinken durchgefuhrt, um die Reaktionen des Feld L Komplexes auf synthetische und naturliche Reize wahrend kritischer Phasen des Gesangslernens zu untersuchen.

- ◆ Bei erwachsenen Vogeln gibt es sechs verschiedene, funktionelle Areale im Neostriatum. Sie werden entsprechend ihrer anatomischen Gegenstucke im Feld L NA-L, NA2a, NA2b, NA2c, NA3 und NA4 genannt. Festgelegt werden diese Gebiete anhand ihrer tonotopen Gradienten, d.h. wie Reintone von tiefen zu hohen Frequenzen abgebildet werden, und ihrer Antwortverhalten auf Reintone. Die Gebiete sind in beiden Hemispharen vorhanden.
- ◆ Gebietsgroen und -volumina werden bestimmt, mit dem Ergebnis, dass die groten Areale (NA-L, NA3) geeignet sind, um spektrale Inhalte des Schalls zu kodieren, wahrend kleinere Gebiete (NA2b, NA2c) geeignet sind, temporale Aspekte zu kodieren.
- ◆ Alle Gebiete reagieren auf harmonische Komplexe, aber nur 1,14% der Ableitorte sind fahig die Tonhoe der fehlenden Basisfrequenz aus den Harmonischen zu erkennen.
- ◆ Zum ersten Mal wird eine Gesangsselektivitat fur Individualgesang im Feld L Komplex gezeigt. Diese neuronale Selektivitat kann nur in den kompletten neuronalen Reaktionen ganzer Gebiete gesehen werden und nicht in den Reaktionen einzelner Zellen oder Ableitorte.
- ◆ In jungen, 30 tagigen Vogeln konnen die drei Gebiete NA-L₃₀, NA2b₃₀ und NA2c₃₀ unterschieden werden. Bei 60 tagigen Vogeln werden die funf Gebiete NA-L₆₀, NA2a₆₀, NA2b₆₀, NA2c₆₀ und NA3₆₀ festgelegt. Gebietsgroen andern sich nicht wahrend der Ontogenie.

- ◆ Die unterschiedlichen Gebiete juveniler Tiere zeichnen sich schon durch ausgereifte neuronale Verhaltensweisen aus, was bedeutet, dass die Myelinisierung vollständig ist und synaptische Eigenschaften adulte Charakteristika zeigen. Bei Jungtieren kann keine neuronale Gesangsselektivität gezeigt werden.
- ◆ Gebiete mit grundlegenden Schallkodierungseigenschaften, wie spektrale und temporale Analyse, existieren schon bei 30 tägigen Vögeln, während Gebiete mit komplexeren Aufgaben, wie der Kodierung von Frequenzmodulationen, erst später in der Entwicklung auftauchen. Spektrale und temporale Eigenschaften von Schallreizen können aber durchaus parallel in einem Gebiet verarbeitet werden. Die Verzweigungen und Verbindungen der Dendriten von Einzelzellen scheinen in einem gereiften Zustand zu sein, so bald das entsprechende Gebiet aktiviert wird.

Eine Vorverarbeitung von unterschiedlichen Gesangstypen wird im adulten Feld L Komplex, afferent zum Gesangssystem, durchgeführt. Die unvollständige funktionelle Organisation des Feld L Komplexes junger, 30 tägiger Vögel mag ein Grund für die neuronale Unspezifität im Gesangssystem dieser Altersgruppe sein.

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