Neural correlations increase between consecutive processing levels in the auditory system of locusts

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running head: Increasing correlations on consecutive processing levels
ABSTRACT
Nervous systems may encode information about sensory stimuli using the temporal relations in spiking patterns between neurons. By conducting simultaneous intracellular recordings on pairs of auditory neurons we measured the strength of correlations between elements of the first three processing levels in the metathoracic auditory network of the locust. We quantified the degree of spike synchronization and rate covariations that occur among auditory neurons during acoustic stimulation. In addition to the acoustic stimulation, current pulses were injected into both neurons to study the connectivity within this network. Our findings support the view that the metathoracic auditory system is a hierarchically organized feed-forward network. Strong synaptic connections were observed only between consecutive processing levels, while there was no indication for strong connections between elements of the same processing level. Both spike synchronization and rate covariations were increased among neurons on higher processing levels. We further investigated the consequences that correlations may have on the common estimates of neuronal variability. For example, rate covariations caused by strong synaptic coupling between two neurons may lead to an overestimation if the variability is measured trial-by-trial regarding only single neurons. For the vast majority of cell pairs tested, however, no strong synaptic coupling could be demonstrated. Thus we could show that in most cases no serious errors are made if one determines variability by following the usual procedure on the basis of single cell recordings.
INTRODUCTION

The task of sensory systems is to extract relevant information from a plethora of input signals, which are represented by spike trains in the nervous system. A meaningful interpretation of the environment usually requires the comparison of spiking responses from different neurons (e.g., Georgopoulos et al. 1986). The population’s response considered as a whole may represent sensory stimuli better than the sum of individual neuron response contributions (Warland et al. 1997; Oram et al. 1998; Reich et al. 2001). For a population code the temporal relations in the spiking patterns among elements are crucial (Krahe et al. 2002a). The investigation of such a coding scheme, therefore, requires simultaneous recordings of at least two elements. Temporal interactions among neurons are commonly measured as spike synchronization or rate covariations between pairs of neurons (Averbeck and Lee 2004). It is mainly the time scale of evaluating the interactions which represents the difference between both measures. Rate covariations are derived from trial-by-trial variability in spike counts, thus representing interactions on long time scales. In contrast, spike synchronizations indicate the temporal relation between the spike times of two neurons on short time scales. They are often estimated on basis of the cross-correlation function (Aertsen and Gerstein 1985).

The consequences that correlations may have on neural coding were subject of many studies (Shadlen and Newsome 1994, 1998; König et al. 1996; Abbott and Dayan 1999, Nirenberg et al. 2001; for review see Averbeck and Lee 2004). An important issue concerns the relationship between strength of correlations and neural variability (Zohary et al 1994; Mazurek and Shadlen 2002). In general, variability of spiking responses is measured trial-by-trial for single neurons (Vogel et al. 2005). However, due to possible rate covariations between parts of a neuronal network this method may overestimate the variability that a nervous system faces at a given time (Ronacher et al. 2004). Thus the quantification of correlations across neurons is a way to obtain a more realistic estimate of relevant spike train variability.

In order to assess the magnitude and possible impact of intrinsic spike train variability in a model sensory pathway, we focused on the metathoracic auditory system of acridid grasshoppers, which is an intensively studied model system for investigating the processing of acoustic stimuli (e.g., Stumpner et al. 1991, Machens et al. 2001, Schaette et al. 2005, Gollisch and Herz 2005, Vogel et al. 2005, Rokem et al. 2006).
The metathoracic auditory network is characterized by a separation into two hemispheres and a hierarchical organization consisting of receptor neurons (RE), segmental (SN), and ascending interneurons (AN), respectively. Auditory neurons can be identified as individuals on the basis of their characteristic morphology and physiology. Fig. 1 shows basic principles of information flow between different processing levels. More than 50 receptor neurons per ear converge onto 10-15 segmental interneurons, which are connected to another 15-20 ascending interneurons (Jacobs et al. 1999; Römer and Marquart 1984; Stumpner and Ronacher 1991; Stumpner and von Helversen 2001). The latter transmit information to the brain where the final evaluation of acoustic information takes place (Ronacher et al. 1986; Bauer and von Helversen 1987). Hence, all relevant information about the acoustic environment available to the brain must be encoded by the set of ascending auditory neurons, which exhibit rather different response characteristics.

The aim of our study was to investigate the strength of correlations across neurons and its relationship to neuronal variability in the metathoracic auditory network of *Locusta migratoria*, an acridid grasshopper. For that purpose we quantified the degree of spike synchronization and rate covariations among auditory neurons. Simultaneous intracellular recordings were performed within the first three processing levels (RE, SN and AN) as well as between neurons of different levels to investigate whether correlations persist or develop across processing levels. The strength of correlations across neurons was evaluated during acoustic stimulation. To exclude as far as possible correlations that are induced by the stimulus we used simple, rectangular modulated sound pulses. Those acoustic stimuli effectively activated the auditory neurons without introducing additional stimulus-related temporal information to the spiking responses; therefore any correlations across neurons should depend primarily on the properties of the network. In addition to the acoustic stimulation we injected current pulses in either of both neurons to study the connectivity within the auditory network.
MATERIAL AND METHODS

Animals and Electrophysiology

Animals were adult female and male locusts (Locusta migratoria), which were obtained from a commercial supplier, and held at room temperature (22-25°C). After removal of head, legs and wings the animals were fixed with their dorsal side up onto a holder. The thorax was opened dorsally and the metathoracic ganglion was exposed and stabilized by a small NiCr-platform. The whole torso was filled with locust Ringer solution (Pearson and Robertson 1981). The temperature of the preparation was adjusted by means of a Peltier element at 30 (± 2) °C.

Intracellular recordings from auditory receptors and interneurons were obtained in the auditory nerve and the frontal auditory neuropil of the metathoracic ganglion, respectively. Since for some cells the recording site was far from the spike initiation zone the spike amplitudes were rather small in those cases (see Fig. 2 and 3). For simultaneous recordings of two neurons we used standard electrophysiological equipments (Krahe and Ronacher 1993). After amplifying the intracellular voltage signal (Bramp-01, NPI) it was filtered with through a 10 kHz low -pass filter. The tips of the glass microelectrodes (Clark Electromedical Instruments) were filled with a 3-5 % solution of Lucifer yellow (Aldrich) in 0,5 M LiCl. This dye was injected after completion of the physiological recordings by applying hyperpolarizing current. After an experiment the thoracic ganglia were fixed in 4 % paraformaldehyde, dehydrated, and cleared in methylsalicylate. Stained cells were identified under a fluorescence microscope based ony their characteristic morphology (terminology after Römer and Marquart 1984). Although both neurons were filled with the same dye, an unambiguous identification was possible by combining the knowledge about response characteristics and recording sites.

Acoustic stimulation

The preparation was placed in a Faraday cage lined with reflection-attenuating pyramidal foam and was acoustically stimulated via two loudspeakers (D2905/9700a, Scanspeak), situated laterally at a distance of 30 cm from the preparation. Sound intensities were calibrated with a Brüel & Kjær microphone (1/2 inch), positioned at the place of the animal, and a Brüel & Kjær measuring amplifier (type 2209). Intensities are given in dB re 2*10^-5 N/m^2 (dB SPL). All stimuli were stored digitally
and delivered by custom software (Labview, National Instruments) using a 100 kHz D/A-converter (PCI-MIO-16E-4, National Instruments).

Neurons were considered as auditory if their spike rate depended on acoustic search pulses. To obtain spike rate-versus-intensity curves, we used broad-band noise stimuli (bandwidth: 0.5 – 30 kHz) of 100-ms duration including 2 ms ramps at intensities ranging from 30 to 70 dB increasing in 10 dB steps. The stimuli were separated by 300 ms inter stimulus intervals and repeated 10 or 15 times at each intensity.

Electrical stimulation

After the completion of the acoustical stimulation protocol, depolarizing current pulses were injected in either of both neurons to test for synaptic interactions. The rectangular current pulses increased from 1 to 4 nA in 1 nA steps. Just before each depolarizing pulse, which lasted 90 ms, the neuron was hyperpolarized for 10 ms at –0.5 nA. Not in all neurons the depolarization elicited spikes. However, due to the limited recording time it was not possible to apply extensive stimulation programmes to increase the success rate. The analysis of the synaptic connectivity was performed off-line on the basis of spike triggered superpositions of the voltage traces of both neurons (see Fig. 2). In general, only the first spike elicited by a current pulse was considered in the superposition since no previous spikes should have affected the postsynaptic potential. If this was not possible only spikes with a minimal interspike interval of 15 ms were accepted. In a few cases, in which the current injection failed to elicit spikes we triggered on spontaneous spikes.

Data analysis

Spiking responses were digitized on-line with 0.05 ms precision (A/D-converter, PCI-MIO-16E-4, National Instruments). From the digitized recordings the spike times were determined off-line by means of a voltage threshold criterion.

Spike synchronization

For the cross-correlation procedure spikes were binned in 1 ms classes such that in each trial we collected at most one spike per bin. Empty bins got a value of zero. To quantify the temporal correlation between two simultaneously recorded auditory neurons, we calculated the coherence function, which is based on the cross-
correlation (see Kimpo et al. 2003, Rosenberg et al. 1989). The coherence of both cells during acoustic stimulation was calculated in a time window of 130 ms after stimulus onset. As an additional control, the coherence was assessed in the stationary part of the spiking response. For that purpose the first part of the spike trains of both neurons were removed and a time window from 65 – 130 ms was analysed.

In a first step we determined the cross-correlation function, which represents a basis to assess the presence of interactions between spike trains. The cross-correlation $C(\tau)$ of a spike train $R_i(t)$ relative to a second spike train $R_j(t)$ as a function of $\tau$ [a time shift between the two spike trains] is given by the following expression:

$$C(\tau) = \left\langle \frac{1}{T} \sum_{t=0}^{T} R_i(t)R_j(t+\tau) \right\rangle$$

where $T$ is the duration of the signal being analysed and $\langle \cdot \rangle$ indicates averaging over all trials.

Before considering a peak in this so called “raw” cross-correlogram to be relevant the covariations in firing rate of the two stimulated cells must be removed. The easiest way to “correct” for these stimulus induced effects is the shuffle corrector (Brody 1999). The shuffle corrector (SC) results from a cross-correlation of the PSTHs of both neurons, where the PSTHs $\langle R_i(t) \rangle$, $\langle R_j(t) \rangle$ are simply the spike trains averaged over trials.

$$SC(\tau) = \frac{1}{T} \sum_{t=0}^{T} \langle R_i(t) \rangle \langle R_j(t+\tau) \rangle.$$  

The shuffle corrector eliminates coincidences, which occurred by chance due to high spike rates. The shuffle corrector corresponds to a simplified version of the shift predictor, which results from the cross-correlation between all not simultaneously recorded trials (Aertsen et al. 1989).

After subtracting the shuffle corrector from the raw cross-correlogram the resulting equation is called cross-covariance. The cross-covariance (CC) of neuron i and j is
then defined by:

\[
CC_j(\tau) = \frac{1}{T} \sum_{t=0}^{T} R_i(t) R_j(t + \tau) - \frac{1}{T} \sum_{t=0}^{T} \langle R_i(t) \rangle \langle R_j(t + \tau) \rangle.
\] (3)

The temporal structure of firing may pose another possible source of cross-covariance between two neurons that does not reflect true neuronal interactions between these cells. Assuming that a spike in neuron i triggers a spike in neuron j and neuron i is a bursting neuron, which has a high probability of firing again after it has fired once. Thus the second spike in the burst of neuron i will also be correlated to the spike in neuron j, although the spike was actually triggered by the first spike in i. To correct for this type of correlation, we calculated the coherence function (γ) (Rosenberg et al. 1989). The coherence function is normalized by the auto-covariance function of each of the two spike trains. This normalization takes into account bursting or other temporally structured behaviour in either neuron i or j (or both) that would result in additional or artificially large and wide peaks in the cross-covariance functions. In practice, the coherence is calculated in the frequency domain. The coherence is given by:

\[
\gamma(\omega) = \frac{CC_j(\omega)}{\sqrt{AC_i(\omega) \cdot AC_j(\omega)}}
\] (4)

where \(CC_j(\omega)\) is the Fourier transform of the cross-covariance between the responses from i and j, and \(AC_i(\omega)\) and \(AC_j(\omega)\) are the Fourier transforms of the auto-covariance \((AC, \text{see formula (3)})\) of activity from neurons i and j, respectively. For plotting purposes, the coherence in the time-domain is then calculated by taking the inverse Fourier transform of Equation 4.

**Rate covariations**

To quantify the rate covariations between two simultaneously recorded neurons covariations (RC) of spike count were measured on a trial-by-trial basis. Spikes were counted within a time window of the stimulus duration, to which the response latency was added. Rate covariations are given by the correlation coefficient:
\[
RC_i = \frac{\langle c_i \cdot c_j \rangle - m_i \cdot m_j}{\sqrt{\langle (c_i - m_i)^2 \rangle \langle (c_j - m_j)^2 \rangle}}
\]

(5)

where \([c_i, c_j]\) represent the spike count of cell \([i]\) and \([j]\) per trial, \(\langle \rangle\) indicates averaging over all trials and \([m_i, m_j]\) are the mean spike counts of both neurons.

**Rate covaritions and variability**

In most investigations the spike count variability is measured for a single neuron based on several stimulus repetitions. However, nervous systems consist of many elements, which may also exhibit temporal correlations such as covariations of spike rate. In our analysis we tried to quantify the effect of such covariations on the commonly used measure of variability, by comparing the responses of two simultaneously recorded neurons instead of a single cell. First, we calculated the expected standard deviation (SD\textsubscript{expected}) of spike count under the assumption that both neurons would respond in a stochastically independent manner. In this case the expected standard deviation is calculated according to the law of error propagation (Sachs 1999):

\[
SD\textsubscript{expected} = \sqrt{SD_i^2 + SD_j^2},
\]

(6)

where \([SD_i]\) and \([SD_j]\) are the standard deviations of spike count for cell \([i]\) and \([j]\), respectively, derived from a trial-by-trial protocol.

As a second measure we calculated the actual standard deviation (SD\textsubscript{measured}) of the differences in spike count between the two simultaneously recorded neurons. For that purpose the difference of spike counts \([c_i, c_j]\) for both cells was determined for each trial, and then the standard deviation of this distribution was calculated.

\[
SD\textsubscript{measured} = \pm SD \text{ of } \langle c_i - c_j \rangle,
\]

(7)
In case of strong covariations this difference remains roughly constant over different trials, yielding a rather small $SD_{measured}$ (see Fig. 9). As an additional control the $SD_{measured}$ was calculated after shuffling the response trials of cell 1 (see Fig. 9D).

**RESULTS**

A total of 43 paired recordings of identified auditory neurons were analysed. We obtained responses from 13 pairs of receptor neurons (RE), 9 pairs: receptor – segmental neuron (SN), 5 pairs: segmental – segmental interneuron, 8 pairs: segmental – ascending interneuron (AN) combinations, and 8 pairs of ascending interneurons (compare Fig. 1). Before analysing spike time and spike count correlations within and between processing levels of the auditory network we will focus on its connectivity to facilitate the interpretation of further results.

*Connectivity within the neural network tested with electric stimulation*

Depolarizing currents were injected in order to explore synaptic connections between pairs of neurons. To present knowledge, no synapses exist between receptor neurons from different sides nor between those from the same side (Rehbein 1976, Marquart 1985b). Of the remaining 30 cell pairs evidence for a strong synaptic contact was found in only four cases. Consistent with the information flow proposed in Fig. 1, these strong synaptic connections were observed between successive elements in the hierarchy. One connection was observed between a receptor and a segmental neuron (RE – SN), and two connections between a segmental and an ascending interneuron (SN – AN; one connection was observed twice with the same combination of individual neurons). Altogether 17 out of 30 cell pairs consisted of successive elements.

In Fig. 2A the spike triggered superpositions are shown for a cell pair, where an ascending neuron showed a reliable response to the activity of a segmental neuron. The upper traces indicate the current-elicited action potentials of the presynaptic neuron and the lower traces show the corresponding postsynaptic response. Sometimes a postsynaptic potential did not occur until the second or third presynaptic spike. The response latency was ~2.5 ms. In most recorded cell pairs, however, the spikes of one cell had no detectable effect in the other neuron – neither excitatory nor inhibitory. An example is illustrated in Fig. 2B for a pair of two ascending interneurons.
Although strong synapses were observed only rarely within the metathoracic auditory network, the existence of weak synaptic connections can not be ruled out (see Discussion). Weak connections, however, may be detected by an analysis of temporal correlations, see below.

**Correlations between neuronal responses elicited by acoustic stimulation**

In the following we will analyse the correlations between simultaneously recorded neurons that were investigated with auditory stimuli instead of current pulses. The response characteristics of two simultaneously recorded cell pairs are illustrated in Fig. 3. The left side of Fig. 3 represents the synaptically connected cell pair that is shown in Fig. 2A. The response characteristics of two ascending interneurons, for which no indication of a strong synaptic connection could be found, are illustrated in the right part (compare Fig. 2B). Individual spike trains recorded at a stimulus intensity of 50 dB are shown in Fig. 3A and B. While the spike raster plots below indicate the spike times for several stimulus repetitions (Fig. 3C and D), the PSTHs show the average response to the acoustic stimulus for both cell pairs (Fig. 3E and F). The intensity response functions illustrate the mean spike rate as a function of the stimulus intensity (Fig. 3G and H).

**Spike synchronization**

Temporal correlations are observed if a spike occurring in one neuron is associated with an increased probability of a spike occurring in the other neuron. To quantify the temporal relations between the spike times of two simultaneously recorded cells, we calculated the coherence function (see METHODS). In Fig. 4 the coherence functions are illustrated for the same cell pairs as in Fig. 3. The cell pair that exhibited a strong synaptic connection (compare Fig. 2A) showed a pronounced peak at a time shift of \( \sim 3 \) ms in the coherence functions, indicating that the AN neuron often spiked around 3 ms after a SN spike (Fig. 4A). Compared to Fig. 4A the peak in the coherence function in Fig. 4B is less highlighted from the baseline both with respect to its height and its width.

To quantify the strength of correlations across neurons we calculated the peak amplitude relative to the twofold standard deviation of the coherence function (shaded area) (see arrows in Fig. 4A). We chose the relative peak height as an indicator for cell-to-cell connections since a reliable temporal relationship between
spikes should result in a peak that clearly rises above a noisy baseline (compare Fig. 4A). We were interested in temporal relations occurring on short timescales, and, therefore determined relative peak values within a time shift (\( \tau \)) of \( \pm 25 \) ms. However, the standard deviation of the coherence function was calculated over the entire function (\( \pm 50 \) ms).

In Fig. 5 the mean relative peak values are summarized over all simultaneous recordings for positive (Fig. 5A) and negative correlations (Fig. 5B). The cell pairs were classified with respect to the three processing levels mentioned above. The shaded area (value of one) in Fig. 5 corresponds to the twofold standard deviation of the coherence function. First, we will focus on positive correlations (Fig. 5A). Obviously, the spike time correlations were highest between segmental and ascending interneurons (mean value 1.8). This can be mainly attributed to the cell pairs that were synaptically coupled and thus increased the average value. For those cell pairs the peaks exceeded the chance level up to threefold (values see legend of Fig. 5A). But we also found significant correlations between receptors and segmental interneurons (bars in Fig. 5 indicate 99% confidence intervals). In both classes, RE – SN and SN – AN information is transferred to next processing level. Rather unexpected was the increased correlation between pairs of ascending neurons since no strong synaptic connections were observed within this group (Fig. 5A, AN – AN). In contrast, correlations between two receptors or two segmental interneurons showed only small (though in the first case significant) deviations from chance level.

We further checked whether the increasing correlations persist if only the stationary part of the spiking response is analysed. By restricting the data evaluation to a time window between 65 and 130 ms possible transient responses at the stimulus onset were excluded. A comparison of the maximal relative peaks for the long and the short time window showed the same pattern of increasing correlations at higher processing levels (compare Fig. 5A and C). However, the differences between the classes became more pronounced when analysing a longer spike train segment.

In Fig. 5B the mean relative peaks are shown for negative values. Negative peaks in the coherence indicate an increased probability for the absence of a postsynaptic spike in response to a presynaptic one. In contrast to the results of Fig. 5A this analysis did not yield pronounced deviations from the chance level for any neuronal
The 99% confidence interval exceeded the chance level in two classes only, namely RE – SN and AN – AN. Only minor deviations from chance level were found as well if the analysis was confined to the stationary part (65 – 130 ms) of the spiking response (Fig. 5D).

So far we considered only the peak amplitudes but not the time shift of the peak values or the peak width. If spike synchronization reflects synaptic interactions both should occur on short timescales. We restricted the investigation of peak positions and width on maximal peaks corresponding to positive correlations since only these showed strong deviations from chance (Fig. 5). The relative peak values were determined in a $\tau$-range of $\pm 25$ ms. The distribution of the absolute $\tau$-positions in Fig. 6A clearly shows an accumulation of maximal peaks within a time window of 4 ms (at $\tau = 0 – 0.99$ ms: 10% and at $1 \leq \tau < 4$ ms: 34% of all peaks). Up to 10 ms the distribution is still dense ($0 \leq \tau < 10$: 65% of all peaks) and turns into a noisy distribution beyond 10 ms. We therefore can assume those peaks at $\tau$-values above 10 ms to arise from a noisy baseline. The distribution of peak widths measured at half-amplitude is shown in Fig. 6B. In 73% the peak width was below 2 ms (median: 1.4 ms).

**Rate covariations**

Rate covariations of simultaneously recorded neurons indicate common input or co-modulations of spike count rather than an exact relation of spike times. Noise correlations were derived from trial-by-trial variability of spike counts (see METHODS). For each stimulus presentation, the spike counts of both neurons were plotted against each other, as illustrated in Fig. 7 for the two cell pairs already shown in Fig. 2 to 4. The correlation coefficients are shown for each sound intensity. Significant correlations occurred almost over the whole intensity range if both neurons were synaptically connected (Fig. 7A, all correlations significant ($p<0.01$) except at 30 dB), while high correlations between the spike counts of both cells were only rarely found for the other cell pair (Fig. 7B, all correlations n.s. except at 50 dB).

As for the quantification of the spike time correlations (Fig. 5) the mean values obtained for the different cell classes are shown in Fig. 8. For the spike time correlations in Fig. 5 the mean values were calculated separately for positive and negative peaks. In contrast, the correlation coefficients for spike count combine
positive or negative correlations in a single expression (Fig. 8). Therefore, the chance level is represented by a value of zero in this analysis. There was a gradual increase of spike count correlations between neurons at higher processing levels, but significant deviations from zero were found only in the classes SN – AN and AN – AN (see 99% confidence intervals). In order to demonstrate that rate covariations of simultaneously recorded neurons indicate common input or co-modulations of spike count rather than stimulus induced covariations we calculated the correlation coefficients after shuffling the response trials of one of both neurons (Fig. 8B). The mean correlation coefficients now fluctuate around zero for all cell classes. This indicates that the covariations observed in Fig. 8A were not caused by an influence of the stimulus onset.

**Impact of spike count correlations on estimates of variability**

A standard quantification of the variability of spike counts is to determine the variance of a spike count distribution obtained from several stimulus repetitions. But this procedure entails a problem. Natural stimuli are, as a rule, not repetitive, which precludes the strategy to enhance reliability by averaging across many trials. Rather, nervous systems will be forced to exploit the information present in parallel neuronal elements with a single stimulus presentation. This difference between our usual experimental protocols – single cell recording combined with many stimulus presentations, and the one-shot information processing capacities of nervous systems may lead to a distorted picture of the “true” variability of neuronal responses. Consider, for example, the case that there exist changes in general excitability of a neural network on time scales of seconds to minutes. Then our usual experimental protocols will result in a massive overestimate of the variability that is relevant for the one-shot stimulus processing. The simultaneous recordings from two neurons applied in this study now enable us to assess the errors that will be introduced by the single cell recording paradigm into our estimate of neuronal variability.

In Fig. 9 the spike count covariations are illustrated for both cell pairs that were introduced above (compare Fig. 7; 60 dB). Treating each cell independently as is the case in single cell recordings, one can derive the expected standard deviation (SDexpected) according to formula (6) (see METHODS). This prediction is shown as left column in Fig. 9A (wide stripes). The right column (narrow stripes) reflects the
measured standard deviation ($SD_{\text{measured}}$) under consideration of the rate covariations between two neurons. This measure was derived from the spike count differences between both neurons (see formula (7) in METHODS). A comparison of Fig. 9A and B underlines the impact of correlations across neurons. In agreement with the weak covariations of spike count in Fig. 9B both variability measures yield similar values. In contrast, the strong covariations between two neurons would lead to an overestimation of the trial-by-trial variability in case of single cell recordings and repeated stimulation (Fig. 9A).

A compilation of all data is shown in Fig. 9C. The standard deviations expected from single cell recordings and the respective standard deviations measured in simultaneous recordings are compared. Although the majority of points is grouped around the 45° line there seems to be a tendency towards slightly lower standard deviations than expected from single trials. The trial-to-trial method yields a substantial overestimation of the instantaneous variability mainly in these cases where the activity of two neurons correlates strongly (compare open stars in Fig. 9C which correspond to the cell pair in Fig. 9A). This impact becomes obvious when comparing Fig. 9C and D. In Fig. 9D the $SD_{\text{measured}}$ was calculated after shuffling trials and thus removing covariations. Data points of those cell pairs with strong correlations now clustered around the 45° line. A comparison of Fig. 9C and D further shows that in most cases the trial-to-trial variability derived from single cell measurements yields a reasonable estimate of the actual variability within this nervous system.
DISCUSSION
The main goal of the present study was to elucidate the functional organization of the metathoracic auditory network of the locust by means of simultaneous intracellular recordings. This approach allowed to cover three aspects: (1) to quantify the degree of correlations across neurons at the first three processing levels, (2) to obtain a measure of variability that takes into account correlations across neurons, and (3) to detect the existence and degree of synaptic connectivity between elements of this circuit.

Connectivity and general information flow within the metathoracic auditory network

The auditory pathway of *Locusta migratoria* represents a very well studied model system to investigate the processing of acoustic stimuli (Stumpner and Ronacher 1991, Machens et al. 2001, Schaette et al 2005, Gollisch and Herz 2005, Vogel et al. 2005). Response characteristics of many individually identified thoracic auditory neurons are extensively documented (Römer and Marquart 1984; Ronacher and Römer 1985; Stumpner and Ronacher 1991; Stumpner et al. 1991; Franz and Ronacher 2002; Krahe et al. 2002b). However, only a few studies examined the connectivity within the auditory network (Marquart 1985a; Boyan 1991, 1992, 1999).

Our current injection protocols aimed at detecting synaptic connections. Of a total of 30 potentially connected cell pairs only 4 clear synaptic links were found. Without exception, these synapses were observed between elements of successive processing levels. In contrast, between elements of the same processing level we found no indication of strong synapses. However, a methodological remark is necessary. While positive evidence, such as PSPs or spikes that are time-locked to the spikes of another cell, demonstrates a strong synaptic connection between these cells, the reverse conclusion does not hold in case of “negative” evidence. Weak synaptic contacts may have been undetected by this procedure, e.g. because the recording sites are too far away to observe PSPs. Hence, a “negative” result, as shown in Fig. 2B cannot rule out the existence of weaker synaptic connections between a cell pair. This asymmetry must be kept in mind when considering possible causes for correlations across neurons.
In conclusion, our findings support the view of a hierarchically organized feed-forward network (Fig. 1). Based on current evidence there appear to exist only few strong connections between processing levels. This evidence, however, does not rule out the possible existence of many weak synapses that contribute to auditory processing.

*Increase of correlations at higher processing levels*

Neuronal correlations may be caused by different sources: (1) the temporal structure of the external stimulus, (2) strong synaptic connections between neurons, by which mainly one cell drives the spike generation in another cell or (3) common synaptic input that drives the activity of both simultaneously recorded neurons. The last case can be further separated into: (3.1) both simultaneously recorded neurons are mainly driven by a single presynaptic neuron or (3.2) they share a pool of many presynaptic elements.

By using rectangular modulated stimuli we tried to reduce stimulus induced correlations as far as possible (compare also Fig. 8B, 9D). In accordance with (2) we observed both spike synchronization and rate covariations between SN and AN (see values for cell pairs (b) and (c) in legend of Fig. 5 and 8). In contrast, the spike count correlations for the synapse between RE and SN were very weak, indicating that the impact of this particular receptor neuron on the SN during acoustic stimulation was small compared to the amount of other synaptic inputs affecting this SN neuron.

At the level of ascending interneurons we observed an increase of spike synchronization and rate covariations which could be due to either (2) or (3). Rate covariations may result from interactions on a long timescale, which could, e.g., result from sharing many presynaptic elements (3.2). Spike synchronization, in contrast, reflects a distinct relationship between the spike times of two neurons probably caused by strong synapses (2 or 3.1). However, since we did not detect any strong synaptic connections between pairs of ascending interneurons the origin of spike synchronization at this level remains unclear. One could speculate that only a few elements of the lower processing level, the segmental interneurons, have a strong presynaptic impact on large number of ascending neurons. Apart from AN(b) and (c) (see Figure legend 5 and 8) which appears to be driven mainly by a single SN it seems unlikely that certain segmental neurons caused the higher correlations
between ascending interneurons. The complex and highly diverse response patterns of ascending interneurons make this scenario improbable. The complex response patterns in addition to the limited number of neurons within the metathoracic network and the increase of correlations across neurons, favour our former suggestion of synaptic cross-links, which are strong only in exceptional cases (Fig. 2A). Thus the correlations between ascending interneurons could be caused by a sharing of several presynaptic elements (3.2).

In a convergent network architecture correlations between elements of the same processing level can occur only if they already exist at the presynaptic processing level. In contrast, a divergent architecture supports the new appearance of correlations due to common presynaptic elements. Which neural substrate is available for the appearance of correlations in the metathoracic network? Within the metathoracic network a substantial convergence exists between receptors and segmental interneurons while between segmental and ascending interneurons the network is probably rather divergent (Fig. 1). The rate covariations, which occurred between segmental interneurons, were not transmitted from the presynaptic level since the receptor neurons responded in an uncorrelated way (Fig. 8). Thus the correlations between segmental interneurons may have been caused by interconnections within this processing level. The situation is different at the level of ascending interneurons. In view of the relatively small number of presynaptic elements it is rather likely that the spike time correlations and the rate covariations observed among ascending interneurons are due to the sharing of common inputs (Fig. 5 and 8, column AN – AN).

For a rough estimate we assumed a number of 10 segmental and 10 ascending interneurons. If each ascending interneuron receives input from half of the segmental interneurons (according to the main information flow; Fig. 1) then the probability that two ascending interneurons receive common input from three segmental interneurons is about 40 % (pers. comm. M. Neumann). Thus correlations could be induced at this processing level even if the presynaptic neurons responded in an uncorrelated way (Shadlen and Newsome 1998).

The results reported here show interesting parallels to other sensory systems. Increasing correlations have also been reported for consecutive processing levels in the visual pathway of vertebrates (Bair et al. 2001; Fries et al. 2002) as well as in the vertebrate auditory system (Albeck und Konishi 1995, Keller und Takahashi 1996).
the olfactory system of locusts an increase of correlations across neurons was observed as well (Laurent 2002; Perez-Orive et al. 2002).

**Correlations and variability**

The relevance of correlations across neurons for a neural code and its impact on neuronal variability still remains open for debate. Spike count correlations could, for example, induce a bias in the traditional methods that measure variability from many stimulus repetitions. In the metathoracic auditory system of grasshoppers we could show that such a bias leading to an overestimate of the “true” variability, depended on the presence of high correlations and hence occurred only between cell pairs with a strong synaptic connection. For the majority of cell pairs tested (26 out of 30) no strong synaptic coupling could be demonstrated, and their correlation coefficients did only rarely exceed 0.4 (Fig. 8). This leads to the conclusion that in most cases no too serious errors will be made if one estimates variability by the usual procedure, on the basis of many stimulus repetitions derived from single cell recordings. However, this conclusion must be tested also in other systems.

A central theme in neural coding is the question of whether neurons transmit information in their average firing rate or in the precise timing of their spikes. In both concepts the coding reliability is affected by response variability and correlations across neurons in different ways. Remarkably, Vogel et al. (2005) observed an increase of variability at higher processing levels of the metathoracic auditory network, which parallels the increase of correlations across neurons reported here. According to Shadlen and Newsome (1994, 1998), the low variability and low correlation values of receptor neurons and segmental neurons both argue for a rate coding scheme at these levels since (1) a high presynaptic variability would decrease the reliability of the instantaneous average spike rate at the postsynaptic level, and (2) only a rising number of stochastically independent input elements, according to the \( \sqrt{n} \)-rule, can counteract this effect. Thus the relatively large number of sensory cells may improve the reliability of signal transmission by a rate code. In contrast, at the level of ascending interneurons both higher variability and higher correlation values will adversely affect information transmission by a rate code, based on single neurons. Moreover, the limited number of ascending neurons provides only little capacity to improve reliability under a rate coding regime. Therefore, high variability
due to filter processes and high correlation values at this level favour a population code. This view is supported by the fact that the ascending interneurons have rather different response characteristics and likely encode different aspects of a stimulus. From this point of view the correlations between ascending interneurons might be helpful since they could reduce the instantaneous variability in the nervous system and increase the mutual information (Oram et al. 1998; Lestienne 2001).

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ABBREVIATIONS
AN ascending neuron
EPSP excitatory postsynaptic potential
IPSP inhibitory postsynaptic potential
PSP postsynaptic potential
RE receptor neuron
SN segmental neuron
REFERENCES


Keller CH and Takahashi TT. Binaural cross-correlation predicts the responses of neurons in the owl’s auditory space map under conditions simulating summing localization. *J Neurosci* 16: 4300-4309, 1996.


FIGURES

Figure 1. Schematic diagram of the information flow in the auditory pathway of *Locusta migratoria*. Only one of the two mirror image hemispheres is shown. More than 60 receptor neurons (RE) converge onto a much lower number of segmental (SN) or bisegmental interneurons (BSN, in the following also included to the SN) which then serve as presynaptic elements for ascending interneurons (AN). Only the ascending interneurons project to the brain. Thus they represent a bottleneck in information transfer.

Figure 2. Evidence of synaptic connections revealed by current injection. A EPSPs in AN triggered by spikes in SN demonstrate the connection between a segmental and ascending interneuron (SN – AN). B Missing evidence for a synaptic link between to ascending interneurons (AN – AN). Shown are superpositions (n=5) of the current induced spiking responses (upper traces) as well as the simultaneously recorded responses from the other neuron (lower traces). The time zero is determined by the presynaptic spike (vertical line) or the current induced spikes.

Figure 3. Response characteristics of two pairs of simultaneously recorded auditory interneurons: a segmental interneuron SN with an ascending interneuron AN (same as in Fig. 2A) and the two ascending interneurons from Fig. 2B. A, B Spike traces of both cell pairs. The neurons were stimulated with a 100-ms rectangularly modulated noise pulse at 50 dB SPL. C, D Spike raster plots for 10 of the 15 stimulus repetitions at 50 dB. The spike traces in A and B correspond to the 10th (from bottom) (A) and the third (B) stimulus repetition, respectively. E, F PSTHs for each neuron of a pair. The PSTHs are normalized to 1 stimulus repetition (y-scale), bin width 1 ms, n=15 (A) and n=10 (B). G, H Intensity response functions, which illustrate mean spike rates and standard deviations at each intensity (n=15).

Figure 4. Coherence functions of the two pairs of simultaneously recorded auditory interneurons as in Fig. 3. A Segmental – ascending interneuron (SN – AN). B Ascending – ascending interneuron (AN – AN), both pairs at 50 dB. The coherence is plotted versus the time shift (\( \tau \)) between both spiking responses (time window: \( \pm 50 \) ms). The shaded area represents the twofold standard deviation of the coherence function (see METHODS). For both coherence functions maximal and minimal peak
positions were determined within a \( \tau \)-interval of \( \pm 25 \) ms (peaks: gray points). The arrows in A illustrate the ratio between the maximal peak and the twofold standard deviation of the coherence function. Insets show the auto-correlations of both neurons.

**Figure 5.** Synopsis of peak amplitudes in the coherence function relative to the twofold standard deviation of the coherence function (compare Fig. 4). Positive (A and C) and negative (B and D) relative peak values are illustrated for the analysis of the entire spiking response (130 ms, A and B) and for the stationary part (last 65 ms, C and D). The cell pairs were classified with respect to the processing levels. For each class mean values (column) and the 99% confidence intervals (bars) are shown. We averaged the mean values from different cell pairs. The mean values from a single cell pair were calculated from different intensities, given that both neurons fired above 10 Hz. Therefore, the mean relative peak values were based on a single up to 5 values (30 – 70 dB, see METHODS). The number (n) indicated above each column represents the number of mean values, which were averaged, corresponding to the number of cell pairs (note, that it is not the number of intensities, which contributed to the mean for each cell pair). The shaded areas represent the chance level, corresponding to 2 SDs of the coherence. A and C Only in the class SN – SN the confidence interval (99%) did include the value one. B and D The confidence interval (99%) did include the value one for three classes in the negative relative peak paradigm (B: RE – RE, SN – SN, SN – AN and D: RE – SN, SN – AN, AN – AN). Mean positive correlations in A for cell pairs with strong synaptic connection: RE – SN: 1.8; SN – AN(a): 2.0; SN – AN(b): 3.1; SN – AN(c): 2.1. SN – AN(b) and (c) were pairs with the same individual neuron combination. SN – AN(c) is shown in Fig. 2A to 4A.

**Figure 6.** Timing and half-width of the maximal peaks of the coherence function. A Frequency distribution of the temporal occurrence of the peak amplitudes. For each coherence function the absolute tau-positions \([ |\tau| \) of the maximal peak amplitude are plotted on the x-axis. Total count: 161; bin width: 1 ms. B Frequency distribution of the peak width at 50 % peak amplitude. Median: 1.4 ms; total count: 161; bin width: 0.5 ms.
**Figure 7.** Covariations of the spike-counts of the two pairs of simultaneously recorded auditory interneurons as in Fig. 3. A Segmental – ascending interneuron (SN – AN). B Ascending – ascending interneuron (AN – AN). The spike counts of both neurons are plotted against each other. Each point represents a single trial (stimulus presentations per intensity n=15 (A) and n=10 (B)), the different symbols indicate different stimulus intensities. For each intensity the correlation coefficients, based on 15 or 10 data points, respectively, are given (note that some points may conceal others with identical values). In A, all correlations except that at 30 dB are significant (p<0.01), while in B all are n.s., except the correlation at 50 dB. The intensity response functions of both cell pairs are shown in Fig. 3G and H (those curves are based on the mean spike count of the single neurons). Sample spike traces are shown in Fig. 3A and B.

**Figure 8.** Synopsis of spike count covariations. Mean trial-to-trial (A) and mean shuffled-trial correlation coefficients (B) are illustrated. The cell pairs were classified with respect to the neuron types and soma positions. For each class mean values (column) and the 99% confidence intervals (bars) are shown. The calculation was performed as described in Fig. 5. In contrast to Fig. 5 the chance level for spike count correlations is zero. This value is exceeded only in by the classes SN – AN and AN – AN (99 % confidence interval). Mean spike count correlations for cell pairs with strong synaptic connection: RE – SN: 0.03; SN – AN(a): 0.1; SN – AN(b): 0.9; SN – AN(c): 0.68. SN – AN(b) and (c) were pairs with the same individual neuron combination. SN – AN(c) is shown in Fig. 2A to 4A. In B the trials of cell 1 for each cell pair and each intensity were shuffled before calculating the correlation coefficient with cell 2.

**Figure 9.** Actual variability measured in simultaneous recordings compared to expected variability derived from successive stimulation of single neurons. A Segmental – ascending interneuron (SN – AN). B Ascending – ascending interneuron (AN – AN). The covariations of spike count are illustrated at 60 dB (compare Fig. 7). Squares and bars indicate mean and standard deviations of spike count (compare Fig. 3G and H). The columns on the right represent the standard deviation expected (SD_{expected}) in case of stochastic independence of both responses (left, wide stripes) and the standard deviation (SD_{measured}) of the spike count...
differences between the two cells, measured in simultaneous recordings (right, narrow stripes). C The \( \text{SD}_{\text{expected}} \) and the \( \text{SD}_{\text{measured}} \) are plotted against each other. Same symbols represent the respective neuronal classes, whereas the single points reflect individual intensities. Filled symbols indicate cell pairs for which a synaptic connection was observed. In addition, a linear regression is shown for the cell pair SN – AN from Fig. 2A to 4A, which was shown to be synaptically connected. D The trials of cell 1 for each cell pair and each intensity were shuffled before calculating the spike count difference with cell 2 and the \( \text{SD}_{\text{measured}} \).