Increase of neuronal response variability at higher processing levels as revealed by simultaneous recordings

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Abstract
A key problem for neuronal information processing is the variability of spike trains, which is likely to constrain the encoding of sensory signals. We measured interspike interval variability (coefficient of variation) as well as spike count variability (Fano factor) in the metathoracic auditory system of locusts. We performed simultaneous intracellular recordings at the first three processing levels in order to establish identical physiological conditions. This allows us to assess whether variability is generated anew or is reduced during synaptic transmission and processing. Both, the interspike interval variability as well as the spike count variability revealed similar trends and showed an increase from the periphery to higher processing levels. This result was confirmed by single cell recordings. A comparison of ascending interneurons coding for sound direction and those encoding sound patterns showed that the latter respond more reliably to repeated stimulus presentations. In general, the variability of spiking responses was much lower than expected from a Poisson process. Furthermore, we observed a strong dependence of variability on the spike rate, which differed at the three levels investigated. The differences in spike rates account for most of the differences in variability observed between processing levels. For auditory receptors we found a good agreement between the Fano factor and the squared coefficient of variation, suggesting similarities to a renewal process of spike generation at the periphery. At the level of interneurons the Fano factor was lower than the squared coefficient of variation, which indicates a higher reliability than expected from the interspike interval distribution.
Introduction

Sensory systems are specialized to recognize relevant signals from the environment. An ultimate limit on the recognition of sensory signals may be imposed by the intrinsic variability of spike trains (Machens et al. 2001, 2003; Ronacher et al. 2004). Intrinsic variability results from stochastic processes during sensory transduction, spike generation and synaptic transmission (Lestienne 2001). In order to quantify intrinsic neuronal variability two measures of spiking responses are commonly employed, that is, the interspike interval distribution and the spike count distribution (Gabbiani and Koch 1998; Dayan and Abbott 2001). In numerous studies, especially on the visual systems of vertebrates, the spike count variability was quantified by the Fano factor (FF) (e.g. Vogels et al. 1989; Kara et al. 2000). As a standard quantification of the interspike interval variability the coefficient of variation (CV) is calculated (Softky and Koch 1993). While the FF reflects the response reliability for multiple stimulus presentations, the variability of an ongoing neuronal response is expressed by the CV. If spike generation follows a renewal process, the FF should correspond to the square of the CV (Gabbiani and Koch 1998). Thus, the relation between these two measures may help to characterize a neuron’s spiking response.

It is important, however, to note that in spite of their general use the two measures of variability suffer from a possible drawback: they rely on very long stimuli or on manifold repetitions of an identical stimulus. This is in contrast to the normal operation of nervous systems, which usually have to process sensory inputs quickly (Ronacher et al. 2004). Hence, by measuring variability over time, it is quite conceivable that one obtains a misleading picture of neuronal variability. Slow changes of the internal physiological state, for example, may lead to an overestimation of the actual variability, which is relevant for the task at hand. If the magnitude of variability is compared along successive stages in a sensory pathway it might be problematic to compare data which were recorded at different times. A reasonable strategy to circumvent such an overestimation of neuronal variability is to perform simultaneous recordings from at least two neurons at successive processing levels. Although the investigation of variability across different processing levels with simultaneous recordings does not relieve the problem of long stimulation times it allows a comparison under identical physiological conditions.
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 (Ronacher et al. 1986; Stumpner et al. 1991). The metathoracic auditory network is characterised by a separation into two hemispheres and a hierarchical organization consisting of receptor neurons, segmental, and ascending interneurons, which can be identified as individuals on the basis of their characteristic morphology and physiology (fig. 1 A). Receptor neurons project onto segmental interneurons, which then serve as presynaptic elements to ascending neurons (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, the set of ascending neurons constitutes a bottleneck for the transmission of auditory information (fig. 1 A). At the level of ascending interneurons a functional separation in elements coding for the sound pattern (upper two traces in fig. 1B, ascending interneurons) and those carrying directional information (AN1 – third trace in fig. 1 B) has been reported (Ronacher and Stumpner 1993). The patterns of spiking responses differ substantially between receptors, segmental and ascending interneurons (fig. 1 B).

The aim of our study was to investigate the neuronal variability of spiking responses at the first three processing levels in the auditory pathway of locusts. We performed simultaneous intracellular recordings from two neurons of successive processing levels under identical physiological and stimulus conditions. This method allows us to assess whether variability is generated anew or is reduced during information processing. In addition to simultaneous recordings single cell responses were analysed to estimate the effect of changes in the internal state on the neuronal variability. In order to distinguish intrinsic variability from that induced by temporal modulations of the stimulus we used simple rectangularly modulated acoustic stimuli. Those stimuli effectively activated the auditory neurons without modulating their spiking responses. Thus, our experiments yield a basic characterization of the variability present within the auditory pathway, which results from its network properties.
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. For simultaneous recordings of two neurons we used standard electrophysiological equipment (Krahe and Ronacher 1993). After amplifying the intracellular voltage signal (Bramp-01, NPI) it was fed 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 on 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 loud speakers (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/m² (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 the occurrence of an acoustic search pulse. Two different stimulus paradigms were applied:

(1) **Intensity response paradigm (short stimulus paradigm):** For intensity-response curves rectangular pulses of 100 ms duration (including 2 ms rising and falling ramps) filled with white noise (bandwidth: 0.5 - 30 kHz) were presented at intensities ranging from 30 to 70 dB increasing in 10 dB steps. At each intensity the stimuli were repeated 10 or 15 times. The stimuli were separated by 300 ms interstimulus intervals. Data from a total of 16 cell pairs were obtained. In addition, single-cell recordings were used to complete the results of dual recordings. Altogether the responses from 33 low-frequency receptor neurons, 25 segmental and 21 ascending interneurons were recorded.

(2) **Long stimulus paradigm:** The stimuli of this set of experiments consisted of 400 ms (including 2 ms rising and falling ramps) rectangular pulses filled with white noise carrier (bandwidth: 0.5 - 30 kHz). In contrast to the short stimulus paradigm, these stimuli were presented at a single intensity only. This intensity was adjusted at around 20 dB above the neuron’s response threshold (in most cases between 60 and 70 dB, in a few cases at 50 dB SPL). The stimuli were repeated 8 times with stimulus intervals of 1 s. We analysed the single-cell recordings of 18 receptor neurons, 27 segmental and 31 ascending interneurons.

**Data analysis**

Spiking responses were digitized with 0.05 ms precision (A/D-converter, PCI-MIO-16E-4, National Instruments). From the digitized recordings the spike times were determined by means of a voltage threshold criterion. The resulting spike trains represented the basis for all subsequent analysis procedures. The statistical analyses considering the spike train variability were based on the evaluation of the interspike interval and the spike count distributions at each intensity. Mean spike count and variance were computed by counting the spikes trial-by-trial within a time window of the stimulus duration, to which the response latency was added. The interspike interval distribution was determined by joining all consecutive trials. The mean interspike interval and standard deviation were determined from this distribution. The spike rate was evaluated within the entire time window of analysis.
Spike train statistics

The coefficient of variation (CV) of the interspike interval distribution is defined as the quotient of standard deviation and mean value. The Fano factor (FF) was calculated as the quotient of variance and mean value of spike number per trial. Since most of the distributions of CV and FF were not fitted by a normal distribution, we applied the non-parametric Mann-Whitney-U-test or the Wilcoxon signed-rank test. However, for reasons of clarity in most cases we plotted mean and standard deviation of the CVs and FFs in the figures. A Bonferroni correction was applied if more than two tests were performed (Sachs 1999).

In order to increase the reliability of the CV estimates a number of at least 50 interspike intervals were demanded for the interspike interval distribution. This lower threshold corresponds to a mean spike rate of around 50 Hz at a stimulus duration of 100 ms and a repetition number of 10 (6 spikes or 5 ISI) or 15 (> 4 spikes or 3,3 ISI), respectively. In the following it is termed the “50 Hz-criterion” (see fig. 2). If at a certain intensity a neuron fired fewer spikes so that the interspike interval distribution was based on less than 50 events the respective CV value for this intensity was excluded from further analysis. Compared to vertebrate neurons the spike rate criterion of 50 Hz may appear rather high as a lower limit. However, the maximum spike rates of grasshopper auditory interneurons range between 150 and 300 Hz, and at spike rates below 50 Hz the firing becomes highly irregular. For simultaneous recordings we plotted the CVs for those intensities where both neurons fired at least at 50 Hz (fig. 3 A and D). The same criterion was applied to the calculation of the FF. Most of the auditory neurons show only a little spontaneous activity (Stumpner and Ronacher 1991). However, in a few cases the spontaneous activity was higher than 50 Hz (fig. 1 B, AN4). Such cases did not influence our data analysis and conclusions, since a confounding influence of spontaneous activity would have been relevant only at very low stimulus intensities, while our statistical analysis is based on stimulus intensities evoking maximal spike rates (fig. 3 B and E) or spike rates of 140 or 210 Hz (fig. 7), or were obtained at distinctly supra-threshold intensities (20 dB above threshold for the long stimulus paradigm, fig. 8).

Results

One main goal of the present study was to investigate the variability of spiking responses at the first three processing levels in the auditory pathway of locusts by
using simultaneous recordings and to compare these findings with those from single cell recordings.

**Variability of Interspike Intervals**

The variability of the interspike interval distributions of simultaneously recorded neurons was quantified by the CV (fig. 3 A). The majority of the paired recordings showed an increase of CV from one processing level to the next (data of simultaneously recorded cells are connected by lines). However, the increase of variability from receptors to segmental neurons is clearly more pronounced than the increase from segmental to ascending interneurons. In this latter comparison one third of the data even exhibited a reduction of variability (fig. 3 A, right). Figure 3 A contains data between 1 to 5 intensities for each cell pair tested (data from one cell pair are indicated by the same symbols). The wide spread of data points along the y-axis results from differences in spike rates between different neurons of the same type on one hand and on the other hand for the same neuron at different intensities corresponding to their intensity-response (rate-level) functions. In figure 2 the intensity-response functions of two simultaneously recorded cells are shown. Auditory neurons exhibited very different response characteristics with respect to response thresholds, maximal spike rates, or the overall shape of the response functions. While receptor neurons typically exhibit a saturation response type of intensity-response curve (Römer 1976), interneurons in the auditory pathway of locusts often show peaked optimum curves (fig. 2). Since both neurons of a pair rarely showed the same intensity dependency of the spike rate, it was not possible to obtain the CV values in a simultaneous recording for both neurons at their respective “best” intensity. Taking this into account, it turned out that a drop of CV-values between segmental and ascending neurons was correlated with the fact that in 7 out of 9 cases the ascending interneuron fired at higher rates than the respective segmental interneuron (see fig. 3 A). This finding indicates a strong impact of the spike rate on the variability of neuronal responses and will therefore be examined in more detail below.

A statistical examination of the data set in figure 3 A is problematic, since dependent data (same cell pair at different intensities) and independent data (different cell pairs) are combined in this plot. However, the analysis of single cell recordings does not
suffer from this problem. Here the data from all neurons (see material and methods) were pooled to calculate the mean CV for receptors, segmental and ascending interneurons, respectively. For each neuron only the CV calculated at the maximal spike rate was included (fig. 3 B, see also fig. 2). This kind of evaluation confirmed the picture emerging from fig. 3 A, and allowed the application of standard statistical tests since now each neuron contributed only a single value. The CV increased significantly from receptor neurons to segmental interneurons and ascending interneurons (see legend fig. 3; mean values of 0.20 for receptor neurons, 0.37 for segmental and 0.48 for ascending interneurons, respectively). For comparison, figure 3 C gives the complete CV data of these neurons, obtained at different sound levels, with the same 50 Hz-criterion as in fig. 3 A (see also fig. 2 and methods). The same tendency is visible as in figure 3 B, however, the mean CVs are shifted to somewhat higher values.

**Spike Count Variability**

The variability of the spike count reflects the reliability of neuronal responses with respect to successive stimulus repetitions. Figures 3 D-F are organized in the same manner as figures 3 A-C, with the same recordings as data basis and the same evaluation criteria. Again one can observe an increase of variability from peripheral to higher processing levels (fig. 3 D-F). The increase of spike count variability was significant between receptor neurons and segmental and ascending interneurons, respectively (fig. 3 E; mean values of 0.04 for receptor neurons, 0.08 for segmental and 0.19 for ascending interneurons, respectively).

As mentioned above, at the level of ascending interneurons one can distinguish two types of cells: neurons, which are specialized in the processing of sound direction, and, on the other hand, neurons with low directionality, obviously suited to encode features of the sound patterns (Ronacher and Stumpner 1993; Stumpner and Ronacher 1994). Examples of spiking responses of a direction coding (AN1) and a sound pattern coding ascending interneuron (AN11) are shown in figure 4. In general, the spiking patterns of direction coding interneurons are characterized by strong inhibitory inputs resulting in highly variable spiking responses (Rheinlaender and Mörchen 1979; Römer and Marquart 1984). Although pattern coding interneurons may receive inhibitory inputs as well, this inhibition usually is more precisely timed (Franz and Ronacher 2002). In contrast to the analysis of the
interspike intervals the spike count variability between ascending interneurons coding for direction and pattern differed significantly (fig. 3 E, p<0.05). In the examples of figure 4 the FF of AN1 was more than three times as high as that of AN11.

**Influence of spike rate on variability**

A comparison of figures 3 B and E with 3 C and F indicates that the spike rate had a strong impact on the variability of neuronal responses, and suggests a negative relationship between spike rate and variability. Such a negative correlation is not unexpected since at high spike rates the refractory period delimits spike intervals and may lead to more regular spike trains. In the context of the increasing variability at higher processing levels it is important to note that the maximal spike rates decreased considerably from a mean of 300 Hz for receptor neurons to 250 Hz for segmental and 170 Hz for ascending interneurons (fig. 5 A, compare also fig. 2). As these differences in spike rates may have a profound influence on neuronal variability, this parameter is taken into account in the following. In a first step we explored whether the reduction of spike rates at the level of ascending interneurons may have been caused by a prolonged refractory period in these neurons. The minimal interspike intervals, which were determined at each cell’s maximal spike rate even showed significantly higher values at the level of receptor neurons than at the level of segmental interneurons (fig. 5 B). Thus, there was no indication that the decrease of spike rate was caused by a change of the refractory period.

The relationship between spike rate and response variability is summarized in figure 6. For each neuron the CV and the FF is plotted versus the spike frequency (fig. 6 A-C and D-F). Each line represents the data of a single neuron, obtained at those intensities which exceeded the 50 Hz-criterion. For receptor neurons the CV depends almost linearly upon the spike rate (fig. 6 A). In order to quantify this dependence the correlation coefficient was determined for each curve that comprises at least three data points, and the average was calculated, which gives a coarse estimate of the mean dependence of variability on spike rate. For the interspike interval variability the mean coefficient of correlation decreased significantly from $-0.98 \pm 0.02$ (n=27) for receptor neurons to $-0.77 \pm 0.27$ (n=18), for segmental neurons (RE – SN: p<0.001), and $-0.43 \pm 0.61$ (n=16) for ascending interneurons (RE – AN: p<0.001, SN – AN: n.s.). This decrease indicated a less pronounced correlation between spike rate and
CV at higher processing levels. The same analysis was applied to the spike count statistics (fig. 6 D-F). Negative correlations were also found for spike rate and spike count variability. Again, the mean coefficient of correlation decreased from \(-0.94 \pm 0.06\) (n=27) for receptor neurons to \(-0.63 \pm 0.47\) (n=18) for segmental neurons (RE – SN: p<0.01) and \(-0.02 \pm 0.68\) (n=16) for ascending interneurons (RE – AN: p<0.001, SN – AN: p<0.05). In conclusion, the spiking variability of receptor neurons depends very strictly on spike rate, while no consistent dependence was observed for the population of ascending interneurons.

In order to reduce the impact of spike rate on response variability, we then restricted the analysis to data from neurons at different processing levels that exhibited roughly the same spike rates. In figure 7 the results are shown for two ranges of spike rates (140 Hz \(\pm\) 20 Hz and 210 Hz \(\pm\) 20 Hz) for both interspike interval and spike count variability. Under these restrictions the increase of interspike interval variability between processing levels was clearly reduced, however, one can still observe a rising tendency for both spike rate ranges (fig. 7 A, compare with fig. 3 B, E). The picture emerging for the spike count variability was similar, although at a spike rate of \(\sim\)210 Hz the ascending interneurons failed to show a tendency for higher mean values (fig. 7 B). In no case significant differences between the processing levels were found, hence the differences in spike count variability between processing levels observed before (fig. 3) can be attributed mostly to differences in spike rates.

**The impact of the evaluation time window on variability**

Previous studies of spike train variability reported a relationship between the time window for analysis and the measured variability (Gabbiani and Koch 1998; Kara et al. 2000). The stimuli used so far had a duration of 100 ms and were presented at different intensities. With a set of long stimuli (400 ms) we investigated the impact of the evaluation time window on variability. For the long stimulus paradigm the interval between the stimulus presentations was 1 s since this time span has been shown to suffice for a extensive recovery from adaptation (Ronacher and Krahe 1998; Benda 2001). The same increase of variability at higher processing levels was observed as with the short stimuli (compare fig. 3 C and F with fig. 8 A and D). However, the absolute values were shifted to higher values for the long stimulus paradigm (fig. 3 B and E). This may be due to the fact that the intensity which was used for stimulation
did not necessarily elicit the cells maximal spike rate. Furthermore, with long stimulus durations adaptation may influence the neurons response more strongly. The analysis of interspike interval variability in time windows ranging from 25 to 400 ms shows a decrease of variability for very small time windows. The CV reached a saturation at a window size of 100 ms whereas the difference between the three processing levels persisted over all window sizes (fig. 8 B). In contrast to the saturating interspike interval variability the enlargement of the evaluation time window did not result in a saturation for the spike count variability, which showed a steady increase with the window size (fig. 8 E). As already observed for the short stimulus paradigm both measures of variability yield different results when separating ascending interneurons into pattern and direction coding types. While no difference between both groups of neurons was observed for the interspike interval variability (fig. 8 C) the pattern coding ascending interneurons showed significant lower FF values than direction coding interneurons (fig. 8 F).

A comparison of interspike interval and spike count variability

So far, for both measures of spike train variability, CV and FF, an increase was observed from the periphery to higher processing levels. If the spike generation follows a renewal process, the FF should approximately correspond to the square of the CV (Gabbiani and Koch 1998). To see whether the variability data followed this rule, individual FF and CV^2 values are plotted in figure 9. Indeed, for the short stimulus paradigm the data of individual receptor neurons scatter around the 45°-line (fig. 9 A). In contrast, for segmental interneurons, the FF was in most cases smaller than the expectation derived from CV^2 (fig. 9 A). To illustrate this, we calculated the ratio between the individual FF and CV^2 values and plotted the median of this distribution in figure 9 E (left part). As can be seen in this figure both receptors and segmental interneurons showed median ratios lower than one. In the case of the segmental interneurons the deviation from one was highly significant while for receptor neurons no significant difference was found (compare the 25 % or 75 % interquartile distances in fig. 9 E). Interestingly, the two groups of ascending interneurons differed considerably in this respect (fig. 9 B and E). While for direction coding ascending interneurons the ratio between FF and CV^2 was larger than one, for the pattern coding interneurons the FF was by a factor 3 lower than CV^2 (fig. 9 E). In the latter case there even existed a significant difference between FF and CV^2.
An important assumption when formulating a renewal process is that the spike rate does not change over time (Gabbiani and Koch 1998). For real neurons this assumption is not realistic since adaptation reduces the neuronal activity even when a constant stimulus is presented. Benda et al. (2001) have shown that in auditory receptors adaptation occurs mainly during the first 80 – 100 ms. Thus, for the short stimulus paradigm the assumption of stationarity of the spike rate certainly was not fulfilled (fig. 9 A and B). Therefore, we considered data from the long stimulus paradigm and rejected the first 200 ms of a 400 ms neuronal response to get rid of adaptation-caused spike rate changes. Indeed, the ratio between FF and CV² is very similar to the short stimulus paradigm for all the groups of neurons and clearly below one (fig. 9 E right part), although there was no significant difference from one in any case (fig. 9 C and D). However, it has to be mentioned that pattern coding ascending interneurons almost approached significance (p<0.06). Thus, it remains to be seen whether a comparison with a renewal process may be an appropriate description for this group of neurons.

Discussion
The aim of this study was to investigate the variability of spike responses at three processing levels of the auditory pathway of grasshoppers. A main result was the increase in variability from receptor neurons to ascending neurons, which was demonstrated both in simultaneous recordings from two neurons, as well as in single-cell recordings (fig. 3). The fact that both approaches yielded the same result is another important finding, which shows that the effect of slow changes of the internal physiological state on variability is not very strong. Thus, no serious errors will be made by estimating variability on the basis of single cell recordings.

The higher variability of ascending neurons was unexpected since these neurons form a bottleneck for the information transfer to the brain, and therefore one may have expected that the precision rather than the variability of spike responses should increase at this level. Before focusing on these findings and the consequences for neural processing, however, a more general technical question concerning our approach to measuring variability shall be discussed.
Do our procedures capture the relevant variability?
Performing simultaneous recordings in the auditory system of locusts was important with regard to establishing identical physiological conditions for a comparison of the neuronal variability at different processing levels. However, the measures that were used to quantify the magnitude of variability may not be ideally suited to capture the influence variability has on the processing tasks, since they determine variability from repeated stimulus presentations (FF) or from sustained stimuli (CV). This is a situation quite different from the actual processing tasks performed by sensory systems, which normally have to rely on single events, and have to come to quick decisions. The long stimulation periods used for determination of FF and CV therefore may lead to an overestimation of the actual variability relevant for the animal. Even by performing simultaneous recordings it is conceivable that the excitability of only one of both simultaneously recorded neurons changes slowly (Pollack 1986, 1988; Römer and Krusch 2000). Moreover, differences might exist in the synaptic coupling strength between different pairs of neurons. A strong excitatory synapse between both neurons, for example, is likely to cause covariations of their spiking responses over time. In this case the animals would have to cope with distinctly lower neuronal variability than expected from a longer measurement series with repeated stimuli as we have done in our study.

In the context of pattern recognition the interspike interval variability possibly does not capture the most important aspects of neuronal coding. Here, the processing of signal features could enforce, as a byproduct, large differences in interspike intervals, while the recognition may be based on the reliability of spike count and, possibly also, spike timing (Berry et al. 1997; De Ruyter van de Steveninck et al. 1997; Warzecha and Egelhaaf 1999; Warzecha et al. 2000; Reinagel and Reid 2002). However, the evaluation of interspike interval variability in the nervous systems is of particular interest for theoreticians, since they may gain insight into neural processing mechanisms or into the properties of synaptic transmission (Softky and Koch 1993; Teich et al. 1997).

Influence of spike rate on variability
The data presented in figures 5 to 7 suggest that the increase in variability from the periphery to consecutive processing levels can to a large degree be attributed to a
parallel reduction of mean and maximal spike rates between receptors and ascending neurons. A negative correlation between interspike interval variability and spike rate has been reported for the visual system (Rodieck 1967; Barlow and Levick 1969; Frishman and Levine 1983; Kara et al. 2000). The reduction of the interspike interval variability at high spike rates is at least partly due to the influence of the refractory period that regularizes the spike distances. Obviously, this was the case for receptor neurons at higher spike rates (fig. 1 B). Interestingly, the strength of correlation between response variability and spike rate changed from a very strong one in receptor neurons to a weak or non-existing correlation in ascending interneurons. The lower spike rates in ascending neurons did, however, not result from physiological limits such as an extended refractory period (fig. 5 B). Rather it appears that the lower spike rate of ascending interneurons is a consequence of signal processing mechanisms. To what extent this assumption is corroborated by further observations will be discussed in the following.

**Implications of the observed variability for information processing**

With the stimuli used here both variability measures yielded values clearly below one, which would be expected if spikes were generated according to a Poisson process. Moreover, it is very unlikely that a Poisson process yields a good description for the spike generating mechanisms in real nerve cells since this model lacks a refractory period (Lestienne 2001). A very simple model, which incorporates a refractory period is the renewal process (Cox 1962; Gabbiani and Koch 1998). Schaette et al. (2005) have shown that the spiking responses of auditory receptor neurons in locusts are well described by a renewal process. The comparison of FF and CV² is a simple indicator of whether the spike train corresponds to a renewal process. For receptor neurons the present evaluation revealed a very close agreement with the work of Schaette et al. (2005) (fig. 9 C). Even the adaptation effective within the first 100 ms did not result in a significant difference between FF and CV² (fig. 9 A). Schaette et al. (2005) found minimal CV values of ~0.2 for the interspike interval distribution in response to constant stimulation. This value corresponds well to the CVs found in our study (see fig. 3 B). For segmental interneurons the relationship between FF and CV² showed a pronounced deviation from one. However, this deviation was significant only for the short stimulus paradigm (fig. 9 E compare left and right part). This means that the neurons’ responses in the adapted state resemble a renewal process while a
strong onset response does not match this theoretical model. For pattern coding ascending interneurons the same tendency as for segmental neurons was observed. The ratio between FF and \( CV^2 \) remained very low even in an adapted state, although significance was just missed. However, at present it remains speculative if there exist active mechanisms in spike generation which increase the reliability of neuronal responses.

Apart from the strong tendency of pattern coding ascending neurons to emphasize the onset of a stimulus (see fig. 1B and fig. 4B) the responses of most ascending interneurons are shaped by both excitation and inhibition (fig. 1B, Römer et al. 1981; Römer and Dronse 1982; Franz and Ronacher 2002). Inhibitory inputs seem to be indispensable for information processing in the auditory system of grasshoppers, as well as other animals (for review see Hennig et al. 2004). The increasing impact of inhibition may be a major reason for both decreasing spike rates and increasing interspike interval variability at higher processing levels. However, these inhibitory inputs do not necessarily also decrease the spike count reliability. This can be deduced from a comparison of ascending interneurons that code for sound direction and for sound pattern (fig. 3). For both classes of ascending neurons the interspike interval variability was virtually the same (fig. 3B). In contrast, the FF of neurons coding for the sound pattern was only marginally larger than that of receptor neurons, and significantly lower than that of neurons coding for sound direction (fig. 3E, \( p<0.05 \)). Hence, the pattern coding interneurons show a remarkable reliability in the spike count for different presentations of a stimulus, in spite of rather variable interspike intervals (fig. 3B). Again, inhibitory inputs seem to be responsible for these differences. In the group of pattern encoding neurons the inhibition occurs rather precisely in time, while in directional neurons without need for precise timing strong inhibitory inputs appear to reduce the spike count on one side (Römer et al. 1981; Römer and Dronse 1982). The directional information can then be represented simply by the differences in spike count between mirror image counterparts. As long as the difference of spike count between both neurons is sufficient, the precise number and timing of spikes may not be a crucial factor (Ronacher et al. 1986; see also Römer and Krusch 2000). In an extreme case this would result in lateralization, that is, excitation from one side and inhibition from the other. Indeed, in grasshoppers this kind of lateralization has been shown for a direction coding interneuron as well.
as in the behavior of these animals (von Helversen 1997; for review see Hennig et al. 2004).

Finally, the pooling of data from different identified neurons into larger classes deserves a comment, in particular for the class of pattern coding ascending neurons. The classes of receptors, local and ascending neurons are anatomically defined, and correspond also to the stages of information flow within the metathoracic processing module (fig. 1 A). Among ascending neurons, the distinction between directional and pattern encoding neurons was introduced earlier, based on their different response properties (Ronacher and Stumpner 1993). The two direction coding interneurons known so far resemble each other in their response properties. Both neurons are excited from one hemisphere and inhibited from the other, and, in view of their variable responses, seem hardly suited to transmit information about the song pattern (Stumpner and Ronacher 1991, 1994). The class of pattern coding neurons is larger and encompasses more different response characteristics, but these neurons share a common feature in that they transmit little or no directional information (Ronacher and Stumpner 1993). In particular, the small standard deviation for the FF of pattern coding elements (fig. 3 E and fig. 8 D) is a hint that pooling of these neurons captures some more general properties of the auditory network.

Comparison with vertebrate sensory systems

The results reported here show interesting parallels to the visual system of vertebrates. Increasing spike count variability has also been reported for successive processing levels in the cat primary visual pathway on the basis of simultaneous recordings in retinal, thalamic and cortical neurons (Kara et al. 2000). This increase of variability was also accompanied by a decrease of mean spike rate in the respective neurons. However, the responses of cortical neurons are often highly variable with respect to the spike number and their temporal occurrence. Numerous studies in the visual cortex of cats report FFs larger than one (Heggelund and Albus 1978; Skottun et al. 1987; Bair and O’Keefe 1998; Buracas et al. 1998; Oram et al. 1999). In other studies FFs smaller than one were found (Gur et al. 1997; Gershon et al. 1998; Kara et al. 2000). Kara et al. (2000) discuss the ineffectiveness of stimuli as a reason for a higher neuronal variability as observed in former investigations. In our study we deliberately chose noise pulses without amplitude modulation, in order to
separate the intrinsic sources of variability from stimulus-induced ones. The responses of neurons to amplitude modulated stimuli will be the subject of another paper (Vogel and Ronacher, in prep).

In comparison to the grasshopper auditory system, where the processing of different stimulus features occurs in a network of ascending interneurons, in the vertebrate nervous system the encoding of different stimulus parameters is often organized in different anatomical nuclei. However, in contrast to the visual system of vertebrates and to the results of our study deWeese et al. (2003) found most reliable spiking responses in the auditory cortex, which is the highest stage of the auditory pathway. This reliability is based on a binary spiking mode, which means “spike” or “no spike” in response to an acoustic stimulus. For some stimuli those auditory neurons fired with the highest reliability one spike per trial. In this case FF is zero. However, this kind of representation requires high functional separation between the neurons. This means that every cell is coding a certain limited aspect of the stimulus (Takahashi et al. 1984; Covey and Cassaday 1991; Viete et al. 1997; Oertel 1999). Besides a functional separation of ascending interneurons in the metathoracic ganglion we also found a pronounced decrease of the spike rate at this processing level. This decrease is accompanied by an increase of the phasic response component. However, it remains speculative whether at higher processing levels in the brain of the grasshopper this mechanism is further intensified finally leading to a kind of binary spiking and thus to a massive decrease of variability. In another sensory system of the locust such a decrease of spike rate at higher processing levels was observed. The Kenyon cells which represent the highest stage of the odour processing system respond with only a single spike when their respective odour is presented (Stopfer et al. 2003). In this context it seems worth to investigate the response characteristics of auditory brain neurons in the future.
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Abbreviations
AN ascending neuron
AN_{dir} ascending neuron encoding sound direction
AN_{patt} ascending neuron encoding sound pattern
CV coefficient of variation
FF Fano factor
ISI interspike interval
RE receptor neuron
SN segmental neuron
SpC spike count
Literature


Figures

A

Metathoracic ganglion

RE 60 - 80  SN 10 - 15  AN 15 - 20

Brain

Information flow

B

receptors  (bi)segmental interneurons  ascending interneurons

SN2  AN11

SN1  AN4  BSN1  AN1

10 mV
50 ms
Figure 1. The auditory pathway in the metathoracic ganglion of *Locusta migratoria*. A Schematic diagram of the information flow in the auditory pathway. 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. B Spike traces of three representatives of each processing level are shown (from top: 3 low-frequency tuned receptor neurons; SN: SN2, SN1 and BSN1; AN: AN11, AN4 and AN1). All neurons were stimulated with a 100-ms rectangularly modulated noise pulse at 60 dB SPL. Vertical scale bars: 10 mV, horizontal bar: 50 ms.
Figure 2. Intensity response functions of two simultaneously recorded auditory neurons. Mean spike rates and standard deviations are shown (n=15 stimulus repetitions). Two criteria for the analysis are indicated in this plot: (1) The lower limit of 50 Hz (dotted line). Data points below this threshold were not analysed. (2) Maximal spike rate, which occurred at different intensities for both neurons in this example.
Figure 3 Interspike interval (ISI) and spike count (SpC) variability for the intensity-response stimulus paradigm. A, B, C Coefficient of variation (CV) as a measure of interspike interval variability. D, E, F Fano factor (FF) as a measure of spike count variability. A, D Simultaneous recordings of receptor neurons and segmental interneurons or segmental and ascending interneurons. Data from simultaneous recordings are connected by lines. Same symbols represent data from the same cell pair at different stimulus intensities, different symbols represent data from different cell pairs. Data are shown only for intensities that caused a spike rate of at least 50 Hz. For reasons of clarity the symbols within a column were shifted against each other. B, E Mean CV and FF with the respective standard deviations from single-cell recordings. For each neuron only data for the intensity that elicited the maximal spike rate were included (see fig. 2). Mann-Whitney-U-Test: CV: RE - SN: p<0,001, RE - AN: p<0,001, SN - AN: p<0,01, ANdir – ANpatt: n.s.. FF: RE - SN: p<0,001, RE - AN: p<0,001, SN - AN: n.s., ANdir – ANpatt: p<0,05, RE - ANdir: p<0,001, SN – ANdir: p<0,01, RE – ANpatt: p<0,05, SN – ANpatt: n.s.. C, F Mean CV and FF with the respective standard deviations from single-cell recordings. In this example the 50 Hz-criterion was applied. Thus, data from the same cell at different stimulus intensities and data from different cells were mixed to calculate the mean. ANdir: ascending neuron encoding direction (n=10), ANpatt: ascending neuron encoding pattern (n=11).
A  AN 1 (direction sensitive)  

B  AN 11 (pattern sensitive)
Figure 4. Spiking responses of two different ascending interneurons. A Ascending interneuron coding for the sound direction (AN1). The directionality of AN1 becomes obvious when stimulating the neuron from the left and the right side. While the neuron is excited from the soma contralateral side it receives a strong inhibition from the other side (data not shown). B Ascending interneuron coding for the sound pattern (AN11). This neuron shows a pronounced onset response while its directionality is not very pronounced (Stumpner & Ronacher, 1994). The stimulus intensity was 60 dB SPL for AN1 and 50 dB for AN11, respectively. For both neurons 5 consecutive trials in response to the acoustic stimulus are shown and the number of spikes is indicated for each trial. The spike count variability (FF) was larger for the AN coding for the sound direction (AN1: FF = 0.54; n=10) than the AN coding for the sound pattern (AN11: FF = 0.16, n=10).
Figure 5 Firing rate and minimal interspike interval. A Mean values of maximal spike rates of receptor neurons, segmental and ascending interneurons. For each neuron only data from the intensity that elicited the maximal spike rate are included. The spike rate was evaluated over the entire stimulus-driven response. Mann-Whitney-U-Test: maximal spike rate: RE - SN: n.s., RE - AN: p<0,001, SN - AN: p<0,01, ANdir – ANpatt: p<0,01. B The same data as in A were used to calculate the minimal interspike interval. One segmental and three ascending interneurons were excluded from the data pool, since the stimulus conditions did not evoke minimal intervals. RE: receptor neurons, SN: segmental interneurons, AN: ascending interneurons, ANdir: ascending neuron coding direction (A: n=10, B: n=7), ANpatt: ascending neuron coding pattern (n=11). minimal interspike interval: Mann-Whitney-U-Test: RE - SN: p<0,05, RE - AN: n.s, SN - AN: n.s., ANdir – ANpatt: n.s.
Figure 6 Dependence of interspike interval (CV) and spike count (FF) variability on the mean spike rate. A and D CV and FF, respectively, versus firing rate for receptor neurons. Connected points represent data for the same neuron. Only data for those intensities are shown, which caused a spike rate of at least 50 Hz. B and E for segmental interneurons. C and F for ascending interneurons. Due to the different shapes of the intensity response curves the same firing rates were evoked at different stimulus intensities.
Figure 7 Response variability for stimuli that elicited the same spike rate. A interspike interval variability. B Spike count variability. A CV as a measure of interspike interval variability for receptor neurons, segmental and ascending interneurons that responded with 140 Hz ± 20 Hz and 210 Hz ± 20 Hz. B Same as in A with the FF as measure of spike count variability. The high mean FF in B at 140 Hz and its large standard deviation are due to a single neuron (a direction coding AN) that showed an extremely high FF value (1,96). If this single value is excluded the mean and SD change from 0,39±0,51 (n=12) to 0,24±0,16 (n=11)
Figure 8 Response variability for the long unmodulated stimulus paradigm. A - C Mean CV as a measure of interspike interval variability. D - F Same as in A - C with the mean FF as measure of spike count variability. A, D In this example the 50 Hz-criterion was applied. In contrast to figure 3 C and F for each neuron only data from a single intensity were included to the distribution. Thus, a statistical evaluation was possible. B, C, E, F In order to investigate the impact of the time window of analysis on the variability both measures of variability were plotted as a function of the time window. Here the 50 Hz-criterion was not applied, since for short time windows this criterion was untenable. For that reason the number of elements within a distribution differs between A, D and B, C, E, F. Typical standard deviations are shown for each graph. B, E Receptor neurons and segmental interneurons. C, F The ascending interneurons are divided in pattern coding and direction coding types. RE: receptor neurons (n=18), SN: segmental interneurons (n=27, at 25 ms: n=23), AN: ascending interneurons (n=31, at 25 ms: n=26), AN\textsubscript{patt}: ascending neuron encoding sound pattern (n=17, at 25 ms: n=14), AN\textsubscript{dir}: ascending neuron encoding sound direction (n=14, at 25 ms: n=12). Mann-Whitney-U-Test at 400 ms: A CV: RE - SN: p<0,001, RE - AN: p<0,001, SN - AN: p<0,01, AN\textsubscript{dir} – AN\textsubscript{patt}: n.s.. D FF: RE - SN: p<0,01, RE - AN: p<0,001, SN - AN: n.s., AN\textsubscript{dir} – AN\textsubscript{patt}: p<0,05.
**Figure 9** Relationship between interspike interval and spike count variability. A, B, C, D The CV² of the interspike interval variability was plotted versus the FF of the spike count variability on logarithmic scales. A, B Short stimulus paradigm (100 ms). Values were taken from the maximal spike rate data (compare fig. 3 B and E). RE (n=33), SN (n=25), AN (n=21), AN_patt (n=11), AN_dir (n=10). C, D Long stimulus paradigm (400 ms). To eliminate the onset responses only the last 200 ms of a 400 ms response were used for the estimation of FF and CV². RE (n=18), SN (n=23), AN (n=22), AN_patt (n=13), AN_dir (n=9). A, C Receptor neurons and segmental interneurons. B, D Ascending interneurons are divided in pattern coding and direction coding types. E For all groups of neurons **medians of the ratio** between FF and CV² are plotted for both stimuli paradigms. The error bars correspond to the 25 % and 75 % interquartile, respectively. The 75 % interquartile distance for AN_dir for the long stimulus paradigm was 2.64. The distributions of FF and CV² were tested for differences with the Wilcoxon signed-rank test. For the short stimuli a significant deviation from FF = CV² (or a ratio of 1, see vertical line) was observed for segmental (p<0.001) and pattern coding ascending interneurons (p<0.01). None of the groups showed significant differences between FF and CV² for the long stimulus paradigm although the value for AN_patt approaches significance (p<0.06).