Comparative post-hearing development of inhibitory inputs to the lateral superior olive in gerbils and mice

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Running head: Development of inhibition to the LSO after hearing onset

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Abstract

Interaural intensity differences are analyzed in neurons of the lateral superior olive (LSO) by integration of an inhibitory input from the medial nucleus of the trapezoid body (MNTB), activated by sound from the contralateral ear, with an excitatory input from the ipsilateral cochlear nucleus. The early postnatal refinement of this inhibitory MNTB-LSO projection along the tonotopic axis of the LSO has been extensively studied. However, little is known to what extent physiological changes at these inputs also occur after the onset of sound evoked activity.

Using whole-cell patch-clamp recordings of LSO neurons in acute brainstem slices we analyzed the developmental changes of inhibitory synaptic currents evoked by MNTB fiber stimulation occurring after hearing onset. We compared these results in gerbils and mice, two species frequently used in auditory research. Our data show that neither the number of presumed input fibers nor the conductance of single fibers significantly changed after hearing onset. Also the amplitude of miniature inhibitory currents remained constant during this developmental period. In contrast, the kinetics of inhibitory synaptic currents greatly accelerated after hearing onset. We conclude that tonotopic refinement of inhibitory projections to the LSO is largely completed before the onset of hearing, whereas acceleration of synaptic kinetics occurs to a large part after hearing onset and might thus be dependent on proper auditory experience. Surprisingly, inhibitory input characteristics as well as basic membrane properties of LSO neurons were rather similar in gerbils and mice.
Introduction

The superior olive, an auditory brainstem structure comprising several distinct nuclei, is the first site of binaural interaction in the brain. One of its main nuclei, the lateral superior olive (LSO) receives excitatory inputs from the ipsilateral anteroventral cochlear nucleus (AVNC) and inhibitory inputs from the ipsilateral medial nucleus of the trapezoid body (MNTB) (Tollin 2003). In agreement with these anatomical features, LSO neurons are excited by sounds presented to the ipsilateral ear and are more and more inhibited when the sound intensity increases at the contralateral ear (Boudreau and Tsuchitani 1968; 1970; Kavanagh and Kelly 1992; Moore and Caspary 1983). This subtraction mechanism enables these neurons to analyze interaural intensity differences (IIDs), the main cue for the localization of high frequency sounds.

The accurate analysis of IIDs requires that the excitatory and inhibitory inputs from each ear are mutually adjusted during development. The frequency tuning and the temporal characteristics of the inhibitory and excitatory inputs need to be matched and the relative input strength of excitation and inhibition needs to be balanced. Experiments over the recent years indicate that the developmental adjustments of the inhibitory inputs is largely accomplished before the onset of hearing (P12), thus in the absence of sound evoked activity. Before hearing onset LSO neurons become functionally disconnected from the majority of their MNTB inputs which results in a sharpening of the tonotopic projection from the MNTB to the LSO (Kandler and Gillespie 2005; Kim and Kandler 2003; 2010). At the same time the efficacy of the remaining inhibitory inputs increases almost 12-fold. Nevertheless, further refinement has been suggested to occur after hearing onset and could therefore also depend on auditory experience (Kandler et al. 2009; Kandler and Gillespie 2005; Sanes and Friaufl 2000). For example, morphological analysis suggests that after hearing onset further pruning of the MNTB axons projecting to the LSO occurs along the tonotopic axis (Sanes and Siverls 1991), a process that is dependent on appropriate neural activity levels (Sanes and Takacs 1993) Whether this structural refinement is a delayed consequence of the functional denervation observed before hearing onset or whether an additional pruning of functional MNTB-LSO synapses occurs after hearing onset has been a matter of debate.

Using whole-cell patch-clamp recordings from acute auditory brainstem slices, we characterized physiological changes of the inhibitory MNTB-LSO projection after hearing onset. To our surprise no further developmental refinement of the inhibitory inputs to the LSO after hearing onset was revealed, except for a considerable acceleration of the inhibitory current kinetics. Moreover, we did not observe a decrease of short-term depression after hearing onset, as previously described for the Calyx and Endbulb of Held (Oleskevich et al. 2004; Taschenberger and von Gersdorff 2000; Wang and Manis 2008). In addition, we investigated whether species specific differences in the development and the function of the inhibitory inputs to the LSO exist, since the previous studies on the development of inputs to LSO neurons have been carried out on various rodent species (Kim and Kandler 2003; Sanes 1993; Youssoufian et al. 2008). The use of mice as experimental animals would also allow a further detailed analysis of the molecular mechanisms underlying these developmental changes.
Methods

All experiments were performed in accordance with the rules laid down by the EC Council Directive (86/89/ECC) and German animal welfare legislation.

Slice Preparation

Recordings were made from LSO neurons in coronal slices (190µm) of the auditory brainstem in animals aged P 9/10 (referred to as P10), P17/18 (referred to as P18) and P22/23 (referred to as P23). Both gerbils (*Meriones unguiculatus*) and mice (Balb/C) were used as experimental models. Although Balb/C mice show significant early-onset hearing loss, the developmental period studied (younger than 1 month) is well before the onset of hearing loss in this mouse strain (after 4 months) (Willott et al. 1998). Before decapitation animals were deeply anesthetized with Isoflurane and the brain was quickly removed and immersed in ice-cold oxygenated (95%O₂, 5%CO₂) sucrose replacement solution containing (in mM): 85 NaCl, 2.5 KCl, 1.3 NaH₂PO₄, 25 NaHCO₃, 75 sucrose, 25 glucose, 0.5 CaCl₂ and 4 MgCl₂. The block of tissue was glued with cyanoacrylic glue onto a bath chamber that was filled with ice-cold sucrose replacement solution. Slices comprising the LSO were cut on a vibrating microtome (VT1000S; Leica, Wetzlar, Germany) and subsequently incubated at 32°C for 30 min in oxygenated artificial cerebrospinal fluid (ACSF) solution containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 25 glucose, 2 CaCl₂ and 1 MgCl₂ (pH 7.4). Afterwards slices were stored at room temperature (22±2°C) for a maximum of 4–5 h. For recordings slices were transferred to the recording chamber and superfused continuously with oxygenated ACSF at a rate of 1-2 ml/min. Slices were viewed with an upright microscope (Zeiss Axioskope; Oberkochen; Germany) using infrared-differential interference optics. All recordings were made at 32±1°C.

Whole-cell recordings and experimental procedures

Whole-cell current- and voltage-clamp recordings were made from neurons in the LSO and using a Multiclamp700A amplifier (Axon Instruments; Foster City; CA). Borosilicate glass electrodes (GC150F-10, Harvard Apparatus, Edenbridge, UK) were pulled on a DMZ Universal Puller (Zeitz Instruments, Germany) yielding a final tip resistance of 3–5 MΩ. For all current-clamp recordings electrodes were filled with an internal solution containing (in mM) 130 K-gluconate, 5 KCl, 10 HEPES, 1 EGTA, 2 Na₂ATP, 2 Mg-ATP, 0.3 Na₂-GTP, 10 Na-phosphocreatin (adjusted to pH 7.3 with KOH; Osmolarity: 280mOsm). For voltage-clamp recordings of evoked inhibitory currents electrodes were filled with solution containing (in mM): 135 Cs-methylsulfonate; 5 KCl; 10 HEPES; 1 EGTA; 2 Na₂ATP; 2 MgATP; Na₂GTP; 10 Na-Phosphocreatin (adjusted to pH 7.3 with CsOH; Osmolarity: 300mOsm). For the recordings of miniature IPSCs the electrode solution contained: 135 Cs-Cl; 5 KCl; 5 mM TEA-Cl, 10 HEPES; 1 EGTA; 2 Na₂ATP; 2 MgATP; Na₂GTP; 10 Na-Phosphocreatin (adjusted to pH 7.3 with CsOH; Osmolarity: 300mOsm). During current-clamp experiments the bridge balance was applied. For voltage-clamp recordings series resistance was compensated by 70 to 80% and monitored throughout the experiment. Recordings were discarded if series resistance exceeded 10 MΩ or had changed more than 20% during the course of the experiment. If not otherwise stated the holding potential in voltage-clamp recordings was usually −55 mV. Junction potentials for different electrode solutions were
determined using ClampFit (Version 10; Molecular Devices) (K-glucophone: 11.6mV; Cs- 
Methylsulfonate: 9mV), and were corrected for throughout the manuscript.

In mice neurons were selected from the entire LSO. In contrast, in gerbils only LSO neurons were 
selected from the medial limb which represents neurons that have characteristic frequencies above 5 
kHz (Sanes et al. 1989), similar to the presumed best frequency range of neurons found in the mouse 
LSO. Neurons were selected upon their bipolar and fusiform shape of their somata and large size with 
a compensated capacitance larger 20pF in gerbils and above 18pF in mice. Lateral olivocochlear 
(LOC) neurons, which are intermingled with principal cells in the LSO in gerbils (Kaiser et al. 2011) 
and mice (Sterenborg et al. 2010), have capacitance of only 50% compared to principal neurons (in 
our experiments around 10pF) and display a depolarized resting membrane potential, a delay-type 
firing pattern and specific inward and outward current properties (Sterenborg et al. 2010). These 
criteria were used to exclude LOC neurons in the current-clamp mode. In voltage-clamp mode only 
capacitance measurements could be employed for the distinction between LOC and principal neurons.

To isolate inhibitory synaptic currents during voltage-clamp recordings, the following pharmacological 
agents were used (Tocris-Cookson, Bristol, UK): 10µM DNQX (6,7-dinitroquinoxaline-2,3-dione), 
50 µM DL-APV (DL-2-amino-5-phosphonopentanoic acid). For the isolation of miniature IPSCs also 
1 µM tetrodotoxin (TTX) was added to the ACSF. Drugs were dissolved in dH2O stored at −20°C, 
diluted prior to the experiment and added to the perfusate during the experiment.

MNTB fibers were stimulated by placing a glass electrode (tip opening 1-2µM) filled with 2M NaCl in 
the MNTB fiber tract about 150µm medial to the LSO. An analog isolated pulse generator (BSI 950, 
Dagan Corporation, Minneapolis, MN, USA) triggered a bipolar (+/−) stimulus pulse at a rate of 0.1- 
0.3 Hz with pulse durations between 200 and 400 µs. Stimulation intensities between 5 and 99V were 
used in these experiments. Threshold for synaptic responses was usually between 10 and 20V. For 
the analysis of input number the stimulation intensity was gradually increased in increments of 5V 
between 5 and 40V and thereafter in increments of 10V. Maximal evoked currents were only 
determined if the amplitude of the current had not increased between 70V and 90V stimulation 
intensity. In a number of neurons low stimulus intensities evoked IPSCs with very small amplitudes 
(<50 pA) similar to the amplitudes of mIPSC. These IPSCs were not considered to be directly evoked 
by fiber stimulation and excluded from the analysis. Single-fiber IPSC amplitude was therefore defined 
as the first average IPSC amplitude larger than 50pA.

Data analysis

The signals were filtered with a low-pass 4-pole Bessel filter at 10 kHz, sampled at 50 kHz, and 
digitized using a Digidata 1322A interface (Axon Instruments). Traces were digitally filtered at 2-5 kHz. 
Stimulus generation, data acquisition and offline analysis of data were performed using pClamp 
(Version 10.2, Axon Instruments) or IGOR (Version 6.0, Wavemetrics) together with the custom written 
package NeuroMatics (Jason Rothman, UCL, London).

Input resistance was calculated from the voltage deflection evoked by a -100pA current injection at the 
negative peak of the voltage deflection and at the steady state voltage, 800ms after the beginning of 
the current injection. Membrane time constants were determined by fitting a single exponential function
to the voltage change in response to a -100pA current injection. Inhibitory conductances were derived from the inhibitory current amplitudes and the driving force. Since cesium in the pipette can influence the activity of the K⁺/Cl⁻-exchanger (Kakazu et al. 1999), the chloride reversal potential can change during these recordings. Therefore, we measured the chloride reversal potential by determining the synaptic current amplitude at various holding potentials at different ages. Chloride reversal did not change for different ages (data not shown). IPSC amplitudes in response to train stimulation were determined by fitting an exponential function to the decay of each IPSC and calculating the difference between this function and the current trace at the time point when the maximum of the next IPSC occurred.

To objectively determine the number of groups of different IPSC values, which presumably reflects activation of individual fibers, cluster analysis of the IPSC peaks was used. This analysis was performed using R statistical software (R-foundation). The number of clusters was obtained using the fpc-protocol, which determines cluster numbers by calculating Mahalanobis distances using linear regression analysis. The mean amplitude of each cluster was determined by K-means clustering, with the number of clusters determined by the fpc-protocol.

In the figures stimulation artifacts have been deleted for clarity. Results are expressed as mean ± standard error of mean (S.E.M.). Significant differences are marked with a single asterisk for values of $P<0.05$, with a double asterisk for $P<0.01$ and with a triple asterisk for $P<0.001$. $P$-values in this study were obtained by using Student’s two-tailed paired or unpaired $t$ test.
Results

Basic membrane properties of LSO principal cells are similar in gerbils and mice

We characterized the membrane properties of principal neurons in mice and gerbil LSO in response to hyper- and depolarizing current injections. The intrinsic properties of LSO principal neurons have been reported to differ among various species in terms of firing pattern, input resistance and membrane potential changes upon hyperpolarization (Adam et al. 2001; Adam et al. 1999; Barnes-Davies et al. 2004; Hassfurth et al. 2009; Kandler and Friauf 1995; Leao et al. 2006; Sanes 1993). However, these studies were carried out under different conditions such as various compositions of ACSFs and electrode solutions, different temperatures and ages of animals. We therefore tested to what extent intrinsic properties of LSO principal neurons differ between gerbils and mice if experimental conditions are exactly the same. To compare neurons with similar best frequencies, neurons in mice were chosen from the entire LSO, whereas in gerbils only neurons from the medial, high frequency limb of the LSO (Sanes et al. 1989) were included. About 10 days after hearing onset at P23 almost all neurons of both species displayed an onset type firing pattern in response to a depolarizing step current injection (Fig.1A). Moreover, at this developmental stage input resistance measured in response to small hyperpolarizing current injections was very similar for both species at the peak (gerbil: 42.5 ± 7.9 MΩ, n=7; mouse: 36.9 ± 9.1 MΩ, n=7; p=0.65) and at the steady state (gerbil: 18.9 ± 2.9 MΩ, n=7; mouse: 23.4 ± 7.2 MΩ; n=7; p=0.61) of the voltage response (Fig.1B,C). Similarly, no species difference was observed for the membrane time constant (gerbil: 2.6 ± 0.5 ms, n=7; mouse: 1.7 ± 0.4 ms, n=7; p=0.12) and the resting membrane potential (gerbil: -62 ± 1.3 mV, n=7; mouse: -61 ± 0.7 mV, n=7; p=0.7) at this developmental stage.

The onset of acoustic experience at P12 profoundly changes activity levels in the auditory brainstem and can thereby trigger a number of changes in neuronal properties. In gerbil LSO neurons peak and steady state input resistance and membrane time constant decreased significantly just after hearing onset between P9/10 and P17/18 (Fig. B,C, D), whereas in mice the decrease was much smaller and for input resistance not significant. No further decrease in input resistance and membrane time constant was observed afterwards (at P23), indicating that input properties of LSO neurons are mature around P18. In both gerbils and mice the resting membrane potential slightly depolarized during the first 10 days after hearing onset (Gerbil: P10: -66 ± 1.4 mV, n=10; P23: -62 ± 1.2 mV, n=7; p≤0.05; Mice: P10: -63 ± 0.5 mV, n=12; P23: -61 ± 0.7 mV, n=7; p≤0.05) (Fig.1E). To find out whether intrinsic properties differ along the tonotopic axis of the mouse LSO we also compared intrinsic properties of neurons in the medial (n=8) and lateral (n=8) part of the LSO of a P18 old mouse in the same brain slice. Intrinsic properties of neurons did not differ between neurons from the medial and lateral part of the LSO in this species (Rin peak: medial: 42 ± 10 MΩ, lateral 38 ± 9 MΩ; Rin SS: medial 20 ± 6 MΩ, lateral 20 ± 6 MΩ; tau: medial 2.1 ± 0.5 ms, lateral 2.2 ± 0.5 ms; Vrest: medial -60 ± 1 mV, lateral -60 ± 1 mV). These results suggest that membrane properties of LSO neurons in adult gerbils and mice are extremely similar and previously described species specific differences have most likely arisen from technical issues or from variations in age distribution.
No change in inhibitory fiber number and strength occurs after hearing onset in mice and gerbils.

The inhibitory inputs from the MNTB to the LSO undergo major functional adaptations before the onset of hearing (Kim and Kandler 2003). To analyze whether further functional refinement of the inhibitory MNTB-LSO projections proceeds also after hearing onset, we recorded pharmacologically isolated inhibitory synaptic currents evoked by MNTB fiber stimulation in LSO neurons just before (at P10) and several days after hearing onset (P18). MNTB fibers were stimulated with various stimulation intensities (10-99V) that were increased in small increments (see methods). Despite the gradual increase in stimulation intensities, IPSC amplitudes increased stepwise usually with 3 to 5 steps total (Fig.2 A,B). This stepwise increase in IPSC amplitude in response to increasing stimulus intensity is generally interpreted as an additional recruitment of inhibitory fibers projecting to the neuron.

Examples of IPSCs evoked by a large range of stimulation intensities are shown in Figure 2Ai (P10) and 2Bi (P17). To quantify the number of inhibitory fibers connecting to one LSO neuron, the amplitude distribution of IPSCs was plotted against the stimulus intensity (Fig.2Aii,Bii) and groups of amplitudes were determined by cluster analysis (see methods) (Fig.2Aiii,Biii). Before hearing onset (at P10) the average number of clusters (presumed inhibitory fibers) amounted to three to four in both species (Fig.2C), very similar to the number of inhibitory fibers reported in rats in the same age range (Kim and Kandler 2003). At P18 the average number of presumed inhibitory input fibers was slightly smaller compared to prehearing animals in both species, but this difference was not significant (gerbil: P10: 3.1 ± 0.4, n=10; P18: 2.8 ± 0.4, n=8, p=0.54; mouse: P10: 4 ± 0.6, n=10, P18: 3.45 ± 0.3, n=11, p=0.3) (Fig.2C). Furthermore, no significant change in the single-fiber conductance was observed during this developmental period in gerbils (P10: 19 ± 2 nS; n=10; P18: 23 ± 5 nS; n=8, p=0.47) and mice (P10: 20 ± 4 nS; n=10; P18: 18 ± 5 nS; n=11, p=0.79) (Fig.2D). Again, these single-fiber conductances were comparable to the ones reported in rats and mice for the age range P8-P14 (Kim and Kandler 2003; 2010; Noh et al. 2010). Calculating the number of fibers by dividing the maximal conductance by the single-fiber conductance gave slightly larger number of fibers for cases, since the conductance of the first fiber was on average smaller than the conductance of the additional fibers recruited. However, no developmental changes were observed in the number of fibers for both species using this method (gerbil: P10: 5.4 ± 0.7, n=10; P18: 6 ± 1.3, n=8, p=0.66; mouse: P10: 6.3 ± 1.8, n=10, P18: 7.6 ± 1.2, n=11, p=0.56). These results indicate that functional refinement of the inhibitory MNTB-LSO projection seems to be mostly completed before hearing onset, and the previously observed structural refinement might lag behind the functional refinement by several days.

Miniature IPSC frequency is larger whereas miniature IPSC conductance is smaller in mice compared to gerbils.

To further investigate developmental changes of the inhibitory input properties such as the number of release sites and the quantal content, we recorded miniature inhibitory postsynaptic currents (mIPSCs) in the presence of the Na⁺-channel blocker TTX in gerbils and mice (Fig.3A,B). At all developmental stages, there was a pronounced difference in the frequency and amplitude of mIPSCs between gerbils and mice. In mature animals at P22/23 mIPSC frequency was almost three times larger in mice.
compared to gerbils (gerbil: 3.8 ± 0.5 Hz, n=9; mouse: 10.2 ± 0.5 Hz, n=9; p≤0.001) (Fig.3C), whereas
the peak conductance of mice mIPSCs was about 50% smaller compared to gerbils (gerbil: 2.9 ± 0.4
nS, n=9; mouse: 2.1 ± 0.2 nS; n=9; p≤0.05) (Fig.3D). This difference in spontaneous quantal release
and quantal amplitude suggests that LSO neurons in mice receive more but weaker inputs compared
to gerbil LSO neurons.
Developmental changes in mIPSC frequency and conductance were not as clear. In both gerbils and
mice mIPSC frequency increased between P10 and P23 (gerbil: P10 2.4 ± 0.8 Hz, n=10, P23: 3.8 ±
0.5 Hz, n=9, p=0.15; mouse: 5.9 ± 1.3 Hz, n=9, P23: 10.2 ± 0.5 Hz, n=9, p≤0.05). However, variability
of mIPSC frequency was large between neurons. Conductance of mIPSC did not change in mice,
whereas in gerbils there was a slight but non-significant decrease in the conductance between P18
and P23 (P18: 3.5 ± 0.2 nS, n=9, P23: 2.9 ± 0.4 nS, n=9, p=0.06). This was again due to the large
variability between neurons. Nevertheless, this increase in mIPSC frequency clearly speaks against an
activity dependent further elimination of inhibitory inputs after hearing onset in both gerbils and mice.

The kinetics of inhibitory synaptic currents accelerates after hearing onset

Short and temporally precise excitation and inhibition is crucial for the analysis of binaural temporally
patterned sounds (Tollin 2003). We therefore quantified the kinetics of evoked single-fiber IPSCs
eIPSCs) and of mIPSCs. The 10-90% rise time and the exponential decay time constant of IPSCs
were determined at three developmental stages: in prehearing animals (P10) and after hearing onset
(P18 and P23). As previously observed in neurons of the MSO (Magnusson et al. 2005), another
target nucleus of the MNTB fibers, there was a striking acceleration in the rise and decay kinetics of
both eIPSCs (Fig. 4A,B) and mIPSCs (Fig. 4C,D) after hearing onset. This acceleration of the
inhibitory currents continued up to P23, indicating that temporal synapse maturation exceeds the
period of synapse refinement by more than a week.

Since transmitter phenotype, which changes from mixed GABA/glycine to purely glycine mostly before
hearing onset (Kim and Kandler 2010; Kotak et al. 1998; Nabekura et al. 2004), we tested whether a
decrease in GABA<sub>A</sub> receptor contribution accounts for the faster kinetics of inhibitory currents in more
mature animals. However, pharmacological blockade of GABA<sub>A</sub> receptors (10µM SR95531) in P10
ergibils only marginally decreased the tau decay (6±4%, n=4) and the amplitude (6±4%, n=4) of
eIPSCs (Fig. 5A), indicating that other factors are mainly responsible for the several-fold decrease in
the decay kinetics of IPSCs after hearing onset. This minor contribution of GABA<sub>A</sub> receptors at this age
is in line with a previous study in mice (Kim and Kandler 2010). However, a larger GABA<sub>A</sub> receptor
component at a similar age range has been previously reported in the gerbil MNTB-LSO connection
(Kotak et al. 1998), the same species as used in the present study. One plausible explanation for this
discrepancy is variations in developmental maturity of the animals used in the two different studies.

It is also unlikely that a selective pruning of dendritic inputs as seen in the MSO (Kapfer et al. 2002),
and therefore a decrease in dendritic filtering is a major factor for the acceleration of decay kinetics,
since mIPSC rise times are differentially distributed in P10 and P23 gerbils and mice (Fig. 5B). This is
also supported by the lack of correlation between amplitude and rise time of IPSCs in P10 animals (data not shown) as would be expected for dendritic filtering.

Comparing the kinetics of mIPSCs of gerbils and mice at P23 revealed that rise and decay times were significantly smaller in mice compared to gerbils, (rise time: gerbil: 0.22 ± 0.01 ms, n=9; mouse: 0.17 ± 0.01 ms, n=9, p≤0.001; tau decay: gerbil: 1.2 ± 0.1 ms, n=9; mouse: 0.75 ± 0.02 ms, n=9, p≤0.001) (Fig.4E), with values which are among the fastest kinetics reported for inhibitory synaptic currents (Awatramani et al. 2004; Stuart and Redman 1990). Interestingly, kinetics of eIPSCs did not differ significantly between gerbils and mice, indicating that lower release synchrony and a larger quantal content per fiber counteracts the faster kinetics of mIPSCs in mice (Fig.4E).

**Short-term synaptic plasticity at the MNTB-LSO synapse**

In a natural sound environment LSO neurons receive trains of inhibitory inputs, with intervals depending on the primary like spike pattern of the presynaptic MNTB neuron (Kopp-Scheinpflug et al. 2003; Kopp-Scheinpflug et al. 2008; Smith et al. 1991). Thus, the short-term dynamics of the synaptic input is an important parameter determining the output of LSO neurons during periods of prolonged stimulation. We analyzed developmental changes in short-term synaptic dynamics of the MNTB input in gerbils and mice by stimulating MNTB fibers for a prolonged period (20 stimuli) with different stimulation frequencies (50-200 Hz). At both ages inhibitory synaptic currents showed substantial depression at stimulation frequencies of 100 Hz with a steady state depression of around 30% of the initial IPSC amplitude (Fig. 6A). Interestingly, in gerbils this synaptic depression was not different before and after hearing onset (P10: 0.27 ± 0.03 (n=9); P18: 0.27 ± 0.03 (n=7); p=0.87) (Fig. 6B,C), and in mice synaptic depression even slightly increased during this developmental period (P10: 0.31 ± 0.02 (n=6); P18: 0.25 ± 0.02 (n=7); p≤0.05). Furthermore, short-term synaptic depression was very similar in mature gerbils and mice (Fig. 6C). This observed depression is considerably stronger than the one described for excitatory synaptic inputs at the synapse preceding the MNTB-LSO connection in mature animals (Taschenberger and von Gersdorff 2000). We therefore analyzed pharmacologically isolated excitatory postsynaptic currents (EPSCs) in LSO neurons of gerbils evoked by repetitive stimulation of the fibers coming from the ipsilateral AVCN and compared short-term depression between the inhibitory and the excitatory inputs to the LSO. Also the excitatory input to LSO neurons showed significant depression (Fig. 6D). However, this depression was slightly but significantly smaller for excitation compared to inhibition for all stimulation frequencies tested (50 Hz: EPSCs: 0.43 ± 0.02; IPSCs: 0.32 ± 0.01; ps0.01; 100Hz: EPSCs: 0.4 ± 0.05; IPSCs: 0.26 ± 0.02; ps0.05; 200 Hz: EPSCs: 0.3 ± 0.05; IPSCs: 0.21 ± 0.02; ps0.05) (Fig. 6D,E). This suggests that the relative EPSC and IPSC amplitudes might dynamically change during periods of prolonged stimulation.
Discussion

In the present study, we investigated post-hearing developmental changes of the inhibitory MNTB-LSO input. To our surprise, neither the number of inhibitory input fibers from the MNTB to the LSO, nor the quantal amplitude of inhibitory miniature events significantly changed after hearing onset in both species gerbils and mice. This indicates that the onset of sensory experience does not trigger a major refinement of this projection. Similarly, unlike at the Calyx synapse in the MNTB, short-term synaptic depression did not change during the same developmental period. In contrast, the kinetics of inhibitory currents largely accelerated in both species after hearing onset with time constants that are among the fastest ever reported (Awatramani et al. 2004).

Comparison of intrinsic properties and inhibitory inputs of LSO neurons in gerbils and mice

A comparison of previous studies has suggested that membrane properties and the firing pattern to depolarizing current injections vary between different species (Adam et al. 2001; Adam et al. 1999; Barnes-Davies et al. 2004; Hassfurth et al. 2009; Kandler and Friauf 1995; Sanes 1993; Sterenborg et al. 2010). This was generally interpreted as species specific adaptations to the required sound localization behavior. However, one has also to consider that these studies were performed at different developmental stages and under various experimental conditions. It is generally known that the major ion channels which determine the intrinsic membrane properties of LSO neurons, namely various K-channels and the HCN channels, can be modulated by neuronal activity via the activation and deactivation of second messengers, or can be affected by the composition of the electrode solution or by recording conditions such as temperature (Leao et al. 2006). The data in the present study support that membrane properties and firing patterns of LSO neurons are indeed very much alike in gerbils and mice, and the variations so far reported are likely to be of technical or developmental origin.

On the contrary, the inhibitory projections from the MNTB to the LSO differ between the two species. The mean amplitude of mIPSC, which reflects the mean quantal content, is much smaller in mice compared to gerbils whereas mIPSC frequency, an estimate for the relative number of functional synapses with similar release probability, is larger in mice compared to gerbils. Thus, more synaptic contacts with smaller quantal size and faster decay kinetics in mice generate similar eIPSC characteristic to gerbils where MNTB –LSO inputs have fewer contacts that have larger quantal size and slightly slower decay kinetics. Since the variance of the inhibitory response is dependent on both the quantal size and the number of functional synapses a quantitative analysis of variance would be required to gain insight into the nature of amplitude fluctuations in gerbils and mice (Clements 2003). Nevertheless, evoked inhibitory transmission from the MNTB to the LSO is in general surprisingly similar in adult gerbils and mice as well as during development (see also (Kim and Kandler 2010).

Lack of a functional refinement of inhibitory inputs after hearing onset

LSO neurons receive ipsilateral excitatory and contralateral inhibitory projections that are both tonotopically matched (Sanes and Rubel 1988). Previous studies have shown that the inhibitory projection from the MNTB to the LSO undergoes a major refinement during postnatal development (Kandler et al. 2009; Sanes and Friauf 2000). This includes a major reduction of the number of active
inhibitory input fibers from the MNTB to the LSO before hearing onset (Kim and Kandler 2003; 2010), and a subsequent morphological refinement of the inhibitory LSO-MNTB axonal arbors (Sanes and Siverls 1991). The present study provides strong evidence that no further physiological refinement of the inhibitory inputs occurs after hearing onset, which is in line with the idea that synaptic silencing precedes the anatomical pruning by several days (Kandler et al. 2009; Kim and Kandler 2003). Already at hearing onset, frequency tuning of the excitatory and the inhibitory inputs is matched (Sanes and Rubel 1988), and thus the general tonotopic alignment of inputs occurs independent of sound experience. Various guidance molecules, most notably the Ephrins, seem to play an important role in this process (Huffman and Cramer 2007; Miko et al. 2007). Comparing the development of the frequency response areas of the inhibitory and excitatory inputs to LSO neurons, however, suggest a further but small functional tonotopic refinement of the inputs which occurs after hearing onset (Sanes and Rubel 1988). Our data indicate that this tonotopic refinement occurs upstream of the MNTB-LSO projection such as in the cochlea or in the cochlear nucleus. Indeed, a narrowing of frequency tuning directly after hearing onset has also been reported from MNTB neurons of mice (Sonntag et al. 2009), and could thus account for observed frequency changes in the LSO. It is also possible that an additional minor adjustment of input fiber arrangement after hearing onset is obscured by technical issues such as a reduction in the number of intact input fibers due to the slicing procedure.

The lack of change in quantal amplitude of individual synapses in combination with an increase in the frequency of inhibitory miniature events speaks against an elimination of inhibitory inputs after hearing onset that is dependent on sensory experience. But why is this different at the other target nucleus of the MNTB the medial superior olive? Anatomical data from this adjacent nucleus suggest a reduction of input fibers to each neuron (Werthat et al. 2008) and a spatial refinement of glycinergic synapses after hearing onset (Kapfer et al. 2002). In addition, physiological data, which show a decline in mIPSC frequency and an increase in quantal amplitude, support this hypothesis (Magnusson et al. 2005). One possible explanation is that the MSO circuit requires a temporal fine-adjustment of its inhibitory inputs which is dependent on binaural auditory experience. This might be different in the LSO circuit where binaural temporal adjustment is probably less crucial. Therefore the binaural matching of excitatory and inhibitory inputs in the LSO might not be dependent on precisely timed auditory activity; nevertheless, some correlation of activity between the ears is required. Before hearing onset spontaneous activity bursts are independently generated in each ear by ATP release of supporting cells which activate inner hair cells (Tritsch et al. 2007) generating uncorrelated excitatory and inhibitory inputs to the LSO. Whether the medial olivo-cochlear efferent neurons, which transiently and bilaterally project to the inner hair cells and inhibit inner hair cell firing via acetylcholine receptors (Glowatzki and Fuchs 2000; Kros 2007), could potentially synchronize binaural excitatory and inhibitory activity patterns before the onset of sound evoked activity, remains to be shown.

The developmental acceleration of inhibition in the LSO in gerbils and mice and its implication for the function of inhibition in the LSO

Similar to neurons in the medial superior olive and the medial nucleus of the trapezoid body (Awatramani et al. 2005; Magnusson et al. 2008) both rise and decay times of inhibitory currents in LSO neurons considerably accelerated after hearing onset. One possible explanation for this
acceleration of IPSC kinetics is a change in the subunit composition of the glycine receptor. Glycine receptors containing the alpha1 subunit display the fastest time constants compared to receptors containing other alpha subunits (Lynch 2009) and expression levels of the alpha1 subunit have been shown to increase in the LSO until several days after hearing onset (Piechotta et al. 2001). However, it is unlikely that the subunit composition is the only factor governing the kinetics of IPSCs. Previous studies have suggested a number of other factors that can contribute to the time course of inhibitory synaptic currents. This includes changes in the anatomical receptor localization, the synchrony of vesicle release from a single fiber transmitter and glycine uptake mechanisms (Magnusson et al. 2005; Wall et al. 2002). Since in our experiments the kinetics of mIPSCs showed a similar developmental acceleration to eIPSCs the contribution from an increase in release synchrony seems to be small. However, a change in the architecture of the synapse (Cathala et al. 2005), a shift in the affinity of the receptor to glycine (Schofield and Huguenard 2007) or the co-release of GABA (Lu et al. 2008) could possibly all lead to an acceleration of IPSC kinetics at this synapse. Most importantly, glycine transporter activity has been shown to speed the decay kinetics of the synaptic currents (Beato 2008; Bradaia et al. 2004). However, whether a developmental up-regulation of glycine transporters in the LSO contributes to the observed acceleration of IPSC kinetics and what different roles the glycine transporters GlyT1 and GlyT2 play for this very fast glycinergic inhibition remains to be shown.

But is the maturation of IPSC kinetics in the LSO indeed dependent on auditory experience? Experiments in the auditory brainstem and the barrel cortex support the idea that sensory deprivation can influence IPSC kinetics. However, this change can go both ways, either accelerating or slowing IPSCs (Leao et al. 2004; Li et al. 2009) and are in some cases paralleled by a change in the alpha subunit composition of the glycine receptor (Li et al. 2009). Nevertheless, some of the above discussed factors most likely also contribute to this effect.

One important remaining issue is the functional implication of this very fast inhibition of the inputs from the MNTB to the LSO with decay kinetics of IPSCs around one millisecond. A similar fast time course of the effective inhibition has been estimated from in vivo recordings of LSO neurons in the Mexican free-tailed bat by changing the relative timing of very rapid frequency sweeps between the ears (Park et al. 1996). Similar fast time constants of effective inhibition can also be derived from two studies in the cat (Joris and Yin 1995; Tollin and Yin 2005) and in the chinchilla (Moore and Caspary 1983) LSO neurons responding to low frequencies. Thus the fast time course of inhibition in the LSO might be essential to improve sound localization accuracy for low frequency sound processing in the LSO or when envelope ITDs of high frequency sounds are used.
Figure legends

Figure 1: Developmental changes in membrane properties of LSO neurons before and after hearing onset in gerbils and mice. (A) Representative voltage responses of LSO neurons at P10 and P23 of gerbils (upper traces) and mice (lower traces) in response to 1 s long current step injections. (B) Summarized changes in input resistance measured at the peak of the voltage response to -100pA. (C) Summarized changes in input resistance measured at the steady state of the voltage response to -100pA. (D) Summarized changes in membrane time constant fitted to the voltage change to -100pA current injection. (E) Developmental changes in resting membrane potential (Vrest) in gerbils and mice measured directly after obtaining the whole-cell configuration. Sample sizes (n) are between 7 and 12. Statistical significance was tested with the unpaired student t-test.

Figure 2: Inhibitory synaptic currents recorded from LSO neuron in response to MNTB fiber stimulation at various stimulation intensities (10 to 99V) in gerbils before (A) and after (B) hearing onset. (Ai) Inhibitory postsynaptic currents (IPSCs) of a gerbil aged P10 evoked by MNTB fiber stimulation at gradual increasing stimulation intensities (10-99V). (Aii) IPSC amplitude and stimulus amplitude plotted against the stimulus #. (Aiii) Histograms of IPSC amplitudes of the same neurons and the number of presumed inhibitory fibers as obtained by cluster analysis depicted on top. (Bi) Inhibitory postsynaptic currents (IPSCs) of a gerbil aged P17 evoked by MNTB fiber stimulation at gradual increasing stimulation intensities (10-99V). (Bii) IPSC amplitude and stimulus amplitude plotted against the stimulus number. (Biii) Histograms of IPSC amplitudes of the same neurons and the number of presumed inhibitory fibers as obtained by cluster analysis depicted on top. (C) Summary of the developmental changes in the number of steps (presumed inhibitory fibers innervating one LSO neuron) in gerbils and mice. (D) Summary of developmental changes in single-fiber inhibitory conductance in gerbils and mice obtained when only one fiber (threshold) was stimulated. Numbers in bars indicate sample size (n). Statistical significance was tested with the unpaired student t-test.

Figure 3: Developmental changes in the amplitude and frequency of miniature glycinergic currents in gerbils and mice. Example traces of miniature IPSCs in LSO neurons of P17 gerbils (A) and mice (B) at low (upper trace) and high temporal (lower trace) resolution. (C) Summary of developmental changes in mIPSC frequency in gerbils and mice. (D) Summary of developmental changes in mIPSC amplitude in gerbils and mice. Numbers in bars indicate sample size (n). Statistical significance was tested with the unpaired student t-test.

Figure 4: Kinetics of evoked and miniature IPSCs (eIPSCs and mIPSCs) in gerbils and mice before and after hearing onset. (A) Example traces of normalized eIPSCs in gerbil and mice at P10 and P23. (B) Example traces of normalized mIPSCs in gerbil and mice at P10 and P23. Summary of 20-80% rise times tau decay of evoked IPSCs (C) and mIPSCs (D) in gerbils and mice. Each open dot (o) represents the kinetics of one LSO neuron. The bar represents the average rise and decay time for the various ages. (E) Comparison of eIPSC and mIPSC rise and decay times between gerbil and mice in P23 animals. Statistical significance was tested with the unpaired student t-test.
Figure 5: (A) Change in amplitude, paired-pulse ratio and decay time constants of evoked IPSCs by blocking GABA\(_A\) (10µM SR95531) receptors in P10 Gerbils (1 indicates no change). Both amplitude and decay time constants decreased by approximately 5% indicating a small remaining GABAergic component at this age. (B) Distribution of mIPSC rise times for P10 and P23 mice (A) and gerbils (B). The shift in the peak and the entire population of mIPSC rise times indicates that a selective elimination of dendritic receptors, which would preferentially reduce longer rise times, only plays a minor role in the speeding of IPSC kinetics.

Figure 6: Developmental changes in short-term depression of IPSCs evoked by 100 Hz MNTB fiber stimulation. (A) Example traces of IPSC currents in a P10 and P18 LSO neurons of gerbils in response to 100 Hz train fiber stimulation. (B) Normalized and averaged IPSC amplitudes to each stimulus of the 100 Hz train in P9/10 and P17/18 gerbils. (C) Summary of IPSC steady state depression (amplitude of 1st IPSC/ average amplitude of last five IPSCs) before and after hearing onset in gerbils and mice. (D) Normalized and averaged IPSC and EPSC amplitudes to each stimulus of a 100Hz train stimulation in P17/18 gerbils. EPSCs were evoked by stimulation of fibers from the cochlear nucleus. For EPSC recordings glycinergic and GABAergic currents were blocked with strychnine (0.5µM) and SR95531 (10µM). (E) Summary of steady state depression of EPSCs and IPSCs (amplitude of 1st PSC/ average amplitude of last five PSCs) in gerbils before and after hearing onset for three different stimulation frequencies (50Hz, 100Hz, 200Hz). Numbers in bars indicate sample size (n). Statistical significance was tested with the unpaired student t-test.
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Fig. 1

A

-69 mV

+ 600 pA

-500 pA

+ 800 pA

B

R_{m, peak} (MΩ)

P10  P18  P23  P10  P18  P23

gerbil  mouse  gerbil  mouse

C

R_{m, SS} (MΩ)

P10  P18  P23  P10  P18  P23

gerbil  mouse  gerbil  mouse

D

τ_{membrane} (ms)

P10  P18  P23  P10  P18  P23

gerbil  mouse  gerbil  mouse

E

V_{rest} (mV)

gerbil  mouse
Fig. 2

A  
(i) 
(ii) 
(iii) 

B  
(i) 
(ii) 
(iii) 

C  

D  

<table>
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<tr>
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<th>P10</th>
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<tr>
<td>gerbil</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>mouse</td>
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Fig. 3

A. P17 gerbil

B. P17 mouse

C. Bar graphs showing mIPSC frequency (Hz) for Gerbil and Mouse at P10, P18, and P23.

D. Bar graphs showing mIPSC conductance (nS) for Gerbil and Mouse at P10, P18, and P23.
Fig. 5

A

B

% IPSC with blocked GABAARs

amplitude

PPR

tau decay

rise time (ms)

mice P10

mice P23

norm. # IPSCs

gerbil P10

gerbil P23

rise time (ms)